



Minocycline Protects Against Lipopolysaccharide-Induced Cognitive Impairment and Oxidative Stress: Possible Role of the CREB-BDNF Signaling Pathway

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Abstract

The oxidative stress-induced dysregulation of the cyclic AMP response element-binding protein- brain-derived neurotrophic factor (CREB-BDNF) cascade has been linked to cognitive impairment in several studies. This study aimed to investigate the effect of minocycline on the levels of oxidative stress markers, CREB, and BDNF in lipopolysaccharide (LPS)-induced cognitive impairment. Fifty adult male Sprague Dawley rats were divided randomly into five groups. Group 1 was an untreated control group. Groups 2, 3, 4 and 5 were treated concurrently with LPS (5 mg/kg, i.p) once on day 5 and normal saline (0.7 ml/rat, i.p) or minocycline (25 and 50 mg/kg, i.p) or memantine (10 mg/kg, i.p) once daily from day 1 until day 14, respectively. From day 15 to day 22 of the experiment, Morris Water Maze (MWM) was used to evaluate learning and reference memory in rats. The levels of protein carbonyl (PCO), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) were determined by enzyme-linked immunosorbent assay (ELISA). CREB and BDNF expression and density were measured by immunohistochemistry and western blot analysis, respectively. LPS administration significantly increased escape latency to the hidden platform with decreased travelled distance, swimming speed, target crossings and time spent in the target quadrant. Besides, the hippocampal tissue of LPS rats showed increased levels of PCO and MDA, decreased levels of CAT and SOD, and reduced expression and density of BDNF and CREB. Treatment with minocycline reversed these effects in a dose-dependent manner, comparable to the effects of memantine. Both doses of minocycline treatment protect against LPS-induced cognitive impairment by reducing oxidative stress and upregulating the CREB-BDNF signalling pathway in the rat hippocampus.

Keywords Neuroinflammation · Learning and reference memory · Minocycline · Oxidative stress · Memantine

Introduction

Lipopolysaccharide is an endotoxin derived from the cell wall of Gram-negative bacteria called *E-coli*. Its cerebroventricular and intraperitoneal injection commonly induce neuroinflammation [1]. As a result, it has been widely employed

in mice and rats as a model of neuroinflammation [2]. LPS binds to the toll-like receptor-4 (TLR-4) found on microglia and astrocytes and forms the LPS/TLR-4 complex. The complex triggers the release of proinflammatory cytokines and chemokines, which further activates the TLR-4/NF-kB inflammatory pathway, leading to the release of reactive oxygen and nitrogen species (ROS/RNS) and depletion of antioxidant enzymes, resulting in oxidative imbalance and activation of the oxidative stress pathway [3].

The activation of the oxidative stress pathway leads to amyloid accumulation and neurofibrillary tangle formation [4]. Amyloid deposition lowers phosphorylated cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), which in turn lowers brain-derived neurotrophic factor (BDNF), leading to dysregulation of the CREB-BDNF synaptic signaling pathway, neuron loss, and cognitive impairment [2, 4, 5]. CREB is a member of

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the leucine zipper transcription factor family, and it can be phosphorylated by a variety of upstream signaling pathways [6]. These pathways are disrupted in cognitively impaired patients, and they have also been shown to be altered by anti-cognitive medication [7, 8]. CREB regulates gene expression such as BDNF, which is important for synaptic and neuronal survival [9–11]. The involvement of the CREB-BDNF pathway in conferring neuroprotection and cognitive functions leads us to study the role of the CREB-BDNF signalling pathway in mediating neuroprotection against LPS-induced oxidative stress and cognitive disturbances in minocycline-treated rats.

Minocycline is a second-generation semisynthetic tetracycline antibiotic that has been used to treat Gram-negative bacterial infection for decades. As a result of its high solubility and small size (495 KDa) that allow its passage through the blood–brain barrier (BBB) and accumulation in the central nervous system (CNS). Many studies are conducted to assess its potential neuroprotective effects on neuroinflammation. It has shown neuroprotective properties in several neurodegenerative diseases in experimental and clinical studies and these beneficial effects were attributed to its ability to deactivate oxidative stress and upregulate BDNF-CREB pathways [12–14]. This study is significant because it sheds light on the antioxidant properties of minocycline upregulated BDNF-CREB signalling pathway in the hippocampus of LPS injected rats, improving learning and reference memory performance.

Material and Methods

Animals

Fifty Sprague Dawley male rats were obtained from the animal research and service centre (ARASC) unit in Universiti Sains Malaysia (USM). The rats were 3 months old and their mean weighed was 270 ± 20 g. Prior to the experiment, they were housed individually in polypropylene cages ($32 \times 24 \times 16$ cm) to habituate to a new environment for 1 week with free access to water and standard pellet (Altromin, Germany). The rats were exposed to 12 h light/dark cycle in a well-ventilated laboratory environment at relative humidity $50 \pm 5\%$ and room temperature 23°C . The experimental protocols were performed according to international guidelines for laboratory animal use and care, and approved by the Research and Ethics Committee of USM (USM/IACUC/2018/ (942) (114)).

Experimental Design

The experimental timeline is summarized in Fig. 1. The rats were allowed to acclimatize to the new environment for 7 days. If no abnormalities were found during the acclimatization period, the rats were randomly divided into five groups ($n = 10/\text{group}$). The groups were as follows; (i) untreated control, (ii) LPS (5 mg/kg; treated with normal saline (0.7 ml/rat, i.p), (iii) LPS treated with minocycline (25 mg/kg; [15]), (iv) LPS treated with minocycline (50 mg/kg; [15]) and (v) LPS treated with memantine (10 mg/kg; [16]) once daily for 14 days. Both minocycline and memantine were obtained from Twinbrook Pkwy, Rockville, MD, USA. LPS was obtained from Sigma-Aldrich, St. Louis,

