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Original Article

The Effects of Rosmarinic Acid on Hippocampal Oxidative Stress Markers in LPS-induced Neuroinflammation Rats

Atiyeh Ahmadi-Zohan, Mohammadmehdi Hassanzadeh-Taheri, Mehran Hosseini*

Cellular and Molecular Research Center, Department of Anatomical Sciences, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran.

Abstract

Neuroinflammation (NI) plays a pivotal role in the pathogenesis of several neurodegenerative diseases. It has been believed that alleviating NI can be a valuable approach in controlling the progress of neurodegenerative diseases. The generation of reactive oxygen and nitrogen species could trigger and deteriorate NI. According to previous studies, rosmarinic acid (RA) could exhibit neuroprotective potential. Therefore, this study aimed to evaluate the effect of RA on hippocampal oxidative stress markers in lipopolysaccharide (LPS)-induced NI in rat brain. A total of 24 adult male Wistar rats were randomly allocated into four equal groups (n=6 each) and NI was induced in three of them by intracerebroventricular (ICV) injection of LPS (50 µg/20µl; 10 µl into each ventricle). RA (2.5-5 mg/kg i.p.) was injected 30 min before the LPS injection and continued once per day up to 48 h. On day 3, animals were sacrificed and their hippocampi were dissected. Then, hippocampal concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), and nitric oxide (NOx) were determined. RA prevented hippocampal MDA elevation in a dose-dependent-manner and increased SOD activity but did not affect NOx content. In conclusion, the results of the present study demonstrated that systemic administration of RA could effectively ameliorate oxidative stress induced by LPS in rat's brain. This potential might be one of the underlying mechanisms through which RA mitigates NI.

Keywords: Innate Inflammatory Response, Neurodegenerative diseases, Hippocampus, Oxidative Stress, Lipopolysaccharides, Rosmarinic Acid.

Corresponding Authors: Mehran Hosseini, Cellular and Molecular Research Center, Department of Anatomical Sciences, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran
Tel: +98-5632381511
Email: mehranhosseiny@bums.ac.ir
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1. Introduction

An accumulating number of recent experimental and clinical studies has been conducted on the role of neuroinflammation (NI) in the pathophysiology of several neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease

(PD), and amyotrophic lateral sclerosis (ALS) [1]. Therefore, NI has become a hot topic in modern neurosciences. NI is defined as an inflammatory response within the central nervous system (CNS) in which glial cells become activated. Subsequently, the activation leads to an ongoing pathologic process in the CNS by releasing several inflammatory mediators such as cytokines and chemokines, as well as generating reactive oxygen/nitrogen species [2]. During prolonged NI, producing reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl free radicals and nitrogen intermediates such as nitric oxides (NOx) and peroxynitrite can cause neuronal damages [3].

Rosmarinic acid (RA) is a class of aqueous phenolic compounds that are naturally found in the *Lamiaceae* family such as rosemary, sage, lemon balm mint, and sweet basil [4]. Several *in vitro* and *in vivo* studies support the idea that RA has anti-inflammatory potential. The results of several preclinical studies also showed the beneficial effects of RA on many inflammatory diseases such as arthritis, colitis, atopic dermatitis, asthma, allergic rhinitis, periodontal diseases, acute pancreatitis, and mastitis [5]. Moreover, RA and its metabolites like caffeic acid (CA) and 3(3,4-dihydroxyphenyl)lactic acid (DHPLA, also known as Danshensu) exhibited valuable antioxidant activities in both *in vivo* and *in vitro* experiments [6-8]. In this regard, the neuroprotective effects of RA and its metabolites have been shown in systemic administration, suggesting that they might cross or affect the blood-brain barrier (BBB) [9, 10].

The hippocampus is a brain area vulnerable to damages like NI particularly at the early stages of several neurodegenerative diseases such as AD and MS [11]. Some typical features such as extensive hippocampal demyelination, neuronal loss, apoptosis, and oxidative imbalance have been described in different neurodegenerative diseases [12]. Therefore, innate immune responses, specifically in the hippocampus, are extensively studied in LPS-induced NI models.

