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" In Rats, Minocycline Prevents LPS-Induced Neuronal Death and Memory Impairment."

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ABSTRACT-

Numerous animal models have shown that minocycline has strong impacts on the architecture and functioning of the nervous system. Its neuroprotective qualities, however, after a single lipopolysaccharide (LPS) injection in an adult rat model remain unclear. This study examined the neuroprotective properties of minocycline in a rat model of LPS-induced neuroinflammation.. **Methods:** Five sets of fifty-year-old male Sprague Dawley rats were created: (i) LPS treated with distilled water, (ii) LPS treated with 25 mg/kg minocycline, and (iv) LPS treated with 50 mg/kg minocycline. treated LPS and (v) LPS treated with 10 mg/kg memantine. Day 5 was a single intraperitoneal injection of LPS (5 mg/kg), followed by daily injections of minocycline and memantine for a period of 14 days.

Results: It was discovered that minocycline treatment significantly attenuated the effects of LPS on β-amyloid peptide buildup, neuronal damage, and memory loss. According to these findings, LPS causes neuronal damage and β-amyloid peptide buildup in the cortex and hippocampus, which subsequently affects cognitive memory. Additionally, we examined the administration of minocycline and memantine. The results indicated that, although minocycline (50 mg/kg) therapy produced superior outcomes, minocycline

(25 mg/kg) and memantine (10 mg/kg) treatment were comparable. It lessens memory loss, neuronal damage, and beta-amyloid peptide formation brought on by LPS..

Conclusions: These results suggest that minocycline might be a promising therapy option for neuroinflammatory conditions including Alzheimer's disease (AD).

Keywords: Alzheimer's disease, memantine, neuroinflammation, minocycline, and cognitive decline etc

1. Introduction-

"Over 46 million people worldwide suffer from dementia.". It is projected that the number of cases of dementia in Japan would increase to 7 million by 2025 and 131.5 million by 2050. About 60% of instances of dementia are caused by Alzheimer's disease (1), making it the most prevalent neurological illness. It is more common in older adults; most cases affect those 65 years of age and beyond. After the age of 65, the chance of developing the condition doubles every five years, and after the age of 85, the risk approaches 50% (2). A toll-like receptor-4 (TLR-4) is activated by the endotoxin lipopolysaccharide (LPS), which initiates the cascade of the systemic inflammatory response. When the LPS-TLR-4 complex attaches to the surface of microglia, it initiates many signaling pathways, including phosphoinositide NF-κB is activated by rapamycin mammalian target (mTOR), mitogen-activated protein kinase (MAPK), and 3-kinase/protein kinase B (PI3K/AKT). Pro-inflammatory chemokines and cytokines, as well as inducible enzymes like cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), are created when NF-κB is activated . As a result of these processes, β-amyloid peptide, intraneuronal neurofibrillary tangle (NFT) development, neuroinflammatory damage, and cognitive impairment appeared as clinical and pathological AD hallmarks (3, 4, 5). For more than 30 years, minocycline—a second-generation tetracycline antibiotic—has been utilized to combat both gram-positive and gram-negative bacteria. It can be utilized to treat a range of CNS disorders since it is a highly lipophilic molecule that easily crosses the blood-brain barrier (BBB) and accumulates in CSF and central nervous system (CNS) cells. According to earlier research, minocycline exhibits "anti-inflammatory effects in various AD rat models," "anti-amyloidogenic" , and "neuroprotective" (7). Consequently, the current research tested the neuroprotective effects of two distinct minocycline doses and contrasted them with the conventional medication, memantine, a clinically licensed N-methyl-D-aspartate (NMDA) receptor antagonist, using the LPS-induced neuroinflammation rats' model.

MATERIALS AND METHODS

Animals

We acquired fifty adult male Sprague Dawley rats from Animal Research and Service Centre (ARASC). All of the rats were kept in 32 x 24 x 16 cm polypropylene cages in a 23°C environment with 12-hour light/dark cycles and free access to food and drink. The research and ethics committee of this approved the experimental design, which adhered to internationally recognized standards for the use and care of laboratory animals.

