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Original Research Article

Modulation of Nitric Oxide and Interleukin-6 Levels in Lipopolysaccharide (LPS) - Induced Neurotoxicity in Wistar Rat Models

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Abstract: Neuroinflammation plays a central role in neurodegenerative diseases, with lipopolysaccharide (LPS) serving as a widely used experimental model for inducing neurotoxicity. This study investigates the modulation of nitric oxide (NO) and interleukin-6 (IL-6) in LPS-induced neurotoxicity using wistar rat models. 20 Wistar rat models weighing 150 - 180g were used and randomly assigned into four groups: Group 1 (Control), Group 2 (Low dose LPS - 0.25mg/kg), Group 3 (Medium dose LPS - 0.5mg/kg), Group 4 (High dose LPS - 1.0mg/kg). LPS was administered intraperitoneally, and neuroinflammatory markers were quantified using biochemical assays. Neurobehavioral activities (Navigation test, Object Recognition, and Barnes maze test) were recorded and analyzed using ANOVA. Results showed that NO and IL-6 levels increased in a dose- dependent manner following LPS exposure. The High-dose LPS group exhibited the highest levels of NO and IL-6, correlating with significant neuronal damage in the hippocampus. Elevated NO production suggests oxidative stress-mediated neurotoxicity, while increased IL-6 levels indicate an amplified inflammatory response contributing to synaptic dysfunction. Histopathological analysis revealed neurodegeneration, particularly in the hippocampal CA1 and CA3 regions, further supporting the role of inflammation in cognitive impairment. These findings highlight the interplay between NO and IL-6 in LPS-induced neurotoxicity and suggest that targeting these pathways could offer therapeutic potential for neuroinflammatory disorders, including Alzheimer's disease and Parkinson's disease. Understanding how these inflammatory mediators contribute to cognitive dysfunction may lead to novel strategies to mitigate neurodegeneration and improve cognitive outcomes in neuroinflammatory conditions.

Keywords: Lipopolysaccharide, Neuroinflammation, Nitric Oxide, Interleukin-6, Neurotoxicity.

INTRODUCTION

Neuroinflammation has emerged as a key contributor to cognitive dysfunction and neurodegenerative diseases. Inflammatory mediators such as Nitric Oxide (NO) and interleukin-6 (IL-6) play crucial roles in modulating neuroinflammatory responses. Nitric Oxide is a signaling molecule involved in neurotransmission and synaptic plasticity, but excessive NO production contributes to oxidative stress, mitochondrial dysfunction, and neuronal apoptosis (Garthwaite, 2008). IL-6, a pro-inflammatory cytokine, influences synaptic remodeling and is implicated in both neuroprotection and neurotoxicity (Gruol, 2015).

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is commonly used to model neuroinflammation due to its ability to activate Toll-like receptor 4 (TLR4) and induce cytokine production (Skrzypczak-Wiercioch & Sałat, 2022). While LPS-induced neuroinflammation has been linked to cognitive deficits, the specific modulation of NO and IL-6 in response to varying LPS doses remains underexplored.

Nitic Oxide plays a dual role in brain physiology. Under normal conditions, it facilitates synaptic plasticity, but during inflammation, Nitric Oxide overproduction leads to oxidative stress,

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disrupting cellular homeostasis and triggering neuronal death (Zhang *et al*., 2023). Similarly, IL-6 is involved in immune responses and neurodevelopment, but chronic elevation of IL-6 has been associated with neurodegeneration and impaired synaptic function (McKim *et al.,* 2016).

Elevated levels of NO and IL-6 are observed in neurodegenerative diseases such as Alzheimer's and Parkinson's disease, where chronic neuroinflammation exacerbates neuronal loss (Wen *et al*., 2016). The purpose of this study is to investigate how Nitric Oxide (NO) and Interleukin -6 (IL-6) levels affect the brain when induced with lipopolysaccharide (LPS). Investigating their modulation in LPS-induced neurotoxicity can provide insights into therapeutic interventions aimed at mitigating neuroinflammation-related cognitive dysfunction.