The present study was conducted regarding the anti-inflammatory, antioxidant, and neuroprotective activities of RA. This research aimed to evaluate the effect of RA on hippocampal oxidative stress markers including lipid peroxidation, superoxide dismutase (SOD), and NOx in lipopolysaccharide (LPS)-induced rat neuroinflammation model.

2. Materials and Methods

2.1. Animals

All procedures involving animals were according to the guides and rules in care and use of Laboratory Animals in Scientific Affairs with the Iranian Ministry of Health and Medical Education (2019) based on the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Moreover, the animal experiments were approved by the Birjand University of Medical Sciences Ethics Committee (permit code: Ir.bums.REC.1398.316).

Male adult Wistar rats weighing 200-230 g (8-week old) were used in the present study. All animals were housed in polypropylene cages in a temperature-controlled room (24 ±2℃) with

30% to 35% relative humidity, and a 12-h light/dark cycle. Rats were given ad libitum access to water and standard chow during the study period.

2.2. Intervention

A total of 24 rats were randomly allocated into the following four equal groups (n=6 each): sham-operated group (SO), LPS model group (LPS), LPS plus RA at the dose of 2.5mg/kg (LPS+RA2.5), and LPS plus RA at the dose of 5mg/kg (LP+RA5).

For the intracerebroventricular (ICV) injection of LPS, rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (45 mg/kg) and placed on a stereotactic apparatus (Stoelting, Wood Dale, IL, USA). The injection sites [Medial/Lateral: 1.8 mm, Anterior/Posterior: -0.8 mm, Dorsal/Ventral: 3.8 mm from the Bregma] were marked with a Hamilton syringe (26G, Hamilton Company, Reno, NV, USA) connected to a stereotaxic device and 0.5 mm diameter holes were made using a mini electric drill (WL800, Shanghai, China)[13]. Then, LPS (O127: B8, Sigma Aldrich, St Louis, MO, USA) was dissolved in artificial cerebrospinal fluid (CSF) (50 µg/20µl) and injected into the lateral ventricles (10 µL each ventricle) [14] using a cannula needle connected to a syringe pump (EN-300, New Era Pump Systems, Farmingdale, NY, USA) with the infusion rate set as 1 µL/min (Figure 1). The control group only received ICV injection of artificial CSF (10 µL each ventricle). The groups LP+RA2.5 and LP+RA5 received the RA (2.5 mg/kg, 5 mg/kg i.p., respectively) (R4033, Sigma Aldrich, St Louis, MO, USA) dissolved in ethanol and then diluted in the 0.9% saline (the final concentration of ethanol was <0.5%) just half an hour before LPS infusion (the first injection) and continued daily up to 3 injections. SO and LPS groups only received 0.9% sterile saline containing 0.5% ethanol (i.p.). These doses of RA were selected based on previous efficiency studies [15, 16].

2.3. Tissue Collection and Sample Preparation

On day 3 and 24-hour after the last administration, all animals were sacrificed under sodium pentobarbital (60 mg/kg i.p.) anesthesia [17]. The brains were carefully taken out, kept over a Petri dish placed on ice, and then promptly their hippocampi were dissected and homogenized for further assessments.

2.4. Assessment of Protein Concentrations and SOD Activity

The hippocampi were homogenized in 900 µL of cold phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 4500 g for 15 min. Supernatants were used to determine protein concentration using Bradford's assay. Accordingly, 20 µL supernatant was dissolved to 200 mL Coomassie brilliant blue reagent (G250), incubated at room temperature for 10 min, and the absorbance was read at 595 nm. The standard curve was determined with different concentrations (10-100 µg/mL) of bovine serum albumin solutions.

