Original Article

Minocycline Acts as a Neuroprotective Agent Against Tramadol-Induced Neurodegeneration: Behavioral and Molecular Evidence

Abstract

Background: Previous evidence indicates that tramadol (TRA) can lead to neurodegenerative events and minocycline (MIN) has neuroprotective properties. **Aim of the Study:** The current research evaluated the neuroprotective effects of MIN for TRA-promoted neurodegeneration. **Methods:** Sixty adult male rats were placed into the following groups: 1 (received 0.7 ml/rat of normal saline, IP), 2 (received 50 mg/kg of TRA, i.p.), 3, 4, 5 (administered TRA as 50 mg/kg simultaneously with MIN at 20, 40, and 60 mg/kg, IP, respectively), and 6 (received MIN alone as 60 mg/kg, IP). The treatment procedure was 21 days. An open field test (OFT) was used to measure motor activity and anxiety-related behavior. Furthermore, oxidative stress; hippocampal inflammation; apoptotic parameters as well as activity of mitochondrial complexes I, II, III, and IV; ATP levels; and mitochondrial membrane potential (MMP) were evaluated. In addition, histomorphological alteration was assessed in two regions of the hippocampus: Cornu Ammonis (CA1) and dentate gyrus (DG). **Results:** MIN treatment could inhibit TRA-induced anxiety and motor activity disturbances ($P < 0.05$). In addition, MIN could attenuate reactive oxygen species (ROS), H_2O_2, oxidized glutathione (GSSG), and malondialdehyde (MDA) level ($P < 0.05$), while there was increased reduced glutathione (GSH), total antioxidant capacity (TAC), ATP, MMP, and BCL2 levels ($P < 0.05$) and also elevation of SOD, GPX, GSR ($P < 0.05$), and mitochondrial complexes I, II, III, and IV activity ($P < 0.05$) in TRA-treated rats. In consistence with these findings, MIN could reduce TNF/TNF-α, IL1B/IL1-β, BAX, and CASP3 levels ($P < 0.05$) in TRA-treated rats. MIN also restored the quantitative ($P < 0.05$) and qualitative histomorphological sequels of TRA in both CA1 and DG areas of the hippocampus. **Conclusions:** MIN probably has repositioning capability for inhibition of TRA-induced neurodegeneration via modulation of inflammation, oxidative stress, apoptosis, and mitochondrial disorders.

Keywords: *Anxiety, minocycline, neurodegeneration, stress, tramadol*

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Introduction

Tramadol (TRA) is a pain killer and analgesic agent, abuses of which are associated with consequences such as mood and behavioral disturbances, hallucination, and euphoria.[1,2] This property of TRA has resulted in increases of its improper use during recent decades.[1-3] Long-term TRA administration or abuses can causes neurodegeneration, which is mediated by activation of oxidative stress, neuro-inflammation, and apoptosis or neural cell-related signaling cascades.[4-7] Evidence indicates that mitochondrial dysfunction is involved in TRA-induced neurodegeneration.[8-10] Many studies show that the chronic use of TRA increases the production of free radicals, as well as the production of ROS and reactive nitrogen species and the activation of lipid and protein peroxidation.[11-13] In addition, the results show that TRA inhibits the function and level of antioxidants and inhibits the cellular defense against oxidative agents by inhibiting SOD (superoxide dismutase), CAT (catalase), GPX (glutathione peroxidase), and GSR (glutathione-disulfide reductase) enzymes and glutathione-derived antioxidants.[11-14] In this regard, previous works have shown that tramadol destroys nerve cells by activating inflammatory and proinflammatory messengers, cytokines, signaling related to prostaglandins, inflammatory kinases, and nitric oxide and affects many pathways.[12,15-17] Activation of neuronal cell death pathways caused by tramadol has also been demonstrated by other studies, and it has been shown that this compound plays a serious role in inducing neurodegeneration by activating cell signaling related to apoptosis, autophagy, and necrosis.[17,18]

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In spite of all this direct and indirect evidence about TRA-induced neurodegeneration, the relevant signaling cascades and a clear mechanism of action have not yet been characterized.[5,7-10] To date, the entirety of the molecular dimensions of the neurotoxic effects of TRA has not been determined and needs to be investigated; also, limited efforts have been made, in the form of a few studies, toward the management of TRA neurotoxic effects and neurodegeneration.[8,18] Based on this concept, the introduction of new agents with neuroprotective properties or the repositioning of conventional drugs with the capability of neuroprotection is necessary for the management of drug abuse-induced events such as neurotoxicity and neurodegeneration.

Minocycline (MIN) is an antibiotic from the tetracycline group, which has protective antioxidant and anti-inflammatory effects in neuronal cells.[19-21] MIN treatment for effects on behavioral disturbances such as anxiety, depression, and cognition was previously approved.[22-24] Molecular evidence suggests that MIN can be considered for repositioning therapy for some neurodegenerative disorders and disease events such as Alzheimer and Parkinson diseases, multiple sclerosis, cerebral ischemia, and drug (such as psychostimulant, alcohol, and nicotine) abuse-induced neurotoxicity.[25-30] Previous studies indicated the protective role of MIN in the reduction of free radicals, and inhibition of production of ROS and reactive nitrogen species, and also confirmed its effects in the reduction of lipid and protein peroxidation in neurodegenerative events.[31,32] MIN enhances the level of aforementioned antioxidants and glutathione function during neurodegeneration.[32,33] Previous studies have indicated that MIN can restore nerve cell function and life by inhibition of inflammatory and proinflammatory cascades, reduction of cytokine levels, and signaling related to prostaglandins, inflammatory kinases, nitric oxide, and other related pathways in neurodegenerative events.[33-35] The use of MIN due to its capability in the inhibition of neuronal cell death pathways such as apoptosis, autophagy, and necrosis during neurodegeneration was approved previously.[21,33,36] It was suggested that the neuroprotective properties of MIN are mediated via mitochondria,[26,27,37-40] and it seems a significant part of MIN's protective effects occurs via activation of mitochondrial membrane and respiratory chain enzymes function.[41,42] Despite the data which introduce MIN as a neuroprotective agent, its role in drug abuse, such as that involving TRA-induced neurodegeneration, and the mechanism through which it acts remain unclear. Thus, the effects of MIN against TRA-induced inflammation, oxidative stress, mitochondrial dysfunction, and apoptosis in hippocampal tissue need further assessment. Thus, in the present study, we analyzed the potential neuroprotective effects of MIN against TRA-induced neurodegeneration. To better understand the mechanism behind the interaction of these two agents, the levels and occurrences of oxidative stress, inflammation, mitochondrial dysfunction, and apoptosis as well as changes in the histology in hippocampal tissue in the CA1 and DG areas were evaluated.

Methods

Animals

Sixty adult male Wistar rats weighing an average of 200–250 g (Experimental Research Center, Veterinary College at Tehran University) were used in the study. The animals were housed under standard laboratory conditions at 22 ± 0.5°C with 30–70% humidity and 12-h light/dark cycles. All animals were able to freely access water and rat pellet food. The methodologies received approval from the Committee on Ethics in Shahid Beheshti University of Medical Sciences, Masih Danshvari Hospital (Ethical Code: IR.SBMU.NRITLD.REC.1402.001). It should be noted that experimental procedures were performed based on technical and ethical guidelines of ARRIVE (Animal Research: Reporting of *In vivo* Experiments) guidelines.[43,44]

Drugs

Tramadol (TRA) (CAS number: 36282-47-0) and minocycline (MIN) (CAS number: 13614-98-7) were purchased from Sigma-Aldrich company (MD, USA). Both TRA and MIN were freshely dissolved in normal saline just before use.

Experimental protocols and design

Sixty adult male rats were divided into six groups as follows:

- Group 1 was considered as a sham group, which only received normal saline (0.7 ml/rat, intraperitoneally [i.p.]) for 21 days, and no other treatment process was performed on these animals.
- Group 2 received only TRA (50 mg/kg, i.p.) for 21 days.
- Groups 3, 4, and 5 were considered as experimental groups and received TRA (50 mg/kg, i.p.) and MIN (20, 40, and 60 mg/kg, i.p.), respectively, simultaneously for 21 days.
- Group 6 received only MIN (60 mg/kg, i.p.) for 21 days and was considered as a minocycline control group.

An illustration of the experimental protocols is schematically detailed in Figure 1.

The doses of MIN as a neuroprotective agent and also TRA as a neurotoxic agent were chosen based on previous work.[4,5,26,27,33,45-50]

On day 22, open field tests (OFTs) were used to provide an assessment of anxiety and motor activity disorder. After behavioral assessment, on day 23, hippocampal tissues of all animals were isolated according to previous guidelines.[26,27] These hippocampus tissues were used to monitor parameters of mitochondrial function, oxidative

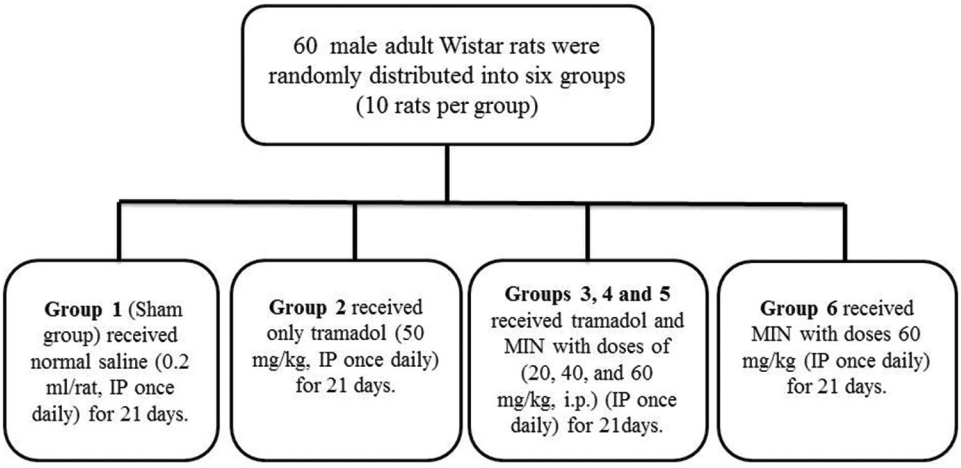

Figure 1: The experimental protocols used in this study are illustrated schematically

stress, apoptosis, inflammation, and also histological changes. Right hemispheres of the hippocampi were used for evaluation of hematoxylin and eosin staining (H and E), and also left hemispheres of the hippocampi were used for evaluation of mitochondrial function, oxidative stress, apoptosis, and inflammation. Doses of MIN for conferring neuroprotection and TRA for induction of neurodegeneration or neurotoxicity were selected based on previous similar studies.[26,27,45,51] The timeline for experimental procedures is indicated in Figure 2.

