ORIGINAL RESEARCH

Med J SDU / SDÜ Tıp Fak Derg ▸ 2025:32(1):27-35 ▸ doi: 10.17343/sdutfd.1518104

The Effect of Nebivolol on Acute Brain Damage in a Rat Model of Lps-Induced Inflammation

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Cite this article as: Oguzoglu AS, Senol N, Doğuç DK , Agirca S, Erzurumlu Y, Asci H. The Effect of Nebivolol on Acute Brain Damage in a Rat Model of Lps-Induced Inflammation. Med J SDU 2025;32(1):27-35.

Abstract

Objective
This research seeks to determine whether nebivolol (NEB) can prevent brain damage caused by lipopolysaccharide (LPS).

Material and Method
The thirty-two female Wistar Albino rats were divided into four groups, each containing eight rats: LPS (5mg/kg single dose i.p.), Control, LPS+NEB, and NEB (1 ml of 10 kg mg/kg given by oral gavage every day for three days). In brain tissues, the following parameters were measured: tumor necrosis factor-alpha (TNF-α), vascular endothelial growth factor A (VEGFA), caspase-3 (Cas-3), total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI).

Results
In the LPS group, there was an increase in the levels of TOS, VEGFA, Cas-3, and TNF-α. NEB therapy markedly reduced TOS, VEGFA, TNF-α, and Cas-3 levels. In both the control and NEB groups, histopathological analysis of the brain, hippocampus, and cerebellum demonstrated normal tissue architecture. In the LPS group, there were mild to moderate hemorrhages and severe hyperemia in the meningeal and parenchymal arteries of the brain and cerebellum. The LPS + NEB group's histopathology results were significantly ameliorated by NEB treatment.

Conclusion
NEB treatments anti-inflammatory, anti-apoptotic, and antioxidant characteristics helped mitigate the brain damage caused by LPS. NEB may help to reduce the severity of LPS-induced damage.

Keywords: LPS, Brain, Nebivolol, oxidative stress, inflammation, apoptosis

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Received: 04.09.2024 • Accepted: 25.11.2024
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Introduction

Neuroinflammation is an important factor contributing to cognitive impairment, various neurodegenerative diseases, and brain injury (1,2). Hypoxia-ischaemia (HI), insulted as a result, exaggerates the brain injury. Loss of oligodendroglial precursors, myelination abnormalities, astrogliosis (especially in the periventricular regions), and bilateral ventricular dilatation are the hallmarks of the inflammatory response. Furthermore, inflammation is the most powerful predictor of brain lesions (3,4). Inflammatory cells and their mediators can directly reach the central nervous system (afferent fibers of the abdominal vagus nerve, non-tight connection areas of the blood-brain barrier, etc.), activate cellular death mechanisms, and destroy cellular functions (5).

A significant part of the gram-negative bacterial wall, lipopolysaccharide (LPS) is an endotoxin that can trigger the creation of immune cells, pro-inflammatory cytokines, and cytotoxic agents like TNF-α, nitric oxide (NO), and interleukin-1 (IL-1), leading to numerous severe inflammatory disorders and dementia (6-9). Therefore, it is used in experimental inflammation models to mimic the systemic inflammatory response (10). Translocation of mitogen-activated protein kinases (MAPK), an upstream regulator of nuclear transcription factor-κB (NF-κB), is crucial for developing this neurodegeneration. The production of factors like VEGF and NO, as well as inflammatory cytokines like TNF-α, IL-6, and IL-1β, is stimulated by translocated nuclear factor-κB (NF-κB) (8).

Vascular Endothelial Growth Factor (VEGF) and its primary isoform VEGFA are pivotal signaling proteins in both physiological and pathological angiogenesis. VEGFA, commonly called VEGF, is primarily responsible for promoting blood vessel formation by interacting with specific receptors on vascular endothelial cells. This signaling pathway is crucial for vascular development during embryogenesis and tissue regeneration and repair processes in adults. VEGFA has important roles in angiogenesis and neuroprotection processes in the brain (11). It is also known that VEGFA enhances the permeability between cells and increases inflammation (12). Therefore, an imbalance in the VEGFA / VEGF receptor pathway has been predicted as an indicator of prognosis in inflammation (13,14).