The SOD activity was evaluated according to the reduction rate of WST-1 [4-(3- (4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1, 3-benzene disulfonate]. Briefly,

50 µL supernatant was transferred into each well and then 250 µL of the reaction mixture was added to the well and mixed appropriately. The mixture contained 45 µL of assay buffer (50 mM Na3PO4, 0.1 mM diethylenetriamine pentaacetic acid, and 0.1 mM hypoxanthine in 20 mL) with 100 µL of 10 mM WST-1 solution, 100 µL of 2 mg/mL catalase, and 5 µL of xanthine oxidase (with the final concentration of 4.5 mU/ml). After 5 min incubation at room temperature, the absorbance was read at 405 nm and the SOD activity was expressed in terms of U/mg protein [18].

2.5. Assessment of Lipid Peroxidation

Lipid peroxidation in the hippocampus homogenate was determined by measuring the amounts of malondialdehyde (MDA), the end product of the lipid peroxidation process. Briefly, 100 µL supernatant was added to 600 µL o-H3PO4 (1 %) and 200 µL of 0.67 % thiobarbituric acid. Next, the mixture was heated at 90℃ for 45 mi and the reaction was stopped by placing samples on ice. Afterward, 800 µL N-butanol was added, vortexed, and centrifuged for 20 min at 5000 g. The resulting supernatant was removed (200 µL) and its optical absorbance was measured at 532 nm. Finally, MDA concentration was expressed as nmol/mg protein [17].

2.6. *Determination of NO Production*

The NO production was determined by the Griess method in the hippocampus tissue homogenates. First, 50 µL of Zinc sulfate (15 mg/mL) was added to each sample and was shaken vigorously. The suspension was re-centrifuged for 20 min at 14000 g and the supernatant (100 µL) was transferred to a micro-plate well. After that, 100 µL of vanadium III chloride (8 mg/mL) was added to each well. Eventually, 100 µL of Griess reagent containing 50 µL sulfanilamide (2%) and 50 µL N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1%) was added to each well. The plate was incubated for 30 min at 37℃ and absorbance was read at 530 nm. Sodium nitrate (0-150 µM) was used as a standard [19].

2.7. Statistical Analysis

Data were analyzed using SPSS software, version 22, and were expressed as mean ± standard deviation in all groups. The homogeneity of data variance was checked by the Shapiro–Wilk test. The differences between groups were determined with ANOVA and Dunnett's T3 post hoc tests. Statistical significance was inferred at $p<0.05$.

3. Results and Discussion

3.1. Results

3.1. 1. Effect of RA on LPS- induced Hippocampal SOD Change

The results of hippocampal SOD activity are presented in Fig. 2. Compared to the SO group, the hippocampal SOD activity was significantly reduced in the LPS group (58.50 ± 3.14 vs. 41.16 ± 2.56 U/mg protein, $p<0.001$). RA treatment only at the dose of 5 mg/kg (56.83 ± 4.57 U/mg protein) could effectively prevent hippocampal SOD from decreasing. So, it was kept at the normal level as there was no significant difference between this group and the SO group ($p=0.84$). RA at the lower dose

could not exhibit a significant effect on reducing SOD activity in the LPS rats (p=0.10).

3.1.2. Effect of RA on LPS-induced Hippocampal MDA Change

The hippocampal MDA concentration of LPS injected rats was statistically higher compared to their counterparts in the SO group (0.63 ± 0.04 *vs.* 0.32 ± 0.05 nmol/mg, p<0.001) (Figure 3). RA treatment at both doses could significantly keep MDA concentration lower than the LPS group (0.51 ±0.07 in RA2.5mg/kg and 0.36 ± 0.03 nmol/mg protein in RA5 mg/kg). RA only at the higher dose could keep the MDA level close to the normal level so that there was no significant difference between LPS+RA5 and SO groups (p=0.43). Meanwhile, RA at the lower dose (2.5 mg/kg) could slightly prevent hippocampal MDA elevation in LPS rats (0.51 ± 0.07 nmol/mg protein). This level was still higher than that of the SO group (p<0.001).

