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Original research article

Neuronal nitric oxide synthase has a role in the detrimental effects of lipopolysaccharide on spatial memory and synaptic plasticity in rats

Akbar Anaeigoudari [a], Mohammad Soukhtanloo [b], Mohammad Naser Shafei [c], Hamid Reza Sadeghnia [d], Parham Reisi [e], Farimah Beheshti [a], Sepehr Behradnia [c], Seyed Mojtaba Mousavi [c], Mahmoud Hosseini [a,*]

[a] Neurocognitive Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Department of Biochemistry, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Neurogenic Inflammation Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Pharmacological Research Center of Medicinal Plants, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[e] Department of Physiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

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ABSTRACT

Background: The role of neuronal nitric oxide synthase (nNOS) in lipopolysaccharide (LPS)-induced memory and synaptic plasticity impairment was investigated.
Methods: The rats were divided and treated as follows: (1) control (saline), (2) LPS, (3) 7NI (7-nitroindazole as a nNOS inhibitor)-LPS and (4) 7NI.
Results: In a Morris water maze, the LPS group took a longer amount of time and traveled a greater distance to reach the platform, this was prevented by 7NI. Malondialdehyde (MDA) and nitric oxide (NO) metabolites in the hippocampus of the LPS group were higher while the total thiol, superoxide dismutase and catalase were lower than that of the controlled specimen. Pre-treatment using 7NI prevented the changes in the biochemical criteria. The slope and amplitude of the field excitatory post-synaptic potential (fEPSP) in the LPS group decreased, whereas in 7NI–LPS group they increased.
Conclusion: It is suggested that inhibition of nNOS by 7NI improves the deleterious effects of LPS by reducing NO metabolites and the brain tissues oxidative damage.

Introduction

Both inflammation [1] and oxidative stress [2] are considered to have an important role in memory loss. Systemic inflammation and overproduction of multiple inflammatory cytokines in the brain, such as tumor necrosis factor-α (TNFα), interleukin1-β (IL-1β) and interleukin-6 (IL-6), have been shown to disrupt working memory and long-term potentiation (LTP) induction in the hippocampus. These contribute to memory loss in neurodegenerative diseases including Alzheimer's disease (AD) [3–7]. In addition, oxidative stress, which is accompanied with increased levels of reactive oxygen species (ROS) superoxide, has been proposed to have a crucial role in neuronal death and memory impairment [8].

Lipopolysaccharide (LPS), a particle extracted from the cell wall of Gram-negative bacteria, has been shown to trigger excessive production of free radicals and inflammatory cytokines that are accompanied with neuroinflammation, neuronal death and memory deficits [9]. Even a single systemic injection of LPS impairs spatial memory and long-term potentiation (LTP) and declines neurogenesis in the hippocampus [10]. It is suggested that the activation of cytokines is followed by overproduction of nitric oxide (NO) through the activation of nitric oxide synthase (NOS) isoforms which lead to superoxide anion formation cell death [11,12]. It is suggested that expression of iNOS increases after injection of LPS [13], however, the role of nNOS still needs to be evaluated. The aim of this study was to further investigate the effects of nNOS on LPS-induced learning, memory and LTP impairments in rats.

Materials and methods

Animals and drugs

Fifty-six Wistar rats were divided into four groups: (1) control, (2) LPS, (3) 7NI–LPS and (4) 7NI ($n = 8$ in each group). LPS was injected (1 mg/kg; *ip*) 2 h prior to the behavioral and electrophysiological experiments [14]. 7NI (30 mg/kg; *ip*) was injected 30 min

* Corresponding author.
E-mail address: hosseinim@mums.ac.ir (M. Hosseini).

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before LPS or saline in the 7NI–LPS and 7NI groups, respectively. In the LPS and control groups, the animals were treated with saline, supplemented with dimethyl sulfoxide (3%, DMSO) (2 ml/kg) instead of 7NI. Thirty-two of the animals were treated by drugs or vehicles for 6 constitutive days and used in a behavioral test. The remaining animals (24) were used for electrophysiological experiments after receiving a single dose of drugs or vehicles. LPS and 7NI were purchased from Sigma (Sigma–Aldrich Chemical Co., St. Louis, MO, USA). Other chemicals such as DMSO, and those which were used for biochemical assessments, were purchased from Merck Company (Darmstadt, Germany).

Morris water maze (MWM) test

Spatial learning and memory were investigated using the MWM test as described in our previous studies [15]. Briefly, the rats were placed into the MWM tank for 4 trials each day for 6 days. In the first 5 days, an escape platform was hidden 2 cm beneath the surface of the water in the center of the quadrant of the apparatus and the rats were given 60 s to find it in each trial. The time spent and distance traveled in order to reach the platform was recorded. On the last day, the platform was removed and a probe test was performed. The time spent and the traveled path in the target quadrant (Q1) was compared between groups.