Behavioral studies

Open field test

An OFT was used to evaluate anxiety- and motor activity-related neurobehavioral changes in mice and rats.[52,53] For evaluation of this test on day 21, after the drug treatments, the animals were transferred to the behavioral laboratory to adapt to the conditions of this laboratory. Also, on this day, each of the animals was subjected to the OFT box test for 5 min to become familiar with this test and to know its conditions. The process was performed to train the animal for the OFT. The main test was performed on day 22. This test was performed using a special apparatus (Bionic-Mobin Company, Tehran, Iran): The main apparatus was a box having a square arena of 60 × 60 × 60 cm. The bottom was black and divided into 16 equal squares delineated by bright white lines, and the walls were opaque. A central square, which was marked with red lines, was drawn in the bottom of this box. A 100 W bulb was located 110 cm above the apparatus and illuminated the OFT box during the experiment; all parts of the room in which the box was situated were kept dark during the experiment. A camera was placed 2.1 m above the equipment, and this camera was connected to a camera-based tracking system (Limelight, Actimetrics, Wilmette, IL, USA) and video tracking software, EthoVision (XT model, Wageningen, The Netherlands) for analysis of animal behavior. Four standard behaviors were recorded in this system:

1. Ambulation distance: the number of times a rat crossed a line, and the total distance which each rat crossed along grid lines.
2. Time spent in the center square: the total amount of time spent by each animal with all four paws contained within the central red square.
3. Center square entries: the number of central square entrances of each animal.
4. Rearing number: the frequency of an animal standing on its hind legs on the bottom of the OFT box.[52,53]

Molecular studies

Tissue preparation, protein extraction, and determination of mitochondrial function, oxidative stress, inflammation, and apoptosis

For evaluation of molecular changes, sodium thiopental (50 mg/kg, i.p.) (DNA Biotech Co, Tehran, Iran) was injected into each animal, and according to animal surgical standards, their hippocampus was removed and dissected.[54] For preparation, the hippocampus tissue was homogenized in cold homogenization buffer (25 mM 4-morpholinepropanesulfonic acid, 400 mM sucrose, 4 mM magnesium chloride [$MgCl_2$], 0.05 mM ethylene glycol tetraacetic acid [EGTA], pH 7.3) (DNA Biotech Co, Tehran, Iran). The homogenized hippocampus was centrifuged for 15 min at 450 g. Subsequently, the supernatant was removed and recentrifuged at 5000 g, 12 min. The centrifuged pellet was resuspended in the homogenization buffer and kept at 0°C. The Bradford method (Bio-Rad Co., CA, USA, Dc-Bio-Rad, catalog number: 5000001) was used for evaluation of the protein level of the suspension: 1 portion of Bradford reagent: 4 portions of dH_2O were mixed with serial dilutions (0.1-1.0 mg/ml) of bovine serum albumin (BSA; Sigma-Aldrich Co, MD, USA, CAS number: 9048-46-8) to generate a standard curve of concentration vs optical density. In the next step, serial dilutions (10, 15, 20, 25, and 30 μl) of the aforementioned homogenized hippocampal protein suspension were mixed with Bradford reagent,

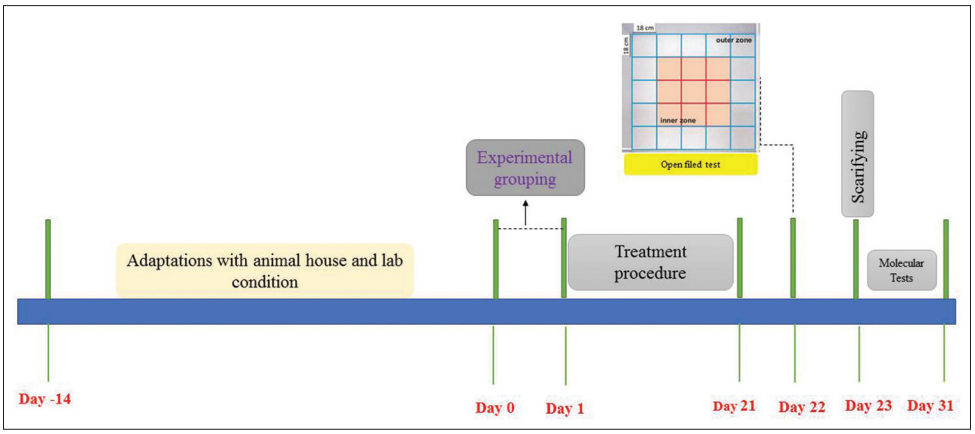

Figure 2: Timeline of drug treatments, behavioral assessments and neurochemical evaluations

and the color/optical density at 630 nm was determined with a plate reader. Finally, the standard curve was used to calculate the protein content of the hippocampus suspensions, and this measurement was applied for evaluation of mitochondrial parameters, oxidative stress, inflammation, and apoptotic indicators.[55-57]

Measurement of lipid peroxidation

Malondialdehyde (MDA; CELLBIOLAB Co, San Diego, CA, USA, catalog number; STA-330-5), a byproduct of lipid membrane peroxidation, was used as an indicator. For measurement of the MDA level in samples, 10 µL of standard sample solution or 10 µL MDA was added in wells of microplates and then 10 µL of SDS lysis solution (an ionic denaturing detergent) was added to each of the wells, which were shaken gently and incubated at 37°C. In the next phase, 25 µL of thiobarbituric acid (TBA; CELLBIOLAB Co, San Diego, CA, USA, Catalog number; STA-330-5) reagent was mixed into each well and kept for 45–65 min at 95°C. All wells were centrifuged at 1000 × g for 20 min, and their supernatants were isolated. Thirty µL of the supernatants were mixed with 30 µl of n-butanol (CELLBIOLAB Co, San Diego, CA, USA, Catalog number; STA-330-5) and centrifuged at 7000 g for 5 min. Absorbance of the mixture was read at 532 nm. The results were recorded and reported as nmol/mg protein.[58,59]

Measurement of reactive oxygen species levels

Hippocampal ROS levels were monitored with the use of fluorescein-labeled dye and conversion of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Chemodex Ltd, Gallen, Switzerland, CAS number: 4091-99-0) to fluorescent 2,7-dichlorofluorescein (DCF) via a de-esterification and oxidation reaction.[60,61] 2,7-dichlorodihydrofluorescein diacetate was add to 50 µL of homogenate of hippocampus tissue and kept at 37°C for 50 min. The fluorescence intensity of DCF was read at 535 nm. The ROS level was recorded and reported as fold change compared to the control.[60-62]

Measurement of hydrogen peroxide (H_2O_2) levels

The measurement of H_2O_2 production was conducted using the following setup: 20 µL of hippocampal tissue homogenate was added to 80 µL of reaction mixture. The reaction mixture consisted of 250 µM sulfate, 25 mM H_2SO_4, and 100 µM xylenol orange (DNA Biotech, Tehran, Iran). The mixture was then vortexed for 10 s and kept undisturbed in a protected area away from light for 45 min. Following this incubation period, a GENESYS 10 UV/Vis scanning spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure the formation of the reaction product at a wavelength of 580 nm. To determine the concentration of H_2O_2 produced, a curve using known concentrations of H_2O_2 ranging from 0 to 100 nM was created, and the results were expressed as nMol H_2O_2/gram of tissue.[63]

Measurement of SOD2/manganese superoxide dismutase activity

For measurement of SOD2 activity, Winterbourn's method was applied.[64,65] The potency of SOD for inhibition of nitroblutotrazolium/NBT reduction by superoxide ion is a basic principle of this test. Twenty µl of the hippocampus homogenate supernatant was mixed with 10 µl of 0.1 M sodium cyanide (MERCK Co, Darmstadt, Germany, CAS number: 143-33-9) and 5 µl of 3 M EDTA (MERCK Co, Darmstadt, Germany, CAS number: 60-00-4), and then, 10 µl of 1.5 M nitroblutotrazolium (Cayman Chemical, CAS Number: 298-83-9) was added. The mixture was kept in a special cuvette and incubated for 5 to 10 min at 37°C. Riboflavin (MERCK Co, Darmstadt, Germany, CAS number: 83-88-5), and potassium phosphate buffer (0.67 M; pH 8.7) (DNA Biotech Co, Tehran, Iran) was added to the cuvette and incubated at 37°C for 12 to 15 min. Activity of the enzyme was reported in units per milliliter/milligram protein.[64-66]

Measurement of GPX activity

GPX activity was measured according to previous standard protocols.[67,68] The mixture to be analyzed

(980 µl) contained 0.25 mM NADPH (MERCK Co, Darmstadt, Germany, CAS number: 2646-71-1), 2.1 mM reduced glutathione (GSH; Cayman Chemical Co, MI, USA, CAS Number: 70-18-8), 0.5 units/mL of GSR (glutathione-disulfide reductase) (MERCK Co, Darmstadt, Germany, CAS number: 9001-48-3)), and 30 mM tert-butyl hydroperoxide (Sigma-Aldrich Co, MD, USA, CAS number: 75-91-2) and was mixed with 10 µl of hippocampal homogenate. Absorbance of the mixture was read every 30 s, for 2 min at 340 nm, and the results were reported as units, per ml/mg of protein.[66-68]

Measurement of GSR activity

Twenty µl of hippocampal homogenate was added to 1980 µl of reaction mixture, which consisted of 0.1 M phosphate buffer, pH 7.6 (DNA Biotech Co, Tehran, Iran), 0.5 mM EDTA (MERCK Co, Darmstadt, Germany, CAS number: 60-00-4), 1.0 mM oxidized glutathione (GSSG; MERCK Co, Darmstadt, Germany, CAS Number: 27025-41-8), 0.1 mM NADPH (MERCK Co, Darmstadt, Germany, CAS number: 2646-71-1), and 10 µl of phenazine methosulfate (MERCK Co, Darmstadt, Germany, CAS number: 299-11-6). The basic principle of this reaction is the disappearance of NADPH, which was read at 340 nm. The results were reported as per ml/mg of protein.[69]

Measurement total antioxidant capacity (TAC)

TAC levels were measured using a special kit (Sigma-Aldrich Co, MD, USA, CAS number: MAK 187) and evaluated the capacity to convert Cu^{2+} from its oxidized to its reduced form Cu^{+}. According to measurement principles, Cu^{+} can chelate with a special colorimetric probe. The absorbance of the complex of Cu^{+} with the colorimetric probe can be detected at a wavelength of 570 nm, and the results were reported as nM/µg on homogenates of hippocampus.[70,71] All procedures were conducted according to the kit instructions.