MATERIALS AND METHODS

Experimental Animals

Experimental rats were purchased from the animal house of the Faculty of Basic medical sciences, Abuja campus, University of Port Harcourt. The animals were housed in steel cages and kept at room temperature. The rats had no history of drug consumption, that is; they had not been used for any previous investigation. The rats were put on standard rat pellet (feed) and pure drinking water and allowed to get acclimatized for 21 days before the start of the experiment. The study was done in accordance with the guidelines for animal use of the Faculty of Basic Medical Sciences, University of Port Harcourt.

Ethical Approval

Ethical approval was obtained from the faculty of basic medical science, Abuja campus, University of Port Harcourt. Rat handling and treatment conform to the guideline of the National Research Council (2011) for care and use of laboratory animals.

Chemicals and Reagents

The chemicals and reagents used for this study were purchased from GGI Intl' Nigeria Ltd. located at GGI Place, Plot 8 GGI Crescent, (Opp. Mikab Filling Station), Port Harcourt, Rivers State, Nigeria. The chemicals and reagents include:

a. **Sodium nitrite ($NaNO_2$)** - Used as a standard for nitric oxide (NO) estimation.
b. **Interleukin-6 (IL-6) -** Used for the determination of IL-6 concentrations in serum and hippocampal tissue.
c. **Lipopolysaccharide (LPS)** - Used to induce neuroinflammation in Wistar rat models.
d. **Griess Reagent** – Employed for the quantification of NO levels in serum and brain tissue.
e. **Phosphate -buffered saline -** Used for sample dilution and washing steps in ELISA assays.
f. **Formalin (10% neutral buffered formalin) -** Used for tissue fixation before histopathological analysis.
g. **Paraffin wax** - Used for embedding brain tissue sections.
h. **Hematoxylin and eosin (H&E) stains** - Used for histological examination of hippocampal tissue.
i. **Primary and secondary antibodies for IL-6 detection** - Used in immunohistochemistry to assess IL-6 expression in hippocampal tissues.

All reagents used in the experiment were of analytical grade and were used according to the manufacturer's recommendations to ensure consistency.

Experimental Design

The research design employed for this study was a Randomized Control Trial (RCT), using Wistar rats as a model. The animals were divided into four groups: Group 1 (Control), Group 2 (Low dose LPS - 0.25mg/kg), Group 3 (Medium dose LPS - 0.5mg/kg), Group 4 (High dose LPS p 1.0mg/kg). LPS was administered intraperitoneally (IP) once daily for seven days and blood samples were taken from the rats for biochemical analysis at specific time intervals. A succinct overview of the experimental design is shown below.

Table 1: The Experimental Design Showing the Group, LPS Dose, Treatment and Behavioural Tests

Groups	LPS Dose	Treatment	Behavioural Tests
Group 1	None	Control	Navigation Test, Object Recognition, Barnes Test.
Group 2	Low (0.25mg/kg)	LPS + Tests	Navigation Test, Object Recognition, Barnes Test.
Group 3	Medium (0.5mg/kg)	LPS + Tests	Navigation Test, Object Recognition, Barnes Test.
Group 4	High (1.0mg/kg)	LPS + Tests	Navigation Test, Object Recognition, Barnes Test.

Induction of LPS-Induced Neurotoxicity

LPS, a neurotoxic agent, was administered to the rats via the Intraperitoneal route. The dosage was stratified into low: 0.25mg//kg, medium: 0.5mg/kg, and high: 1.0mg/kg, doses to mimic varying degrees of neuroinflammation. The Intraperitoneal (IP) route was chosen due to its common use in similar studies and its ability to induce a consistent and measurable inflammatory response.

The administration protocol adhered to ethical standards, ensuring the welfare of the animals. The timing of LPS administration was carefully controlled to synchronize with the experimental design.

Blood Collection

Blood samples were collected at specific time points from the tail vein during the experiment to assess systemic markers, including cytokines. The collection process adhered to ethical guidelines and minimized stress on the animals.

Histopathology

Hippocampal sections were examined using toluidine blue staining to assess neuronal integrity. Tissue samples were collected for histopathological examination to identify structural changes in the brain associated with LPS-induced neurotoxicity. Brain sections were stained with toluidine blue and examined for neuronal damage, focusing on the hippocampus (CA1, CA3, and dentate gyrus regions). Neuronal loss and microglial activation were quantified.

Biochemical Analysis: Brain tissue homogenates were analyzed for NO levels (Using Griess reagent) and IL-6 levels (Using ELISA).