Acute inflammation may be accompanied by an overabundance of a situation that is beneficial to the organism in small or medium amounts, which causes oxidative stress, often referred to as reactive oxygen species (ROS), to be produced at the cellular level. The shift of the balance between the pro-oxidant / antioxidant system can incline to the direction of oxidants, due to excessive ROS production and can damage the tissues. (15, 16). In the evaluation of the oxidant/antioxidant system, TOS and OSI are used as oxidant parameters, TAS are used as antioxidant parameters, and these parameters are oxidative stress indicators (16-18).

NEB is a beta-1 adrenergic receptor antagonist that is used to treat hypertension (19). NEB has antioxidant, vasodilator, and anti-inflammatory properties. NEB inhibits NF-κB, which results in a decrease in the levels of various pro-inflammatory cytokines. It also has antiapoptotic effects (20-22).

In the light of this study aims to investigate the ameliorative role of NEB in LPS-induced brain injury, considering its effect in reducing oxidative damage, inflammation, and apoptosis.

Material and Method

Animals and Ethical Acceptance

32 female Wistar Albino rats weighing 250-350 grams lived in a room with controlled temperature (21°C to 22°C) and humidity (60 ± 5%), as well as a 12:12 h light/dark cycle. All rats were given regular commercial chow (Korkuteli Yem, Antalya, Turkey).

Experimental Design

The four groups of rats—Control, LPS, NEB, and LPS+NEB groups—each had eight rats. (Figure 1)

1-Control Group (n = 8); Once a day for three days, a single dosage of one milliliter (ml) of saline was given orally via gavage, and thirty minutes later, a single dose of one milliliter of PS was given intraperitoneally (i.p.).

2-LPS Group (n = 8); 1 ml of saline was administered by oral gavage on 1-3 days and dissolved in saline and i.p. 5 mg/kg LPS (048K4126, Sigma Aldrich, USA) was administered 30 minutes after the last oral gavage saline administration (23).

3-NEB Group (n = 8); A single dose of 1 ml i.p. saline was given 30 minutes after the last oral gavage NEB treatment, and 1 ml of 10 mg/kg NEB was given by oral gavage every one to three days (22).

4-LPS + NEB (n = 8); 30 minutes after the last oral gavage NEB treatment, i.p. 5 mg/kg LPS (048K4126, Sigma Aldrich, USA) was given. 1 ml of 10 mg/kg NEB

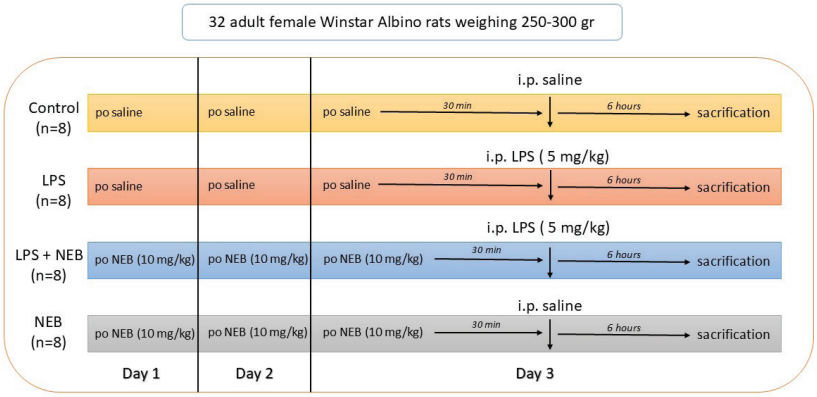

Figure 1
Experimental Design.
Po, Per Oral; i.p., İntraperitoenal; LPS, lipopolysaccharide; NEB, nebivolol.

was provided by oral gavage every one to three days and dissolved in saline.