Experimental design

The rats were divided into five groups, each with ten rats: (i) LPS-treated with distilled water, (ii) control (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, respectively. On the fifth day of the experiment, a single intraperitoneal LPS injection was administered (11). The rats in the memantine and minocycline groups received intraperitoneal injections of both drugs once a day for a duration of 14 days. The rats in the memantine and minocycline groups received intraperitoneal injections of both drugs once a day for a duration of 14 days. From day 15 to day 19, every rat was put through the Novel Object Recognition Test (NORT). After a 24-hour period of NORT, the rats were euthanized using sodium pentobarbital (100 mg/kg; Alfasan, Woerden, Holland). Brain tissues were immediately extracted and preserved in a 10% formalin solution for histological examination.

Novel object recognition test (NORT)

The rats were transported to the behavioral room two hours before to the experiment so they could become acclimated. The experiment ran from eight in the morning to twelve in the evening. On the first and second days, all rats were allowed to freely explore the open arena (60 x 60 x 30 cm) for ten minutes each session in order to acclimate them to it. " On the third day, two identical items (A1 & A2) were positioned and secured symmetrically on the arena's left and right sides, around 10 cm from the wall. These objects (A1 & A2) had the same textures, colors, and sizes but distinct shapes since they were constructed from plastic toys that were 5 cm in height. The rats were free to investigate both of the objects for 10 minutes. After ten minutes of exploration of both items (A1 & A2) on the fourth day, the rats were put back in their cage. Two hours later, the rats were given the opportunity to investigate both items (B & A2) for ten minutes each, after which the novel object B was substituted for object A1 in the STM test. The rats were given ten minutes to investigate both novel items (C & A2) during the fifth day's LTM test, which was carried out 24 hours following the STM test. Novel object B was swapped out for novel object C. The length of time devoted to examining the things was carefully recorded. The duration of time spent examining every object was captured by a camera. Every piece of equipment was cleaned with 70% alcohol in between sessions to eliminate fragrance signal discrimination. To avoid location preference (right and left), the item position was altered. Sitting on the object was not deemed exploration, but using the rat's nose to sniff or touch it was. During the trial, the examiner, who was blind to

all experimental groups, manually determined the discrimination index. The discrimination index, which is displayed below, was created by recording the whole amount of time spent examining both items.

Discrimination index = Total time exploring both / Time exploring new item – time exploring the familiar item

The index's purpose was to assess memory for recognition. A high score indicates that people enjoy the new item. While a low score indicates respect for the recognized object and suggests memory loss, a high score suggests that the known thing is recalled well Figure 1A displays the schematic depiction of the NORT.

Histopathological analysis

Rats' cortical and hippocampus tissues were fixed using intracardiac perfusion fixation using 0.1 M phosphate-buffered saline (PBS) for two minutes and 4% paraformaldehyde (PFA) for three minutes (Fisher Scientific,. Subsequently, following the perfusion fixation procedure, ten right brain hemispheres per group were harvested, post-fixed in 10% formalin solution (Fisher Scientific, USA), and kept at room temperature until paraffin sectioning in accordance with standard protocol. The six hippocampi and central mPFC (+3.5 mm anterior to bregma) slices that were used for staining were selected (13). Each slide had tissues that were 5 µm thick.

Congo red staining

The paraffin sections were submerged in xylene 1 and 2 solutions for two minutes each in order to remove the wax. The slides were then hydrated in ethanol dilutions (100%, 90%, and 70%) for two minutes, rinsed with running water for two minutes, submerged in Congo red solution for thirty minutes, and then cleaned with distilled water to get rid of any leftover Congo red. After dipping the slides ten times in an alkaline phosphatase solution, they were given a two-minute rinse under running water. The slides were soaked for 30 seconds in hematoxylin solution, then rinsed for two minutes under running water and dried with progressively dilutions of ethanol.

