Minocycline Ameliorates LPS-Induced Learning and Memory Impairment By Inhibiting Microglia and Astrocytes Activation in the Hippocampus

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ABSTRACT

INTRODUCTION: Many studies support the notion that neuroinflammation involving glial cells may be the fundamental pathogenic event that lead to brain damage and degeneration in Alzheimer's disease (AD). The effects of minocycline on glial cells activation in the lipopolysaccharide (LPS) rat model is studied here. **MATERIALS AND METHODS**: Fifty adult male Sprague Dawley rats were split into five groups: control, LPS, LPS-treated with minocycline 25 mg/kg, LPS-treated with minocycline 50 mg/kg, and LPS-treated with memantine 10 mg/kg. For two weeks, rats were treated with minocycline and memantine intraperitoneally daily, while were injected with LPS intraperitoneally once on day 5. Morris water maze (MWM) test was performed to evaluate the learning and working memory behaviour. Immunohistochemistry and Western blot were performed to measure ionized calcium-binding adaptor molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) expressions in the hippocampal tissue. **RESULTS:** LPS injection caused significant learning and working memory deficits ($p < 0.05$) as well as increased expressions of Iba-1 and GFAP ($p < 0.05$), indicating microglia and astrocytes activation, respectively. Minocycline treatment significantly improved learning and working memory performance ($p < 0.05$) and reduced microglia/astrocytes activation ($p < 0.05$) in a dose-dependent manner, similar to the effect of memantine. **CONCLUSIONS:** Our results suggest that minocycline modulates LPS-induced microglia and astrocytes activation as well as improves learning and working memory comparable to memantine. Thus, minocycline may have a preventive-therapeutic effect in diseases involving neuroinflammation such as AD.

INTRODUCTION

Many studies presently support the notion that neuroinflammation involving glial cells (microglia and astrocytes) may be the fundamental pathogenic event that lead to brain damage and degeneration in Alzheimer's disease (AD).[1,2,3] A previous study has shown that reactive microglia and astrocytes are abundant near senile plaques in AD patients.[4] Neuroinflammation has been found in the early stages of AD, which is responsible for increased neuronal damage, owing to glial cell malfunction. This malfunction leads to a lack of clearance of amyloid-beta (Aβ) peptide, which causes glial cell over-activation. Overall, an inflammatory cascade is initiated, followed by neuronal injury and dysfunction, as well as plaque formation.[5]

As a result, presently the discovery of anti-inflammatory drugs for AD that targets glial cells appears to be promising.[6] In the preclinical stage, a good animal model is important to mimic AD in humans. One of them is lipopolysaccharides (LPS)-induced neuroinflammation in a rat model.[7] LPS is an endotoxin found in the outer cell membrane of gram-negative bacteria, accounting for up to 80% of the membrane of *Escherichia-coli* and *Salmonella sp.* It can stimulate excessive Aβ plaque formation and

neurofibrillary tangles, two hallmarks of AD.[8] This Aβ peptide then triggers glial cells mediated-neuroinflammatory pathway.[9]

Minocycline (glial cells inhibitor) is a broad-spectrum semisynthetic second-generation tetracycline antibiotic with anti-inflammatory, anti-oxidant, and neuroprotective properties in addition to its antibacterial actions.[10] Previous research has found that minocycline reduces the severity of numerous neurological disorders in animal models by suppressing glial cells activation (microglia and astrocytes activation) that mediated neuroinflammation.[11] However, it is uncertain whether minocycline has a protective effect in the LPS-induced neuroinflammation rats model and what the underlying mechanisms are. This study aims to compare the effects of minocycline and memantine, a clinically approved N-Methyl-D-aspartate (NMDA) receptor antagonist, on learning and working memory behaviour as well as microglia and astrocyte expression in the rat hippocampus, in an LPS-induced neuroinflammation rat model.

MATERIALS AND METHODS

ANIMALS

Adult male Sprague Dawley rats were obtained from the Animal Research and Service Centre (ARASC) of Universiti Sains Malaysia (USM). They were housed in polypropylene cages (32 x 24x 16 cm) and were given unrestricted access to a normal pellet rat food (Altromin, Germany) as well as tap water.