All rats were given injections of 10 mg/kg Xylazine (Alfazin, Alfasan IBV) and 90 mg/kg ketamine intraperitoneally (i.p.) (Alfamin, Alfasan International B.V., Netherlands) to induce anesthesia six hours after the LPS was administered. After the trial, After the brain was removed, a sagittal cut at the level of the midbrain was made to separate the two hemispheres. One hemisphere was placed in formaldehyde for histopathological and immunohistochemical analyses, while the other hemisphere was further divided. One part was stored at -20°C for biochemical analyses, and the remaining part was allocated for genetic analyses and stored at -80°C.

Biochemical Analysis
Determination of the Parameters of Oxidative Stress
Rat brain tissues, weighing about 150 mg apiece, were homogenized using a phosphate-buffered saline (PBS) solution. After homogenization, samples were centrifuged at 10,000 rpm for 10 min. Using an automated analyzer fitted with Erel's colorimetric method, the values of the Oxidative Stress Index (OSI), Total Oxidant Status (TOS), and Total Antioxidant Status (TAS) in the samples were determined (Beckman Coulter, USA). After computing [(TOS, µmol/l) / (TAS, mmol Trolox eq/l)×100], the OSI value was determined (24,25).

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)
Total RNA was extracted from rat tissue samples using a GeneJET RNA purification kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The cDNA synthesis was performed from approximately 500 - 1 µg of RNA sample using the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Subsequently, a qRT-PCR assay was carried out using an iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) with conditions consisting of 30 seconds at 95°C followed by 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. A fluorescence signal was detected on a CFX connect instrument (Bio-Rad Laboratories, Hercules, CA). The sequences of the primer were designed to amplify VEGFA (Forward 5'-GGA AGA GAG AGA GAG AGA GAG AC-3', Reverse 5'-GAC TGG TCC GAT GAA AGA TCC-3'). Per cDNA samples were analyzed in triplicates for each PCR. The expression of GAPDH (Forward 5'-CAA GGT CAT CCC AGA GCT GAA-3', Reverse 5'-CAT GTA GGC CAT GAG GTC CAC-3') was used for normalization. The relative gene expression was determined using the 2–ΔΔCt method. The results were presented as a fold change in the graph.

Analysis of Immunohistochemistry and Histopathology
Histopathological Analysis
Brain and cerebellum samples were obtained and stored in a 10% neutral formalin solution. Samples

were routinely processed and paraffin-embedded after fixing. After the paraffin blocks cooled, 5 µm thick sections were stained with hematoxylin-Eosin (HE). Analysis of the samples was done using a light microscope.

Immunohistochemical Analysis:
Furthermore, two series of sections from each block drawn on poly-L-lysine coated slides were immunohistochemically stained for the expression of TNF-α (Anti-TNFα Antibody (52B83):sc-52746, 1/100 dilution) and caspase-3 (Anti-caspase-3 Antibody (E-8): sc-7272) Santa Cruz (Texas, USA) using the streptavidin-biotin technique as directed by the manufacturer. After the sections were treated with the primary antibodies for 60 minutes, biotinylated secondary antibodies and streptavidin-alkaline phosphatase conjugate were used for immunohistochemistry. DISCLOSE The secondary antibody was a Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) (Abcam, Cambridge, UK). As the chromogen, diaminobenzidine (DAB) was employed. Primary antibodies were substituted with antigen dilution solution for the negative controls.

For each antibody, slices were examined independently for immunohistochemical examination. Semiquantitative analysis was utilized to determine the degree of immunohistochemical reactivity of the cells with markers, using a grading score ranging from (0) to (3) as follows: 0 denote no expression, 1 focal and weak staining, 2 diffuse and weak staining, and 3 diffuse and marked staining respectively. In each part, ten distinct locations were inspected under 40X objective magnification for evaluation (Olympus Co., Tokyo, Japan). After being saved, the outcomes were statistically examined.