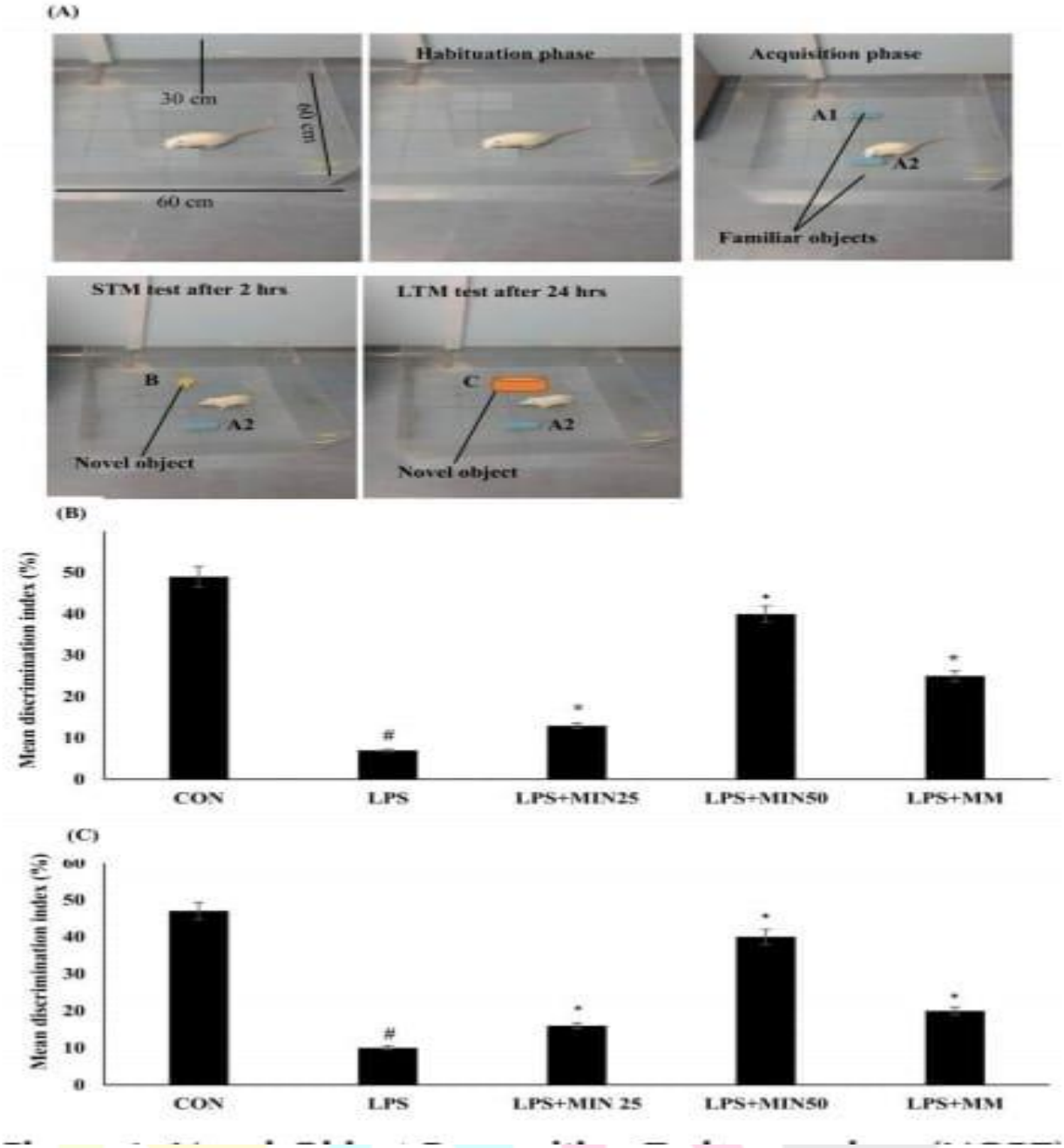

Figure 1: Novel Object Recognition Task procedure (NORT)

(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

"In xylene 1 and 2 solutions, the paraffin sections were dewaxed for two minutes each. After hydrating the slides for two minutes in progressively diluted ethanol, they were immersed in cresyl violet for three minutes, and any leftover cresyl violet was removed by washing them with distilled water. The slides were then soaked in xylene 1 and 2 for two minutes each, dried for thirty minutes, then dehydrated for two minutes in progressively higher ethanol dilutions. Ultimately, the slides were coated with coverslips and mounted in DPX mounting fluid

Slide evaluation

Several cortex and hippocampus tissue slices were selected from each rat using systematic random sampling. Using an Olympus DP21 digital camera, an Olympus CX-31 light microscope, and a computer running Image Proplus image processing software, the cortical and hippocampus slices were captured on camera at a 20x objective lens power magnification.A grid was focused on an image of cortical or hippocampus slices at 4x objective lens power. Then, using a systematic random selection technique, five places were selected using the grid. We tallied the neurons and divided the total by the number of sections. The average of three slices was then used to determine the neuron number for each rat. Body cells that were blurry or shrunken were not included in the count. Two scientists who were blinded assessed the slides.