Electrophysiological study

The results of MWM showed that there was a significant difference between 7NI–LPS and LPS groups but no significant difference was observed between 7NI and control groups. For electrophysiological experiments, 24 of the animals were divided into three groups: (1) control, (2) LPS and (3) 7NI–LPS ($n = 8$ in each group).

The animals were deeply anesthetized with urethane (1.6 g/kg) and two small holes were drilled into the skull using stereotaxic apparatus in order to place stimulating and recording electrodes. Field potential was recorded from the CA1 area of the hippocampus. For this purpose, a bipolar stimulating electrode (stainless steel, 0.125 mm diameter, A-M System, Sequim, WA, USA) was infixed into the ipsilateral Schafer collateral pathway (AP = 3 m, ML = 3.5 mm, DV = 2.8–3 mm) and a unipolar recording electrode was lowered into the stratum radiatum of the right CA1 area of the hippocampus (AP = 4.1 mm, ML = 3 mm, DV = 2.5 mm). The stimulating electrode was connected to a stimulator and the recording electrode was connected to an amplifier. Extracellular field potential was obtained from CA1 area of the hippocampus following the stimulation of the Schafer collateral pathway. These were amplified (100×) and filtered (1 Hz to 3 kHz band pass) using a differential amplifier. A maximum field excitatory post-synaptic potential (fEPSP) was obtained by stimulating the Schafer collateral pathway and recording in the CA1 area. After a 30 min stabilization period, in order to evaluate synaptic potency before induction of LTP, an input–output (I/O) function was exerted by gradually increasing the stimulus intensities with constant current (input) and recording fEPSP (output). A baseline recording was then taken 30 min before the induction of LTP. After ensuring a steady state baseline response, in order to carry out LTP induction, a high frequency stimuli (HFS) protocol of 100 Hz was applied. The stimuli with the intensities which produced 50% of the maximum response were applied to induce LTP. The fEPSP was then recorded for the 90 min after high frequency stimuli. Computer-based stimulation and recording was performed using Neurotrace software version 9 and Eletromodule 12 (Science Beam Institute, Tehran, Iran). The values of the slope and amplitude of the fEPSP were the average of the 10 consecutive traces. Reponses were analyzed using custom software from the same institute.

Biochemical assessment

After completion, the behavioral tests were deeply anesthetized, the blood samples were collected, the hippocampal tissues were removed, weighed and submitted to determine the total thiol (SH) content, malondialdehyde (MDA), NO metabolites (NO_2 or NO_3) concentrations and the activities of superoxide dismutase (SOD) and catalase (CAT).

Determination of MDA, total thiol and NO metabolites

The MDA concentration, as an index of lipid peroxidation and total thiol groups' content were assessed in the hippocampal tissues according to a protocol that we described previously [16]. Briefly, a reaction of thiobarbituric acid (TBA) with MDA results in the production of a red complex which has a peak absorbance of 535 nm After reacting DTNB (2,2′-dinitro-5,5′-dithiodibenzoic acid) as a reagent with the SH group produced a yellow complex, absorbance was read at 412 nm The concentration of NO metabolites (NO_2/NO_3) was determined according to the Griess reagent method. In brief, after adding 100 μL supernatant to the Griess reagent and transferring the contents to a 96-well flat-bottomed micro-plate, the absorbance was read at 520 nm This was done using a micro-plate reader and the final values were computed with standard calibration plots [17–19].

The enzymatic assays

SOD activity was evaluated according to the method of Madesh and Balasubramanian. In a colorimetric assay, the SOD activity was measured at 570 nm One unit of SOD was defined as the amount of enzyme required to inhibit the rate of MTT reduction by 50%. The results were presented as units per milligram of protein. Catalase activity was determined by the method of Aebi with hydrogen peroxide (30 mM) as the substrate. One unit of catalase activity is determined as the micromoles of the hydrogen peroxide consumed per milligram of protein sample.

Determination of serum TNFα content

The content of TNFα in the serum was measured using an enzyme linked immunosorbent assay (ELISA) kit (Rat TNFα Platinum ELISA, e-Bioscience, San Diego, CA, USA) following the instructions of the kit.

Statistical analysis

All data were expressed as a means of ± SEM. For the data of the time and distance during the 5 days of MWM, the experimental design was included two between-subject factors (7NI and LPS), and day as a repeated measure. So, three-way mixed design ANOVAs were used and three-way interactions were also demonstrated (LPS × 7NI × Day). Data of the LTP criteria were compared using a repeated measures analysis of variance (ANOVA) followed by Tukey's *post hoc* comparisons test. The biochemical data collated in the probe trial in the MWM was compared using one-way ANOVA followed by Tukey's *post hoc* comparisons test. Differences were considered statistically significant when $p < 0.05$.

