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Research Article

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N-Nitro-L-Arginine-Methyl ester Exerts Neuroprotection byInhibiting Inflammation in Cerebral Injury Induced by Transient Ischemia/Reperfusion in Rats

Hiba A Awooda*

Al Maarefa University, College of Applied Sciences, Respiratory Care Department, Riyadh, Saudi Arabia.

Email: hsheekh@mcst.edu.sa. Phone number: 00966595550392

Abstract: Cerebral ischemia-reperfusion injury is a pivotal cause of deaths due to cerebrovascular accidents. Further research efforts are needed to reveal the mechanism underlying its aggravation or alleviation. We previously reported the antioxidant effect of N-Nitro-L-Arginine-Methyl Ester (L-NAME), a nonselective nitric oxide synthase (NOS) inhibitor, on rats subjected to transient focal cerebral ischemia-reperfusion (I/R). The aim of this work was to explore further neuroprotective anti-inflammatory effects of L-NAME. This study involved 30 adult male Wistar rats divided into three groups with ten rats in each: sham-operated (control), I/R group of rats subjected to 30 minutes of left common carotid artery (CCA) occlusion followed by 24-hour of reperfusion and test group infused with L-NAME intraperitoneally 15 minutes before the same I/R period. Neurological assessments were evaluated, Western blotting used to estimate Nuclear factor-kappa B (NF-?B), ELISA used to detect Tumor necrosis factor- α (TNF-α), and Nitric oxide metabolites were measured colorimetrically, as well as H&E staining to assess brain damage. Compared with the I/R group, the neurological score, infarction area, and the inflammatory biomarkers NF-?B, TNF-α, and NO were significantly decreased in L-NAME treated rats (P =0.001). As a conclusion from the current study, L-NAME showed potential neuroprotection through it is an anti-inflammatory effect on a rat's model of transient focal cerebral ischemia-reperfusion.

Keywords: Cerebral injury, Transient Ischemia/reperfusion, Nonselective Nitric Oxide Synthase, Neuroinflammation, Neuroprotection, N-Nitro-L-Arginine-Methyl Ester.

***Corresponding Author**

Hiba A Awooda , Department of Respiratory Care, College of Applied Sciences, Almaarefa University, Riyadh, Saudi Arabia.

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1. INTRODUCTION

Cerebrovascular accidents are a leading cause of death and disability globally and particularly in low- and middle-income countries [1]. Although advances have been made regarding treatment methods in recent years, the prognosis of cerebrovascular accidents remains unsatisfactory [2]. Among all types of cerebrovascular accidents, cerebral ischemic disease is an essential topic in clinical medicine due to its high incidence, high mortality, and serious outcomes [3]. Ischemia-reperfusion (I/R) injury also plays a fundamental role in cerebral ischemic disease via complex pathophysiology as tissue damage results from diverse mechanisms with central involvement of neuroinflammation, free radicals' overproduction that eventually results in activation of transcription factors, and alteration in gene expression [4]. Inflammation is critical for focal cerebral ischemia/reperfusion damage and can aggravate the ischemic and anoxic injury, as well as worsening patients' prognosis [5]. A highly pleiotropic inflammatory cytokine, Tumor Necrosis Factor-α (TNF-α) is one of the most studied cytokines related to inflammation in acute ischemic stroke. It assumed to augment or dissuade cellular survival through activation of receptor-mediated signal transduction [6]. Nuclear factor-kappa B (NF-κB) is a transcription factor that amends diverse physiological and pathological phenomena. Activation of NF- κB plays a crucial role in inflammation after ischemic stroke through its ability to induce transcription of proinflammatory genes such as TNF-α and inducible Nitric Oxide Synthase (iNOS) [7]. Nitric oxide (NO) is an uncharged gas that can easily cross biological membranes. It is a critical molecule in the central nervous system with a variety of physiological and pathological effects. Nitric oxide is synthesized by a group of three NO synthases (NOS) from L-arginine, two constitutively expressed isoforms, mainly located in endothelial cells and neurons (eNOS and nNOS, respectively) generate low NO levels, and are constitutively expressed and calcium-dependent. The third isoform, iNOS is expressed in many different cell types, for instance, macrophages, astrocytes, and microglia, and is calcium-independent. Under normal physiological conditions, NO is an essential endogenous vasodilator that can regulate cerebral blood flow and inhibit platelet aggregation and adhesion [8,9]. NO derived from eNOS has been suggested to have neuroprotective effects during cerebral ischemia as it promotes vascular dilation, increases vascular smooth muscle cell proliferation and migration, thereby enhances arteriogenesis after stroke. In contrast to the salubrious effects afforded by eNOS, both nNOS, and iNOS are linked to neurotoxic effects in stroke [10]. Many studies have been conducted to discover new effective drugs to reduce ischemia-reperfusion injury, exposing many promising products that protect against cerebral ischemic injury. Our previous study revealed a potential antioxidant effect of nonselective NOS inhibitor, N-Nitro-L-Arginine-Methylester (L-NAME), in rat's focal cerebral I/R through inhibition of oxidative stress and lipid peroxidation [11]. This study aimed to explore the additional possible anti-inflammatory effect of L-NAME.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats, weighing 150–250 g used in this experiment and conserved at a constant temperature of 22±2°C with a fixed 12:12-h light-dark cycle. Nutritionally balanced pellets and water were freely available. This study was approved by the Ethical Committee of the Alexandria University. All study's examinations conform to the Guide for the care, and use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