Measurement of GSH and GSSG

In each well of a 96-well microplate, we added 25 µL of the glutathione reductase solution (DNA Biotech, Tehran, Iran). Then we introduced 25 µL of a 1X NADPH solution (DNA Biotech, Tehran, Iran). Next, we mixed in a solution consisting of either glutathione or hippocampal samples (100 µL). To start the reaction, we added 50 µL of chromogen (DNA Biotech, Tehran, Iran) mixed it well, and immediately measured the absorbance, at 405 nm. We determined the quantification of GSSG/GSH by referring to a curve. The results are expressed as nmol/mg protein.[26,72]

Measurement of changes in levels of apoptosis and inflammatory protein expression

An enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO, Co, Huston, TX, USA) was used for measurement of BCL2 (BCL2 apoptosis regulator) (CSB-E13604r), BAX (BCL2 associated X, apoptosis regulator) (CSB-EL002573RA), CASP3 (caspase 3) (CSB-E08857r), IL1B (interleukin 1 beta) (CSB-E08055r), and TNF (tumor necrosis factor) (CSB-E11987r) protein expression/levels in the hippocampal homogenates. Briefly, the procedure was carried out as follows: All ELISA kits were washed 3 times with wash buffer which consisted of 0.5 M sodium chloride (MERCK Co, Darmstadt, Germany, CAS number: 7647-14-5), 2.5 mM sodium dihydrogen phosphate/NaH_2PO_4 (MERCK Co, Darmstadt, Germany, CAS number: 7558-80-7), 7.5 mM Na_2HPO_4, and 0.1% Tween 20 (MERCK Co, Darmstadt, Germany, CAS number: 9005-64-5), pH 7.2. In the next phase, 100 µl of ovalbumin (Sigma-Aldrich Co, MD, USA, CAS number: O1641) 1% (w:v) solution was mixed in each well and kept at 37°C for 1 h. Then, all wells were washed 3 times. In the next step, 100 µl of standard solution or homogenized hippocampus was mixed in each well and stored at 50°C for 2 h. Wells were again washed as above, and 100 µl of antirat primary antibody against TNF, IL1B, BAX, CASP3, and BCL2 was added to all wells. All antibodies were diluted in wash buffer 1:999. In the next step, all wells were stored at 37°C for 1 h. Then, wells were washed as above and filled with 100 µl of AvidinHRP (Santa Cruz Biotechnology Co, Dallas, TX, USA, catalog number; *1405-69-2*) and stored 15 min at 37°C. Wells were again washed as above and filled with 100 µl 3,3',5,5'-tetramethyl-benzidine (TMB substrate) (TCI, Zwijndrecht, Belgium, catalog number: T3854) and stored at 37°C for 15 min. In the final step, 100 µl of 1 M H_2SO_4 (MERCK Co, Darmstadt, Germany, CAS number: 7664-93-9) was mixed in, and the absorbance at 450 nm was determined by ELISA reader (Hiperion Microplate Reader, MPR4^{+}, Rayto Company, China). TNF and IL1B levels were reported as ng/ml of hippocampal sample suspension, and BAX, CASP3, and BCL2 were reported as pg/ml of hippocampal sample suspension.[73-75]

Measurement of mitochondrial complex enzymes activity

Mitochondria complexes I, II, III, and IV activities were measured using commercial kits (Abcam Co, Boston, MA, USA, CAS numbers: Ab287844, Ab109908, Ab287844 and Ab109911, respectively). Mitochondrial complex I activity was measured based on the oxidation of NADH to NAD^{+}, and its absorbance was read at 450 nm. The activity of mitochondrial complex II was assessed according to potential catalysis of the electron transfer of succinate to ubiquinone, and its absorbance was read at 550 nm. The reaction speed for the conversion of the oxidized form of CYCS (cytochrome c, somatic) to the reduced form at 600 nm was used for the measurement of mitochondrial complex III activity. Measurement of the oxidation of the reduced form of CYCS at 550 nm was used to monitor mitochondrial complex IV activity. All values were reported as activity/mg of protein/min.[76,77]

Measurement of adenosine triphosphate (ATP)

ATP levels in the brain were assessed utilizing an ATP assay kit (Abcam Co, Cambridge, UK, CAS numbers: Ab83355) as per the manufacturer's guidelines. Ten mg of hippocampal tissue was processed according to the aforementioned protocol, and its homogenate was prepared. In brief, 20 µl of homogenized tissue was diluted with ATP assay buffer. Following neutralization and deproteinization with 2 M KOH, the samples were loaded in triplicate onto a microplate reader. The OD of the mixture was measured at 570 nm using a GENESYS 10 UV/Vis scanning spectrophotometer (Thermo Scientific, Waltham, MA, USA). The ATP levels were determined by referencing a standard curve, and the results were expressed accordingly as nmol/mg of tissue.[78]

Measurement of mitochondrial membrane potential (MMP)

MMP was measured by using JC-1 dye (Cat # 600880, Cayman, MI). This type of dye can enter the mitochondria and cause the formation of J-aggregates (Ex:Em = 560:590 nm; red) or J-monomers (Ex:Em = 485:535 nm; green). The intensity of J-aggregate:J-monomer (red: green) ratio is an indicator of the MMP. High ratios indicate high MMP and vice versa. MMP values were stated as JC-1 ratio (J-aggregate:J-monomers ratio) of the supernatant of hippocampus homogenates.[62,70,79]

Evaluation of histomorphological changes

For evaluation of histological changes, a fixation process was carried out using 4% paraformaldehyde for perfusion throughout the vascular system based on previous standard studies to obtain the finest possible brain conservation for histological evaluations.[80,81] The hippocampus of all the animals in the groups (all 10 animals in each group) was used for hematoxylin and eosin (H and E) staining. All hippocampus samples were washed with normal saline and fixed in paraformaldehyde (12% w: v). Subsequently, fixed tissues were dehydrated with ethanol and then embedded in paraffin. The tissues of all 10 animals in each group were paraffinized, and one section was prepared from each of them for preparing a slide. In the next step, 5 µm thick sections were generated, and slides were prepared; thus, we prepared 10 slides for each group. In the final stage, the slides were stained with H and E, and one image with 400× and/or 100× magnification was prepared from each slide. The aforementioned images were analyzed by using morphometry software (Optikavision pro, Italy), and quality (changes of cell shapes) and quantity (cell density) alterations of all images were evaluated in regions of 1.30 mm of the hippocampal subfield.[82-84]

Statistical analysis

All data were collected and analyzed using special statistical software, Graph Pad PRISM v.7 (CA, USA). Mean ± standard error (SEM) was considered for sets of each data in each experimental group. First, the normality of continuous variables was assessed using the Kolmogorov–Smirnov test. Second, homogeneity of variances between two groups or among more than two groups was evaluated by the Leven's test or the Bartlett's test, respectively. The results of these tests showed the normality of data and indicate that variances were homogeneous between tested groups. Then, differences between the sham and treatment (experimental) groups were measured with ANOVA, and differences between each group were compared by use of the Tukey posttest. Values of $P < 0.05$ were considered as significant.

Results

Results of MIN effects against TRA-induced OFT behavior alteration

The data from all experiments in this study were analyzed using a one-way ANOVA *F*-test with (5,54) degrees of freedom. The number in parentheses after each experimental parameter is the *F* ratio followed by the *P* value. TRA with doses of 50 mg/kg caused a decrease in ambulation distances (53.87; $P < 0.05$) and rearing number (3.400; $P < 0.05$) and also attenuated the time spent in the central square (38.47; $P < 0.05$) and number of central square entries (3.295; $P < 0.05$) when compared to the sham group [Table 1]. In contrast, administration of MIN (20, 40, and 60 mg/kg) improved the number of ambulation distances (53.87; $P < 0.05$) and time spent in the central

Table 1: Effects of various doses of minocycline on tramadol-induced open field exploratory and anxiety-like behavior in rats

Groups	Ambulation distance (cm)	Central square entries (number)	Time spent in central square (sec)	Number of rearing
Sham	364±16	31±5	182±16	18±3
Tramadol (50 mg/kg)	105±14[a]	11±2[a]	35±6[a]	4±1[a]
Tramadol (50 mg/kg) + MIN (20 mg/kg)	145±12[b]	17±1	71±8[b]	13±2
Tramadol (50 mg/kg) + MIN (40 mg/kg)	204±12[b,d]	22±1[b]	109±4[b,d]	13±1
Tramadol (50 mg/kg) + MIN (60 mg/kg)	289±17[b,c]	25±2[b]	149±15[b,c]	15±3[b]
MIN (60 mg/kg)	354±10	27±2	191±3	17±2

All data are given as Mean±SEM, (n=10). [a]Shows significant difference in relation to sham group (P<0.05). [b]Shows significant difference in relation to tramadol (P<0.05). [c]Shows significant difference in relation to tramadol in combination with MIN (40 mg/kg) and or MIN (20 mg/kg) (P<0.05). [d]Shows significant difference in relation to tramadol in combination with MIN (20 mg/kg) (P<0.05). MIN: minocycline

square (38.47; $P < 0.05$) when compared to TRA-treated animals (50 mg/kg) [Table 1]. Administration of MIN (40 and 60 mg/kg) caused increases in central square entries and also (60 mg/kg) in rearing number (3.400; $P < 0.05$) when compared to TRA-treated animals (50 mg/kg) [Table 1]. Administration of MIN (60 mg/kg) in TRA-treated rats caused significant changes in ambulation distances (53.87; $P < 0.05$) and the time spent in the central square (38.47; $P < 0.05$) in comparison to TRA in combination with MIN at 40 mg/kg or 20 mg/kg [Table 1]. Furthermore, MIN at 40 mg/kg in TRA-treated rats could not induce significant changes in ambulation distances and the time spent in the central square behavior in comparison to TRA in combination with MIN at 20 mg/kg [Table 1]. There were no significant changes regarding central square entries and rearing behavior between groups undergoing treatment with TRA in combination with MIN at 20, 40, or 60 mg/kg [Table 1]. Finally, MIN alone (60 mg/kg) did not change the OFT behaviors when compared to the sham group [Table 1].

Results of MIN effects against TRA-induced antioxidant enzymes and TAC

TRA (50 mg/kg) decreased the level of TAC (6.835; $P < 0.05$) and attenuated the activity of antioxidant enzymes such as SOD (4.349; $P < 0.05$), GPX (16.41; $P < 0.05$), and GSR (4.238; $P < 0.05$) activity when compared to the sham group [Table 2]. MIN administration with doses of 40 and 60 mg/kg meaningfully increased the TAC level (6.835; $P < 0.05$) [Table 2]. MIN administration with 60 mg/kg significantly increased SOD (4.349; $P < 0.05$), GPX (16.41; $P < 0.05$), and GSR (4.238; $P < 0.05$) activity when compared to the TRA-treated group (50 mg/kg) [Table 2]. There were no significant changes in the TAC level or SOD, GPX, and GSR activities between groups undergoing treatment with TRA in combination with MIN at 20, 40, or 60 mg/kg [Table 2]. Moreover, MIN (60 mg/kg) alone did not alter the MDA level or SOD, GPX, and GSR activities when compared to the sham group [Table 2].

Results of MIN effects against TRA-induced changes in GSH and GSSG levels

In comparison to the sham group, TRA administration meaningfully attenuated the GSH (12.89; $P < 0.05$) level and elevated GSSG (61.43; $P < 0.05$) levels [Table 3]. In contrast, MIN at all doses decreased the GSSG (61.43; $P < 0.05$) levels and at doses of 40 and 60 mg/kg increased the GSH level (12.89; $P < 0.05$) when statistically analyzed in comparison to TRA-only treated groups [Table 3]. Administration of MIN at 60 mg/kg in TRA-treated rats caused significant changes in GSH (12.89; $P < 0.05$) [Table 3] in comparison to TRA in combination with MIN at 20 mg/kg. In contrast, MIN at 60 mg/kg or MIN at 20 mg/kg in TRA-treated rats did not show significant changes when compared to the group under treatment with TRA and MIN at 40 mg/kg [Table 3].

MIN at 60 mg/kg in TRA-treated rats caused significant changes in GSSG (61.43; $P < 0.05$) levels in comparison to TRA in combination with MIN at 40 or 20 mg/kg [Table 3]. However, MIN at 40 mg/kg in TRA-treated rats did not show significant changes when compared to the group under treatment with TRA and MIN at 20 mg/kg [Table 3]. MIN alone (60 mg/kg) did not affect the GSH or GSSG level when compared to the sham group [Table 3].