Statistical Analysis
The presentation of the variables was as mean ± standard deviation. The groups' biochemical, histopathological, and genetic results were compared using ANOVA (post hoc LSD and Duncan) tests. Statistical computations were performed utilizing the SPSS 18.0 program pack (SPSS Inc., Chicago, IL, USA). A significance threshold of P<0.05 was used.

Results

Oxidative Stress Markers in Brain Tissue
When comparing the LPS group to the control group, the levels of TOS were found to be significantly greater, and when comparing the LPS+NEB group to the LPS group, they were shown to be reduced (p = 0,016 and p = 0,029, respectively). Compared to the LPS group, the NEB group's TOS levels were considerably lower (p = 0.001). OSI levels were significantly greater (p = 0,018) in the LPS group compared to the control group, whereas they were lower in the NEB group (p = 0,005). TAS levels were significantly greater in the NEB group than in the LPS group (p = 0,011) (Table 1).

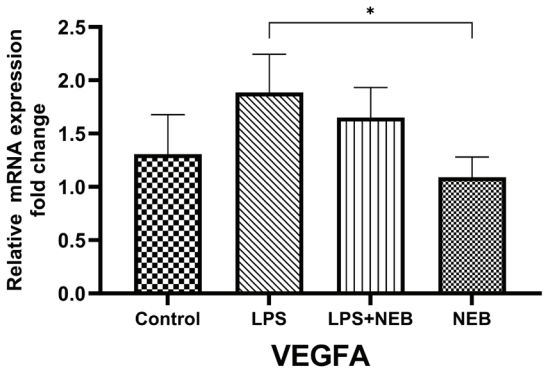

Figure 2
Analysis of VEGFA mRNA expression level by qRT-PCR.
The total RNA was extracted, and the VEGF gene expression was assessed by qRT-PCR assay. Gene expression was standardized based on GAPDH expression, and relative gene expression was calculated using the 2–ΔΔCt method. One-way ANOVA was used to compare the groups, and a post hoc LSD test was used to evaluate the results. The graph's fold change was used to display the results. The control value was 1. The values are shown as means ± SD. Vascular endothelial growth factor, VEGF; * p<0.05; LPS, lipopolysaccharide; NEB, nebivolol.

Table 1 Oxidative stress markers for brain tissues.

	Control	LPS	LPS+NEB	NEB	P value
TAS	0,67 ± 0,05	0,57 ± 0,14	0,65 ± 0,44	0,71 ± 0,10	Control vs. LPS = NS LPS vs. LPS+NEB = NS LPS vs. NEB < 0.05
TOS	8,15 ± 1,05	9,83 ± 1,32	8,32 ± 1,09	7,33 ± 1,68	Control vs. LPS < 0.05 LPS vs. LPS+NEB < 0,05 LPS vs. NEB = 0,001
OSI	1,22 ± 0,16	1,91 ± 1,03	1,28 ± 0,16	1,06 ± 0,30	Control vs. LPS <0,05 LPS vs. LPS+NEB <0,05 LPS vs. NEB < 0,01

Data are means ± SD. Comparison between groups and results of oxidative stress markers were assessed by a one-way ANOVA test that followed by post hoc Tukey's multiple comparison test. LPS, lipopolysaccharide; NEB, nebivolol; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index.