Results

MWM results

Using three-way ANOVA, the results showed that there was a significant effect of LPS on escape latency to reach the platform $f_{(1, 536)} = 25.22$; $p < 0.001$. 7NI also significantly affected the escape

latency to reach the platform $f_{(1,\ 536)} = 18.97$; $p < 0.001$. Day also significantly affected the escape latency to reach the platform $f_{(4,\ 536)} = 36.23$; $p < 0.001$. There was a significant interaction between 7NI and LPS on the escape latency to reach the platform $f_{(1,\ 536)} = 8.99$; $p < 0.01$. There was no significant interaction between LPS and Day on the escape latency $f_{(4,\ 536)} = 0.39$; $p > 0.05$. There was also no significant interaction between 7NI and Day on the escape latency $f_{(4,\ 536)} = 1.80$; $p > 0.05$. There was no significant interaction in LPS × 7NI × Day $f_{(4,\ 536)} = 0.31$; $p > 0.05$. In addition, the results of the *post hoc* test indicated the latency times to reach the platform in the LPS group were significantly higher than the control group ($p < 0.01$ and $p < 0.001$). The animals in the 7NI–LPS reached the platform in a shorter time in comparison to those of the LPS group ($p < 0.01$ and $p < 0.001$) (Fig. 1a).

Using three-way ANOVA, the results showed that there was a significant effect of LPS on the traveled distance to reach the platform $f_{(1,\ 536)} = 23.77$; $p < 0.001$. 7NI also significantly affected the traveled distance to reach the platform $f_{(1,\ 536)} = 32.72$; $p < 0.001$. Day also significantly affected the traveled distance to reach the platform $f_{(4,\ 536)} = 29.54$; $p < 0.001$. There was a significant interaction between 7NI and LPS on the traveled distance to reach the platform $f_{(1,\ 536)} = 20.96$; $p < 0.001$. There was no significant interaction between LPS and Day on the escape latency $f_{(4,\ 536)} = 0.39$; $p > 0.05$. There was also no significant interaction between 7NI and Day on the escape latency $f_{(4,\ 536)} = 1.19$; $p > 0.05$. There was no significant interaction in LPS × 7NI × Day $f_{(4,\ 536)} = 0.56$; $p > 0.05$.

The results of the *post hoc* test indicated the traveled distances to reach the platform in the LPS group was significantly longer than the control group ($p < 0.05$, $p < 0.01$ and $p < 0.001$). The animals in the 7NI–LPS group traveled a shorter distance to reach the platform in comparison to those of the LPS group ($p < 0.05$ and $p < 0.001$) (Fig. 1b).

Using three-way ANOVA, the results showed that there was no significant difference in the speed between the groups (Fig. 1c). Trial-by-trial day analysis of day-1 data of the times also indicated that there was no significant difference between the groups in the first trial (Fig. 1d).

On the probe day, the animals of the LPS group spent less time and traveled shorter distances in the target quadrant (Q1) than the control group ($p < 0.001$ and $p < 0.05$, respectively). The animals in the 7NI–LPS group spent more time and traveled longer distances in Q1 compared to the LPS group ($p < 0.05$). There was no significant difference in the time spent or distance traveled in Q1 between the control, 7NI–LPS and 7NI groups. The results also showed that there was no significant difference in the time spent or distance traveled in the non-target quadrant (Q2, Q3 and Q4) between the four groups (Fig. 2a and b).

