ORIGINAL ARTICLE

Minocycline Protects Against LPS-induced Neuronal Death and Memory Impairment in the Rat

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ABSTRACT

Introduction: Minocycline has been demonstrated to have potent effects on neurologic structures and functions in several animal models. However, its neuroprotective properties following a single injection of lipopolysaccharide (LPS) in an adult rat model have not been clearly elucidated. This study investigated minocycline's neuroprotective effects in the LPS-induced neuroinflammation rat model. **Methods**: Fifty adult male Sprague Dawley rats were split into five groups at random: (i) control, (ii) distilled water-treated LPS, (iii) 25 mg/kg minocycline-treated LPS, (iv) 50 mg/kg minocycline-treated LPS, and (v) 10 mg/kg memantine-treated LPS. On day 5, LPS (5 mg/kg) was given intraperitoneally once, whereas minocycline and memantine were given once daily for 14 days. **Results:** LPS was found to significantly induce β-amyloid peptide deposition and neuronal damage, and impair recognition memory, while administration of minocycline dose-dependently reversed these effects. These data suggest that LPS-induced recognition memory impairment by inducing β-amyloid peptide deposition and neuronal damage in the cortical and hippocampal areas. Furthermore, we compared minocycline with memantine administration, and these data suggested better effects in minocycline (50 mg/kg) and comparable effects between minocycline (25 mg/kg) and memantine (10 mg/kg) treatments in reducing β-amyloid peptide deposition, neuronal damage and recognition memory impairment induced by LPS. **Conclusion:** Minocycline may be a strong contender as an effective preventive-therapeutic drug for neuroinflammatory diseases such as Alzheimer's disease (AD) based on these findings.
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INTRODUCTION

"Dementia affects more than 5 million people in the United States and 46 million people worldwide" (1 p. 22). "Dementia cases are anticipated to reach 7 million in Japan by 2025, increasing to 131.5 million by 2050. Alzheimer's disease (AD) is the most common neurodegenerative disease, accounting for around 60% of all dementia cases" (1 p. 22). Its frequency rises with age, with the majority of instances occurring in those aged 65 and over. The risk of contracting the disease became doubles every five years after 65 years old, and after more than 85 years old, the risk is close to 50% (2). "Lipopolysaccharide (LPS), an endotoxin triggers a toll-like receptor-4 (TLR-4) that starts the systemic inflammatory response cascade. The LPS-TLR-4 complex binds to the microglial surface and activates a variety of signalling pathways, including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), mitogen-activated protein kinase (MAPK) and rapamycin mammalian target (mTOR), all of which activate NF-κB. When NF-κB is activated, pro-inflammatory chemokines, cytokines, and inducible enzymes including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are produced" (3 p. 8). These effects resulted in an appearance of clinical and pathological AD characteristics, including β-amyloid peptide, intraneuronal neurofibrillary tangle (NFT) formation, neuroinflammatory damage, and cognitive impairment (3, 4, 5).

"Minocycline is a second-generation tetracycline antibiotic that has been used against gram-positive and gram-negative bacteria for over 30 years. It's a highly lipophilic chemical that can readily pass the blood-brain barrier (BBB) and accumulate in cerebrospinal fluid (CSF) and central nervous system (CNS) cells, allowing it to be used to treat a variety of CNS illnesses" (6 p. 744). "Previous studies have shown that minocycline has neuroprotective" (7 p. 1442), "anti-amyloidogenic" (8 p. 1090) and "anti-inflammatory effects in various AD rat models" (9 p. 2394). As a result, the present study

used the LPS-induced neuroinflammation rats' model to test the neuroprotective benefits of two different minocycline dosages and compare them to the standard therapy, memantine, a clinically licensed N-methyl-D-aspartate (NMDA) receptor antagonist.