Relative VEGFA mRNA Expression Levels in Brain Tissues

The LPS group's relative VEGFA mRNA level rose significantly in comparison to the NEB group (p=0,013). In the LPS group, relative VEGFA mRNA levels were lower than in the LPS+ NEB group and greater than in the control group, respectively. Nevertheless, these differences did not reach statistical significance (p> 0.05). (Figure 2)

Histopathological and Immunohistochemical Results

In both the control and NEB groups, histopathological analysis of the brain, hippocampus, and cerebellum demonstrated normal tissue architecture. In the LPS group, there were mild to moderate hemorrhages and severe hyperemia in the meningeal and parenchymal arteries of the brain and cerebellum. The brains and cerebellum of the LPS group showed a large number

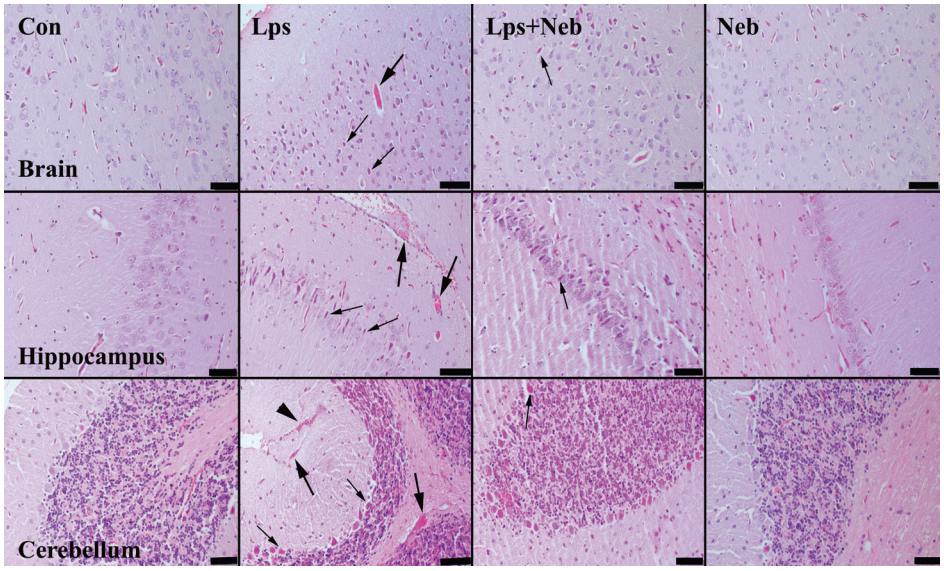

Figure 3

Histopathological appearance of the brain cortex, hippocampus, and cerebellum between the groups. Several degenerative pink coloured neurons in the brain cortex, hippocampus, and cerebellum (thin arrows), severe hyperemia (thick arrows), hemorrhages in the cerebellum (arrowhead), lipopolysaccharide (LPS), NEB (nebivolol), and H&E (scale bars= 50µm)

of deteriorated neurons, whereas normal neurons were observed in the control and NEB groups. Purkinje cells were the most afflicted cells in the cerebellum. The histopathology results in the LPS + NEB group were dramatically improved compared to the LPS group (Figure 3).

Cas-3 and TNF-α immunostained slides were examined using immunohistochemistry, and the LPS group showed significant expression of both markers. Figures 4-5 show that the LPS + NEB group's expressions were reduced compared to the LPS group. The results of the statistical analysis of immunohistochemistry scoring are displayed in Figure 6.