Table 2: Effects of various doses of minocycline on tramadol-induced oxidative stress in rats

Groups	TAC level (nM/ microgram)	SOD (U/ml/mg of protein)	GPX (mU/mg of protein)	GSR (mU/mg of protein)
Sham	1.9±0.2	92±6.3	142±6	145±6
Tramadol (50 mg/kg)	0.8±0.1[a]	51±8[a]	75±3[a]	96±9[a]
Tramadol (50 mg/kg) + MIN (20 mg/kg)	1.4±0.1	71±3	92±6	126±6
Tramadol (50 mg/kg) + MIN (40 mg/kg)	1.6±0.1[b]	76±8	102±8	131±7
Tramadol (50 mg/kg) + MIN (60 mg/kg)	1.8±0.1[b]	81±8[b]	112±12[b]	138±11[b]
MIN (60 mg/kg)	2±0.3	96±11	154±5	152±15

All data are given as Mean±SEM, (n=10). [a]Shows significant difference in relation to sham group (P<0.05). [b]Shows significant difference in relation to tramadol (P<0.05). MIN: Minocycline

Table 3: Effects of various doses of minocycline on tramadol-induced GSH and GSSG content in tramadol-treated rats

Groups	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG
Sham	105.7±9.1	2.43±0.61	43
Tramadol (50 mg/kg)	31.4±7.1 [a]	74±6[a]	0.42[a]
Tramadol (50 mg/kg) + MIN (20 mg/kg)	49.8±8.6	43±5[b]	1.13[b]
Tramadol (50 mg/kg) + MIN (40 mg/kg)	68.6±9.2[b]	31±3[b]	2.9[b]
Tramadol (50 mg/kg) + MIN (60 mg/kg)	89.4±8.2[b,d]	14±2[b,c]	6.3[b]
MIN (60 mg/kg)	102.4±7.6	3.1±0.5	34

All data are given as Mean±SEM, (n=10). [a]Shows significant difference in relation to sham group (P<0.05). [b]Shows significant difference in relation to tramadol (P<0.05). [c]Shows significant difference in relation to tramadol in combination with MIN (40 mg/kg) and or MIN (20 mg/kg) (P<0.05). [d]Shows significant difference in relation to tramadol in combination with MIN (20 mg/kg) (P<0.05). MIN: minocycline

Results of MIN effects against TRA-induced changes in the levels of ROS, MDA, and H_2O_2

TRA (50 mg/kg) significantly elevated H_2O_2 (6.084; $P < 0.05$), ROS (12.20; $P < 0.05$), and MDA (18.10; $P < 0.05$) when compared to the sham group [Figure 3a-c]. MIN (40 and 60 mg/kg), in comparison to TRA-treated rats, reduced the ROS (12.20; $P < 0.05$) and in doses of 20, 40, and 60 mg/kg reduced the MDA level (18.10; $P < 0.05$) [Figure 3a and b]. MIN at 60 mg/kg, in comparison to TRA-treated rats, also reduced the H_2O_2 (6.084; $P < 0.05$) level [Figure 3c]; there were no significant changes in groups treated with MIN at 20 or 40 mg/kg. Administration of MIN at 60 mg/kg in TRA-treated rats caused significant changes in ROS (12.20; $P < 0.05$) and MDA (18.10; $P < 0.05$) in comparison to TRA in combination with MIN at 20 mg/kg [Figure 3a and b], whereas MIN at 40 mg/kg in TRA-treated rats could not induce significant changes in ROS and MDA level in comparison to TRA in combination with MIN at 20 or 60 mg/kg [Figure 3a and b].There were no significant changes in H_2O_2 levels between groups under treatment with TRA in combination with MIN at 20, 40, and 60 mg/kg [Figure 3c]. MIN alone could not change ROS, MDA, and H_2O_2 levels [Figure 3a and b].

Results of MIN effects against TRA-induced changes in inflammatory (TNF and IL1B) biomarkers

Treatment of animals with TRA (50 mg/kg) significantly elevated the IL1B (44.48; $P < 0.05$) and TNF (32.01; $P < 0.05$) levels as inflammatory biomarkers when compared to the sham group [Figure 4a and b]. MIN at 20, 40, and 60 mg/kg, in comparison to TRA-treated rats, reduced IL1B (44.48; $P < 0.001$) and TNF (32.01; $P < 0.001$) levels [Figure 4a and b]. Administration of MIN at 60 mg/kg in TRA-treated rats caused significant changes in both IL1B (44.48; $P < 0.05$) and TNF (32.01; $P < 0.05$) levels in comparison to TRA in combination with MIN at 20 or 40 mg/kg [Figure 4a and b]. In contrast, MIN at 40 mg/kg in TRA-treated rats could not induce significant changes in TNF and IL1B level in comparison to TRA in combination with MIN at 20 or 60 mg/kg [Figure 4a and b]. MIN alone at the indicated doses could not change the levels of these proinflammatory biomarkers [Figure 4a and b].

Results of MIN effects against TRA-induced changes in apoptosis (BAX, BCL2, and CASP3) biomarkers

TRA (50 mg/kg) significantly elevated BAX (44.43; $P < 0.05$) and CASP3 (11.23; $P < 0.05$) levels, increased the BAX: BCL2 ratio (2.636; $P < 0.05$), and reduced the

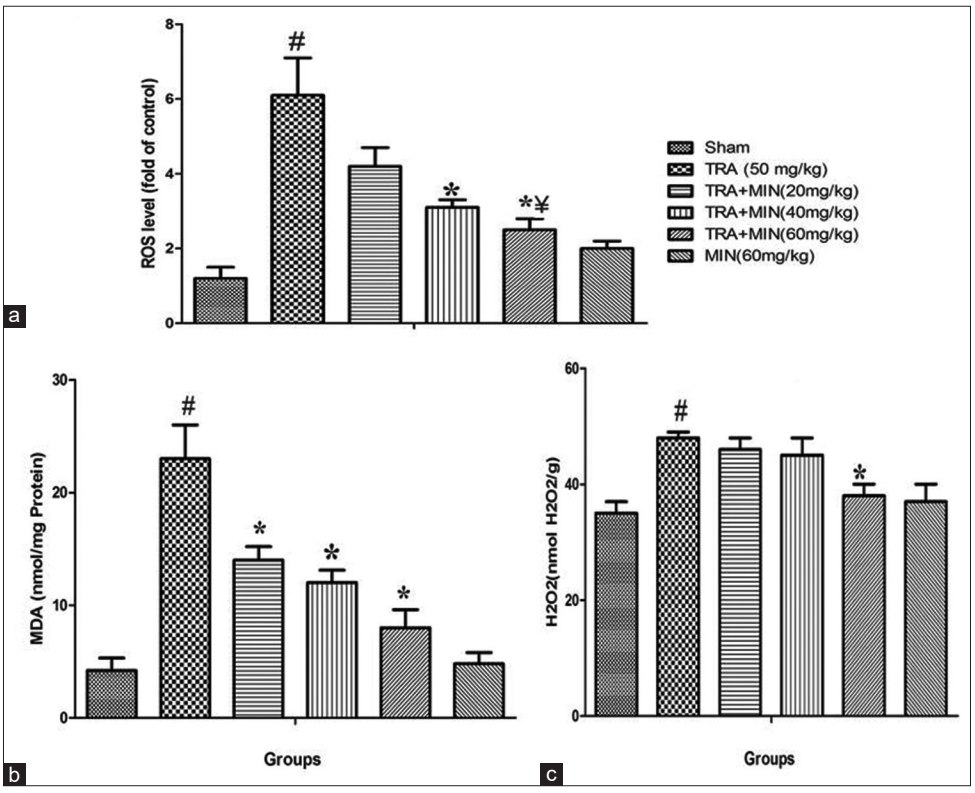

Figure 3: Oxidation effects of TRA and MIN. Alterations of ROS (a), MDA (b) and H_2O_2 (c) in hippocampus in sham group, and groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg of MIN in combination with tramadol. All data are expressed as Mean ± SEM ($n = 10$). # $P < 0.05$ vs. Sham group. * $P < 0.05$ vs 50 mg/kg of tramadol. ¥ $P < 0.05$ vs tramadol in combination with MIN (20 mg/kg). The number of animals in each group of rats was 10, each of which was evaluated once. Therefore, the number of replicates and experiments was one, and the average results of the 10 animals are shown above. TRA: tramadol, MIN: minocycline

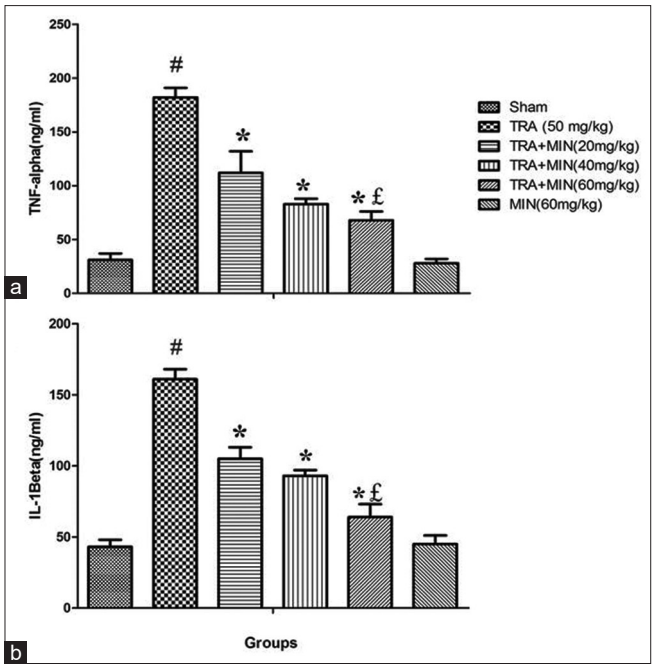

Figure 4: Changes in cytokines in response to TRA and MIN treatment. Alterations of the expression/level (ELISA) of TNF (a) and IL1B (b) in hippocampi in the sham group, and groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg of MIN in combination with tramadol. All data are expressed as Mean ± SEM (*n* = 10). # *P* < 0.05 vs. Sham group. * *P* < 0.05 vs 50 mg/kg of tramadol. £ *P* < 0.05 vs tramadol in combination with MIN (20 mg/kg) and or tramadol in combination with MIN (40 mg/kg). The number of animals in each group was 10 rat, each of which was evaluated once. Therefore, the number of replication and experiment was once, and the average results of their 10 animals are reported in the above table. TRA: tramadol, MIN: minocycline

BCL2 (30.65; $P < 0.05$) levels when compared to the sham group [Figure 5a-d]. MIN (20, 40, and 60 mg/kg), in comparison to TRA-treated rats, reduced BAX (44.43; $P < 0.05$) and CASP3 (11.23; $P < 0.05$) and elevated BCL2 (30.65; $P < 0.05$) levels when compared to the TRA-treated group [Figure 5a-c]. MIN (40 and 60 mg/kg), in comparison to TRA-treated rats, reduced the BAX: BCL2 ratio (2.636; $P < 0.05$) when compared to the TRA-treated group [Figure 5d]. Administration of MIN at 60 mg/kg in TRA-treated rats caused significant changes in BAX (44.43; $P < 0.05$) and BCL2 (30.65; $P < 0.05$) levels in comparison to TRA in combination with MIN at 20 mg/kg [Figure 5a and b]. In contrast, MIN at 40 mg/kg in TRA-treated rats could not induce significant changes in the BAX and BCL2 levels in comparison to TRA in combination with MIN at 20 or 60 mg/kg [Figure 5a and b]. There were no significant changes in the CASP3 level and BAX: BCL2 ratio between groups under treatment with TRA in combination with MIN at 20, 40, or 60 mg/kg [Figure 5c and d]. MIN alone could not change the levels of BAX, BCL2, CASP3, and BAX: BCL2 ratio [Figure 5a-d].