MATERIALS AND METHODS

Animals

Fifty adult male Sprague Dawley rats were purchased from the Animal Research and Service Centre (ARASC) at Universiti Sains Malaysia (USM). All rats were housed in 32 x 24 x 16 cm polypropylene cages in a room with a 23°C temperature and light/dark cycles last 12 hours with free access to food and water. The experimental methodology was authorized by this university's research and ethics committee [USM/IACUC/2018/(942) (114)] and followed globally acknowledged norms for laboratory animal use and care.

Experimental design

"The rats were separated into five groups (n=10 rats/group): (i) control, (ii) LPS-treated with distilled water, (iii) LPS-treated with minocycline 25 mg/kg (11), (iv) LPS-treated with minocycline 50 mg/kg (10) and (v) LPS-treated with memantine 10 mg/kg" (11 p. 334). The single dose (5 mg/kg) of intraperitoneal LPS (Sigma-Aldrich, St. Louis, MO. 297-473-0) injections was given on day 5 of the experiment (11). Minocycline and memantine (USP, Rockville, MD) were given intraperitoneally once daily for 14 days to the rats in the minocycline and memantine groups. All rats were subjected to the Novel Object Recognition Test (NORT) from day 15 to day 19. The rats were sacrificed with sodium pentobarbital (100 mg/kg; Alfasan, Woerden, Holland) after 24 hours of NORT. For histological investigation, the brain tissues were promptly removed and stored in a 10% formalin solution.

Novel object recognition test (NORT)

The experiment was conducted from 8 am till 12 pm, and the rats were brought to the behavioural room 2 hours before the experiment for acclimatization. All rats were habituated to the open arena (60 x 60 x 30 cm) by allowing them to freely explore it for 10 minutes per session on first and second days. " On third day, two equivalent objects (A1 & A2) were placed and fastened in a symmetrical location on the right and left sides of the arena, about 10 cm from the wall. Toys made of plastic with a height of 5 cm were used to create these items with identical textures, colors, and sizes but different forms, and the rats were allowed to explore both objects (A1 & A2) for 10 minutes. On the fourth day, the rats were allowed to explore both objects (A1 & A2) for 10 min and returned backed to their cage. After 2 hr, STM test was conducted where object A1 were replaced by novel object B and the rats were allowed to explore both objects (B & A2) for 10 min. On the fifth days, LTM test was conducted (24 hr after STM test) where novel object B was replaced by novel object C and the rats were allowed to explore both objects (C & A2) for 10 min. The amount of time spent studying the objects was meticulously documented. A camera recorded the amount of time spent inspecting each item" (12 p. 557).

To remove scent signal discrimination, all equipment was cleaned with 70% alcohol between sessions. The item position was changed to prevent location preference (right and left). Sniffing or touching the thing with the rat's nose was considered exploration, but sitting on the item was not.

The discrimination index was calculated manually by the examiner, who was blind to all experimental groups during the trial. The entire time spent exploring both objects was recorded and used to generate the discrimination index, as shown below.

$$\text{Discrimination index} = \frac{\text{Total time exploring both}}{\text{Time exploring new item} - \text{time exploring the familiar item}}$$

"The index was designed to evaluate recognition memory. A high score shows that the new item is well-liked. A positive score implies that the known thing is remembered well, whereas a low score displays admiration for the familiar item and signals memory loss" (12 p. 557). The schematic representation of the NORT is shown in Figure 1A.

Histopathological analysis

"Perfusion fixation with 0.1 M phosphate-buffered saline (PBS) (pH 7.0) for two minutes, followed by 4% paraformaldehyde (PFA) (pH 7.0) for three minutes (Fisher Scientific, USA) were performed through intracardiac to fix rats' cortical and hippocampal tissues" (12 p. 557). Next, the right brain hemispheres (10 per group) were collected immediately after perfusion fixation process, post-fixed in 10% formalin solution (Fisher Scientific, USA), and maintained at room temperature until paraffin sectioned according to the standard technique. The six slices of central mPFC (+3.5 mm anterior to bregma) and hippocampi (2.2 mm to 4.6 mm from bregma) utilised for staining were chosen (13). The thickness of tissues in each slide was 5µm.