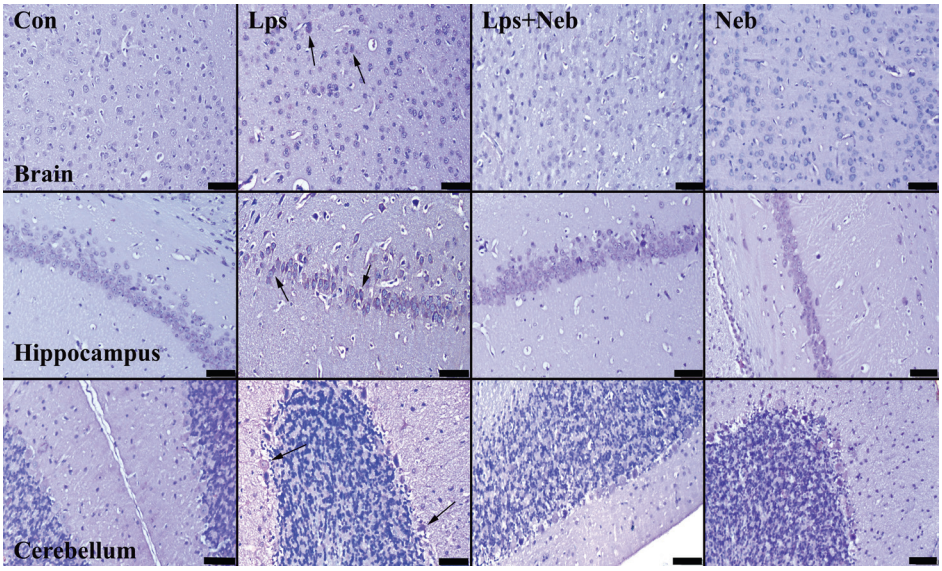

Figure 4

Cas-3 immunohistochemistry findings among the groups.

Increased expressions in neurons (arrows) in the brain cortex, hippocampus, and cerebellum in the LPS group, LPS, lipopolysaccharide; NEB, nebivolol, Streptavidin biotin peroxidase method, scale bars= 50µm

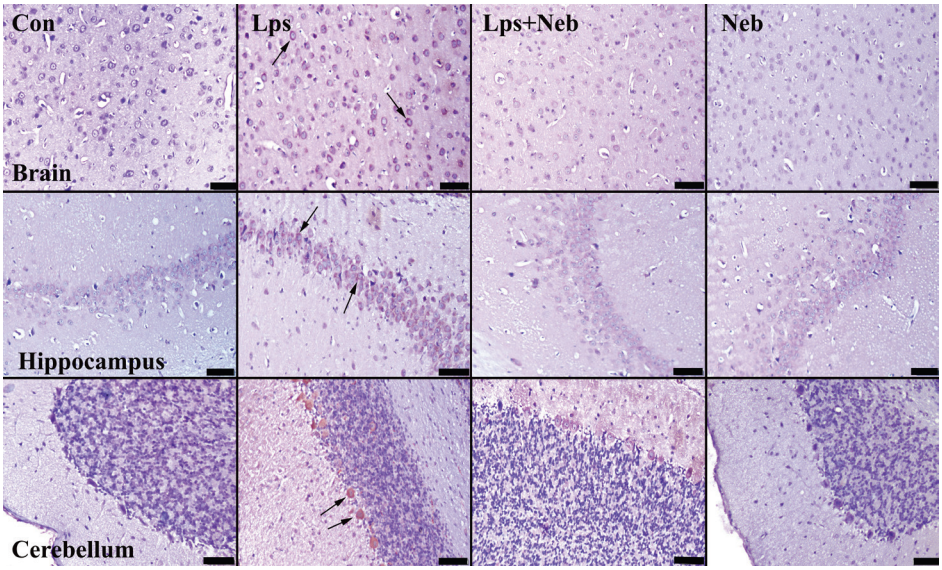

Figure 5

TNF-α immunohistochemistry findings among the groups.

Markedly increased in expressions in neurons (arrows) in brain cortex, hippocampus, and cerebellum in LPS group, LPS, lipopolysaccharide; NEB, nebivolol, Streptavidin biotin peroxidase method, scale bars= 50µm.