Results of MIN effects against TRA-induced changes in mitochondrial enzymatic activity

In comparison to the sham group, TRA administration meaningfully attenuated the activity of mitochondrial complexes I, II, III, and IV [5.230 for complex I, 10.59

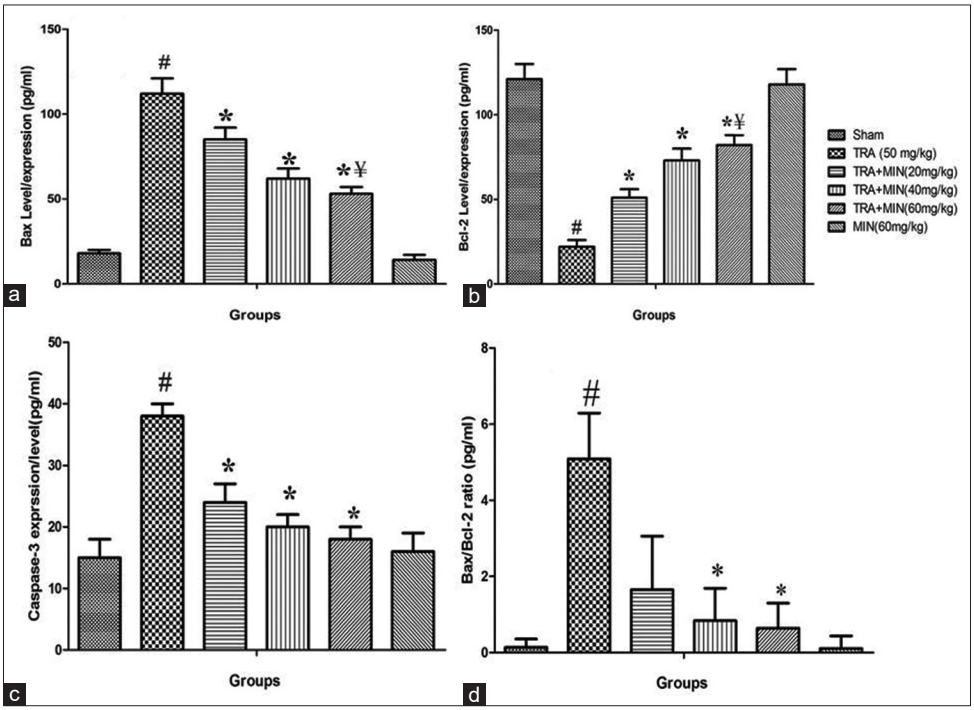

Figure 5: Changes in apoptotic proteins in response to TRA and MIN. Alterations of expression/levels (ELISA) of (a) BAX, (b) BCL2, (c) CASP3, and (d) BAX: BCL2 ratio in hippocampus in the sham group, and groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg of MIN in combination with tramadol. All data are expressed as Mean ± SEM (*n* = 10). # *P* < 0.05 vs. Sham group. * *P* < 0.05 vs 50 mg/kg of tramadol. ¥ *P* < 0.05 vs tramadol in combination with MIN (20 mg/kg). The number of animals in each group of rats was 10, each of which was evaluated once. Therefore, the number of replicates and experiments was one, and the average results of the 10 animals are reported above. TRA: tramadol, MIN: minocycline

for complex II, 7.663 for complex III, and 4.586 for complex IV; $P < 0.05$; Figure 6a-d] in the hippocampus. MIN at 60 mg/kg, in contrast, significantly elevated mitochondrial complexes I and IV [5.230 for complex I and 4.586 for complex IV; $P < 0.05$; Figure 6a and d] activity when compared to TRA-treated rats. MIN at 40 and 60 mg/kg also significantly elevated mitochondrial complexes II and III [10.59 for complex II and 7.663 for complex III; $P < 0.001$; Figure 6b and c] activities when compared to TRA-treated rats. Administration of MIN (60 mg/kg) in TRA-treated rats caused significant changes in mitochondrial complex I [5.230; $P < 0.05$; Figure 6a] in comparison to TRA in combination with MIN at 20 mg/kg. MIN at 40 mg/kg in TRA-treated rats could not induce significant changes in the activity of mitochondrial complex I compared to TRA in combination with MIN at 20 or 60 mg/kg [Figure 6a]. There were no significant changes in mitochondrial complexes II, III, and IV between groups under treatment with TRA in combination with MIN at 20, 40, or 60 mg/kg [Figure 6b–d]. Treatment with MIN alone (60 mg/kg) did not alter the enzymatic activity of any of these mitochondrial complexes [Figure 6a–d].

Results of MIN effects against TRA-induced changes in ATP level and mitochondrial membrane potential (MMP)

The JC-1 assay showed that TRA administration meaningfully reduced the mitochondrial membrane potential (18.75; $P < 0.05$) and decreased ATP levels (7.495; $P < 0.05$) when compared to the sham groupb [Figure 7a and b]. Conversely, MIN (40 and 60 mg/kg) significantly increased mitochondrial membrane potential (18.75; $P < 0.001$) and also MIN at 60 mg/kg significantly increased ATP levels (7.495; $P < 0.05$) when compared to TRA-treated rats [Figure 7a and 7b]. There were no significant changes in ATP level or mitochondrial membrane potential between groups under treatment with TRA in combination with MIN at 20, 40, or 60 mg/kg [Figure 7a and b]. Treatment with MIN alone (60 mg/kg) did not alter the mitochondrial membrane potential or ATP level [Figure 7a and b].

Results of MIN effects against TRA-induced histopathological changes

TRA (50 mg/kg) induced significant cell shrinkage and degeneration in granular cells of the DG and pyramidal cells of the CA1 area of the hippocampus. TRA also reduced cell number and density of the DG (18.10; $P < 0.05$) and CA1 (18.46; $P < 0.05$) when compared to the sham group [Figures 8 and 9] [Table 4]. MIN at 40 and 60 mg/kg in the DG area and MIN at 60 mg/kg in the CA1 areas meaningfully reduced TRA-induced cell degeneration and reduction of cell density (18.10; $P < 0.05$ for the DG and 18.46 for CA1; $P < 0.05$) in granular cells and pyramidal cells when compared to the TRA-only treated group [Figures 8 and 9] [Table 4].

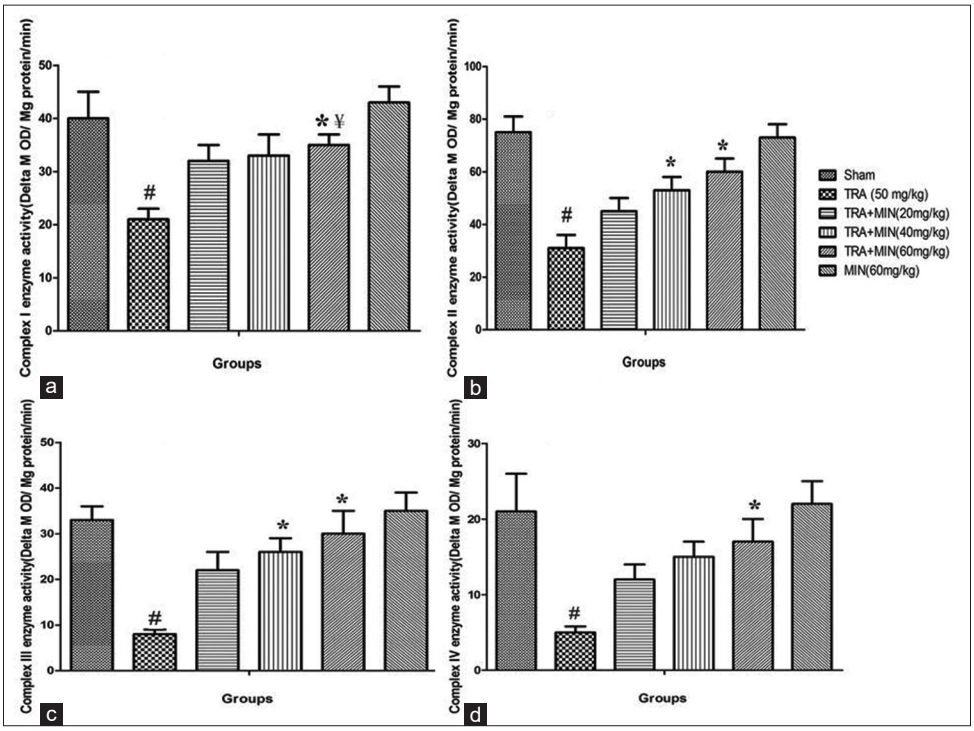

Figure 6: The effects of TRA and MIN treatment on the activities of mitochondrial complexes. Alterations of (a) complex I, (b) complex II, (c) complex III, and (d) and complex IV activity in hippocampus in sham group, and groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg of MIN in combination with tramadol. All data are expressed as Mean ± SEM (n = 10). # $P < 0.05$ vs. Sham group. * $P < 0.05$ vs 50 mg/kg of tramadol. ¥ $P < 0.05$ vs tramadol in combination with MIN (20 mg/kg). The number of animals in each group of rats was 10, each of which was evaluated once. Therefore, the number of replicates and experiments was one, and the average results of the 10 animals are reported above. TRA: tramadol, MIN: minocycline

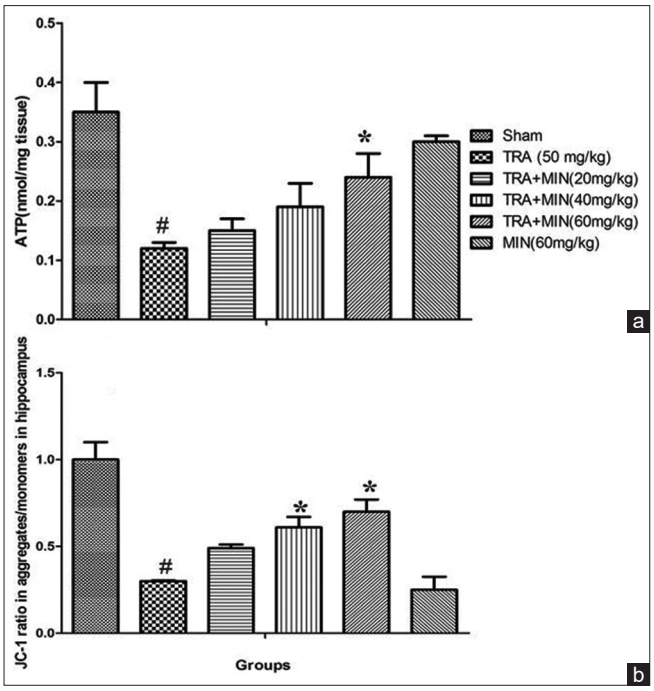

Figure 7: Change in mitochondrial function in response to TRA and MIN. (a) Alterations of adenosine triphosphate (ATP) and (b) mitochondrial membrane potential (MMP) in hippocampi in the sham group, and groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg of MIN in combination with tramadol. All data are expressed as Mean ± SEM (*n* = 10). # *P* < 0.05 vs. Sham group. * *P* < 0.05 vs 50 mg/kg of tramadol. The number of animals in each group of rats was 10, each of which was evaluated once. Therefore, the number of replicates and experiments was one, and the average results of the 10 animals are reported above. TRA: tramadol, MIN: minocycline

Table 4: Effects of various doses of minocycline on tramadol-induced DG and CA1 cell count in rat hippocampus

Groups	Number/ mm in DG	Number/ mm in CA1
Sham	671±28	356±31
Tramadol (50 mg/kg)	426±28[a]	123±17[a]
Tramadol (50 mg/kg) + MIN (20 mg/kg)	512±24	178±15
Tramadol (50 mg/kg) + MIN (40 mg/kg)	548±22[b]	205±16
Tramadol (50 mg/kg) + MIN (60 mg/kg)	608±18[b]	255±19[b]
MIN (60 mg/kg)	688±19	309±18

All data are given as Mean±SEM, (*n*=10). [a]Shows significant difference in relation to sham group (*P*<0.05). [b]Shows significant difference in relation to tramadol (*P*<0.05). MIN: Minocycline

There were no significant changes in quantitative and qualitative histological changes between groups under treatment with TRA in combination with MIN at 20, 40, or 60 mg/kg [Figures 8 and 9] [Table 4]. Treatment with MIN alone (60 mg/kg) did not alter the hippocampal histopathological status [Figures 8 and 9] [Table 4].