Experimental design

The rats were randomly divided into five groups as follows: (i) control rats treated with distilled water, (ii) LPS-treated with distilled water, (iii) LPS-treated with minocycline 25 mg/kg (12), (iv) LPS-treated with minocycline 50 mg/kg [12] and (v) LPS-treated with memantine 10 mg/kg [13], (n=10 rats/group). Minocycline (USP, Rockville) and memantine (USP, Rockville) were administered intraperitoneally once daily for 14 days consequently, starting from day 1 to day 14. LPS was obtained from *E.coli* 0111: B4 (Sigma-Aldrich, St. Louis, MO) and injected intraperitoneally once on day 5 at the dose of 5 mg/kg.[14]

Behavioural assessment

Learning and working memory behaviour were assessed 24 hours after 14 days of treatment with minocycline and memantine using the MWM apparatus. The rats were acclimatised to a behavioural room for 30 minutes prior to testing. After each trial, animals were returned to their cages. Habituation was carried out two days (days 15 and 16) before the training session where the animals were allowed to swim for 60 s in the pool without a platform and the MWM test was performed for six days (days 17 until 22) as described by Pretorius et al (2018).[15] The acquisition training was carried out for five days and the retention test on the 6th day.

Immunohistochemistry

The rats were killed by intraperitoneal injection of sodium thiopental after a 24-hour MWM test. To fix the rats' cortical and hippocampus tissues, intracardiac perfusion with 0.1 M phosphate-buffered saline (PBS) (pH 7.0) and 4% paraformaldehyde (PFA) (pH 7.0) (Fisher Scientific, USA) was performed. The tissues were carefully dissected, post-fixed in 10% formalin solution (Fisher Scientific, USA), and maintained at room temperature before paraffin sectioning, as per the usual technique.

Primary antibody (goat anti-Iba-1 & GFAP; dilution 1:200 and 1:500), secondary antibody (anti-goat Iba-1 & GFAP; dilution 1:1000 each) and 3, 3'-Diaminobenzidine (DAB) were used for staining. The sections known to express Iba-1 and GFAP positive cells were imaged under 40x and 100x magnification using an image analyser connected to a light microscope (Olympus Corporation, Japan). The Iba-1 and GFAP positive cells were counted using Image-J software (http:// imagej.nih.gov/ij).

Western blotting

The Iba-1 and GFAP proteins were extracted from hippocampus tissues using radioimmunoprecipitation assay (RIPA) buffer. The protein's quantifications in each

supernatant were calculated using a Bradford protein assay kit (Bio-Rad, USA). Proteins (60 µg) were denatured with sodium dodecyl sulfate (SDS) sample buffer and separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Membrane Solutions, USA) and then blocked with 5% skim milk for 1 hr at room temperature. Primary antibody (goat anti-Iba-1 and/or anti-GFAP; dilution 1:500 each) and anti-goat secondary antibody (anti-goat Iba-1 & GFAP; dilution 1:5000 each) were used for blotting. The protein bands were detected with Clarity™ Western ECL substrate kits (Bio-Rad, USA). The relative density of the protein bands was evaluated by densitometry using Fusion FX Chemiluminescence Imaging apparatus (Viber Lourmat, Germany) and quantified by Image J software (NIH, USA).