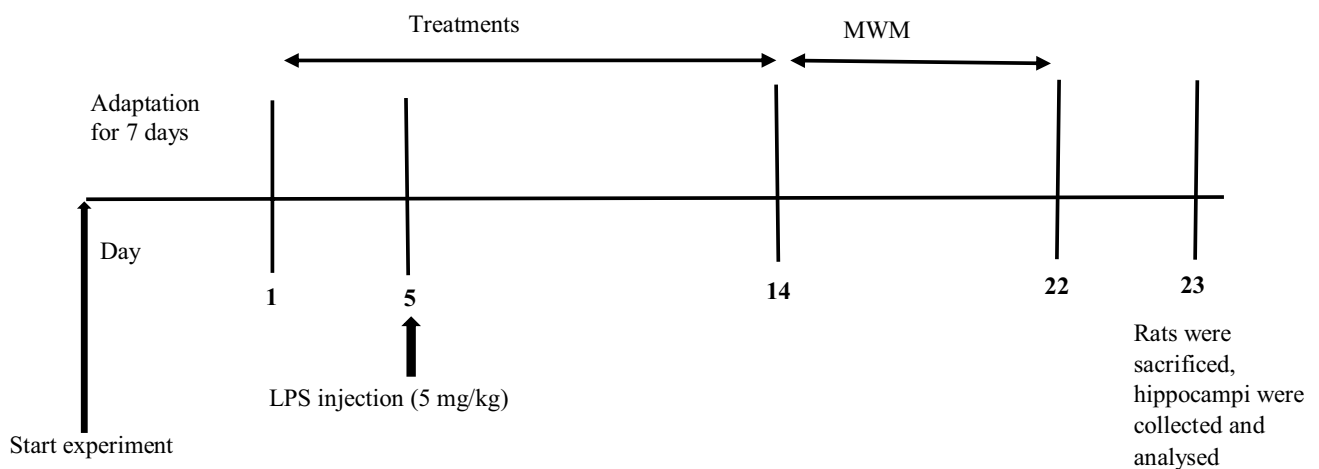


Fig. 1 Experimental timeline

MO, USA and was injected intraperitoneally on day 5 of the experiment.

Morris Water Maze Task (MWM)

Learning and reference memory performance was evaluated in the MWM test using the place navigation test and probe trial, respectively. The MWM apparatus was a black coloured circular tank (150 cm in diameter and 60 cm in height) filled with water at a temperature of 23 ± 1 °C to a depth of 42 cm. Visual-spatial cues (coloured pictures) were placed on the water pool within plain sight of the rats and in the same position during all trials and to eliminate olfactory cues, the water in the pool was stirred after each trial. The MWM test was carried out from 8 a.m. until 3 p.m. on day 15 to day 22 of the experiment. The test was divided into three phases: (i) habituation phase (2 days), (ii) acquisition phase (5 days) and (iii) probe trial (1 day). The rats were taken to a behavioural room for 30 min to acclimate before the test and then returned to their cage after each trial. During the acquisition phase, rats were placed in the pool facing the wall at each of the four different beginning places for 5 days and then allowed to swim freely. Each day, the beginning positions were rearranged in a different order. If the rat located the platform within 120 s, it was allowed to stay on it for another 10 s. If the rat could not find the platform within 120 s, it was placed on it for 10 s. Each trial ended when the rat reached the platform or after 120 s had passed. The escape latency was defined as the time it took to find the concealed platform and stay on it for at least 3 s. During the experiment, each rat's activity was recorded using a digital camera mounted directly above the pool centre and connected to a recording computerised system using a Panlab SMART video tracking system (USA system version 3.0). The escape latency, distance travelled, swimming speed, target crossings and time spent in the target quadrant were recorded for each rat.

Immunohistochemistry

The rats were deeply anaesthetised with an overdose of sodium pentobarbital (60 mg/kg) and the perfusion-fixation was done. The rats were transcardially perfused with phosphate buffer saline (PBS at pH = 7.4) followed by perfusion with 4% paraformaldehyde (PFA at pH = 7.4) in 0.1 M phosphate buffer and then sacrificed. The hippocampi were extracted quickly and preserved in 10% formalin at room temperature. The paraffinised sections were immersed in xylene solutions I and II for 2 min each. The slides then were hydrated in ethanol (100%, 95%, 80% and 70%) for 2 min each. The slides were immersed inside a pressure cooker containing Tris EDTA buffer at 90 °C for up to 3 min for Antigen (Ag) retrieval.

The slides were cooled down in dH₂O for 2 min. The slides were positioned in sequenza immunostainer and three drops of hydrogen peroxide (H₂O₂) blocking agent were added into the slides and the slides were incubated at room temperature for 5 min. The slides were washed with dH₂O and left for 2 min. The slides were immersed in Tris buffer saline-Tween 20 (TBST) buffer two times for 5 min each. Primary antibodies derived from mouse for BDNF and CREB (Santa Cruz, USA with dilution 1:200 and 1:100) were added into sections and slides were incubated 24 h at -4 °C. The slides were immersed in TBST twice for 5 min each. Secondary antibodies anti-mouse BDNF and CREB (Santa Cruz, USA with dilution 1:500 and 1:200) were added into sections and slides were incubated at room temperature for 1 h.

The slides were rinsed in TBST buffer twice for 5 min each and then flooded in 3, 3'-Diaminobenzidine (DAB) at room temperature for 5–10 min. dH₂O was added into slides and left for 2 min and then the slides were dipped for 5 s in haematoxylin solution. The slides were dehydrated in ethanol (70%, 80%, 95% & 100%) for 2 min each. The slides were immersed in xylene I and II solutions for 2 min each, mounted using Cyto Seal and coverslipped.