Discussion

The current study for the first time evaluated the neuroprotective role of MIN against TRA-promoted neurodegeneration. According to our data, MIN neuroprotective properties against TRA-induced neurotoxicity and neurodegeneration in the hippocampus occurred via reduction and inhibition of apoptosis, oxidative stress, mitochondrial dysfunction, and inflammation. The data indicate that MIN treatment could inhibit TRA-induced neurobehavioral changes such as anxiety and motor activity disturbances. In addition, according to the present study, MIN could attenuate TRA-induced oxidative stress and decrease MDA, H_2O_2, ROS, and GSSG levels; caused increases of SOD, GPX, and GSR activities; and elevated GSH and TAC levels. Based on our data, MIN caused a reduction of TRA-induced inflammation and reduced TNF and IL1B levels. MIN treatment could also inhibit TRA-induced apoptosis and inhibit TRA-induced BAX and CASP3 elevation and BCL2 level reduction. MIN treatment also reduced TRA-induced mitochondrial dysfunction. Furthermore, MIN could increase the activity of mitochondrial complexes I, II, III, and IV and resulted in a restoration of mitochondrial membrane potential and ATP level in TRA-treated rats. MIN also restored the histomorphological sequelae of TRA in both the CA1 and DG areas of the hippocampus.

The results of the present study indicated that 50 mg/kg of TRA caused decreases in the time spent in the central square, counts of central square entries, rearing number, and ambulation distances in the OFT. TRA is an analgesic remedy which has sequelae such as mood and behavioral disturbances, hallucination, and euphoria.[1,2] Current results confirm that TRA administration can cause changes in mood and anxiety-like behaviors. Previous data indicated that although acute administration of TRA can reduce anxiety and depressive-like behavior, its long-term administration or abuse can cause symptoms of anxiety and depression.[85,86] Some of these effects of TRA are likely explained due to its effects on the brain monoamine neurotransmitter; TRA abuse can cause depletion of serotonin, norepinephrine, GABA, and dopamine, and this phenomenon probably is responsible for its malicious effects on mood and motor activity behaviors.[15,87] In addition, chronic abuses of TRA can cause cognition impairment, confirming our data about its effects on behavioral performances.[88,89]

Conversely, our data indicated that MIN administration, at various doses, in TRA-dependent rats could cause reduction of TRA-induced motor-related behavior in the OFT. Furthermore, MIN administration by itself could not change the OFT behaviors. These data can be considered along with the properties of MIN, which indicated that MIN administration can reduce depressive and anxiety-like behavior in multiple types of neurobehavioral disturbances.[90-92] MIN is an antibiotic from the tetracycline group, which has protective neurobehavioral and neurochemical effects in neuronal cells.[19-21] In addition, this part of the study can be viewed along with the basic concept that showed that MIN has a powerful potential for modulation of serotonin, norepinephrine, GABA, and

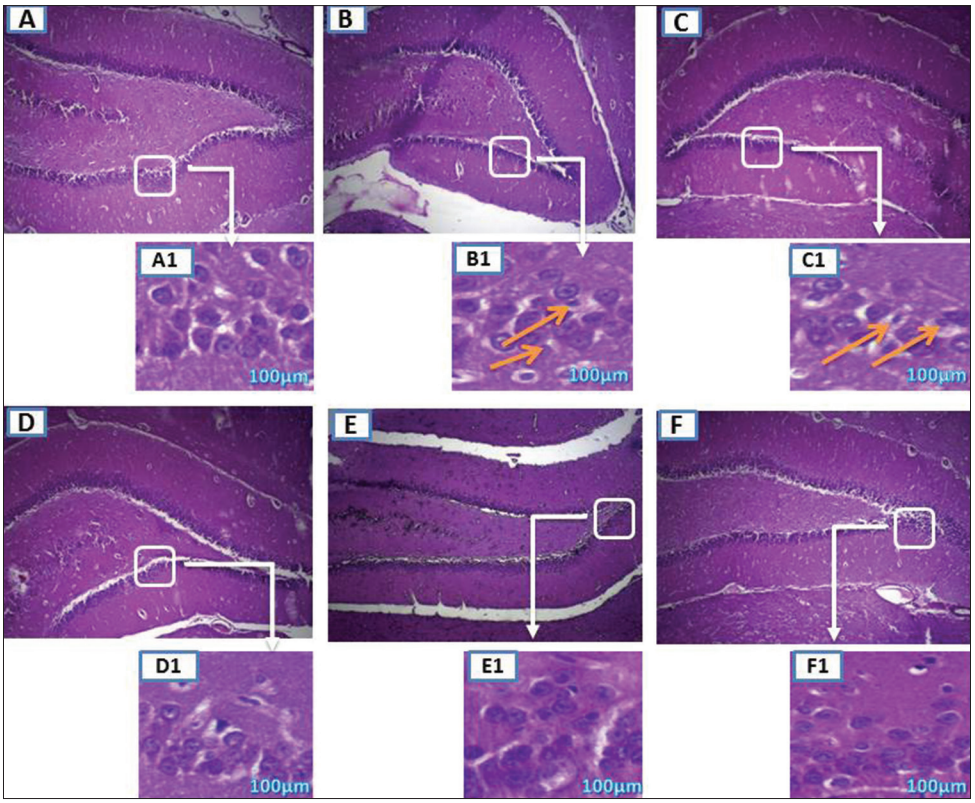

Figure 8: Histological changes in DG area of rat hippocampus. Low power images (magnification × 100) indicted as (A) sham, (B) group received 50 mg/kg of tramadol, (C, D and E) groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg of MIN, respectively, in combination with TRA and (F) group received MIN alone (60 mg/kg) mg/kg. Also, high power images (magnification × 400) are shown as A1 to F1. Orange arrows show damaged or dead cells (scale bar: 100 µm)

dopamine, and probably this mechanism can modulate behavioral disturbances.[93-95] The results of this part of our study also are consistent with previous experimental and clinical studies about the protective effects of MIN against motor activity disturbances.[24,27,33,92,96] These results have been so effective that in the treatment strategies for mood and motor activity management, minocycline is considered as prepositional therapy for these types of disorders.[24,27,33,92,96] Also, our data indicated that the role of MIN in inhibition of TRA-induced anxiety and motor activity disorder is dose dependent; the data from the current study indicated that administration of MIN improved the number of ambulation distances and time spent in the central square (20, 40, and 60 mg/kg), central square entries (40 and 60 mg/kg), and also rearing number (60 mg/kg). These results can be interpreted as indicating that high doses of minocycline can have antianxiety effects, but medium and low doses can stabilize mood and improve movement disorders. Of course, these results and the effects of different doses have not been directly evaluated, but several studies have indirectly confirmed the effect of different doses of this compound against multiple behavioral disorders.[50,92,97,98]

In spites of all the data about the effect of MIN for the management of mood and motor activity disorders, the protective effects of this agent for the management of TRA-induced neurobehavioral disturbances were not approved and the current study has therefore tried to evaluate this capability of MIN; it seems these results can be a clue as to the effectiveness of MIN against behavioral disorders caused by drug abuse.

The molecular aspects of our data indicated that TRA administration can cause activation of oxidative stress events, and this was characterized by elevation of MDA, H_2O_2, and ROS levels and reduction of activity of antioxidant enzymes such as GPX, GSR, and SOD2 along with decreases of TAC. These data are consistent with previous results, which demonstrated that a large part of the malicious and harmful properties of TRA in brain sections is due to the occurrence of oxidative stress.[15,99] According to previous work, TRA administration can cause activation of lipid peroxidation and also production of H_2O_2 and ROS.[11,15,99,100]

Along these lines, TRA administration can lower antioxidant activity and, probably via this mechanism, can induce neuronal cell damage and neurodegeneration.[14,15,99,101] These results confirm our data about TRA's potent effects in reduction of total antioxidant capacity and other antioxidant enzyme activity in the hippocampus. It seems that depletion of the antioxidant defense plays a part in TRA-induced neurodegeneration.[14,15,18,99,101,102] Based on general principles

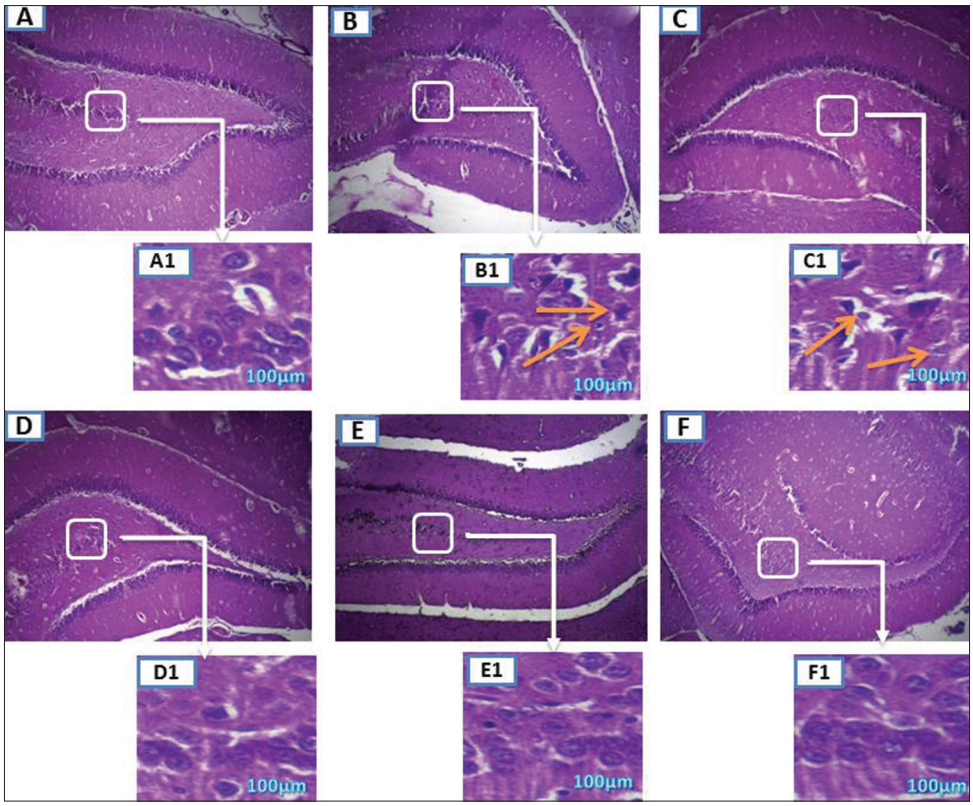

Figure 9: Histological changes in the CA1 area of rat hippocampus. Low power images (magnification × 100) indicted as (A) sham, (B) group received 50 mg/kg of tramadol, (C, D and E) groups treated with 50 mg/kg of TRA and 20, 40 and 60 mg/kg, respectively, of MIN in combination with TRA and (F) group received MIN alone (60 mg/kg) mg/kg. Also high power images (magnification × 400) are shown as A1-F1. Orange arrows show damaged or dead cells (scale bar: 100 µm)