Statistical analysis

A one-way ANOVA was used to analyze the data, and the Bonferroni post hoc test was performed when the sample size of fifteen was established. The data are represented as means and standard errors of the mean (SEM). It was determined that a probability (p) value of less than 0.05 was significant.

RESULTS

Minocycline reduces recognition memory generated by LPS.

The impact of memantine and minocycline treatments on object identification ability is seen in Figures 1B and 1C. When comparing the LPS group to the control group, the discrimination indices of the STM and LTM tests were considerably lower ($p < 0.05$) (Figure

1B and 1C). Additionally, the LTM discrimination score was lower in the LPS group than the STM discrimination index, suggesting that LTM was more impacted than STM. In contrast, the group treated with 50 mg/kg of minocycline had a significantly greater discriminating index ($p<0.05$) in comparison to the LPS group. The memantine and minocycline (25 mg/kg) groups also had comparable results, although their impacts were not as great as those of the minocycline (50 mg/kg) group.

Minocycline reduces the production of β-amyloid peptides generated by LPS.

To verify the existence of β-amyloid peptides, especially in the LPS groups, Congo red staining was employed. The findings showed that all groups, with the exception of the controls, had a significant deposition of β-amyloid peptide formation. Figure 2 illustrates that the cortical and hippocampal tissues of the memantine and minocycline groups exhibited reduced production of β-amyloid peptide deposition.

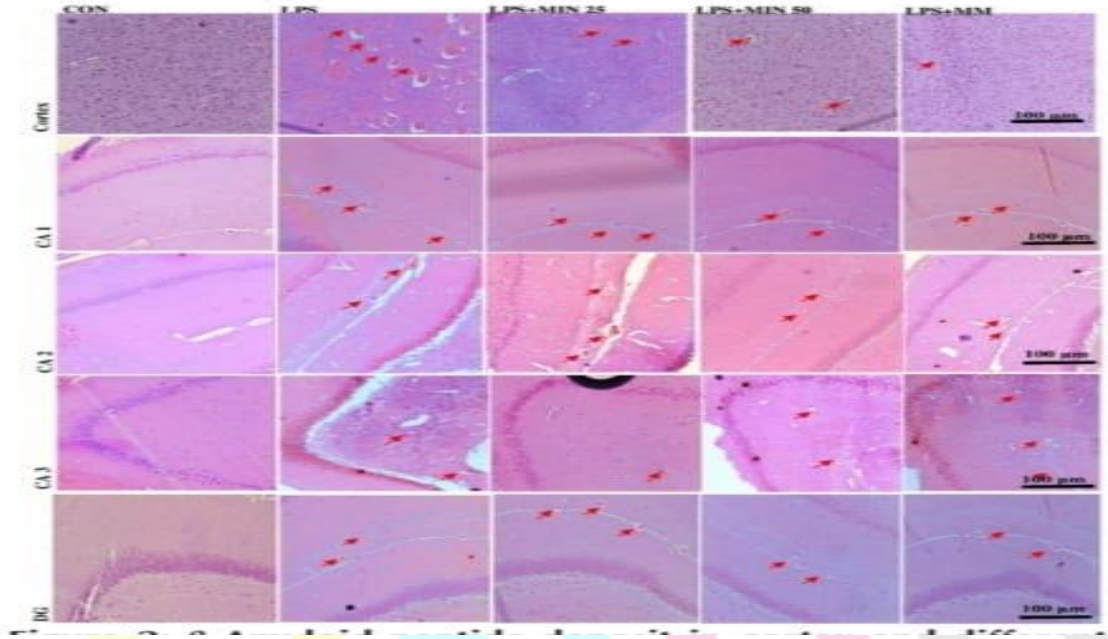

Figure 2: β-Amyloid peptide deposit in cortex and different regions of the hippocampus at 20x objective lens power magnification. Control (CON), lipopolysaccharide (LPS), Li-popolysaccharide +minocycline 25 mg/kg (LPS+MIN 25), Li- popolysaccharide +minocycline 50 mg/kg (LPS+MIN 50) and Lipopolysaccharide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

Minocycline reduces the aberrant neuronal morphology brought on by LPS.