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS) software version 24. The repeated measured Analysis of Variance (ANOVA) was used to analyse the behaviour assessment. One-way ANOVA was used to analyse immunohistochemistry and western blot results. The Bonferroni post hoc test was used to further assess the differences between groups. To show a significant difference, a probability value (p<0.05) was utilised.
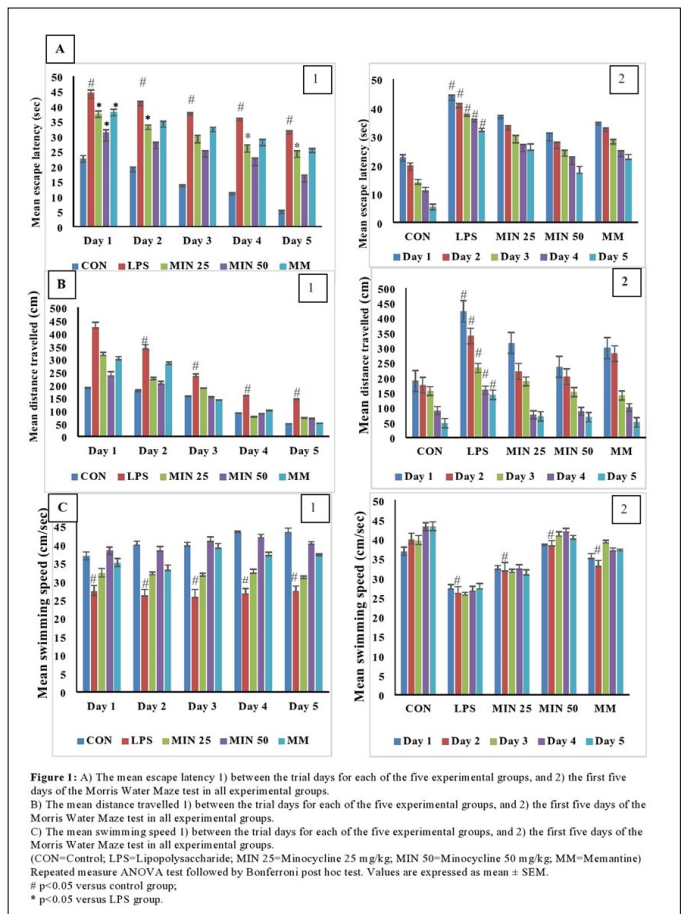
RESULTS

EFFECTS ON SPATIAL LEARNING

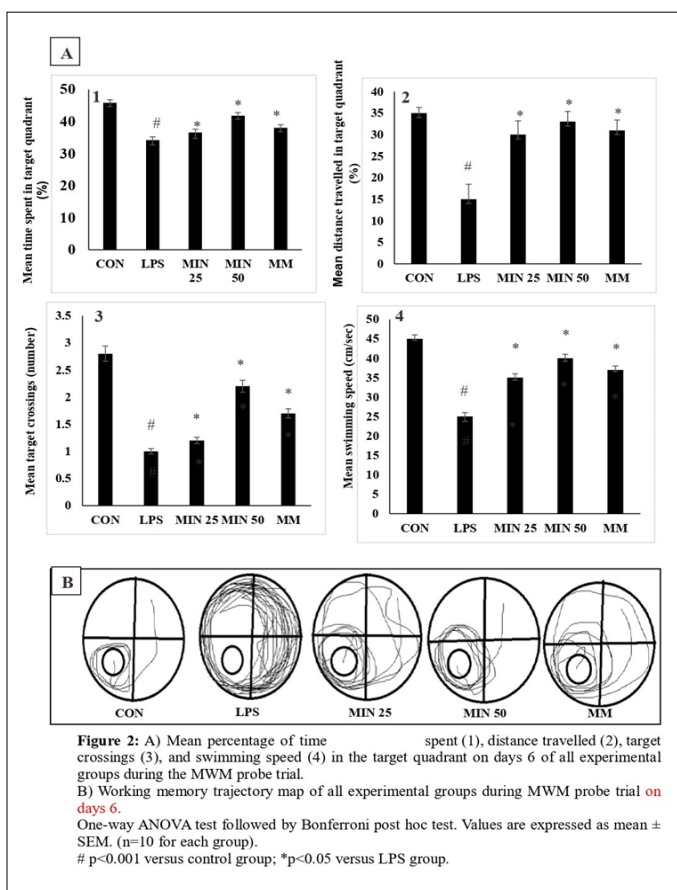#### Escape latency and total travelled distance

The mean values of escape latency and distance travelled during the acquisition phase for five days are presented in Figures 1A and 1B. The escape latency and total travelled distance reduced steadily in all experimental groups. The LPS-injected rats had a longer escape latency and travelled a greater distance. In contrast, LPS rats treated with minocycline at dosages of 25 and 50 mg/kg showed decreased escape latency and total travelled distance, reaching the submerged platform site in 20 to 30 seconds

on the fifth day of trials, comparable to memantine-treated rats.