of cell biology, there must be a balance between the production of free radicals, active species of oxygen and nitrogen, and activity of antioxidant agents and antioxidant enzymes, and any factor that can disrupt this balance provides the basis for cell dysfunction or death. We interpret our findings to indicate that TRA in the toxic and high doses used in the current study can inactivate antioxidant pathways. By inactivating antioxidant enzymes such as SOD, GPX, and GSR, it causes the increase of oxidant agents such as MDA, free radicals such as H_2O_2, and active species of oxygen and nitrogen such ROS. Furthermore, the present study showed that TRA can play its destructive role by reducing the total antioxidant capacity.[9,103] TAC is considered as the main biomarker for evaluation of the cumulative action of all antioxidants present within the cell.[104] Thus, in the present study, the reduction of TAC confirms the effects of tramadol on the reduction of SOD, GPX, and GSR enzymes and also GSH level.[9,103]

Thus, by evaluating changes of TAC, we can more confidently confirm the role of TRA in reducing antioxidants.[11,12,15] Along these lines, it can be speculated that TRA by reducing the SOD, GPX, and GSR enzymes and also GSH level and overall antioxidant capacity provides the opportunity for the activation of oxidant agents such as MDA, H_2O_2, and ROS and thus exerts its neurodegenerative effects.[12,15]

In contrast, administration of 20, 40, and 60 mg/kg of MIN decreased the TRA-induced elevation in H_2O_2, MDA, and ROS levels and also inhibited the reduction of antioxidant enzymes such as GPX, GSR, and SOD activity as well as TAC. Also, MIN alone could not change any of these antioxidant and oxidative biomarkers. These data are consistent with previous data from our lab and others, which demonstrates MIN's neuroprotective properties in the reduction or inhibition of lipid peroxidation and the formation of reactive oxygen species and free radicals.[33,38,105] Previous work also indicated that MIN's neuroprotective effectiveness was mediated via reversing or restoring the activity of GPX, GSR, and SOD activity in neuronal cells.[31,38,95,106,107] The effects of MIN in the activation of TAC and modulation of the redox system in neurodegenerative events were demonstrated previously.[31,107,108] As mentioned above, the TAC level provides an estimation of the overall antioxidant components in a cell.[104] Thus, increasing the level of TAC with multiple doses of MIN can enhance the antioxidant effects of this compound as well as its positive effects on GPX, GSR, and SOD activity. Based on previous studies, the TAC level provides a general view of the antioxidant activity of each neuroprotective compound. Therefore, the results of the present study can be interpreted to mean that MIN can increase the total antioxidant level/capacity of

the cell by increasing antioxidant enzyme (GPX, GSR, and SOD) activity, and through this mechanism, it can reduce oxidant agents such as H_2O_2, MDA, and ROS.[32,33,109,110] In support of these results, a significant relationship between the activity of GPX, GSR, and SOD and the TAC level is observed.[104,109,111]

Thus, it can be considered that a large part of the neuroprotective properties of MIN with regard to neurodegenerative disease or disorder is due to the inhibition of oxidative stress and potentiation of antioxidant defenses and thus activation of TAC.[31,38,95,106,107] In other words, increasing TAC levels is the key parameter to the neuroprotective role of MIN against oxidative damage and injuries, which are induced by TRA.[31,49] In spite of all these direct and indirect lines of evidence regarding the role of MIN in the inhibition of oxidative stress and modulation of the redox system, the protective effects of this neuroprotective antibiotic in the management of TRA-induced oxidative stress in the hippocampus were not approved. Based on the current study, the indicated outcomes suggest the efficiency of MIN against neurochemical disorders caused by drug abuse such as with TRA.

Another debatable and important issue is that according to our data, MIN administration with 60 mg/kg significantly increased SOD, GPX, and GSR and also with doses of 40 and 60 mg/kg caused increases of TAC in TRA-treated rats. Furthermore, MIN with doses of 20, 40, and 60 mg/kg caused reduction of H_2O_2, MDA, and ROS levels. From these results, two relatively complete conclusions can be made. First, the antioxidant effects of MIN are dose dependents and occur more in medium and high doses. Second, some part of MIN's effects against oxidative biomarkers such as H_2O_2, MDA, and ROS are not related to the effects of the mentioned antioxidants and the TAC system because MIN, even at low doses, can probably inhibit H_2O_2, MDA, and ROS levels, which indicates the role and involvement of various miscellaneous protective pathways in the action of MIN antioxidant against TRA-induced oxidative events,[21,31,112] confirming the results of previous studies.[31,35]

Our results also indicate that administration of TRA could reduce the level of the protective form of glutathione (GSH) and also caused increases in the level of the harmful form (GSSG) in the hippocampus. In contrast, MIN at multiple doses could decrease GSSG levels and also increased GSH content in TRA-dependent rats, whereas the administration of 60 mg/kg of minocycline alone could not affect significant changes in GSH and GSSG. Based on previous results, TRA, via increases of GSSG and reduction of GSH, can trigger neuronal cell death.[101,113] In addition, TRA neurodegenerative and neurotoxic effects are mediated via disturbances of the glutathione cycle.[4] Conversely, MIN, probably via attenuation of GSSG and elevation of GSH, can protect neuronal cells from degenerative signals and inhibit neurodegenerative events.[114-116] Thus, it can be considered that MIN by triggering GSR activity can enhance the GSSG-to-GSH conversion and by this effect prevents TRA-triggered disturbances in the glutathione cycle.[114-116] According to our study, MIN effects on GSH and GSSG are dose dependent; this agent in all doses decreased the GSSG level and at doses of 40 and 60 mg/kg increased the GSH level. This can be interpreted to mean that minocycline in low to high doses can have antioxidant effects and reduce the destructive forms of glutathione (GSSG), whereas in medium to high doses, it can exert its neuroprotective role and cause the generation of useful forms of glutathione (GSH).[95,114,117] Several studies have confirmed the role of low doses of minocycline as an antioxidant and high doses of minocycline as a neuroprotective agent, which is consistent with the results of this part of our study.[118,119] This result can give new insight about the role of MIN against drug abuse-induced glutathione dysfunction and can suggest new mechanisms for MIN neuroprotective properties.[114-116]

Other parts of our study indicated that administration of TRA significantly increased the level of proinflammatory cytokines such as IL1B and TNF, whereas MIN administration could inhibit TRA-induced neuro-inflammation; however, MIN alone could not change the TNF or IL1B levels. These data are consistent with previous results which show that TRA can induce inflammation in neuronal cells and causes activation of the cytokine pathway and by this mechanism initiates neurodegeneration.[18,120,121] A similar study indicated that TRA administration can cause the elevation of serum proinflammatory cytokines (TNF and IL6) and overexpression of NFKB/NF-κB, NOS2/iNOS, TNF, and IL6 in rat brains.[15] In contrast, findings about the anti-inflammatory and immunomodulatory effects of MIN demonstrated that this neuroprotective agent causes the attenuation of cytokine formation/production and by this mechanism defends neuronal cells from neurodegenerative events.[29,122,123] Along these lines, MIN administration can inhibit alcohol-induced neuro-inflammation.[124] Another study revealed that levels of GFAP (glial fibrillary acidic protein), AIF1/IBA1 (allograft inflammatory factor 1), and IL6 as inflammatory biomarkers are significantly reduced in a neurodegenerative mouse model after MIN administration.[125] Also, the dose-dependent role of minocycline as an anti-inflammatory agent in reducing inflammatory events with low or high doses has been the focus of previous studies,[126,127] and our study also confirms this effect in the inflammation induction model caused by tramadol. In other words, it has been determined that minocycline has an anti-inflammatory role even in low doses, and in high doses, it will have both anti-inflammatory and anti-oxidant effects.[23,112] Although some of these MIN effects as a neuro-inflammatory inhibitor or as an inflammatory modulator were approved previously, its protective effects as a modulator of neuro-inflammation in TRA-addicted subjects were not indicated; thus, the current study is important in demonstrating the effects

of MIN against TRA-induced neuro-inflammation in the hippocampus and can clarify the neurochemical consequences of both MIN and TRA administration.

Our results indicate that TRA significantly attenuates the enzymatic activity of mitochondrial complexes I, II, III, and IV in the mitochondrial respiratory chain in the hippocampus. Indeed, previous studies suggest that dysfunction in mitochondrial respiratory enzymes are involved in TRA-induced neurodegeneration and neurotoxicity.[8,128] Our results also indicate that TRA administration dramatically decreased mitochondrial membrane potential in hippocampus tissue; this result confirms the effects of TRA on mitochondrial respiratory chain enzyme activities and also indicates that the TRA effect on mitochondrial dysfunction is probably one of the main causes of TRA-induced neurodegeneration.[8,128] In addition, it seems that the detrimental role of TRA on mitochondrial function can cause neuro-inflammation, oxidative stress, and apoptosis.[113,129-132] These effects of TRA on mitochondrial respiratory enzymes and mitochondrial membrane potential can help explain our data, which show that TRA administration caused dysfunction in ATP synthesis, which led to reduction of the ATP level. The effects of TRA on mitochondrial respiratory chain enzymes can further lead to impairment of the mitochondrial membrane potential and reduction of ATP level, which consequently cause neurodegeneration.[113,129-134] As discussed above, hippocampal dysfunction of mitochondria in response to TRA was not previously evaluated, but indirect evidence indicated that mitochondria are responsible for the neuro-pathological events caused by TRA administration.[113,129-134] Thus, it can be suggested that mitochondrial dysfunction can be one of the key factors in TRA-promoted neurodegeneration.