To evaluate the morphology of the neurons, cresyl violet staining was used to the cortical and hippocampal tissues. The results showed a significant ($p <0.05$) increase in the number of aberrant neurons in all tissues of the groups as compared to the controls. As can be seen in Figure 3, there were fewer aberrant neurons in the memantine and minocycline groups. These were corroborated by the fact that these groups had more undamaged neurons (Figure 4).

Compared to the memantine and minocycline (25 mg/kg) groups, the minocycline (50 mg/kg) group showed superior effects.

DISCUSSION

Three significant findings are presented in this study. Firstly, the intraperitoneal treatment of LPS was successful in inducing the production of β-amyloid peptide deposition and aberrant neuronal morphology in a single dose; Secondly, LPS was shown to impair recognition memory, with LTM being more impacted than STM. Third, the effects of LPS-induced β-amyloid peptide deposition, abnormal/loss of cortical and hippocampal neurons, and impairment of recognition memory were reduced by treatments with minocycline, particularly at 50 mg/kg.LPS is a strong molecule that causes inflammation that, whether administered systemically or

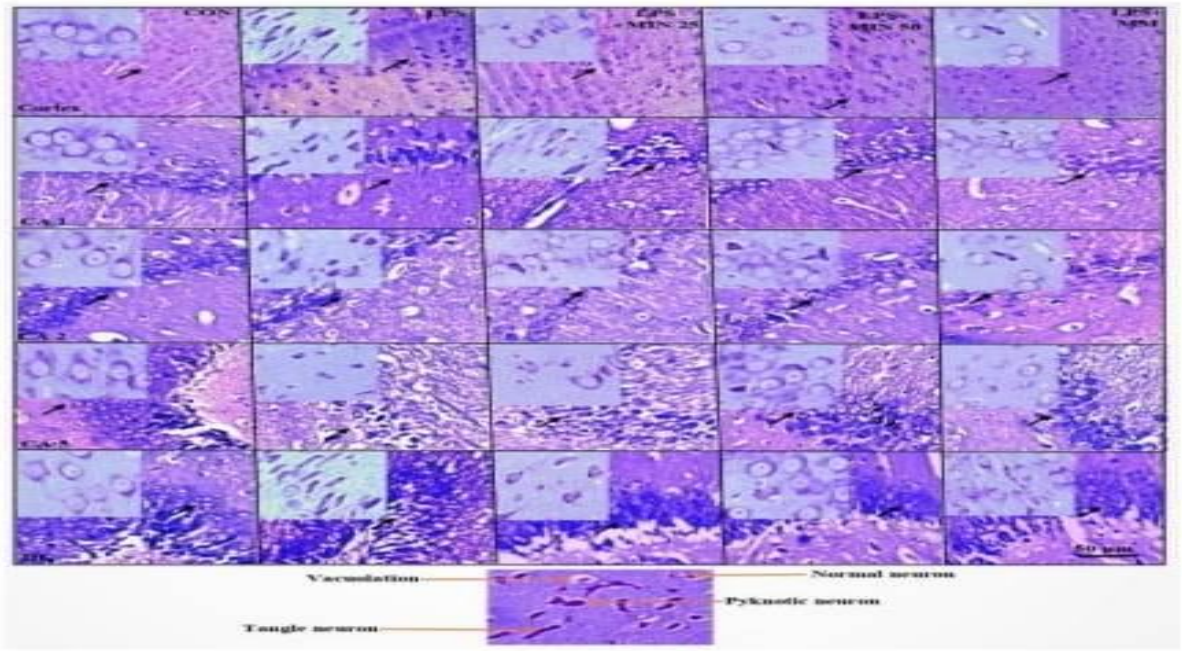

Figure 3: Morphological alterations in the cortex and several hippocampal areas using cresyl violet staining at 20x objective lens power magnification. Control (CON), lipopolysaccharide (LPS), Lipopolysaccharide +minocycline 25mg/kg (LPS+MIN 25), Lipopolysaccharide +minocycline 50 mg/kg (LPS+MIN 50)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