Figure 1: A) The mean escape latency 1) between the trial days for each of the five experimental groups, and 2) the first five days of the Morris Water Maze test in all experimental groups.
B) The mean distance travelled 1) between the trial days for each of the five experimental groups, and 2) the first five days of the Morris Water Maze test in all experimental groups.
C) The mean swimming speed 1) between the trial days for each of the five experimental groups, and 2) the first five days of the Morris Water Maze test in all experimental groups.
(CON=Control; LPS=Lipopolysaccharide; MIN 25=Minocycline 25 mg/kg; MIN 50=Minocycline 50 mg/kg; MM=Memantine)
Repeated measure ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean ± SEM.
# p<0.05 versus control group;
* p<0.05 versus LPS group.

Swimming speed

Figure 1C depicts the mean values of swimming speed during the acquisition phase within five days. Swimming speed was reduced in the LPS-injected rats (p<0.05) than the control group, indicating a problem with working memory. In contrast, LPS rats treated with minocycline at dosages of 25 and 50 mg/kg, as well as memantine, demonstrated enhanced swimming speed (p<0.05), reaching the submerged platform position within 20 to 30 seconds on the fifth day of trials, compared to LPS-injected rats.

EFFECTS ON MEMORY RETENTION

The LPS injected rats showed significantly less time spent in the target quadrant (p<0.05) and target crossings (p<0.001) compared to the control group during probe test. The rats treated with minocycline (25 and 50 mg/kg)

and memantine spent significantly more time in the target quadrant (p<0.05) and displayed significant more target crossings (p<0.001) compared to LPS injected rats. Similarly, LPS injected rats showed significantly less travelled distance (p<0.001) and swimming speed (p<0.001) to reach the target quadrant compared to the control group. Minocycline treated rats displayed significant increased travelled distance (p<0.001) and swimming speed (p<0.001) to reach the target quadrant compared to memantine. These findings suggest that injection of LPS impaired memory retention and treatment of minocycline at both doses ameliorated this poor memory retention.

Trajectory view demonstrated that LPS injected rats swam in all quadrants (aimless swimming), whereas controls, minocycline and memantine treated rats swam mostly in the target quadrant containing platform (Figure 2).

Figure 2: A) Mean percentage of time spent (1), distance travelled (2), target crossings (3), and swimming speed (4) in the target quadrant on days 6 of all experimental groups during the MWM probe trial.
B) Working memory trajectory map of all experimental groups during MWM probe trial on days 6.
One-way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean ± SEM. (n=10 for each group).
# p<0.001 versus control group; *p<0.05 versus LPS group.

Effects on Iba-1 and GFAP positive cells expressions

The LPS injected rats displayed an increased number of the Iba-1 and GFAP positive cells in CA1 (p<0.001), CA2 (p<0.001), CA3 (p<0.001), DG (p<0.001) and hilum (p<0.001) regions of the hippocampus as compared to the control. In contrast, minocycline treatment reduced the number of the Iba-1 and GFAP positive cells in CA1 (p<0.05), CA2 (p<0.05), CA3 (p<0.05), DG (p<0.05) and hilum (p<0.05) regions of the hippocampus which are comparable to memantine effects (Figure 3, 4A and 4B).
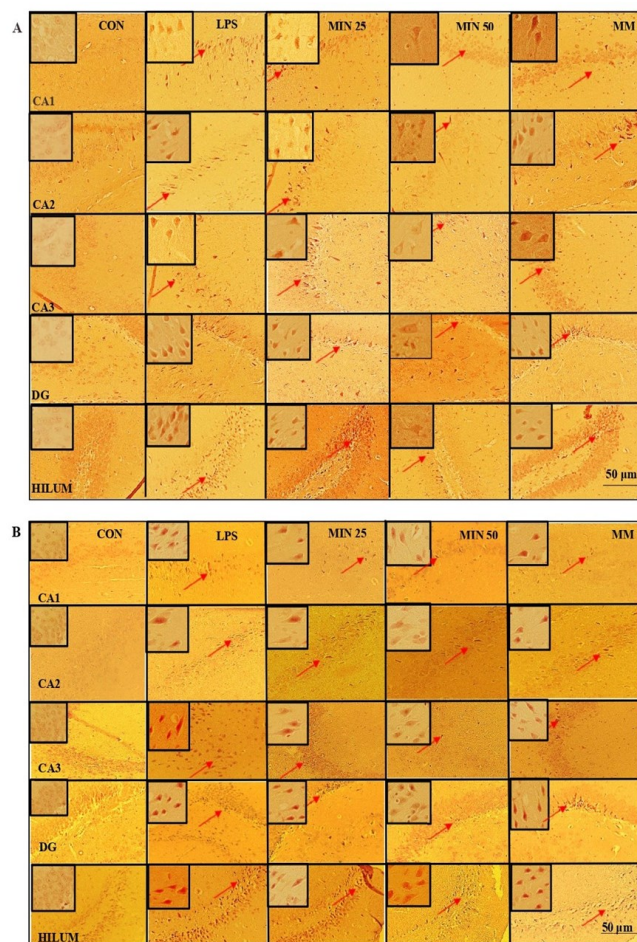