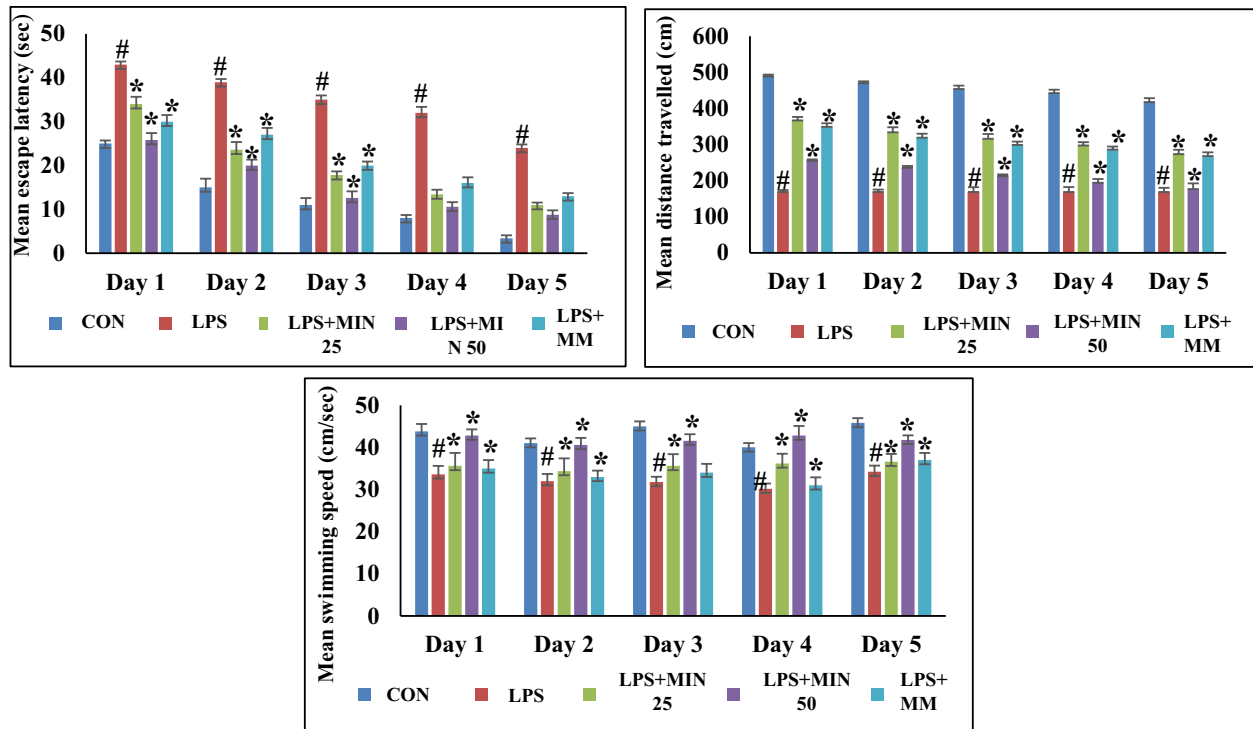
The hippocampal sections that expressed BDNF and CREB positive neurons were imaged at 40× and 100× magnifications via an image analyser attached to a light microscope (Olympus Corporation, Japan). Counting of BDNF and CREB positive neurons was performed using Image-J software (<http://imagej.nih.gov/ij>). The counting was carried out within 100×100 mm grid that was placed in the specific hippocampal tissue using three random sections for each rat. Only clearly visible brown DAB colour cells were regarded as BDNF and CREB positive neurons.

Western Blotting

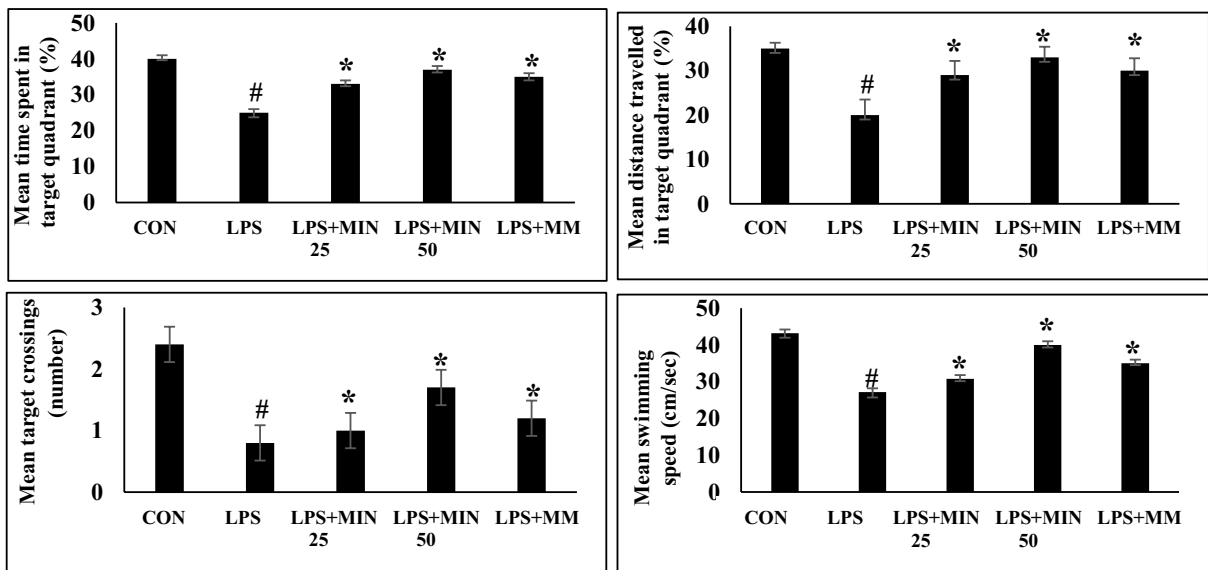
Extraction of BDNF and CREB proteins from hippocampal tissue was performed using radioimmunoprecipitation assay (RIPA) buffer. The obtained homogenates were centrifuged at 12,000 g at -4 °C for 15 min (Hettich Zentrifugen, Germany). Quantification of protein in each supernatant was done using a Bradford protein assay kit (Bio-Rad, USA). Denaturation of protein at a concentration of 60 µg was performed with sodium dodecyl sulfate (SDS) sample buffer and separation of this protein using 10% SDS-polyacrylamide gel electrophoresis (PAGE) was carried out.