3.1 Experimental Protocol

Three randomly divided experimental groups were used, of 10 rats each:
(1) Sham-operated group: which included full surgical preparation without common carotid artery (CCA) occlusion serves as a control group.
(2) Ischemia/ reperfusion (I/R) group: Brain ischemia was maintained for 30 minutes of left CCA occlusion followed by 24 hours reperfusion. All rats in this group received 0.9% normal saline intraperitoneally 15 minutes before the induction of ischemia [12]. The volume of saline-infused was equivalent to the volume of L-NAME received by the third group.
(3) L-NAME (Sigma Aldrich Co., Ltd.) 15 mg/kg over 5 minutes intraperitoneally (i.p) 15 minutes before left CCA occlusion [12].

3.2 Induction of Cerebral Ischemia/Reperfusion (I/R)

Groups of animals were subjected to left common carotid artery occlusion. The animals fasted overnight before surgery with free access to tap water. Anesthesia was induced by ether inhalation and maintained by thiopental sodium (2.5 mg/kg) [13]. Body temperature was kept constant at 36.5±0.5°C using a heating pad. Animals were placed on the back; a midline ventral incision was made in the neck. The trachea of the animal was exposed followed by the left common carotid arteries located. Ischemia was induced by placing non-traumatic microvascular clip-on left CCA just before its bifurcation [14]. During ischemia, rats were monitored for body temperature and respiration patterns. The vascular occlusion was maintained for 30 minutes, and then the clips were removed to resume blood flow to the ischemic region for 24 hours [12]. Sham control animals received the same surgical procedures except left CCA was not occluded. After the completion of the reperfusion period, the animals were assessed for their neuroprotective activity and were sacrificed thereafter. The brains were dissected out for the determination of biochemical parameters, and histopathology study.

3.3 Neurological Evaluation

Neurological assessment of all experimental groups was recorded 24 hours after surgery and before scarifying the rats. For observing the symmetry in the movements of the four limbs, the rats were kept in the air by the tail. The scoring criteria were as follows: (3) when all four limbs extend symmetrically, (2) when not all limbs on the left side extended or they extended slowly than those on the right side, (1) when the limbs on the left side had the minimum motion, and (0) when there was no motion of the forelimb on the left side [15].