Congo red staining

The wax was removed from the paraffin sections by immersing them in xylene 1 and 2 solutions for 2 minutes each. Following that, the slides were hydrated for 2 minutes in decreasing ethanol dilutions (100%, 90%, and 70%), washed with running water for 2 minutes, immersed in Congo red solution for 30 minutes, and then cleaned with distilled water to remove excess Congo red. The slides were then dipped 10 times in alkaline phosphatase solution and washed under running water for two minutes. After 30 seconds in hematoxylin solution, the slides were washed with running water for 2 minutes, dehydrated in increasing ethanol dilutions

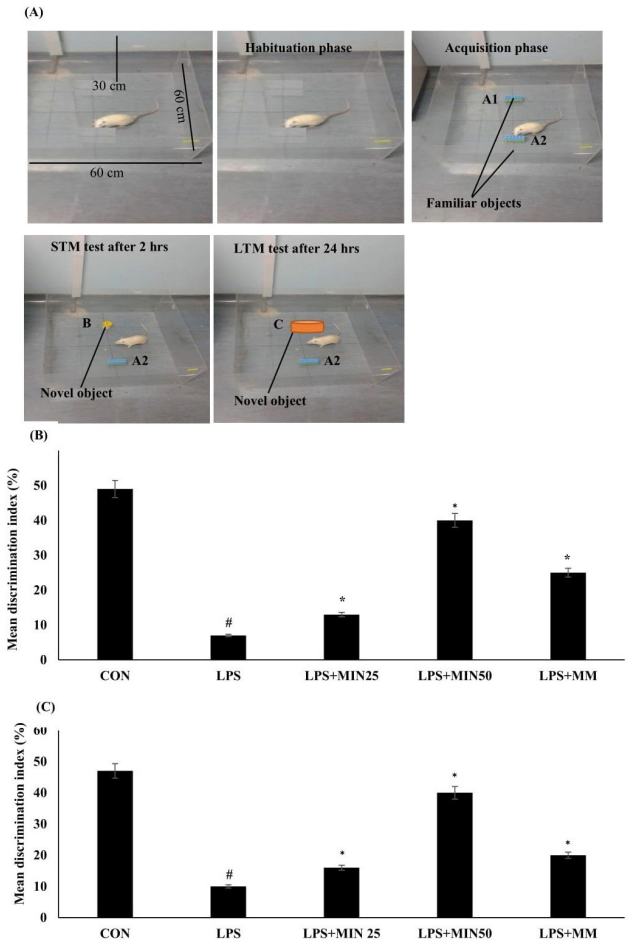

Figure 1: Novel Object Recognition Task procedure (NORT) (A). Mean percentage of discrimination index for long-term (B) and short-term memory (C) during the Novel Object Recognition test. Control (CON), lipopolysaccharide (LPS), Lipopolysaccharide +minocycline 25 mg/kg (LPS+MIN 25), Lipopolysaccharide +minocycline 50 mg/kg (LPS+MIN 50) and Lipopolysaccharide +memantine (LPS+MM). One-way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean ± SEM, n=10 animals in each group. # p<0.05 versus control group; * p<0.05 versus LPS group.

for 2 minutes each, then submerged in xylene 1 and 2 for 2 minutes each before drying for 30 minutes. The slides were then placed in DPX mounting medium (BDH Chemicals, UK) and coated with coverslips (HmbG Inc., Germany) (14). Finally, the slides were analysed by two blinded scientists using a light microscope (Olympus Corporation, Japan) coupled to an image analyzer at 20x objective lens power magnification.