Transferring of protein to a polyvinylidene fluoride (PVDF) microporous membrane (Membrane Solutions, USA) was carried out that was followed by blocking of protein using skim milk (5%) at room temperature for 1 h. Incubation of membranes with primary antibody, namely mouse anti-BDNF and anti-CREB (Santa Cruz USA with dilution 1:500 each) at -4 °C overnight. The membranes then were

(A)



(B)



(C)

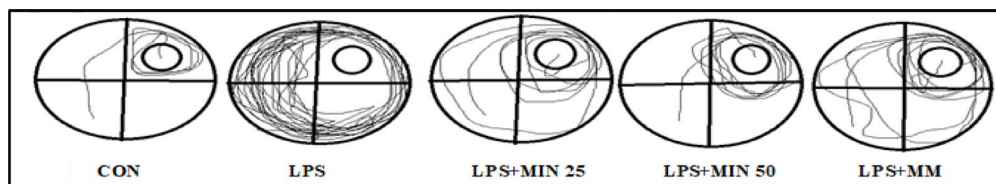


Fig. 2 **A** Mean of escape latency (1), distance travelled (2), swimming speed (3) between the trial days for each of the five experimental groups during the acquisition phase. **B** Mean of time spent (1), distance travelled (2), target crossings (3), and swimming speed (4) in the target quadrant on day 6th of all experimental groups during MWM probe trial. **C** Reference memory trajectory map of all experimental groups during MWM probe trial. CONControl; LPSLipopolysaccharide; MIN 25Minocycline 25 mg/kg; MIN 50Minocycline 50 mg/kg; MMemantine 10 mg/kg Two-way repeated measured ANOVA (acquisition phase) and One-way ANOVA (probe trial) test followed by Bonferroni post hoc test. Values are expressed as mean \pm SEM. M # $p < 0.001$ versus control group; * $p < 0.05$ versus LPS group

incubated with anti-mouse secondary antibody (Santa Cruz USA with dilution 1:5000 each) at room temperature for 1 h. Detection of protein bands on the membranes was done using Clarity™ Western ECL substrate kits (Bio-Rad, USA). Determination of the relative density of the protein bands and quantification of protein were carried out by Fusion FX Chemiluminescence Imaging apparatus (Viber Lourmat, Germany) and Image J software (NIH, USA), respectively. Normalisation of protein density to the corresponding level of β -actin and calculation of integrated density value (IDV) were performed for every sample.

Enzyme-Linked Immunosorbent Assay (ELISA)

Oxidative stress markers in hippocampal tissue of the brain were performed using the ELISA method. The hippocampal tissues were extracted, weighed and homogenised (10% w/v) in ice-cold PBS (0.1 M, pH = 7.4) to prevent degradation of enzymes for 5 min. The homogenised tissues were centrifuged at 10,000 g at -4°C for 10 min (EBA 21, Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatants were allocated into Eppendorf tubes and preserved at -80°C for further biochemical assay. The total protein concentrations in tissues were measured using a Bio-Rad Quick Start™ Bradford Protein Assay kit (Bio-Rad, USA) according to the instructions in Bradford assay (Bradford 1976). The obtained values of total tissue protein were applied in this biochemical parameter as per mg of protein. The PCO, MDA, CAT and SOD levels were measured according to protocol given by the supplier.

Statistical Analysis

Statistical Package for the Social Sciences software (SPSS Inc., USA) version 24.0 was used to analyse the obtained data of this study. Two-way repeated measure ANOVA was used to analyze reference memory function. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used to estimate the mean for probe trial, density and expression of BDNF and CREB proteins and oxidative stress markers between groups. The data were expressed as

means \pm standard errors of mean (\pm SEM) and a probability value less than 0.05 ($p < 0.05$) was used to indicate a significant difference.

Results

Effects of Minocycline on Spatial Learning

After two weeks of treatment, MWM test was carried out to assess spatial learning and reference memory of rats. Escape latency, distance travelled and swimming speed were used to assess reference memory function during the acquisition phase.

Figure 2A demonstrated the mean escape latency, distance travelled and swimming speed throughout 5 days of acquisition phase of the MWM test. The escape latency and travelled distance decreased steadily in all experimental groups. Post-hoc test showed that the mean escape latency was significantly increased ($p < 0.05$) in LPS group during 5 days of acquisition phase compared to control group, indicating LPS induced reference memory impairment. In contrast to this, minocycline (25 and 50 mg/kg) and memantine treated groups showed a significant decrease in escape latency ($p < 0.05$) as compared to LPS rats where they reached the hidden platform in 20–30 s on day 5 of acquisition phase, indicating that minocycline could ameliorate LPS-induced reference memory impairment.