Figure 3: Distribution of A) Iba-1 positive cells B) GFAP positive cells in the CA1, CA2, CA3, DG & hilum regions of hippocampus at 40X & 100X (in small box) magnification. The red arrows indicate microglia and astrocytes positive cells.

Effects on level of expression of Iba-1 and GFAP proteins

The level of expression of Iba-1 and GFAP proteins were determined by Western blot. The densities of Iba-1 and GFAP proteins in the hippocampus tissues of LPS-injected rats were significantly higher (p<0.001) than in controls. In contrast, Iba-1 and GFAP proteins densities in minocycline and memantine treated rats were significantly lower (p<0.05) than in LPS-injected rats (Figure 4C and 4D).
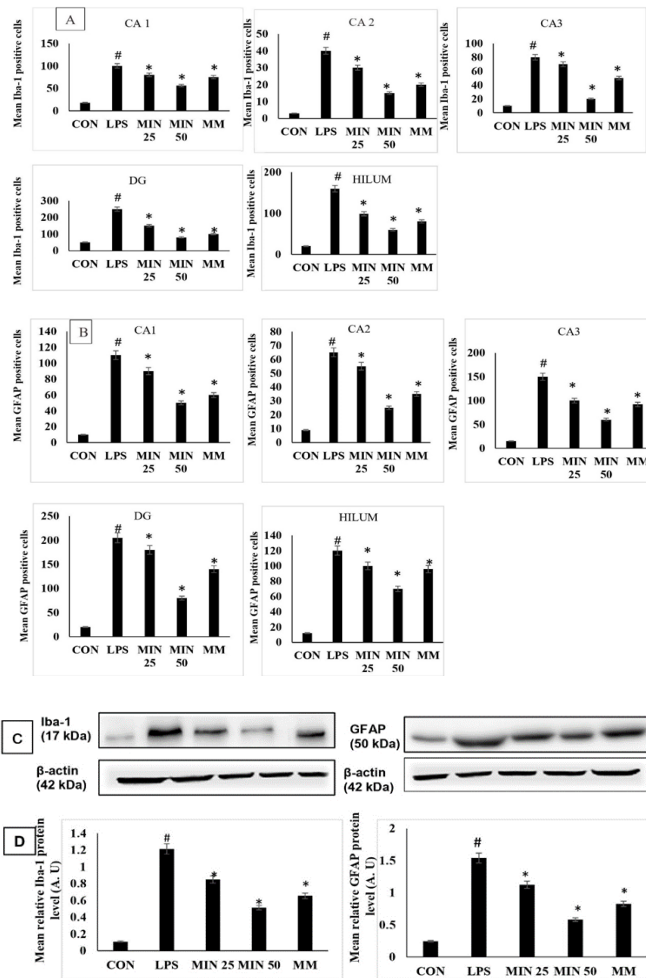
Figure 4: Total number of (A) Iba-1 and (B) GFAP positive cells in CA1, CA2, CA 3, DG & Hilum regions. (C) An example of Western blot results for all groups. The lower panel demonstrates the loading control. (D) Quantification analysis of IDV between the groups. The data were normalized by the control group. One-way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean ± SEM. n= 10 for each group # $p < 0.05$ versus control group; *$p < 0.05$ versus LPS group.