Cresyl violet Staining

"The paraffin sections were dewaxed for 2 minutes each in xylene 1 and 2 solutions. The slides were then hydrated for 2 minutes in decreasing ethanol dilutions, submerged in cresyl violet for 3 minutes, then washed with distilled water to eliminate excess cresyl violet. The slides were then dehydrated for 2 minutes in increasing ethanol dilutions, dipped in xylene 1 and 2 for 2 minutes each, and dried for 30 minutes. Finally, the slides were mounted in DPX mounting media (BDH Chemicals, U.K.) and covered with coverslips (HmbG Inc., Germany)" (12 p. 557).

Slide evaluation

Systematic random sampling was used to choose different slices of cortical and hippocampal tissues from each rat. The cortical and hippocampal slices were photographed using an Olympus DP21 digital camera linked to an Olympus CX-31 light microscope and a computer running image analysis software (Image Proplus, Media Cybernetics) at 20x objective lens power magnification.

At 4x objective lens power, a grid was directed on an image of cortical or hippocampal slices. The grid was then used to choose five locations using a systematic random sampling procedure. The total number of neurons was counted and divided by the number of sections. The neuron number for each rat was then equal to the average of three slices. Body cells that were shrunken or indistinct were excluded and not counted. Two blinded scientists evaluated the slides.

Statistical analysis

The sample size was estimated (15), and " the data were analysed using one-way ANOVA, followed by the Bonferroni post hoc test. The means and standard errors of the mean are used to represent the data (SEM). A probability (p) value of less than 0.05 was considered significant" (12 p. 557).

RESULTS

Minocycline attenuates LPS-induced recognition memory

Figure 1B and 1C depicts the effect of minocycline and memantine treatments on object recognition performance. The discrimination indices of the STM and LTM tests in the LPS group were significantly lower ($p < 0.05$) when compared to the control group (Figure 1B and 1C). Furthermore, the LTM discrimination index was lower than the STM discrimination index, indicating that LTM was more affected than STM in the LPS group. The minocycline (50 mg/kg) group, on the other hand, showed a considerably higher discriminating index ($p<0.05$) than the LPS group. The minocycline (25 mg/kg) and memantine groups showed similar findings; however, their effects were lesser than the minocycline (50 mg/kg) group.

Minocycline attenuates LPS-induced β-amyloid peptide formation

Congo red staining was used to confirm the presence of β-amyloid peptides, particularly in the LPS groups. The results displayed a considerable deposition of β-amyloid peptide formation in all the groups except controls. However, cortical and hippocampal tissues of minocycline and memantine groups showed lesser β-amyloid peptide deposition formation, as shown in Figure 2.