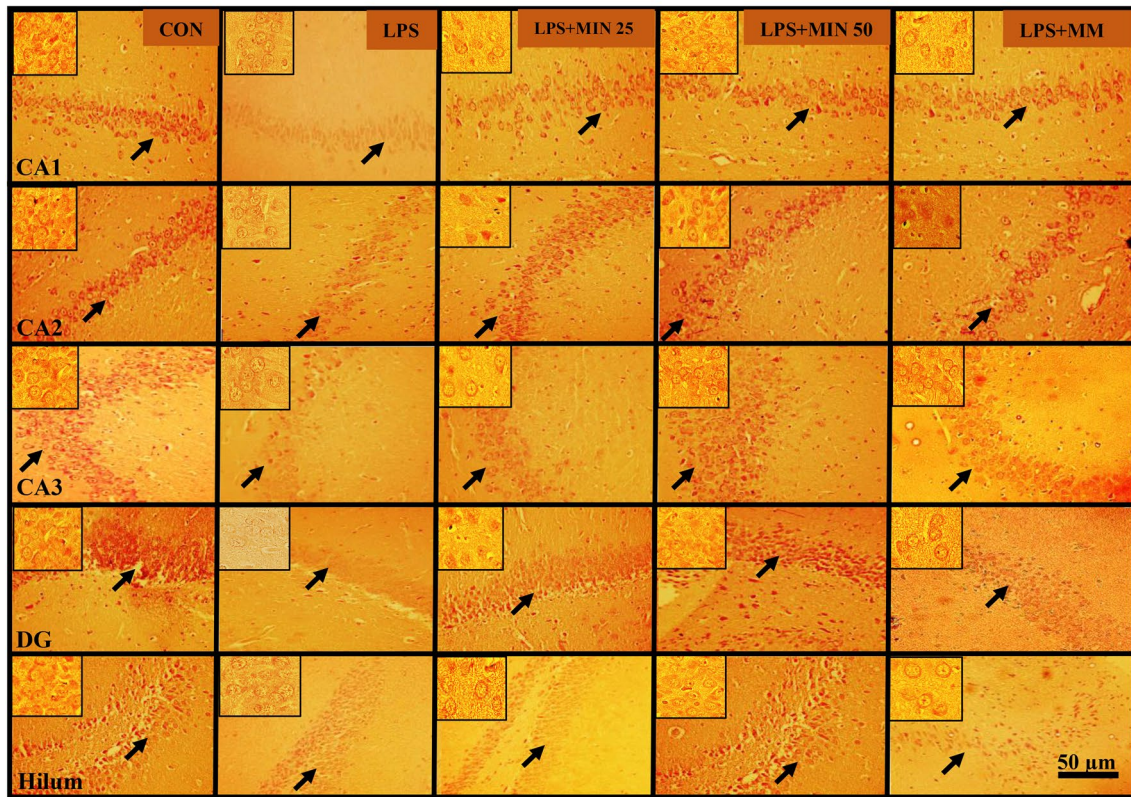
Post-hoc test demonstrated a significant decrease in mean swimming speed and distance travelled in LPS group during 5 days of acquisition phase compared to control group that indicates reference memory impairment. The mean swimming speed and distance travelled were significantly increased ($p < 0.05$ and $p < 0.05$) in minocycline and memantine treated groups during 5 days of acquisition phase in comparison to LPS group where the rats reached the hidden platform within 20–30 s on day 5 of acquisition phase, indicating that minocycline could ameliorate LPS-induced reference memory impairment.

Effects of Minocycline on Reference Memory

The probe trial was conducted to assess reference memory on the 6th day of MWM test. Time spent in the target quadrant, distance travelled, swimming speed to reach platform and target crossings were estimated.

Post-hoc analysis displayed that there was a significant decrease in mean time spent in the target quadrant and target crossings ($p < 0.05$ and $p < 0.001$) in LPS group compared to control group. There was a significant increase in mean time spent in the target quadrant and target crossings ($p < 0.05$ and $p < 0.001$) in LPS group treated with minocycline (25

(A)



(B)

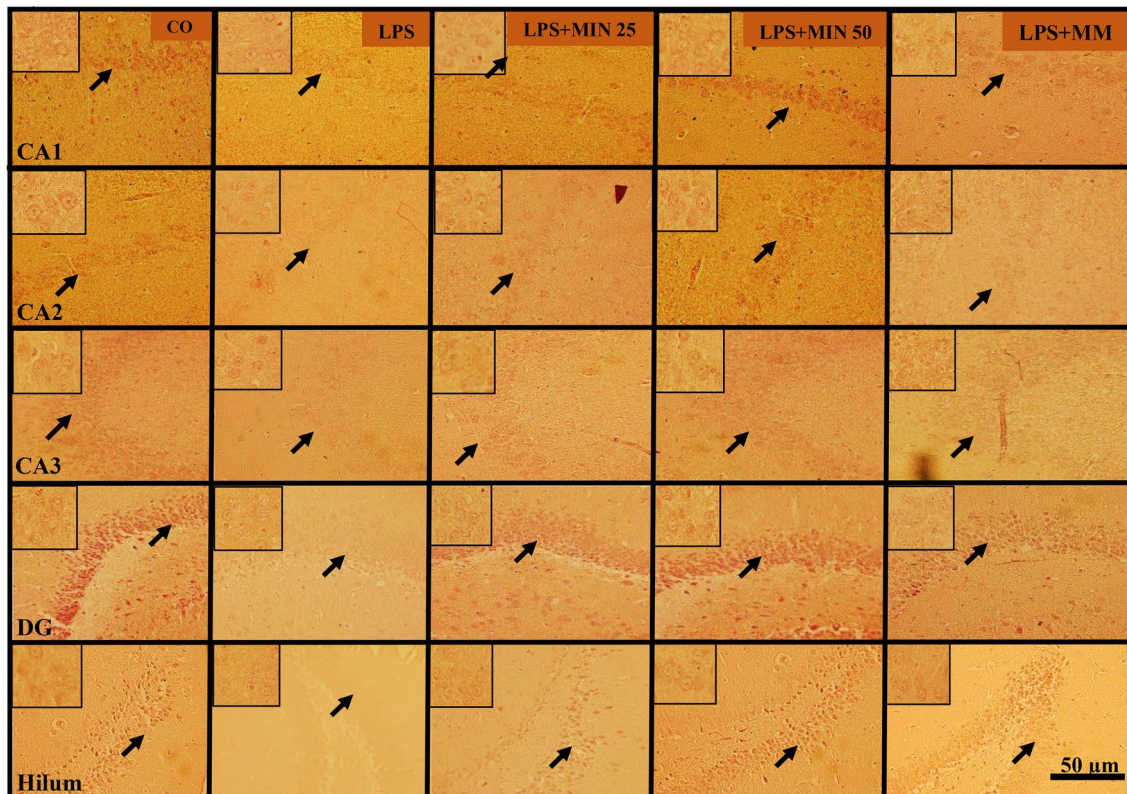


Fig. 3 Distribution of BDNF (A) and CREB (B) positive neurons in the CA1, CA2, CA3, DG and hilum regions of hippocampus at 40× & 100× magnification. The black arrows indicate positive neurons. CON Control; LPS Lipopolysaccharide; MIN 25 Minocycline 25 mg/kg; MIN 50 Minocycline 50 mg/kg; MM Memantine 10 mg/kg

and 50 mg/kg) and memantine as compared to LPS injected group.