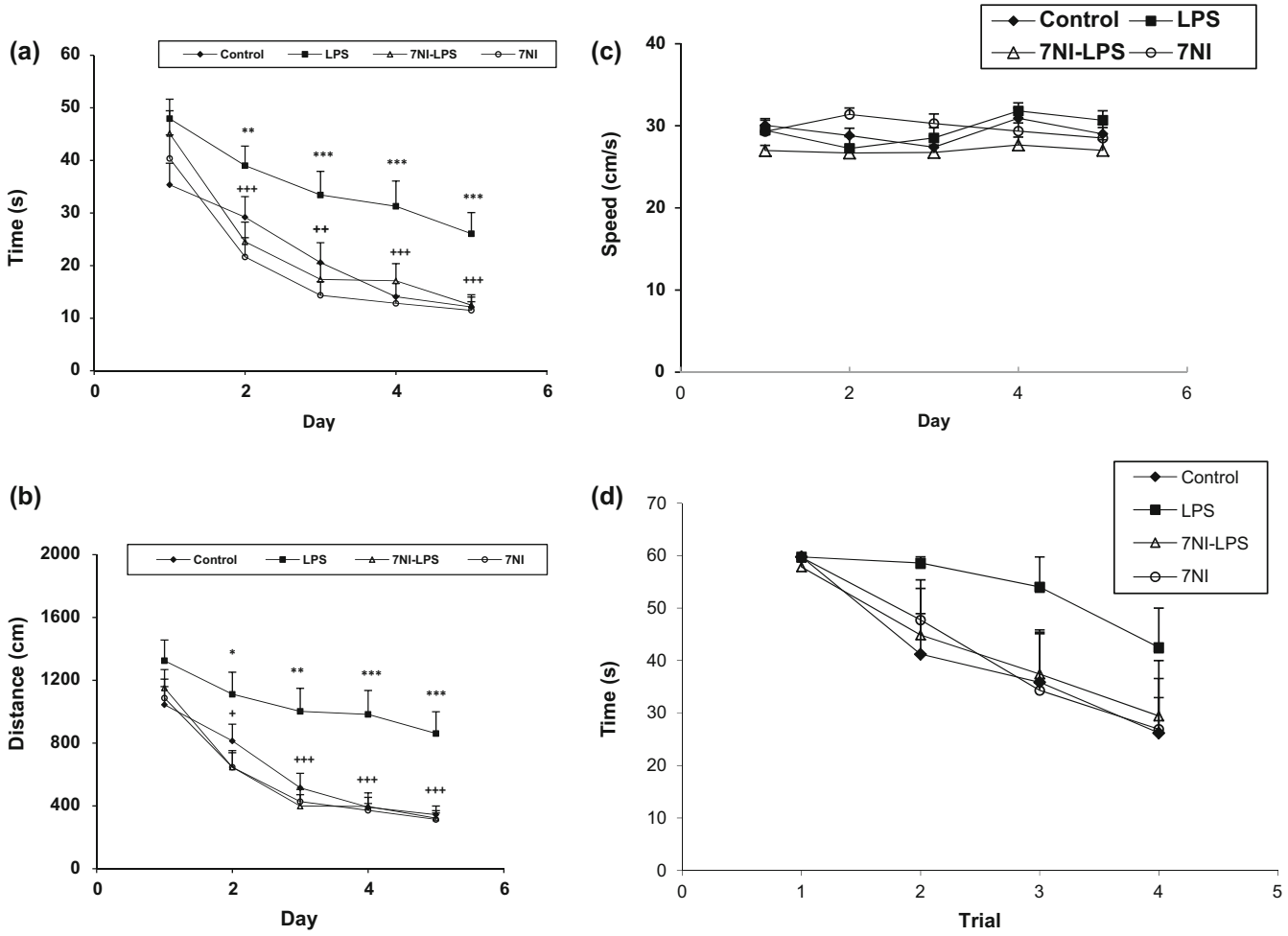

Fig. 1. Comparison of time latency (a) and distance traveled (b) to reach the platform and (c) the swimming speed in the Morris water maze between the four groups ($n = 8$ in each group). The time latency and distance traveled of the LPS group was higher than the control group. The animals of the 7NI–LPS group spent a shorter time and traveled a shorter distance to reach the platform in comparison with LPS specimens. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group. $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to the LPS group. There was no significant difference in swimming speed between the four groups. (d) Trial-by-trial analysis of day-1 data of the time. There was no significant difference in the first trial between groups.

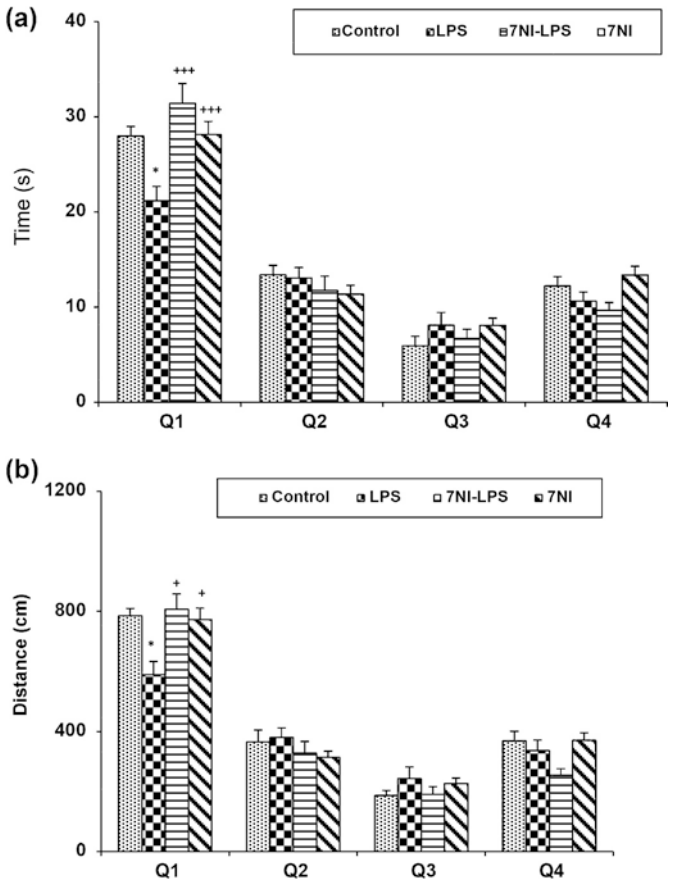

Fig. 2. Comparison of time spent (a) and distance traveled (b) in target quadrant (Q1) during the probe day 24 h after the last learning session. The platform was removed and the time spent in the target quadrant was recorded. Data are shown as mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$ compared to the control group and $^{+}p < 0.05$, $^{+++}p < 0.001$ compared to the LPS group.