Guidelines from the National Research Council on the handling of laboratory animals (National Research Council, 2011) and Principles and techniques of histopathological examination as outlined by Bancroft *et al*., (2004) were followed.

Statistical Analysis: Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS). Descriptive statistics, including means and standard deviations, were calculated for each group. To assess the effects of LPS treatment on spatial behavior, inferential statistics, such as Analysis of Variance (ANOVA) or t-tests, were employed. Post-hoc tests may be used for further comparisons if necessary.

RESULTS AND DISCUSSION

The experimental groups in this study were categorized as follows:

Group 1: Negative Control, Group 2: 0.25mg/kg of LPS (low dose), Group 3: 0.5mg/kg of LPS (medium dose), Group 4: 1mg/kg of LPS (high dose).

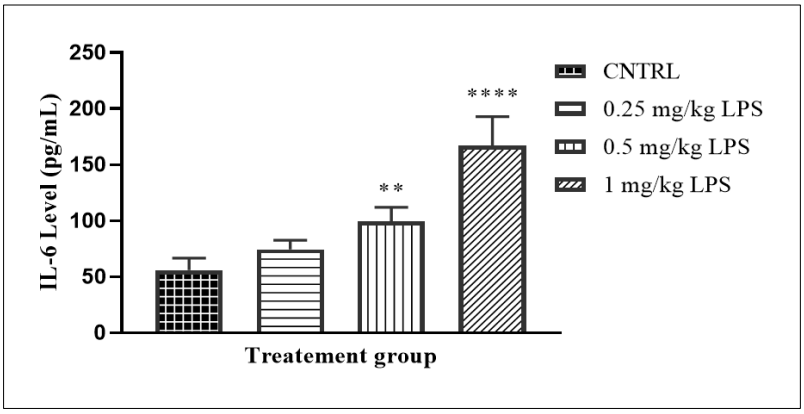

Figure 1: Effect of IP administration of different doses of LPS on interleukein-6 levels on wister rats. Results are presented as mean ± SEM. N=5

The columns represent the mean values and error bars indicate the Standard deviation (SD) of our independent experiments. * Indicates statistical significance of LPS treatments versus the Negative controls * significant at P < 0.05, ** significant at 0.01, *** significant at 0.001 and **** significant at 0.0001.

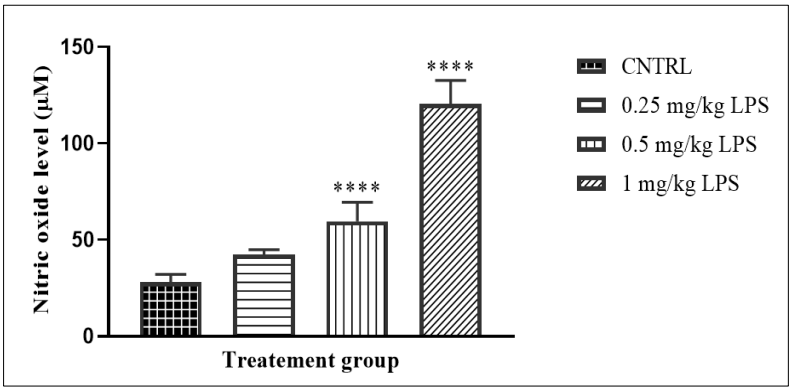

Figure 2: Effect of IP administration of different doses of LPS nitric oxide levels on wister rats. Results are presented as mean ± SEM. N=5

The columns represent the mean values and error bars indicate the Standard deviation (SD) of our independent experiments. * Indicates statistical significance of LPS treatments versus the Negative controls * significant at P < 0.05, ** significant at 0.01, *** significant at 0.001 and **** significant at 0.0001.

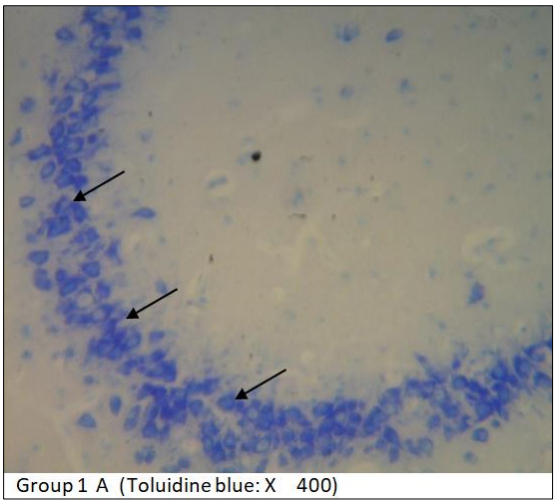

Figure 3: Photomicrograph (Toluidine X400) of the normal Hippocampus showing CA3 region of the cornu ammonis, showing few layers of large pyramidal cells in CA3 region, also with vesicular nuclei (arrows).