Similarly, the mean distance travelled and swimming speed to reach the target quadrant was significantly decreased ($p < 0.001$ and $p < 0.001$) in LPS treated group as compared to control group. Minocycline and memantine treated groups displayed a significant increase in mean travelled distance and swimming speed to reach the target quadrant ($p < 0.001$ and $p < 0.001$) as compared to LPS groups. These findings suggest that LPS impaired memory retention and minocycline at both doses ameliorated LPS-induced memory retention impairment. Trajectory map view demonstrated aimless swimming (swimming in all quadrants) of LPS group, while control, minocycline and memantine treated LPS groups swam mostly in the target quadrant. Figure 2B, C demonstrated the mean escape latency, target crossings, distance travelled, swimming speed and trajectory map during probe trial of MWM test.

Effects of Minocycline on the Expression of BDNF and CREB Proteins

Figures 3 and 4 display the expression and quantification of BDNF and CREB positive neurons. The LPS group showed a more significant decrease in number of the BDNF and CREB positive neurons in the hippocampus compared with control group ($p < 0.05$). The minocycline and memantine treated LPS groups exhibited a more significant increase in BDNF and CREB positive neurons compared to LPS group ($p < 0.05$).

There was a significantly higher number of BDNF and CREB positive neurons in LPS group treated with minocycline (50 mg/kg) ($p < 0.05$) compared to LPS group treated with minocycline (25 mg/kg) and memantine (10 mg/kg) with no significant differences between minocycline (25 mg/kg) and memantine groups in number of BDNF and CREB positive neurons.

Effects of Minocycline on Oxidative Status Markers

Figure 5 displayed the effects of minocycline on mean level of oxidative status markers. Post-hoc test depicted that the LPS injected group exhibited significantly higher mean level of PCO & MDA ($p < 0.05$) and significantly lower mean level of CAT & SOD compared to control group ($p < 0.05$). LPS injected group treated with minocycline (at both doses) and memantine showed more significant decrease in mean

level of PCO & MDA ($p < 0.05$) and significant increase in mean level of CAT & SOD ($p < 0.05$) as compared to LPS group.

The mean level of PCO and MDA in LPS group treated with minocycline (50 mg/kg) group was significantly lower ($p < 0.05$) compared to minocycline (25 mg/kg) and memantine (10 mg/kg) treated groups. The mean level of CAT and SOD in LPS group treated with minocycline (50 mg/kg) was significantly higher compared to minocycline (25 mg/kg) and memantine treated groups ($p < 0.05$). There was no significant difference between minocycline (25 mg/kg) and memantine treated groups in mean level of PCO, MDA, CAT and SOD.

Effects of Minocycline on Mean IDV of BDNF and CREB

Figure 6 demonstrated the protein density and mean IDV value of BDNF and CREB proteins. The mean IDV values of BDNF and CREB proteins in hippocampal tissues of LPS group were significantly decreased compared to control group ($p < 0.05$). The mean IDV values of BDNF and CREB proteins in LPS group treated with minocycline and memantine were significantly increased compared to LPS group ($p < 0.05$).

The mean IDV values of BDNF and CREB proteins in LPS group treated with minocycline (50 mg/kg) was significantly higher ($p < 0.05$) than minocycline (25 mg/kg) and memantine (10 mg/kg) treated LPS rats. There was no significant difference in mean IDV values of BDNF and CREB proteins between minocycline (25 mg/kg) and memantine groups.

Discussion

Minocycline treatment was demonstrated to improve LPS-induced learning and reference memory deficits as well as reduce oxidative stress in the rat hippocampus in this study. The minocycline's neuroprotective effect is most likely due to activation of the BDNF-CREB signalling pathway. Previous studies have shown that single or multiple LPS injections might cause chronic neuroinflammation [17, 18]. LPS binds to TLR-4, activates TLR-4/NF- κ B pathway cascades and releases inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) [19]. As a result of the activation of the inflammatory pathway, ROS and RNS are released, causing oxidative molecular damage to DNA, lipids, and proteins, as well as depletion of antioxidant enzymes, resulting in an imbalance between oxidant molecules and antioxidant enzymes, as well as activation of the oxidative stress pathway [20, 21]. The present study showed that single intraperitoneal LPS