primarily causing mental impairment and encouraging unhealthy behaviors (16). On day 5, the rats in this study had an intraperitoneal injection of 5 mg/kg of lipopolysaccharide (LPS) as a single dosage, which resulted in decreased recognition memory (STM and LTM). "This finding was confirmed by an earlier investigation on the effects of a single dose of LPS (5 mg/kg) intraperitoneal injections on rats' cognitive performance" .Nonetheless, prior research

using lower doses of intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) LPS injections in rats (17, 18, 19) and mice (20, 21, 22) has demonstrated that LPS alters neuronal structure and reduces cognitive performance. Therefore, in contrast to memantine, this study examined the effects of various dosages of minocycline on memory impairment caused by lipopolysaccharide (LPS). Our research showed that administering memantine and minocycline both successfully reduced the LPS-induced impairment of STM and LTM. In comparison to memantine and its lesser dosage of 25 mg/kg, minocycline at a 50 mg/kg dose shown greater memory-improving effects.An inflammation of the brain is brought on by the neurotoxin LPS.

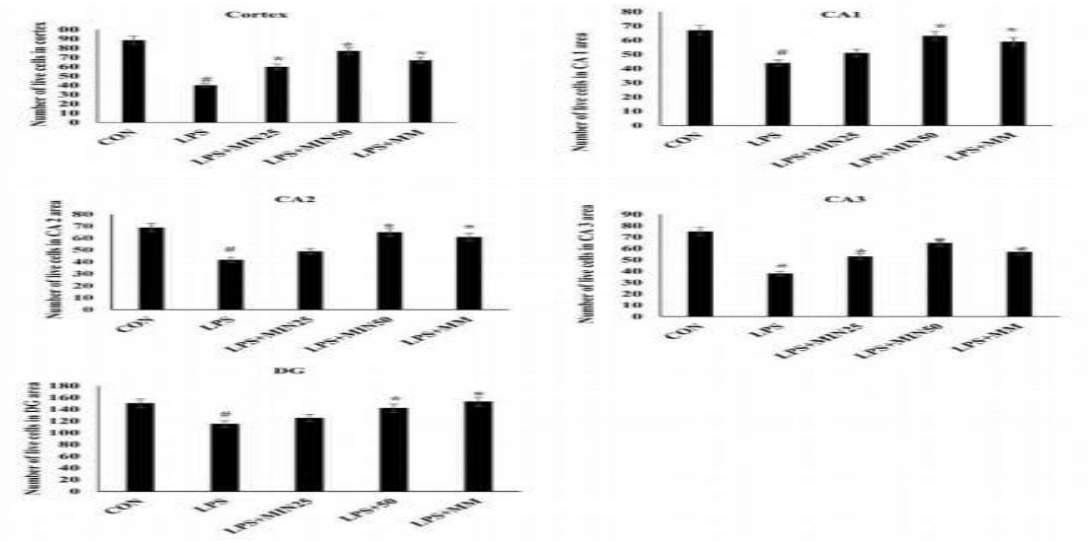

Figure 4: Quantification of undamaged neurons in the cor- tex and the hippocampus's CA1, CA2, CA3, and DG re- gions. CON=Control; LPS=Lipopolysaccharide; LPS+MIN 25= Lipopolysaccharide +Minocycline 25 mg/kg; LPS+MIN50= Lipopolysaccharide +Minocycline 50 mg/kg; LPS+MM= Lipopolysaccharide +Memantine. One-way ANOVA test fol- lowed by Bonferroni post hoc test. Values are expressed as mean ± SEM. # $p<0.05$ versus control and *$p<0.05$ versus LPS