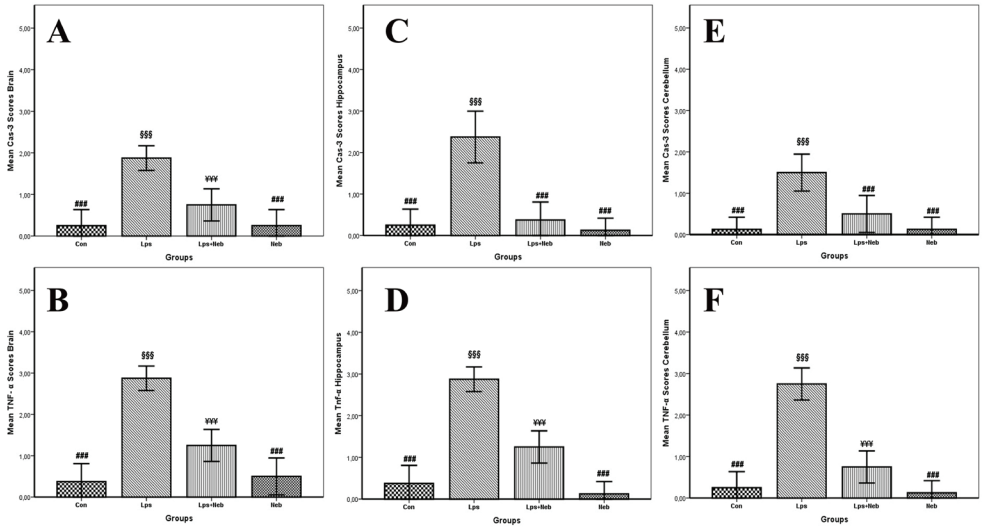

Figure 6

Illustration of the mean values of the immunohistochemical scores between the groups. (A) Cas-3 scores of the brain cortex, (B) TNF-α scores of the brain cortex, (C) Cas-3 scores of the hippocampus, (D) TNF-α scores of the hippocampus, (E) Cas-3 scores of the cerebellum, (F) TNF-α scores of the cerebellum.

Discussion

Endotoxin levels in blood plasma increase in the presence of an infection in the body. In the case of very high endotoxin levels inflammatory diseases may occur, unless not sufficiently cleared from the blood (26). An increased inflammatory response results from the endotoxin LPS, which increases the synthesis of pro-inflammatory cytokines like TNF-α and IL-1 (6–10). In the current investigation, we found that NEB protects brain tissue against oxidative stress, inflammation, and apoptosis caused by LPS.

NEB, a third-generation beta-1 adrenergic receptor antagonist, has higher β-receptor affinity compared to other beta-blockers and has a beta-3 receptor partial agonist function. In addition to beta-1 receptor antagonism in cardiac muscles, beta-3 agonism activates endothelial and neuronal nitric oxide synthase and causes NO-mediated vasodilation (19,20). Rats exposed to cisplatin-induced nephrotoxicity showed nephroprotective effects from NEB, and it was noted that these effects might be mediated by the compound's anti-inflammatory, antiapoptotic, and antioxidant properties. The application of Nx-nitro-L-arginine methyl ester, a non-specific NOS inhibitor, altered the protective effect of NEB in the same trial) (21). Also, NEB has been shown to have a nephroprotective effect by improving renal histopathology and decreasing serum renal function parameters (22). Colak et al reported that, after ischemia reperfusion damage was developed in the ovaries of rats, increased levels of malondialdehyde and TNF-α, oxidative stress and inflammation markers, were reduced by NEB (27).

Kumar et al. evaluated oxidative stress and antioxidant status in sepsis and reported that the balance between oxidants/antioxidants has a key role in sepsis (28). Furthermore, the uncontrolled and exaggerated peripheral inflammatory response that occurs in sepsis may cause neuroinflammation. Along with neuroinflammation, disruption in the blood-brain barrier is accompanied by glial activation, and as a result, proinflammatory cytokines increase (5, 29). Microglia activation is the primary indicator of neuroinflammation, resulting in the release of cytokines like IL-1, TNF-α, and NO (9). LPS binding to TLR-4 receptors in microglia cells activates the NF-κB and mitogen activated protein kinase pathways, leading to the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6). NO and prostaglandin E2 mediators, which further aggravate this inflammatory process, are released as a result of the upregulation of nitric oxide synthase and cyclooxygenase-2 enzymes (30). According to Hun et al., LPS caused the synthesis of prostaglandin E2, TNF-a, IL-1β, and IL-6 (31).