Our data also indicated that MIN administration at the indicated doses could cause activation of mitochondrial complexes I, II, III, and IV in the hippocampus of TRA-treated animals. This is consistent with studies which show the efficacy of MIN to improve mitochondrial function.[42,135-137] MIN-dependent cytoprotection, including neuroprotection, is mediated via activation of mitochondrial function.[42,138] We found that MIN administration could restore mitochondrial membrane potential and also resulted in elevation of the ATP level in the hippocampus of TRA-dependent rats. Furthermore, MIN alone did not change the mitochondrial function parameters. Previous studies indicated that minocycline plays a critical role in mitochondrial biogenesis and by regulation of mitochondrial homeostasis inhibits occurrences of apoptosis, oxidative stress, and inflammation and thus manages neurodegenerative events.[39,95,109,137,139] However, the participation of MIN in other mitochondrial activities is still unclear and needs further study, although mitochondria functions appear to be critical for MIN's neuroprotective effects.[136,139,140] Our study suggests that MIN activation of mitochondrial complexes I, II, III, IV and the ATP synthase and restoration of the membrane potential could reduce neuro-inflammation and increase anti-oxidative defenses; these effects in turn can reduce the formation of free radicals, which leads to inhibition of neuronal cell death and neurodegeneration in the hippocampal tissue in TRA-addicted rats. Also, these properties of MIN on the modulation of nonchemical parameters probably modulate TRA-induced neurobehavioral disturbances such as mood and motor activity disease. In spite of all the data about the MIN effects as a mitochondrial function modulator, its neuroprotective effects in TRA-induced mitochondrial dysfunction were not indicated; thus, our results expand our present understanding with regard to the neuroprotective effects of MIN in response to certain neurotoxic agents such as TRA and propose the potential clinical advantages of MIN to restore mitochondrial function in neurodegenerative diseases and disorders, especially drug abuse-induced neurodegeneration.[141,142]

Another point to be discussed here is that based on the results of the present study, MIN at 60 mg/kg significantly elevated mitochondrial complexes I and IV activities and also in doses of 40 and 60 mg/kg elevated mitochondrial complexes II and III activities. Also, MIN at all mentioned doses increased the ATP level and enhanced mitochondrial membrane potential. It seems that the results of this part of the study further define the role of dose-dependent effects of minocycline in the control of mitochondrial function.[136,140,143] Although a direct study that evaluates the role of multiple minocycline doses on mitochondrial function has not been done, according to the results of the present study, and also previous indirect results, it can be concluded that minocycline in middle and high doses will play a very useful role in strengthening the function of mitochondrial enzymes, increasing ATP production and activating the potential of the mitochondrial membrane.[41,141,143]

According to the results presented here, TRA caused an increase in important apoptotic factors, BAX and CASP3, and also reduced the BCL2 level as an anti-apoptotic parameter. TRA-induced neurotoxicity appears to be mediated by occurrences of apoptosis, and this apoptosis is one of the malicious outcomes of TRA that result in dysregulation of mitochondrial function, oxidative stress and inflammation.[15,18,144] Thus, it can be suggested that TRA-promoted apoptosis is one of the main players of neurodegenerative events which occurs during TRA abuses or chronic administration.[15,18,144] The current study also demonstrated that MIN administration could reduce BAX and CASP3 and also caused elevation of the BCL2 level. Furthermore, MIN alone could not effect significant changes with regard to these apoptosis markers. Similar work indicates that MIN inhibits BAX activation and CASP3 cleavage, and also inhibits nuclear condensation during occurrences of some neurodegenerative disorders and diseases.[39,123,145] However, the effects of MIN in the management of apoptosis and cell death in TRA-administered subjects was not clarified; thus, our results enhance the knowledge about

the mechanism of MIN-mediated neuroprotection against neurodegenerative diseases and disorders such as TRA-induced neurodegeneration. Also, based on the present study MIN effects on apoptosis biomarkers and its anti-apoptotic function are dose dependent. These results can be interpreted as meaning that low to high doses of minocycline can have anti-cell death effects. These results and the effects of different doses of MIN on apoptosis have not been directly evaluated previously, but several studies have indirectly confirmed the effect of different doses of this compound against multiple types of cell death signaling pathways such as apoptosis.[21,146,147]

For further confirmation and validation of MIN's cellular protection against TRA-induced neurotoxicity and neuronal cell death, we carried out cellular staining and assessed the quantitative and qualitative alterations of granular cells in the DG and pyramidal cell in the CA1 regions. Our data showed that TRA could cause considerable decreases of cellular count and quality of cells in both granular (in the DG regions) and pyramidal (in the CA1 regions) cells of the hippocampus. Conversely, MIN at 40 and 60 mg/kg in the DG area and MIN at 60 mg/kg in CA1 areas dramatically reduced the TRA-induced hippocampal cell damage in both quantitative and qualitative aspects. These data are consistent with the effects of MIN against TRA-induced apoptosis and cell death[15,18,144] and can be interpreted with the dose-dependent effects of minocycline as discussed above. In other words, minocycline in medium to high doses can play its anti-oxidative stress, anti-inflammatory and anti-cell death role and exert its positive effects on pathological features.[148,149] TRA effects can be interpreted in light of the conclusion that this agent, possibly via activation of mitochondrial dysfunction, can cause initiation or triggering of neuro-inflammation, neuronal oxidative-stress, and apoptosis which lead to neuronal cell loss and neuronal damage.[11,150] In contrast, MIN inhibits these TRA-induced processes and can promote survival despite the cell damage that results from these TRA-induced neurochemical changes. Previous data have also shown that MIN inhibits degeneration of hippocampal cells, but the exact signaling pathway in this manner remains unknown.[151,152] In spite of the effects of MIN in the management of hippocampal cell death and apoptosis, its effects in TRA-addicted subjects was not yet evaluated; thus, the current results extend our understanding and knowledge about the role of MIN and its mechanism as a neuroprotective agent against TRA-induced neurodegeneration or neurotoxicity.

Conclusions

The results of the present study suggest, for the first time, that MIN acts as potent neuroprotective agent against TRA-induced neurodegenerative sequelae such as mitochondrial dysfunction, neuro-inflammation, neuronal oxidative stress, disturbances in cell defense mechanisms, or apoptosis [Figure 10]. The important point is that it cannot be claimed that low doses of

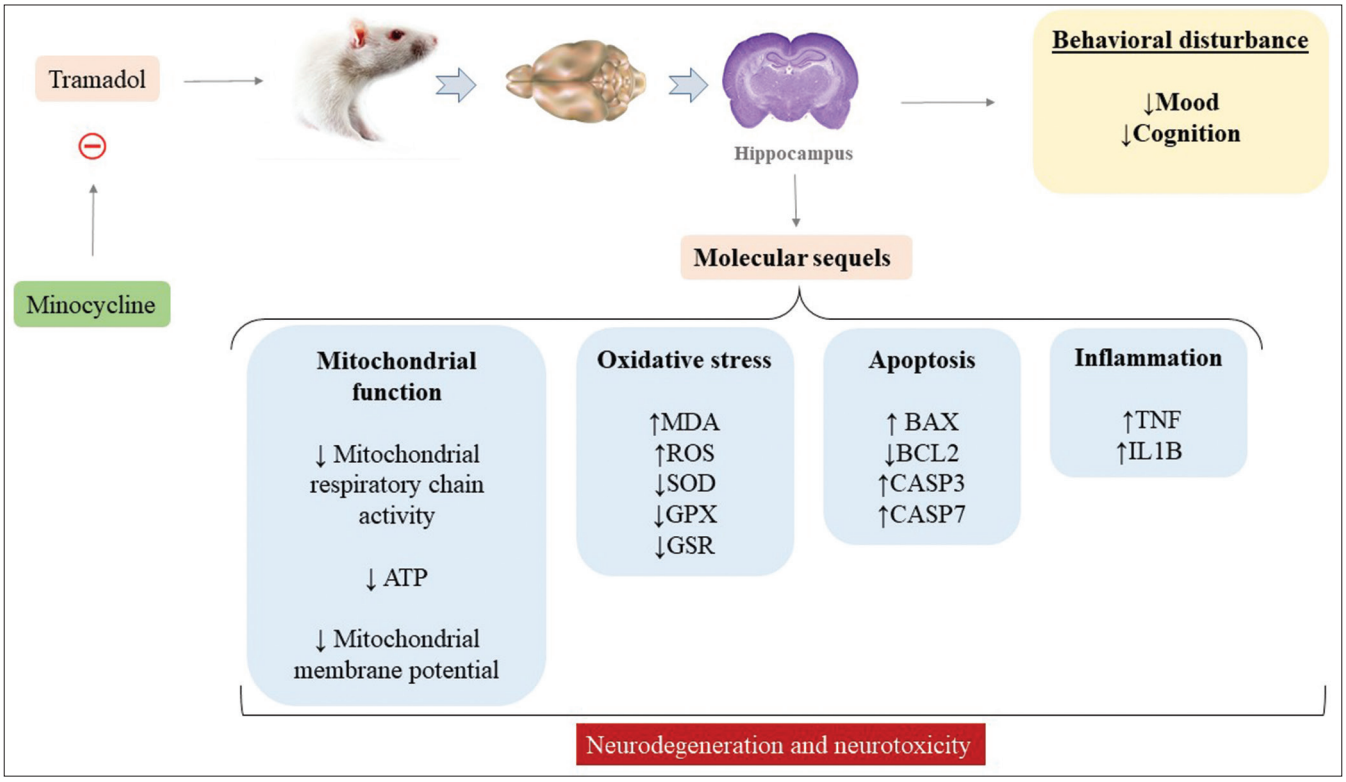

Figure 10: Minocycline restore TRA-induced mood and motor activity, oxidative stress, inflammation, apoptosis mitochondrial respiratory enzyme dysfunction, mitochondrial membrane potential dysfunction, and ATP synthesis inhibition

minocycline necessarily have a neuroprotective role, but based on the present results, moderate to high doses of this agent can definitely play a neuroprotective role by reducing hallmarks of neurodegenerative events. Although all these results offer a new perspective and novel visions into the explanation of mechanisms involved in neurodegeneration induced by TRA and the neuroprotection of MIN, it appears that further evaluation for the specific neurochemical, molecular, and cellular aspects of the mentioned hypotheses and claims need to be carried out especially in human subjects.

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Abbreviations

ATP: adenosine triphosphate; **BSA:** bovine serum albumin; **CA1:** Cornu Ammonis; **DG:** dentate gyrus; **EGTA:** ethylene glycol tetra-acetic acid; **ELISA:** enzyme-linked immunosorbent assay; **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase; **GFAP:** glial fibrillary acidic protein; **GPX:** glutathione peroxidase; **GSR/GR:** glutathione-disulfide reductase; **GSSG:** oxidized glutathione; **GSH:** reduced glutathione; **H_2O_2:** hydrogen peroxide; **H and E:** hematoxylin and eosin; **HPLC:** high-performance liquid chromatography; **IL1B/IL-1β:** interleukin 1 beta; **i.p.:** intraperitoneally; **KOH**: potassium hydroxide, **MDA:** malondialdehyde; **MAPK/JNK:** mitogen-activated protein kinase; **MIN:** minocycline; **$MgCl_2$:** magnesium chloride; **MMP:** mitochondrial membrane potential; **NADPH:** nicotinamide adenine dinucleotide phosphate; **OD:** optical density, **OFT:** open field test; **PC:** personal computer; **PDH:** pyruvate dehydrogenase; **SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **SOD:** superoxide dismutase; **ROS:** reactive oxygen species; **TAC:** total antioxidant capacity; **TBA**: thiobarbituric acid; **TNF/TNF-α:** tumor necrosis factor; **TRA:** tramadol.

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Conflicts of interest

There are no conflicts of interest.

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