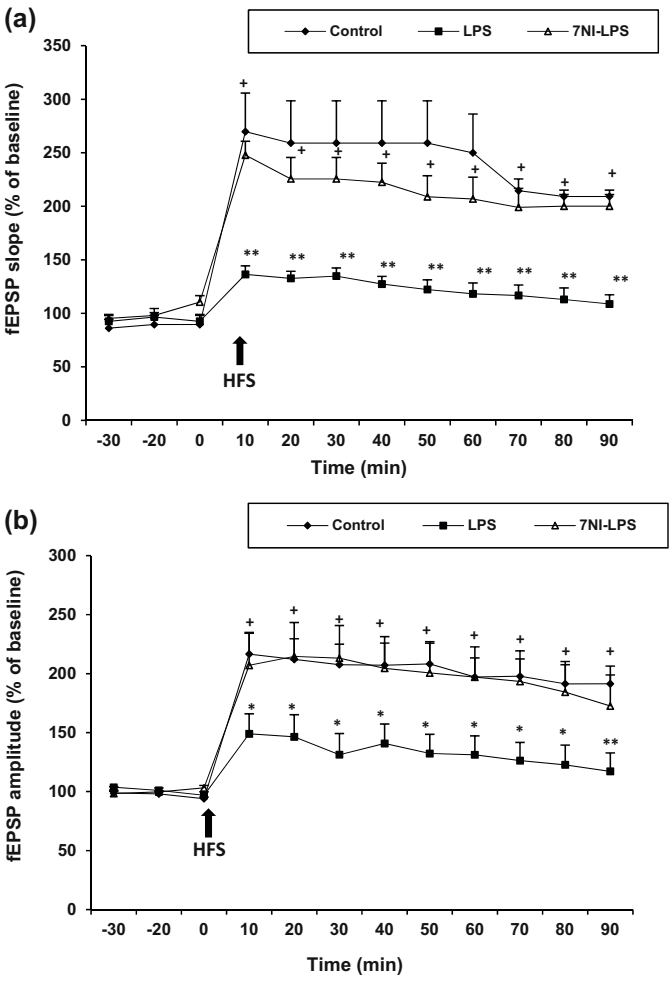

Fig. 3. The results of LTP induction in the CA1 area of the hippocampus: (a) the fEPSP slope and (b) the fEPSP amplitude. Each point shows mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to the control group. $^{+}p < 0.05$ compared to the LPS group. (c) Single traces were recorded before and after induction of LTP in CA1 area of the hippocampus.

Electrophysiological results

After inducing HFS, the mean fEPSP slope in the LPS group decreased significantly in respect to that of the control group ($p < 0.01$). The mean fEPSP slope in the7NI–LPS group was significantly higher than that of the LPS group ($p < 0.05$). There was no significant difference in fEPSP slope between the control and 7NI–LPS groups (Fig. 3a). In addition, after applying HFS, the mean fEPSP amplitude in the LPS group was significantly lower than that of the control group ($p < 0.05$ and $p < 0.01$). Injection of 7NI increased the mean fEPSP amplitude in the7NI–LPS group in comparison to the LPS group ($p < 0.05$). However, there was no significant difference in fEPSP amplitude between control and 7NI–LPS (Fig. 3b).

Biochemical results

The concentration of MDA and NO metabolites, NO_2 or NO_3, in the hippocampal tissues of the LPS group were higher than in that of the control group ($p < 0.001$ and $p < 0.05$, respectively). Injection of 7NI before LPS in the 7NI–LPS group decreased the concentration of MDA and NO metabolites in comparison with the LPS group ($p < 0.001$ and $p < 0.05$). However, there was no significant difference between the control, 7NI–LPS and 7NI groups (Fig. 4a and c). The total thiol concentration in the hippocampal tissues of the LPS group was lower than in the control group ($p < 0.001$). Injection of 7NI increased the concentration of the total thiol in the 7NI–LPS group compared to the LPS group ($p < 0.05$). There was no significant difference between the control, 7NI–LPS and 7NI groups (Fig. 4b).

The activity of SOD and CAT enzymes in hippocampal tissues of the LPS group were lower than that of the control group ($p < 0.05$). Administration of 7NI enhanced the activity of these two antioxidant enzymes in the 7NI–LPS group in comparison with the LPS group ($p < 0.05$). No significant difference between the control, 7NI–LPS and 7NI groups (Fig. 5a and b) was observed.

The serum's TNFα content in the LPS group was higher than in the control group ($p < 0.01$). Injection of 7NI reduced the serum TNFα content in the 7NI–LPS group in comparison to the LPS group ($p < 0.01$). There was no significant difference in serums' TNFα contents between the control, 7NI–LPS and 7NI groups (Fig. 6).