3.4 Laboratory investigations

At the end of the experimental period, the rats were

sacrificed by decapitation. Brains were rapidly removed from the skull and washed with cold saline and stored at −20°C for further analysis. A small part of each brain from the affected hemisphere were dissected into approximately 1-2 mm pieces and they were homogenized in 7 ml of ice-cold extraction buffer contain: (Triton X-100: 1%, MgSO4: 10 mmol/l, EDTA: 1 mmol/l, Dithiothreitol: 1 mmol/l, NaCl: 0.5 mol/l, Protease inhibitor cocktail: 1%, and 20 mmol/l HEPES (pH 7.5) [16]. The homogenate was centrifuged; the supernatant was taken and stored at −20°C before use. A modification of the method of Lowry was used for the determination of protein in the brain homogenate [17]. TNF-α level in the brain and serum was measured using ELISA kits[18].

2.2 Western Blotting Method for Detection of NF-κβ

The brain was quickly removed and was homogenized and sonicated in the homogenizing buffer (250 mM sucrose, 20 mM HEPES, pH 7.4 with KOH, 100 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]). The homogenate was centrifuged (1000 × g, 15 min, 4°C) and the resulting supernatant was used for quantitation. Protein concentrations were determined and equal amounts of protein were loaded per lane after adding the same volume of Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA). Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis was performed on a Tris-glycine gel (Invitrogen) and then transferred to a Nitrocellulose membrane (Invitrogen). Membranes were incubated with primary antibody: Affinity-purified rabbit anti-NFkB antibody (R&D Systems, USA). The antibody was reconstituted in 100 µl of sterile PBS containing 0.02% sodium nitrite (NaN3). Block solution dissolved in TBS, and HRP conjugated-secondary antibody was added and placed on the shaker for 1 hour at room temperature. The membrane was then washed 3 times (5min each) with TBST (0.05%Tween in TBS) on the shaker and then washed again in TBS. DAB substrate solution than hydrogen peroxide 30% was added. After developing the color of the blot, the reaction was stopped after the appearance of the expected bands by pouring out the substrate and rinsing with distilled water repeatedly. Finally, the membrane was dried and placed in the dark and pictures were taken. The pictures were fed to the computer using the Corel paint shop pro X2 software, the color intensity of each band was converted to a number with red green blue (RGB) unit and divided by the protein concentration in each sample to be represented finally with RGB unit/mg protein [19].

2.3 Histopathology study

For histological evaluation, brains from control and experimental groups of global ischemia were removed, post-fixed overnight in paraformaldehyde, processed, and embedded in paraffin. Coronal brain sections (4 µm-thick) were cut. Sections were deparaffinized in xylene and rehydrated in a gradient of ethanol and distilled water. Then hematoxylin and eosin staining were used to examine the brain neuronal tissues [20].

3. STATISTICAL ANALYSIS

Significance differences among groups were evaluated by independent student t-test and the relationships between different variables were assessed using bivariate correlations. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the SPSS statistical package version 23.

4. RESULTS

4.1 L-NAME enhances neurological deficit:

The means of the neurological scores of both the I/R group (2.133±0.115) and L-NAME treated rats (2.5116±0.097) were significantly lower compared to the control group (2.91±0.118, $P < 0.001$). The L-NAME group showed a significant improvement in neurological deficit compared to the I/R group ($P < 0.001$). (Fig. 1).

4.2 L-NAME inhibits NO:

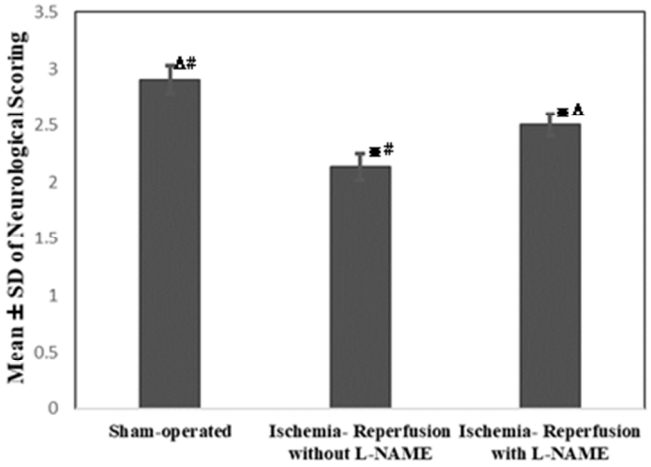
Pre-treatment with L-NAME results in significant inhibition of both serum and tissue level of NO(18.44±0.513µmol/L, 4.47±0.392 nmol/mg protein, $P <0.001$ respectively) when compared to I/R group (42.03±4.558 µmol/L, 8.88±0.572 nmol/mg protein, $P <0.001$ respectively). Furthermore, L-NAME group showed a significant improvement in neurological deficit (M±SD = 15.07±0.584) compared to both I/R and control groups (12.798±0.689, 17.50±0.707, respectively $P <0.001$).