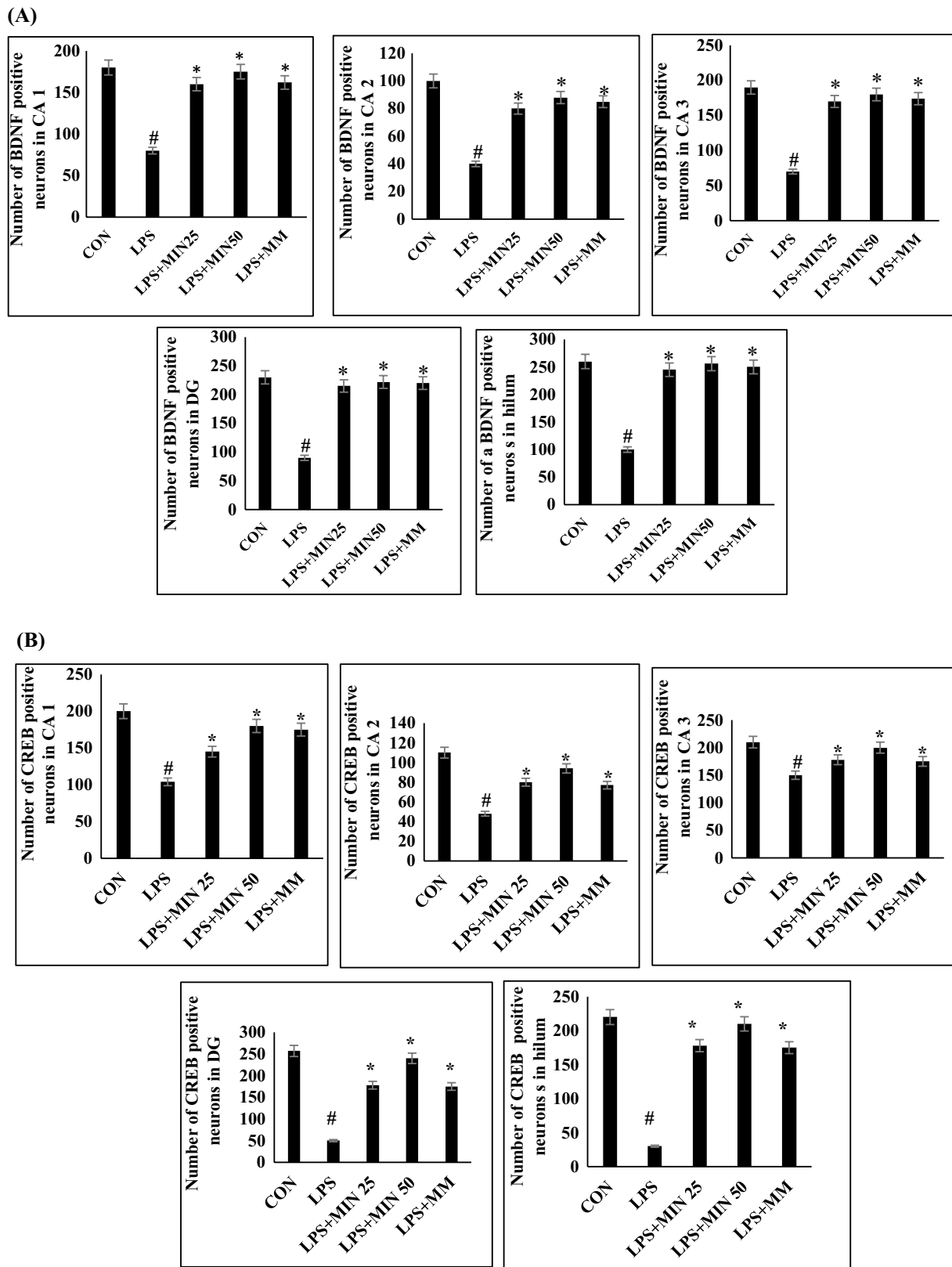


Fig. 4 Total number of BDNF (A) and CREB (B) positive neurons in CA1, CA2, CA3, DG and hilum regions of hippocampus. CON Control; LPS Lipopolysaccharide; MIN 25 Minocycline 25 mg/kg; MIN 50 Minocycline 50 mg/kg; MM Memantine 10 mg/kg. One-

way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean \pm SEM, in each group. # $p < 0.001$ versus control group; * $p < 0.05$ versus LPS group

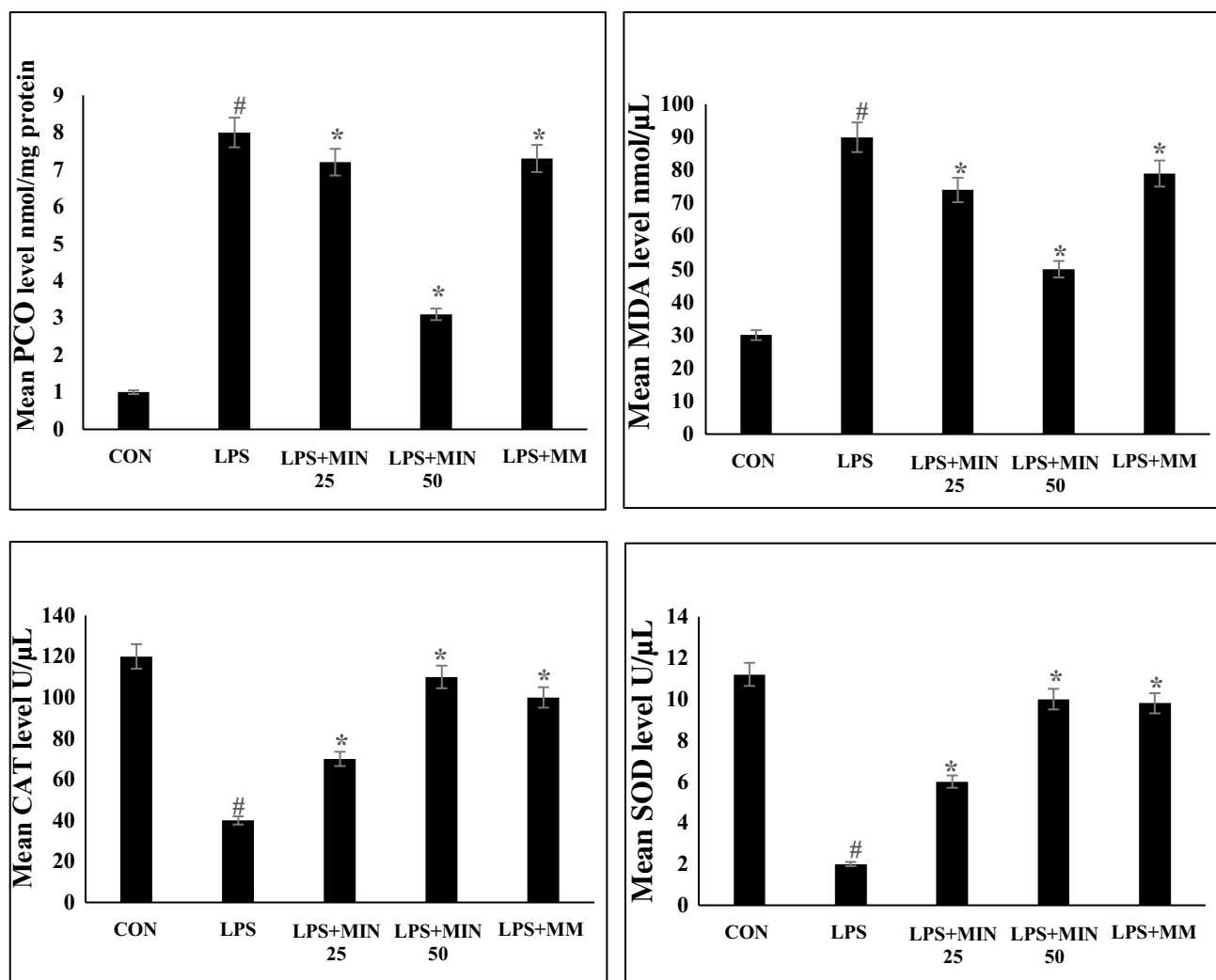


Fig. 5 Effects of minocycline on mean protein carbonyl (PCO), malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) level on hippocampus of LPS injected rats. CON Control; LPS Lipopolysaccharide; MIN 25 Minocycline 25 mg/kg;