Discussion

The results of the present study indicated that the injection of LPS impaired the spatial learning and memory tasks. This was shown by increased latency and longer distances traveled by the animals treated with LPS as they attempted to reach the platform over 5 days. The results were compared against the vehicle treated specimens. The results of the probe trial day also showed that the rats of the LPS group did not remember the location of the platform

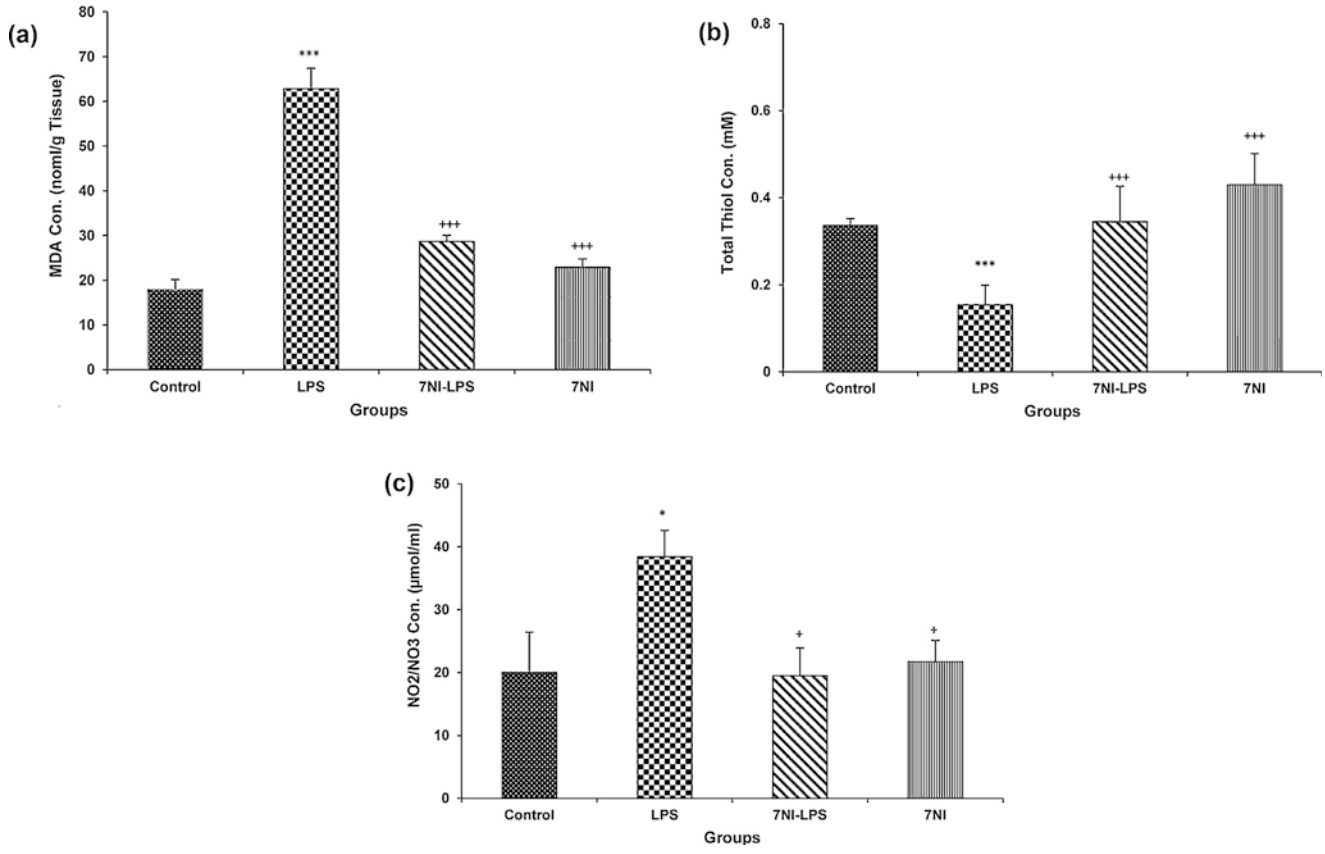

Fig. 4. Comparison of the MDA (a) and the total thiol (b) concentrations and the level of nitric oxide metabolites (c) in the hippocampal tissues. Data are presented as mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the control group. $^{+}p < 0.05$ and $^{+++}p < 0.001$ compared to the LPS group.

and spent less time and traveled less distance in the target quadrant where the platform was previously located. All of these findings agree to our previous studies which proved that LPS affected CNS and impaired learning and memory in the rats [16,20]. In the current study, we did not observe any significant difference in the animals' swimming speed (Fig. 1c). We also compared the time spent to reach the platform on the first day. The result showed that the there was no significant difference between the groups in the first trial. However, the time was increased in days 2–4 (Fig. 1d). These findings confirm that administration of LPS did not impair motor activity in the rats. LPS significantly impaired synaptic plasticity in the CA1 area of the hippocampus which was presented by a decrement in amplitude and slope of fEPSP-LTP in the LPS group compared to the control group.