The impairment of balance between the oxidant and antioxidant system causes increased ROS levels that lead to oxidative damage and oxidative stress via

damaging cell structures such as protein, lipid, and DNA (16). ROS is beneficial for the organism at low concentrations (such as defense against infectious agents, and cellular signaling functions) (15). In Chen et al.'s study, LPS decreased the activity of antioxidant enzymes that cause cardiac damage caused by LPS, such as glutathione peroxidase, catalase, and superoxide dismutase (32). In another study, LPS has been shown to increase ROS production in microglia cells (33). In our investigation, six hours following the application of LPS, TOS levels, and OSI levels statistically rose, but TOS levels were dramatically reduced by NEB administration. Moreover, albeit this was not statistically significant, NEB treatment raised TAS levels. Insufficiency of the administered dose could be the explanation. As such, additional research at varying doses could help clarify NEB's antioxidant action.

As known VEGF, a vasoactive glycoprotein, has important roles in vascularization, angiogenesis, and neuroprotection. Besides increasing microvascular permeability, it induces cell migration and increases endothelial mitogenesis. VEGFA is released by proteases in inflammation and its levels increase in sepsis, which is an exaggerated inflammatory response (11-14, 34, 35). Braile et al. showed activation of VEGFA release from neutrophils via LPS administration (13). In another study, Li et al. reported significantly higher levels of VEGFA in the lungs of the LPS group rats (36). Correlative to the literature in our study, LPS application increased VEGFA levels, and NEB treatment improved increasing VEGFA levels, but this was not statistically significant. In cases where such inflammation occurs, it is known that apoptosis develops with the activation of various intracellular pathways.

Apoptosis is essential to the processes of cell division and death. Cas-3 is essential to the process of apoptosis (37-39). According to Mohamed et al., NEB can protect the heart against doxorubicin-induced cardiotoxicity via modulating TNF-α, inducible nitric oxide synthase, and Cas-3 (40). In this study, LPS administration increased the Cas-3 levels, and a statistically significant decrease was observed with NEB treatment in the LPS + NEB group. According to these findings, we can interpret that the antiapoptotic effects of NEB contribute to the improvement of inflammation induced by LPS.

The limitations of our study include: 1- The use of only female animals, 2- The inability to investigate detailed cellular signaling mechanisms, 3- The lack of assessment of protein-level expression of examined genes due to financial constraints, and 4- The absence of evaluation of NEB's effects over longer periods and at varying doses. In our future studies, we aim to include animals of both sexes to determine whether the neuroprotective effects of NEB are sex dependent. Additionally, it is essential to investigate various signaling mechanisms and document their protein-level expressions to contribute to the scientific literature.

Conclusion

NEB may be considered a potential therapeutic agent in brain damage within neuroinflammation via protective effects due to anti-inflammatory, antioxidant, and anti-apoptotic features. However different dose studies may be useful in elucidating the effects of NEB.

Acknowledgment
This study was supported by the Scientific Research Projects Coordination Unit (project code TSG-2022-8783.) for consumable, analysis, and animal costs required during experimental applications.

Conflict of Interest Statement
Dr. Oguzoglu Ali Serdar and the co-authors have no conflicts of interest to declare in association with this study.

Ethical Approval
In this study, all experiments were performed under the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research of Suleyman Demirel University, Isparta (Ethic No:11.09.2020/06-02).

Funding
This study was supported by Suleyman Demirel University Scientific Research Projects Coordination Unit (project code TSG-2022-8783).

Availability of Data and Materials
A data availability statement should be included.

Authors Contributions
ASO: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing-original draft;Writing- review & editing.

NS: Methodology; Validation; Visualization; Writing-original draft,Writing- review & editing.

DD: Methodology; Validation; Visualization;

SA: Formal analysis; Validation; Visualization

YE: Genetic analysis

HA: Methodology; Validation; Visualization; Writing-original draft;Writing- review & editing.

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