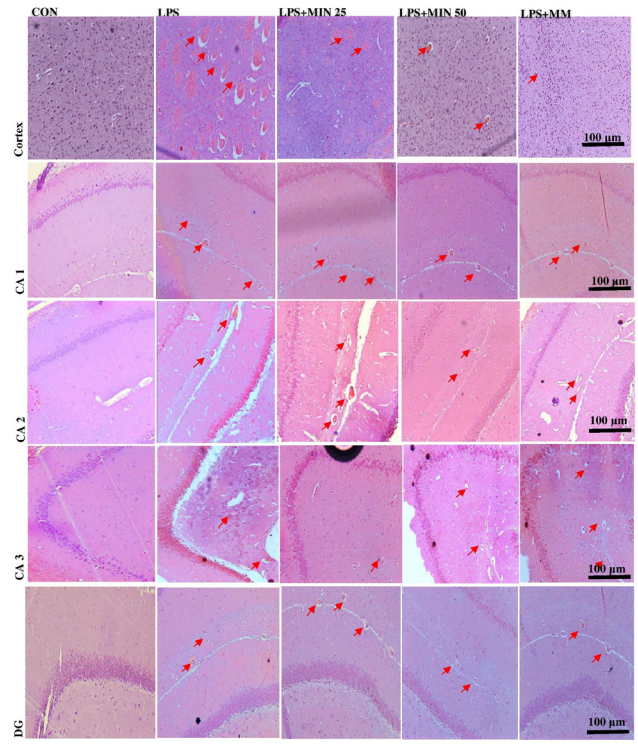

Figure 2: β-Amyloid peptide deposit in cortex and different regions of the hippocampus at 20x objective lens power magnification. Control (CON), lipopolysaccharide (LPS), Lipopolysaccharide +minocycline 25 mg/kg (LPS+MIN 25), Lipopolysaccharide +minocycline 50 mg/kg (LPS+MIN 50) and Lipopolysaccharide +memantine (LPS+MM). β-Amyloid peptide deposit indicated with red arrow. Bar scale 100 µm.

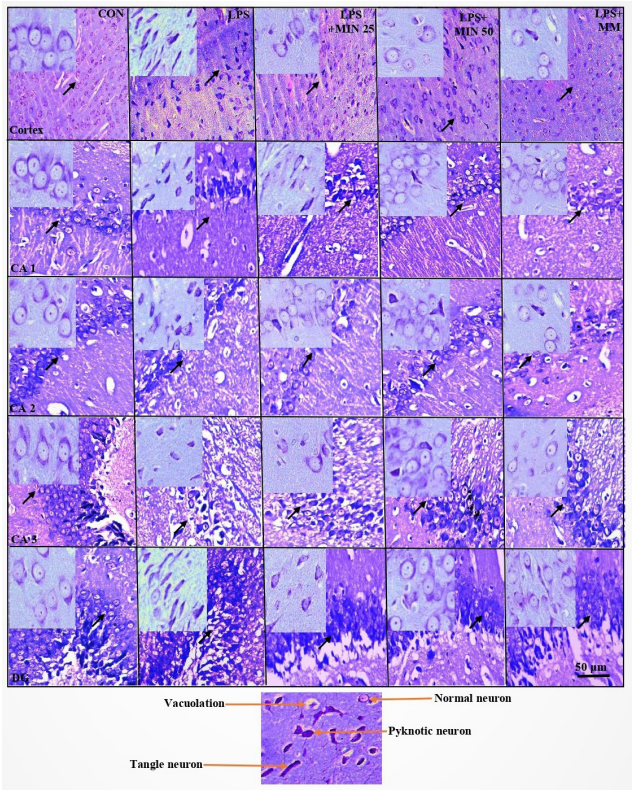

Figure 3: Morphological alterations in the cortex and several hippocampal areas using cresyl violet staining at 20x objective lens power magnification. Control (CON), lipopolysaccharide (LPS), Lipopolysaccharide +minocycline 25mg/kg (LPS+MIN 25), Lipopolysaccharide +minocycline 50 mg/kg (LPS+MIN 50) and Lipopolysaccharide +memantine (LPS+MM) at 40x and 100x lens magnification. The black arrow indicates neuronal damage. Dead cells have a shrinking cytoplasm and a pyknotic nucleus. Higher magnified of normal, pyknotic, tangle-like neurons and vacuolation were shown below. Bar scale 50 µm.

Minocycline attenuates LPS-induced abnormal neuronal morphology

The cortical and hippocampal tissues were stained using cresyl violet to assess neuronal morphology. When compared to the controls, the results revealed a substantial ($p < 0.05$) increase in the number of abnormal neurons in all tissues of the groups. Figure 3 shows that the number of abnormal neurons in the minocycline and memantine groups was reduced. These were supported by the number of intact neurons, which were higher in these groups (Figure 4). The effects were better in the minocycline (50 mg/kg) group than the minocycline (25 mg/kg) and memantine groups.

DISCUSSION

The present study shows three important findings. First, a single administration of LPS intraperitoneally successfully induced β-amyloid peptide deposition formation as well as abnormal neuronal morphology; Second, LPS impaired recognition memory, LTM was more affected than STM. Third, minocycline treatments, especially (50 mg/kg), attenuated LPS-induced β-amyloid peptide deposition, abnormal/loss of cortical and hippocampal neurons, and recognition memory impairment.