The results of our study are supported by other scientific findings which show that injection of LPS impairs learning and memory in various experimental animal models such as contextual fear conditioning [21], MWM and the Y-maze tests [13]. A reduction in the number and the size of neurons in the hippocampus has been suggested to be responsible for spatial learning and memory impairments after administration of LPS [10]. LPS induces an overproduction of cytokines such as TNFα, IL-1β and IL-6 in macrophages [22]. These may play a role in learning and memory as well as LTP impairments in the hippocampus [23].

In the present study, serum content of TNFα was enhanced by LPS. This confirms an induction of inflammatory responses by LPS and its detrimental effects on learning, memory and LTP induction. Previously, we have shown that the brain tissue's oxidative damage plays a critical role in the detrimental effects of LPS on learning and memory [16]. Moreover, using various animal models, the destructive effect of oxidative stress on learning and memory has been reported [24]. Among the various areas of the brain, the cerebral cortex and hippocampus, which both play vital roles in learning and cognition, have shown the highest sensitivity to oxidative stress [25].

In the present study, where LPS-treated animals are compared with control specimens, an increased level of MDA and a decreased content of the brain's total thiols, may confirm the involvement of oxidative damage in harmful effects of LPS on learning and memory as well as synaptic plasticity. Previously, detrimental effects of LPS on the balance of oxidative status and energy metabolism in the brain have been suggested [26]. It has also been indicated that administration of LPS increases the level of MDA and reduces glutathione (GH) [27]. In our study, LPS also lessened the activity of the SOD and CAT enzymes in comparison with control specimens which is also confirmed by other studies [28,29].

NO as a diffusible gaseous neurotransmitter plays a protective role in physiological concentrations and has a cytotoxic role in a high concentration [13]. An overproduction of NO and its derived-oxygen species, including peroxynitrite, can induce lipid peroxidation, protein oxidation, oxidation of thiols and activation or deactivation of various enzymatic systems which damages the brain tissues [30]. LPS has also been observed to induce the production of NO metabolites and several reactive oxygen and nitrogen species including hydroxyl radical ($OH^{\bullet}$) and superoxide ($O_2^{\bullet}$), as well as inflammatory cytokines [31].

In the present study, we have assumed that NO may have a role in the deleterious effects of LPS on spatial learning and memory as well as the impairment of LTP in the hippocampus. The results showed that the metabolites of NO were increased in the hippocampal tissues of LPS group compared to the control. Thus, it seems that the excessive production of NO after the administration

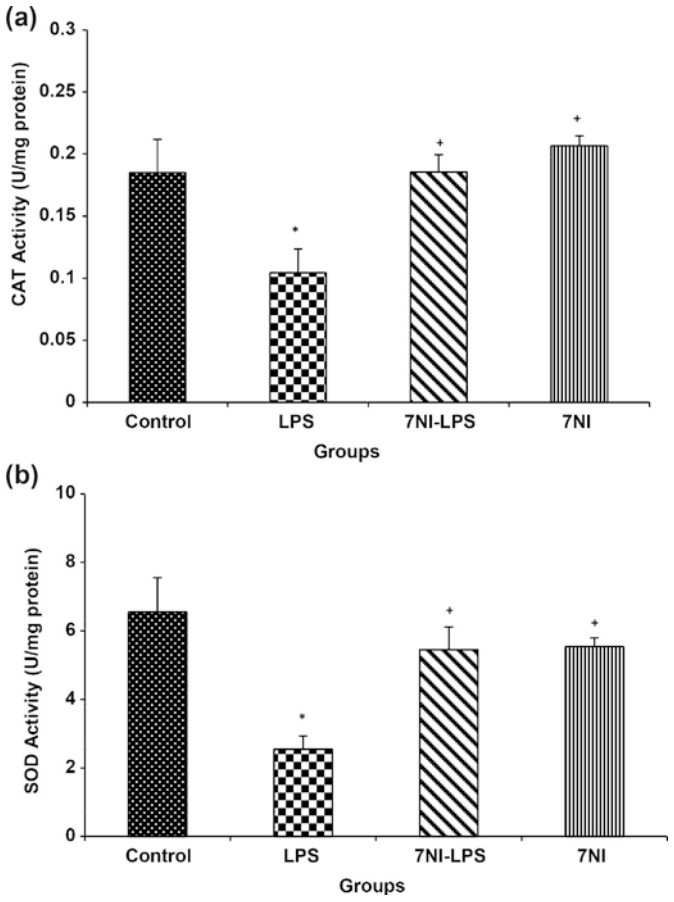

Fig. 5. Comparison of the activity of CAT (a) and SOD (b) in hippocampal tissues between the four groups. Data are shown as mean ± SEM ($n = 8$ in each group). $^{*}p < 0.05$ compared to the control group and $^{+}p < 0.05$ compared to the LPS group.