Reactive oxygen species (ROS) and pro-inflammatory and chemokines (chemotactic cytokines) are among the many inflammatory mediators that are produced by microglia, neurons, and astrocytes in response to the neuroinflammatory response . Disruption and irreversibility of the inflammatory response in AD are strongly associated with elevated levels of pro-inflammatory cytokines and many acute-phase proteins in the blood, CSF, and brains (24). Furthermore, ROS and nitrosative stress are elevated in chronic inflammation. Additionally, it raises the amount of β-amyloid peptide in the brain by converting amyloid precursor protein (APP) into the β-amyloid peptide, increasing the amount of β-amyloid peptide that enters the brain from blood, and lowering the amount that exits the brain from brain . According to a different idea, tangles and β-amyloid peptides both cause an ongoing inflammatory response. A vicious loop is created when inflammatory mediators influence amyloidogenic processing, which leads to amyloid-beta (Aβ) . The ongoing inflammatory

cycle results in neuronal injury, β-amyloid peptide buildup, and cognitive decline (24, 26). One of the well-known models of memory impairments in rodents is amnesia brought on by lipopolysaccharide (LPS) (27). Previous research employing a variety of behavioral tests showed that LPS produced β-amyloid peptide deposit and inflammation in the brain tissue resulted in significant learning and memory impairment (28). Moreover, earlier research has demonstrated that LPS affects Aβ deposition (29) and that anti-inflammatory medications prevent Aβ deposition (30). In the current investigation, the LPS group exhibited a greater degree of β-amyloid peptide deposition compared to the control group.

On the other hand, groups treated with minocycline exhibited a similar reduction in β-amyloid peptide deposition formation in the cortical and hippocampal regions as the memantine group. A second-generation tetracycline with anti-inflammatory properties is minocycline. It has been shown to delay the activation of microglia (31), reduce the production of various inflammatory parameters by microglia, including IL-1, IL-4, IL-10, TNF, and NGF (32, 33), increase the level of BDNF (33), and cause neuronal death through apoptosis in a variety of in vitro and in vivo AD models (34, 35, 36, 37, 38).

Because of increased amyloid peptide buildup in the regions of the cortex and hippocampus, LPS may induce neuronal cell death, which may be related to memory impairment . Furthermore, we found that the brains of LPS-treated rats displayed a higher number of aberrant neuronal morphology and a lower number of intact neurons in vivo, both of which are indicators of accelerated neuronal death. Minocycline and memantine treatment reduced LPS-induced neuronal mortality, indicating that neuroinflammation-induced amyloidosis is the direct cause of LPS-stimulated neuronal apoptotic cell death . Because of the dose-dependent nature of minocycline's effects, greater doses (50 mg/kg) of the drug produced better outcomes than lower doses (25 mg/kg). Interestingly, dosage dependence was also found for the neuroprotective effect of minocycline by Aras et al. (39).

Minocycline treatment has been demonstrated to enhance recognition memory in several animal models of memory impairment (40). Likewise, earlier research expounded upon the anti-inflammatory and anti-amyloid properties of minocycline in a range of neurodegenerative disorders (40). The protective impact of minocycline in acting as neuroprotection, preserving the neuronal structure, and improving memory deficits-induced AD was explained by its inhibitory effects on amyloidogenic and inflammatory pathways (41). Additionally, we contrasted the administration of memantine with minocycline. These results indicated that both therapies had similar effects on lowering neuronal damage, β-amyloid peptide deposition, and recognition memory impairment brought on by LPS.

Our research suggested that minocycline improves cognitive behaviors by reducing β-amyloid peptide deposits and neuronal damage in the cortex and hippocampus through its strong anti-amyloidogenic effects. This is the first investigation into how two different dosages of minocycline affect adult rats' LPS-induced memory impairment. To learn more about the processes by which minocycline enhances memory without harming neurons, more study is required.

CONCLUSION

The study discovered that whereas minocycline (25 mg/kg) had a comparable effect, minocycline (50 mg/kg) had a greater ability to reduce LPS-induced recognition memory impairment by reducing β-amyloid peptide deposition and neuronal death in the rats' cortex and hippocampus. Thus, minocycline may have a potential role in neurodegenerative disease prevention and treatment by regulating the process of neuro inflammation.

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