4.3 L-NAME reduces inflammatory factor release

As shown in figure (2), serum and tissue level of TNF-α were significantly increased in I/R group (734.8±108.9 pg/ml, 110.4±6.2pg/mg protein) compared to the control group (37.18±10.183 pg/ml, 4.9±0.8 pg/mg protein, respectively $P <0.001$), while L-NAME administration resulted in a significant decrease in serum TNF-α (64.36±11.053, 21.60±2.289pg/mg protein respectively $P <0.001$) compared to the I/R group. Regarding NF-κB, the ischemic group demonstrated a significant increased NF-κB (129.2±1.7 RGB unit/mg protein) compared to the control group (53±1.03RGB unit/mg protein $P <0.001$) and L-NAME pretreatment resulted in a significant decrease in NF-κB (44.4±1.3RGB unit/mg protein, $P <0.001$) compared to I/R group (Figure 3).

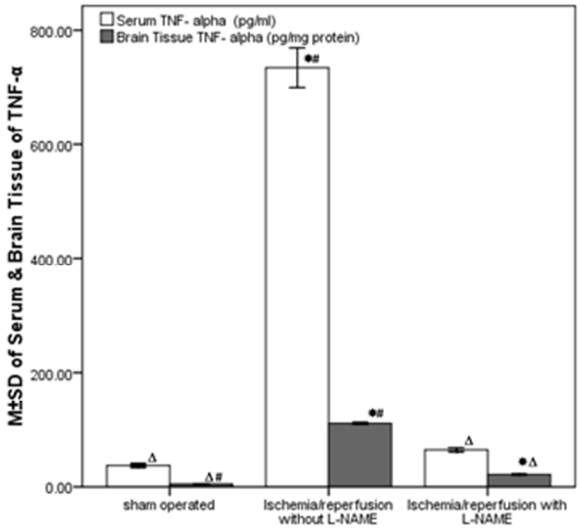
4.4 L-NAME Attenuated Neuronal Injury

The results of H/E staining demonstrated that neurons in the cerebral cortex were clearly visible in the sham-operated group. Noticeable pathological lesions were visible in I/R rats when compared to sham-operated ones, where neurons were showing acute inflammatory responses such as edema, atrophy, and necrosis. These pathological lesions induced by cerebral ischemia were markedly reduced when compared with the L-NAME pretreatment group. The number of neurons with edema, that were atrophic or necrotic, were reduced in the L-NAME pre-treatment group (Figure 4). These findings illustrate that pre-treatment with L-NAME may reduce brain injury induced by focal cerebral I/R in rats.