Diagnosis: Normal Hippocampus.

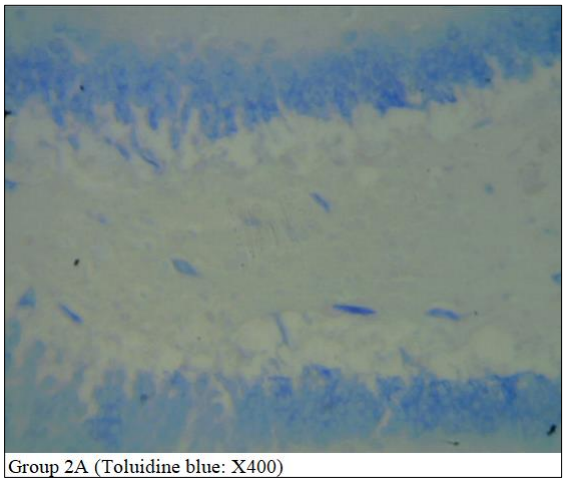

Figure 4: Photomicrograph (Toluidine X400) of the Hippocampus showing moderate pyramidal cell loss with an associated diffuse chromatolysis.

Diagnosis: Mild pyramidal Cell Loss.

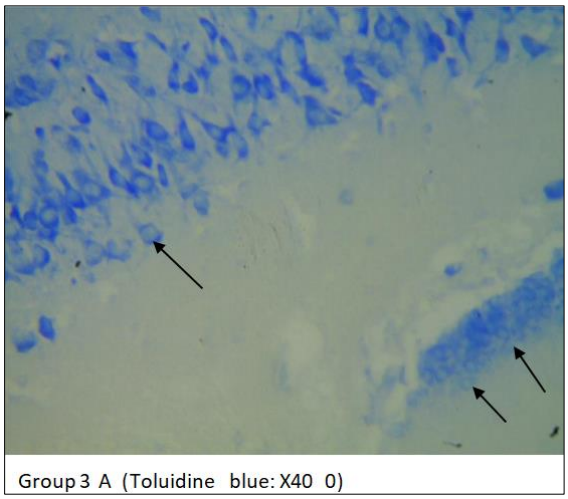

Figure 5: Photomicrograph (Toluidine blue X400) of the Hippocampus showing minimal neuronal cell loss associated with chromatolysis (arrows)

Diagnosis: Minimal Neuronal Cell Loss.

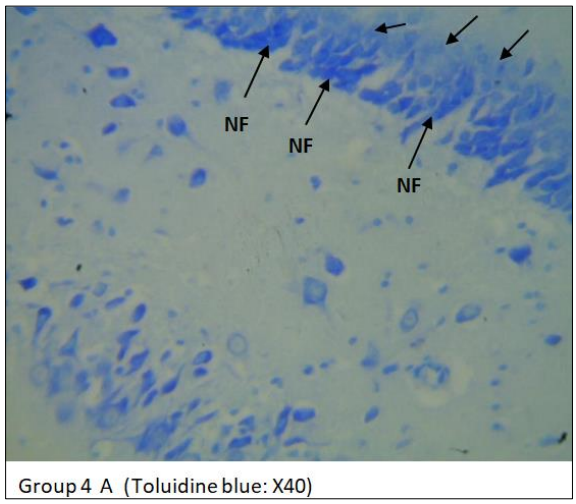

Figure 6: Photomicrograph (Toluidine blue X400) of the Hippocampus showing minimal neuronal darkening and multiple necrotic foci (NF) with associated chromatolysis

Diagnosis: Minimal Neuronal Darkening.

DISCUSSION

Biochemical Findings

NO Levels: Increased in a dose-dependent manner, with the High-Dose LPS group exhibiting the highest NO concentration.