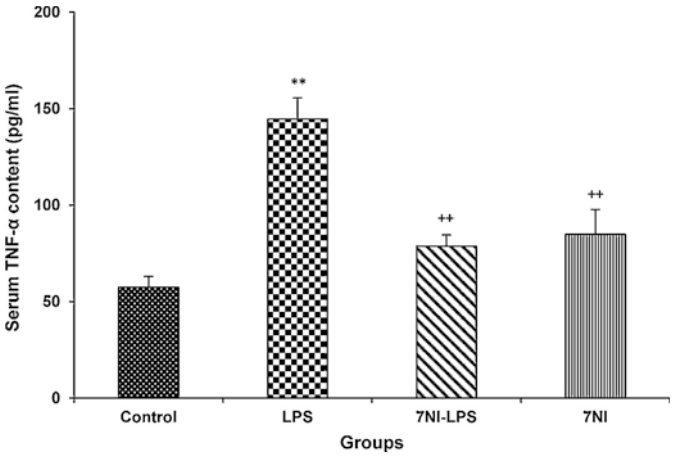

Fig. 6. Comparison of the serum TNFα content between the four groups. Data are shown as mean ± SEM ($n = 8$ in each group). $^{**}p < 0.01$ compared with the control group. $^{++}p < 0.01$ compared to LPS the group.

of LPS induces an oxidative stress procedure which probably plays a role in spatial learning, memory and LTP impairments by LPS. It has been frequently reported that increased activity of nNOS plays an important role in overproduction of NO in the brain [32]. Therefore, it was thought that administration of 7NI as an inhibitor agent of nNOS [33] may change the effects of LPS. The results showed that administration of 7NI before LPS in the 7NI–LPS group shortened the time and the distance traveled to find the platform over 5 days compared to the LPS group. During the probe trial day, the animals of 7NI–LPS group also took more time and traveled longer distances in searching for the location of the platform in the target quarter in comparison to the LPS specimens. With regard to these results, improving effects of 7NI on LPS-induced spatial learning and memory impairment was suggested. This was accompanied with the improvement of oxidative stress criteria as well as lower concentrations of NO metabolites is.

The electrophysiological results also confirmed the results of the MWM test. The results showed that 7NI enhanced amplitude and slope of fEPSP-LTP in the 7NI–LPS group in comparison with the LPS group. In line with these results, it was previously reported that IP injection of 7NI prevents zinc-induced cell death in cerebellar Purkinje cells of rats [34]. It has also been reported that the NOS inhibitors *N*-nitro-L-arginine methyl ester (L-NAME) and 7NI protect the hippocampus against kainate-induced excitotoxicity in rats [35]. With this in mind, a protective effect of 7NI against hippocampal tissues' oxidative damages, for improvement of memory impairments due to LPS, is conceivable.

In the current study, the injection of 7NI before LPS not only lessened the TNFα serum level but also resulted in improved oxidative status by decreasing the level of MDA and increasing the total thiol content in hippocampus tissue of the rats. This was shown in the 7NI–LPS group in comparison to the LPS group. Additionally, 7NI increased the activities of the SOD and CAT enzymes in the 7NI–LPS group compared with the LPS group. According to these results, it seems that the impairing effects of LPS on learning and memory as well as neuronal plasticity, are mediated by increasing of the nNOS activity, promoting inflammatory responses, increased level of NO and the brain tissue's oxidative damage.

To assist with explanation of the present results, it has been previously reported that nNOS acts as a negative regulator of neurogenesis in the brain [36]. It has been documented that several agents including L-NAME, *N*-nitro-L-arginine (L-NA) and 7NI can affect learning and memory in the brain by increasing or decreasing NO concentration in animal models [11].

In general, it seems that the effects of NO on learning and memory processes are controversial [37]. In contrast to the findings, which suggest that overproduction of NO in the brain disturbs learning and memory processes in some behavioral models [38], it is suggested that systemic inhibition of NOS impairs the learning of spatial tasks by the rats [39]. It was also shown that induction of spatial memory was accompanied with an increased level of the activities of nNOS and level of NO in the hippocampus of rats [40].

A further experiment showed that systemic or local injection of NOS inhibitors into the hippocampus had no effect on learning and memory [41]. In this study, we did not observe any significant difference in the time spent or distance traveled in the animals' seeking of the platform in the 7NI group, compared to the control group. Thus it is deduced that 7NI alone did not affect spatial learning and memory. These results are consistent with the findings which have shown that inhibition of NOS does not prevent the induction of LTP or spatial memory and the findings suggest that NO does not play an important role in memory and learning [41].

The results of the biochemical assessment also confirmed the results of the MWM test, in which the serum TNFα content, concentrations of MDA, thiol, NO metabolites and activities of SOD and CAT in the hippocampal tissues of the 7NI group were not changed in compared to the control group.

Finally, it is concluded that the detrimental effects of LPS on spatial learning and memory and synaptic plasticity are mediated by inducing inflammatory responses, overproduction of NO and brain tissue's oxidative damage. It may also be postulated that 7NI,

probably by inhibition of nNOS, improves the effects of LPS. However, this must be investigated further at some point in the future.

Conflict of interest

We declare that there is no conflict of interest.

Acknowledgments

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