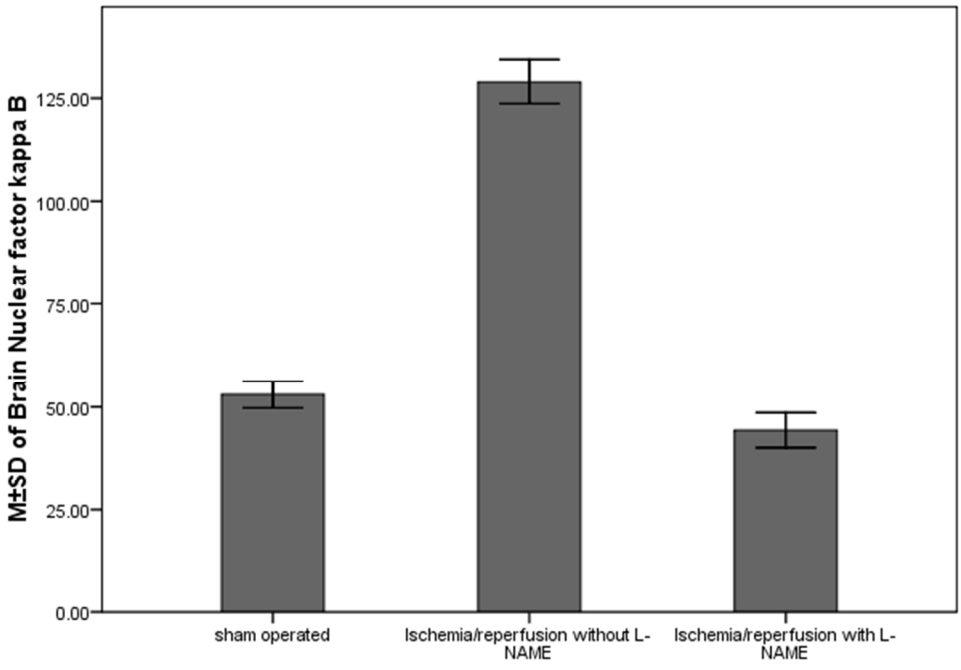
** Significant with group 1, Δ Significant with group 2, # Significant with group 3. * : Statistically significant at p ≤ 0.05.*

Fig.1 Comparison between the different experimental groups according to mean neurological deficit.



Significant with group 1, Δ Significant with group 2, # Significant with group 3. * : Statistically significant at P ≤ 0.05

Fig 2. Comparison between the different experimental groups according to Serum and Brain tissue of TNF-α



**Significant with group 1, Δ Significant with group 2, # Significant with group 3. * : Statistically significant at P ≤ 0.05*

Fig 3. Comparison between the different experimental groups according to Brain NF-κβ RGB /mg protein

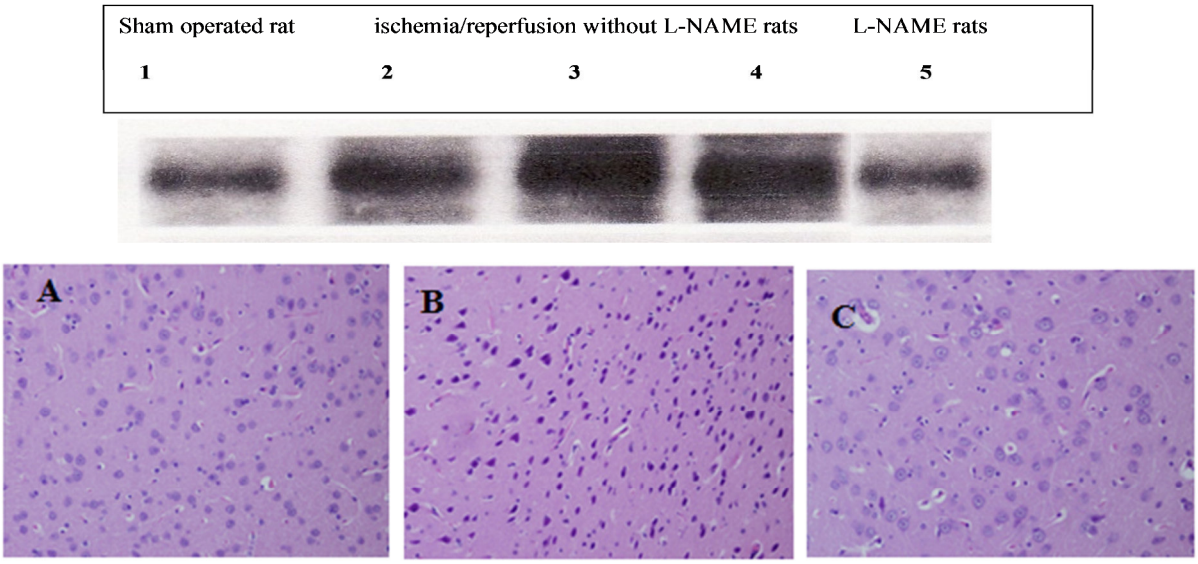


Fig 4. HE staining (×400). (A) Sham group; (B) I/R group; (C) L-NAME group.