DISCUSSION

This study confirmed that LPS injection (5 mg/kg; once) significantly impaired learning and working memory in adult male Sprague Dawley rats, as evidenced by increased escape latency time and distance travelled to the hidden platform, while decreased swimming speed, time spent in the target quadrant and target crossings in the LPS treated group. In minocycline and memantine treated rats, on the other hand, the escape latency time and total distance travelled decreased, but swimming speed, time spent in the target quadrant and target crossings increased, indicating that learning and working memory impairment was improved. Thus, intraperitoneal injections of minocycline at doses of 25 and 50 mg/kg for two weeks alleviated LPS-induced learning and memory impairment

comparable to 10 mg/kg of memantine. Furthermore, if we further compared minocycline with memantine administration, all data suggested better effects in minocycline (50 mg/kg) and comparable effects between minocycline (25 mg/kg) and memantine (10 mg/kg) treatments in alleviated learning and working memory impairment due to LPS injection.

LPS injection also can induce glial cells activation in the hippocampal region, as evidenced by the increased number of Iba-1 and GFAP positive cells and level of expression of Iba-1 and GFAP proteins. Minocycline treatment at 25 and 50 mg/kg inhibited LPS-induced microglia and astrocytes activation, as evidenced by decreased expression of Iba-1 and GFAP positive cells and reduced Iba-1 and GFAP proteins expression level in the hippocampus. The minocycline (25 mg/kg) and memantine groups showed similar findings; however, their effects were lesser than the minocycline (50 mg/kg) group in inhibited microglial and astrocytes activation due to LPS injection.

This effect seen in our study was comparable to a recent study that discovered minocycline can reduce intrahippocampal LPS-induced cognitive deficits in mice by suppressing the activation of microglia and astrocytes and normalising BDNF expression.[16] Aside from having comparable findings, our study methodology only used one dose of LPS intraperitoneally and evaluated working memory in rat, whereas Hou et al., 2016 [16] administered LPS intrahippocampal and measured reference memory in mice.

Previous studies demonstrated that LPS induced β-amyloid peptide deposit and inflammation in the neural tissue resulted in severe learning and memory impairment using various behavioural tasks[17,18,19] The systemic inflammation and neuroinflammation induced by LPS injection, which promotes the formation of Aβ plaque and neurofibrillary tangles, are two hallmarks of AD.[12] Both stimulate continuous hyperactivation of microglia and astrocytes that further activates the release of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1a (IL-1a) and complement protein (C1q). These cytokines also enhance the production of

insulin-degrading enzyme (IDE) in microglia and astrocytes, which activates the NF-κβ inflammatory cascade, serves as a signal for neuronal death, and culminates in excessive neuroinflammation.[13] Inability to reversed this chronic inflammation causes oxidative and nitrosative stress, as well as the accumulation of additional amyloid, which prolonging the inflammatory cycle and causing neuronal death, synaptic dysfunction, and learning and memory impairment all at the same time.[20]

The neuroprotective effects of minocycline were attributed to its anti-inflammatory, anti-oxidant and anti-amyloid properties.[21,22] LPS at a dose of (≥10,000 EU/mg), administered once via the intra-hippocampal route, activated neuroinflammatory pathways and impaired cognitive function in mice. [23] In addition, Zhang et al. (2021) found that 1 mg/kg intraperitoneal LPS injections for three days activated glial cells in a rat model and that 25 mg/kg [24] minocycline administration two hours for four days before LPS injections restored this activation. LPS (5 mg/kg) injection also was shown to increase the production of oxidative stress markers as well as other neuroinflammatory mediators. [25] Thus, more research is needed to elucidate the cellular and molecular mechanisms of neuroinflammation by LPS injection to support our hypothesis.

CONCLUSION

Finding from our results suggest that minocycline dependent on dose improves LPS-induced cognitive deficit through modulation of glial cells activation.

Declaration of conflicting interests

Concerning the research, writing, and/or publishing of this paper, the author(s) reported no possible conflicts of interest.

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