IL-6 Levels: Elevated IL-6 levels were observed in LPS-treated rats, correlating with increased neuroinflammation.

Histopathological Findings

Neuronal Damage: Increased neuronal loss was observed in the High-Dose LPS group, particularly in the CA1 and CA3 regions of the hippocampus.

Microglial Activation: Higher microglial activation was noted in LPS-treated groups, consistent with increased IL-6 levels.

Analysis of Interleukin-6 (IL-6) Levels

IL-6 levels were observed to have increased dose-dependently, with the High-dose group exhibiting the highest concentration. Statistical analysis (ANOVA) indicates significant differences between groups 3- 4 ($p < 0.05$).

A clear dose-response relationship, with IL-6 levels increasing proportionally with the dose of LPS. This suggests that higher doses of LPS result in a more pronounced inflammatory response. Within each group, there is a general trend of increased IL-6 levels over time. This indicates that the inflammatory response induced by LPS persists and may even intensify over the observation period. The control group shows a minimal increase in IL-6 levels. This can be considered within the normal variation and may be attributed to factors unrelated to LPS administration. The transition to high doses of LPS results in a substantial increase in IL-6 levels. This suggests a potential threshold effect where a certain level of LPS is required to trigger a significant neuroinflammatory response. Elevated IL-6 levels are associated with various neurological conditions. The observed increases in IL-6 support the hypothesis that LPS-induced neurotoxicity contributes to neuroinflammation, potentially impacting cognitive functions.

The above findings are similar to that of X. Li *et al*., (2009) who carried out a similar survey and found that LPS dramatically induced astrocytes to secrete IL-6 in a dose-dependent manner.

In conclusion, the IL-6 data strongly indicates a dose-dependent rise, providing valuable insights into the implications of neurotoxicity on the immune environment in the brain. These findings contribute to our understanding of the molecular mechanisms underlying neuroinflammation in the context of LPS exposure.

Analysis of Nitric Oxide Levels (µM)

The result shows similar dose-dependent trend in nitric oxide levels, with the High Dose group having the highest concentration. Statistical analysis indicates significant differences between group 3 and 4 ($p < 0.05$).

The study revealed a discernible dose-dependent response in Nitric Oxide levels. As the LPS dose increased from low to high, there was a progressive elevation in Nitric Oxide concentrations. This implies that the administration of LPS correlates with an increased production of Nitric Oxide in a dose-dependent manner. Statistical analysis demonstrated significant differences in Nitric Oxide levels between the various dosage groups. The high dose groups exhibited notably higher levels compared to the control and low dose groups. This discrepancy underscores the importance of

dosage in influencing Nitric Oxide modulation. Nitric Oxide serves as a key signaling molecule in various physiological processes, including neurotransmission. The observed fluctuations in Nitric Oxide levels bear significance in the context of neural function. Elevated levels may be indicative of heightened cellular activity, inflammation, or response to neurotoxic stimuli.

The findings align with existing literature that associates LPS administration with increased Nitric Oxide production. Studies by Yang *et al*., (2012) have reported similar dose-dependent relationships between LPS exposure and Nitric Oxide synthesis. This consistency strengthens the validity of the current study's results and underscores the reproducibility of these patterns across different experimental setups. However, it is essential to note discrepancies in specific thresholds and magnitudes of Nitric Oxide response, which could be attributed to variations in experimental conditions, species differences, or methodological approaches (Yang *et al*., 2012).

The Nitric Oxide findings provide valuable insights into the nuanced dynamics of LPS-induced neurotoxicity. The dose-dependent response and significant differences between groups emphasize the need for a comprehensive understanding of Nitric Oxide modulation in neuroinflammatory processes. These results contribute to the growing body of literature on neuroimmune interactions and provide a foundation for further investigations into the intricate mechanisms of LPS-induced neurotoxicity.

Histopathology Analysis

The photomicrographs presented in figures 3 to 6, indicates a detailed view of the hippocampus under different conditions, which was captured using Toluidine staining at magnification of X400. The figures tracks and traces changes in the structure of the hippocampus, specifically showing changes in the cornu ammonis (CA) regions CA1 - CA4, the dentate gyrus, and the subiculum. Each photograph displayed has a diagnosis that indicate the extent of necrotic foci, neuronal health, loss and darkening.