5. DISCUSSION

It is apparent from the current data that inflammation is an anticipated outcome in rats' model of focal cerebral ischemia for 30 min followed by 24 hours of reperfusion. The inflammatory biomarkers were significantly decreased by administration of L-NAME prior to cerebral ischemia/ reperfusion. This suggested a probable anti-inflammatory effect of L-NAME which was further supported by the improvement of rats' neurologic assessment and histological evaluation of the brains of rats treated by this medication prior to cerebral ischemia/reperfusion. Previous researches reported inconsistent findings on the effect of L-NAME in rats subjected to focal or global cerebral ischemia [21–23]. We previously reported the possible antioxidant effect of L-NAME in rat's focal cerebral ischemia-reperfusion, through inhibition of oxidative stress and lipid peroxidation [11]. Therefore, the current study used the same dosing and timing regimen of L-NAME. In the current study, L-NAME administration before transient cerebral ischemia/ reperfusion results in significant inhibition of TNF-α overproduction in both serum and brain tissue, and this associated with significantly abolished NF-κβ in the treated group as compared to sham-operated and ischemic rats without L-NAME. These results proposed for the potential anti-inflammatory effect of L-NAME through inhibition of one of the central pro-inflammatory cytokine (TNF-α) and transcription factor (NF-κβ) involved in the pathogenesis of ischemic stroke. Comparable research concerning the anti-inflammatory effect of L-NAME in rats' transient cerebral ischemia/reperfusion are very scarce. According to Zhu *and his* fellows, TNF-α augments the toxicity of NO in the brain capillary endothelial cells culture, which associated with the generation of superoxide and MDA and L-NAME administration completely inhibits TNF-α toxicity [24]. Moreover, cerebral ischemia proved to induce excitotoxicity through impairment of astrocytic glutamate uptake and release of cytokines such as TNF-α. Hence, Ye and others reported that incubation of postnatal rats' hippocampus cultures with TNF-α results in the reduction of astrocytic glutamate uptake and administration of L-NAME repeal TNF-α effect[25]. Previous researches demonstrated that inhibition of DNA binding activity of NF-κβ could interfere through NO-donors. Consequently, Greco and co explored this speculation by using L-NAME (3mg/kg) in rats' model of MCAO, IkB-alpha expression in cerebral ischemic cortices considered as an indicator of NF-κβ activation, and pre-treatment with L-NAME significantly reduced the infarct volume and prevented ischemia-induced by NF-κβ activation. On the contrary, in the same experiment administration of l-NIO (selective eNOS inhibitor) associated with a significant increase in infarct volume and induced NF-κβ activation in the ischemic cortices as compared with sham-operated rats. Therefore, these results imply that NO originated from nNOS and iNOS potentiates NF-β activation via IkB-alpha modulation and mediates ischemic-related damage in the brain following ischemia [26]. In contrast to the current data, Li *et al* experiment investigated the effect of L-NAME on behavioral long-term potentiation and maze learning performance in freely moving rats through intrahippocampal administration of L-NAME, using a combination of electrophysiologic recordings and behavioral tests. L-NAME was found to deteriorate the behavioral long-term potentiation and maze learning performance [27].

6. CONCLUSION

The present study demonstrates the potential anti-inflammatory effect of L-NAME in rat's focal cerebral ischemia/reperfusion. This evident by inhibition of studied inflammatory biomarkers TNF-α and NF-κβ in rats treated by this medication before cerebral ischemia/reperfusion as well as improvement of neurological deficits and histological analysis.

7. ACKNOWLEDGMENT

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8. CONFLICT OF INTEREST

Conflict of interest declared none.

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