LPS, a potent inflammation-causing chemical, mimic the role of live bacteria when delivered systemically or centrally, impairing cognition and promoting sickness behaviours (16). The rats in this present study showed reduced recognition memory, both STM and LTM, after receiving a single dose of i.p. injection of LPS (5 mg/kg) on day 5. "A earlier investigation on the effects of a single dose of LPS (5 mg/kg) i.p injections on cognitive performance in rats confirmed this finding" (11 p. 340). However, previous studies in rats (17, 18, 19) and mice (20, 21, 22) that used smaller doses of intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) LPS injections have shown that LPS damages neurologic structure and impairs cognitive function. As a result, this study investigated the effects of different doses of minocycline in memory impairment-induced by LPS in comparison to memantine. Our study demonstrated that both minocycline and memantine administration effectively alleviated the LPS-induced STM and LTM impairment. Compared to its lower dosage (25 mg/kg) and memantine, minocycline at a 50 mg/kg dose had stronger memory-improving benefits.

"LPS is a neurotoxin that causes inflammation in the brain.

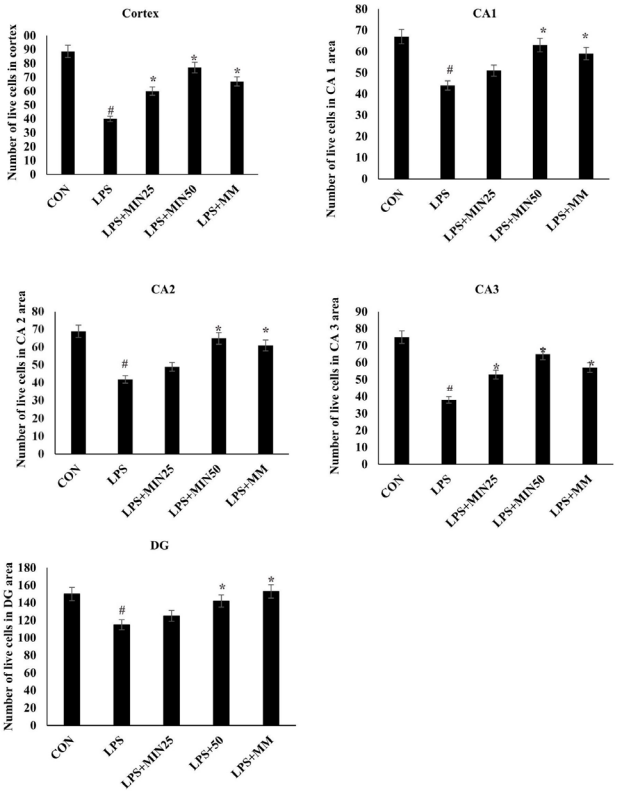

Figure 4: Quantification of undamaged neurons in the cortex and the hippocampus's CA1, CA2, CA3, and DG regions. CON=Control; LPS=Lipopolysaccharide; LPS+MIN 25= Lipopolysaccharide +Minocycline 25 mg/kg; LPS+MIN 50= Lipopolysaccharide +Minocycline 50 mg/kg; LPS+MM= Lipopolysaccharide +Memantine. One-way ANOVA test followed by Bonferroni post hoc test. Values are expressed as mean ± SEM. # p<0.05 versus control and *p<0.05 versus LPS.