Neuronal Damage: Increased neuronal loss was observed in the High-Dose LPS group, particularly in the CA1 and CA3 regions of the hippocampus.

Microglial Activation: Higher microglial activation was noted in LPS-treated groups, consistent with increased IL-6 levels.

Figure 3 has the diagnosis of a normal hippocampus, showing that all cellular layers, especially in the CA3 region, display the expected morphology with no indication of cell loss or chromatolysis. These normal findings in the structure of the hippocampus are in line with Per Andersen *et al*., (2007) studies that details the organized and structural layering of pyramidal cells and their role in cognitive function and processes. Also, the presence of vesicular nuclei is a distinctive characteristic of healthy neurons, as they signify cellular metabolism and active transcription necessary for proper neuronal function (Witter, 2010).

Figures 4 and 5 presents the example of mild to moderate neuronal cell loss. Dissolution of the Nissl body, scientifically known as chromatolysis, normally implies a cellular reaction to injury and is frequently observed in states in which neurons are under stress or damaged (Cowan *et al*., 2004). This coupled with a loss of pyramidal cells could show early signs/symptoms of degenerative processes inside the hippocampus.

The unpleasant effect of the mild to moderate cell loss are important for a number of reasons, one of which is because pyramidal cells are intrinsic to the organization and capacity of the hippocampus. The observed chromatolysis may also indicate cellular stress extending to the molecular level that might result from metabolic demands or excitotoxicity through over-stimulation of neurons that results in neuron death (Krieglstein, 1997).

Minimal Neuronal Darkening and Necrosis

Figure 6 shows little neuronal darkening and includes necrotic focuses (NF) with chromatolysis. Neuronal darkening and necrosis signify very high level of cellular stress or injury and can be best described by cellular changes that feature condensed nucleus and shrunken cytoplasm commonly before the cell death (A. W. Brown *et al*., 1979). Hippocampal necrosis may be due to ischemic conditions, in which insufficient blood circulation leads to a deficiency of oxygen and glucose, or due to excitotoxicity which is typical of acute neurological diseases.

These findings showed in this figure tally with studies that record the securing of necrotic foci and chromatolysis due to oxidative stress, a phenomenon well observed in hippocampal tissue subsequent to hypoxic or ischemic events (Adameova *et al*., 2022).

CONCLUSION

This study delved into the modulation of NO and IL-6 levels in LPS neurotoxicity with Wistar rats as models. The key findings were:

Impact of NO on Neuroinflammation:

The observed increase in NO levels suggests that LPS-induced neurotoxicity involves oxidative stress-mediated neuronal damage. Excess NO disrupts mitochondrial function, leading to apoptotic cell death (Garthwaite, 2008). Previous studies have shown that NO overproduction exacerbates neuroinflammation and contributes to synaptic dysfunction, aligning with our findings (Zhang *et al*., 2023).

Role of IL-6 in LPS-Induced Neurotoxicity:

Elevated IL-6 levels in LPS-treated rats indicate an amplified inflammatory response. IL-6 has been implicated in neurodegenerative diseases due to its ability to disrupt synaptic transmission and promote neuronal apoptosis (McKim *et al*., 2016). The dose-dependent increase in IL-6 supports the notion that chronic neuroinflammation leads to cognitive impairments.

Histopathological Correlates

Histological analysis revealed neuronal loss and microglial activation, consistent with NO and IL-6 elevation. The High-Dose LPS group exhibited the most severe neurodegeneration, suggesting a direct link between inflammatory marker elevation and neuronal damage (Wen *et al*., 2016).

Clinical Implications

Targeting NO and IL-6 pathways could provide therapeutic benefits in neuroinflammatory conditions. Strategies such as NO synthase inhibition and IL-6 receptor blockade may help mitigate neuroinflammation-associated cognitive decline (Gruol, 2015).

In summary, his study demonstrates that NO and IL-6 levels are significantly modulated in LPS-induced neurotoxicity, with dose-dependent increases correlating with neuroinflammation and neuronal damage. The findings provide insights into the role of these mediators in neurodegenerative diseases and highlight potential therapeutic targets for reducing neuroinflammatory damage. Future studies should explore pharmacological interventions to modulate NO and IL-6 pathways in neuroinflammatory disorders.

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