MIN 50 Minocycline 50 mg/kg; MM Memantine 10 mg/kg. One-way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean \pm SEM. # p < 0.001 versus control group; * p < 0.05 versus LPS group

injection at a dose of 5 mg/kg could increase escape latency to hidden platform and decrease travelled distance, swimming speed, target crossings and time spent in the target quadrant, increase PCO and MDA levels, and decrease CAT and SOD levels in the hippocampal tissue. These findings support previous findings that LPS can impair learning and reference memory [4, 22, 23] while also causing oxidative stress in rat brain tissue [24–26].

In line with our findings, a previous study found that giving intraperitoneal minocycline (25 and 50 mg/kg) for 7 days before injecting intrahippocampal LPS ($\geq 10,000$ EU/mg) reduced neuroinflammation and improved cognitive impairment in mice [27]. Another study conducted on neonatal rats, where intracerebroventricular LPS (1 mg/kg) was injected once and intraperitoneal minocycline (45 mg/kg) was administered 12 h before and immediately after LPS

injection and showed that anti-oxidative actions of minocycline ameliorated LPS-induced neurobehavioral dysfunction [28]. To further examine the mechanism by which minocycline improved cognitive impairment and reduced oxidative stress following LPS injection, the expression and density of CREB and BDNF proteins were examined in the rat hippocampal tissue.

Minocycline has a high capability to pass blood–brain barrier and shows neuroprotective effects in various neurodegenerative diseases. Our study showed that both doses of minocycline given for 14 days improve learning and reference memory, reduce oxidative stress as well as increase BDNF and CREB proteins expression in hippocampi of LPS rats. These findings support minocycline's neuroprotective potential as a treatment for neurodegenerative diseases comparable to memantine, indicating its function

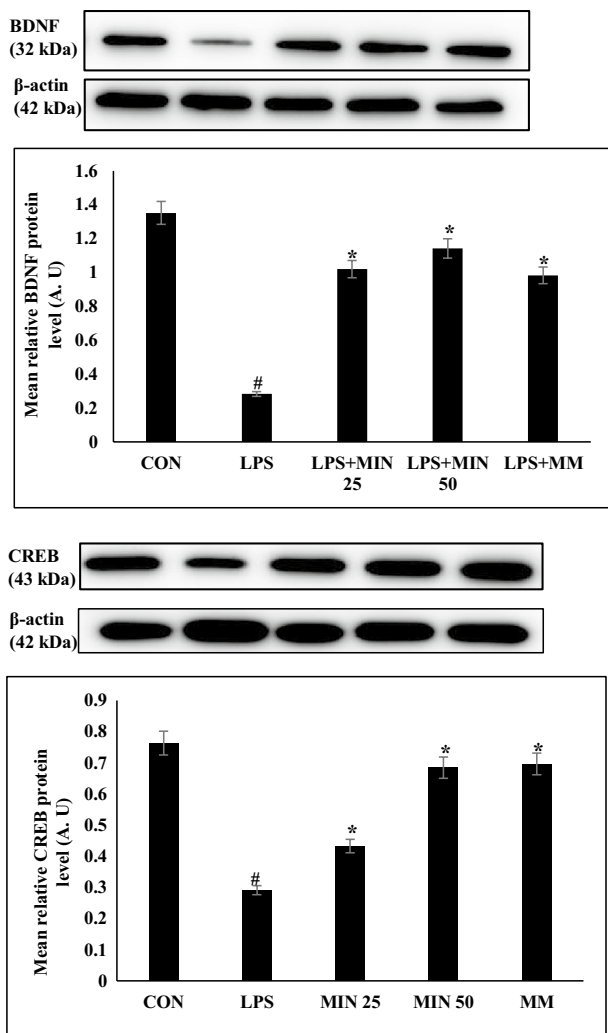


Fig. 6 Mean relative BDNF and CREB protein level in the rats' hippocampal tissue. **A** An example of Western blot results for all groups. The lower panel demonstrates the loading control. **B** Quantification analysis of IDV between the groups. The data was normalized by the control group. CON Control; LPS Lipopolysaccharide; MIN 25 Minocycline 25 mg/kg; MIN 50 Minocycline 50 mg/kg; MM Memantine 10 mg/kg. One-way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean \pm SEM, $n=10$ animals in each group. $\#p < 0.05$ versus control group; $*p < 0.05$ versus LPS group

in the modulation of neuro-regenerative proteins in brain cells [28, 29], as well as its ability to modulate the CREB-BDNF signalling pathway [12, 31, 32]. Thus, it can be suggested that minocycline treatment restores the CREB-BDNF signalling cascade and protects the brain from LPS-induced neurotoxicity. The neuroprotective properties of minocycline (50 mg/kg) were shown to be superior to those of minocycline (25 mg/kg) and memantine (10 mg/kg), with no significant difference between the two. This disparity in minocycline effects could be attributable to the drug's dose-dependent effects.

Conclusion

Our findings showed that minocycline protects against LPS-induced learning and reference memory impairment, probably acting as a neuroprotective agent via a reduction in oxidative stress and upregulation of the CREB-BDNF signalling pathway in the rat hippocampus. Thus, minocycline has a potential to prevent neuronal loss in neurodegenerative diseases, although further human studies are required.

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Data Availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest The authors declared that there were no possible conflicts of interest in the research, writing, or publishing of this paper.

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