The neuroinflammatory response stimulates astrocytes, microglia, and neurons, causing them to generate large amounts of inflammatory mediators such as pro-inflammatory cytokines and chemokines (chemotactic cytokines), as well as reactive oxygen species (ROS)" (23 p. 3). Increased levels of pro-inflammatory cytokines and several acute-phase proteins in the blood, CSF, and brains are closely linked to the disruption and lack of reversibility of the inflammatory response in AD (24). In addition, chronic inflammation stimulates an increase in ROS and nitrosative stress. "Furthermore, it elevates brain levels of β-amyloid peptide by increasing influx of β-amyloid peptide from blood into brain, decreasing efflux of β-amyloid peptide from brain into blood, and processing amyloid precursor protein (APP) to the β-amyloid peptide" (25 p. 507). Another theory proposed that both β-amyloid peptides and tangles trigger a persistent inflammatory response. Inflammatory mediators, in turn, affect amyloidogenic processing, resulting in amyloid-beta (Aβ (1- 42)), creating a vicious cycle (3).

This perpetuation of the inflammatory cycle leads to cognitive impairment, β-amyloid peptide accumulation, and neuronal damage (24, 26). Amnesia induced by LPS in the rodent is one of the well-established models of memory deficits (27). 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 (23,24,28). "In addition, previous studies have shown that LPS impacts Aβ deposition" (29 p. 337) and that anti-inflammatory drugs inhibit Aβ deposition" (30 p. 7504, 20 p. 2). The LPS group formed more significant β-amyloid peptide deposition than the control group in the present study. However, in the cortical and hippocampus areas, minocycline groups showed decreased β-amyloid peptide deposition production, equivalent to the memantine group. Minocycline is a tetracycline of the second generation with anti-inflammatory effects. It has been demonstrated to delay microglial activation (31), diminishes microglial production of different inflammatory parameters such as IL-1, Il-4, IL-10, TNF, and NGF (32, 33), raises BDNF level (33), and neuronal death via apoptosis in various in vitro and in vivo AD models (34, 35, 36, 37, 38).

"LPS may promote neuronal cell death due to increased amyloid peptide accumulation in the cortex and hippocampus areas, which may be linked to memory impairment" (20 p. 12). In addition, we discovered that in vivo, LPS rats' brain showed an increased number of abnormal neuronal morphology and reduced intact neurons, which indicate increased neuronal death. LPS-induced neuronal mortality was decreased by minocycline and memantine therapy, demonstrating that LPS-stimulated neuronal apoptotic cell death directly results from neuroinflammation-induced amyloidosis (20). The effects of minocycline were dose-dependent, whereby a higher dose of minocycline (50 mg/kg) demonstrated better results than its lower dose (25 mg/kg). Interestingly, Aras et al. (2015) (39) also discovered that minocycline's neuroprotective impact is dose-dependent.

Other animal models of memory impairments have shown that therapy with minocycline improves recognition memory (40). Similarly, previous studies elaborated on minocycline's anti-amyloid and anti-inflammatory roles in various neurodegenerative diseases (40–41). The inhibitory effects of minocycline on amyloidogenic and inflammatory pathways explained its beneficial role to act as neuroprotection, preserve the neuronal structure and improve memory deficits-induced AD (41). Furthermore, we compared minocycline with memantine administration. These data suggested comparable effects of both treatments in reducing β-amyloid peptide deposition, neuronal damage, and recognition memory impairment induced by LPS.

Our findings postulated that minocycline acts through its potent anti-amyloidogenic properties to reduce

β-amyloid peptide deposit and neuronal damage in the cortex and hippocampus and improve cognitive behaviours. This is the first study to look at the effects of two distinct minocycline doses on LPS-induced memory impairment in adult rats. More research is needed to better understand the mechanisms through which minocycline improves memory while avoiding neuronal damage.

CONCLUSION

The study found that minocycline (50 mg/kg) had a better impact than memantine (10 mg/kg) in attenuating LPS-induced recognition memory impairment by lowering β-amyloid peptide deposition and neuronal death in the rats' cortex and hippocampus, whereas minocycline (25 mg/kg) had a similar effect. Thus, by modulating the neuroinflammation process, minocycline may have a potential preventive-therapeutic impact for neurodegenerative diseases.

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