3.1.3. Effect of RA on LPS-induced Hippocampal NOx Change

The results of the hippocampal NOx assay are shown in Fig. 4. As can be seen, after ICV administration, the NOx content in rats hippocampi dramatically was increased compared with the SO group (14.17 ± 1.6 nmol/mg protein *vs* 5.84 ± 0.86 nmol/mg protein, p<0.001). RA treatment at both 2.5 mg/kg (14.55 ± 1.36 nmol/mg protein) and 5 mg/kg (12.33± 1.63 nmol/mg protein) did not show any significant NOx reduction efficiency in LPS injected rats (p>0.05 in both). There was no statistically significant difference between RA doses in the hippocampal NOx level (p=0.056).

3.2. Discussion

To shed more light on one of the probable mechanisms of action of RA, NI was adopted in rats by ICV injection of LPS. Animals were treated with RA before and after the LPS injection. The results demonstrated that systemic administration of RA could influence oxidative stress markers of the CNS, particularly the hippocampus.

As the NI plays a pivotal role in the etiology of many CNS disorders, the LPS-induced models of NI and cognitive impairment are frequently used for studying the mechanisms of cognitive impairment of several neurodegenerative diseases such as AD, PD, multiple sclerosis (MS), and ALS [20]. Numerous studies show that prolonged activation of microglia could lead to deleterious effects over neurons and other cells via an increase in the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), Interleukin-1β (IL-1β), prostaglandin E2 (PGE2), and NOx. [21, 22]. LPS through Toll-like receptor 4 (TLR4) could activate microglia. Subsequently, the activation leads to triggering the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, which is associated with the production of pro-inflammatory cytokines through a Myeloid differentiation factor 88 (MyD88)-dependent pathway. In the CNS, TLR4 is expressed in neurons, astrocytes, and microglia and mediates neuronal death [23, 24]. Therefore, the LPS administration frequently is

used to study NI-associated disorders in rodents. This model is a useful tool for studying the pathological mechanisms involved in neurodegeneration and testing potential therapeutic interventions [25].

Considerable evidence has shown that oxidative stress, NI, and neuronal loss are involved in the initiation and progression of cognitive impairments [26, 27]. In agreement with previous studies reporting significant alterations in oxidative stress markers following ICV injection of LPS [20, 25, 27], in the present experiment, 50 µg ICV injection of LPS resulted in a significant increase in hippocampal NOx and MDA levels and a significant decrease in SOD activity.

NO is a pleiotropic Janus-faced molecule involved in the regulation of the cardiovascular, immune, and nervous systems. In the CNS, NO contributes to many physiological functions such as synaptic plasticity, the sleep-wake cycle, neurosecretion, fluid balance, and reproductive processes. However, its double-edged-sword action plays a key role in several neurodegenerative-associated processes such as neuronal death, necrosis, apoptosis, and autophagy [28]. In this regard, physiological amounts of NOx are neuro-protective, while higher concentrations are neurotoxic. In the present study, hippocampal NOx content significantly increased (about 2-fold) in the LPS injected rats. RA treatment could not influence this increase. To the best of our knowledge, there is no *in vivo* study investigating the effect of RA on hippocampal NOx content in local/systemic LPS induced NI. While *in vitro* studies have shown that RA could suppress NOx production in the inflammatory conditions, *in vivo* studies have not reported such effects except in higher doses [29-31]. A previous study demonstrated that 30 days of oral administration of RA at doses below 16 mg/kg could not affect hippocampal NOx content on ethanol-induced learning and memory deficits in rats [32]. Our results are in contrast with those of Wei et al. who investigated the impact of RA on LPS-stimulated mouse microglial cells (BV2 cell line) and on LPS-induced NI in C57BL/6 mice. They found that RA at 50-200 ng/mL concentrations could ameliorate the expression of inducible nitric oxide synthase (iNOS) in the BV2 cell line. Moreover, in the mentioned study, ICV LPS-injected mice were treated (single dose) with 40, 60, or 80 mg/kg RA (i.p.) 30 min before LPS injection and were sacrificed 24 h after the LPS injection. The whole-brain expression levels of iNOS in RA treated mice (all doses) were significantly lower than LPS-untreated animals [33]. Despite presenting some methodological differences between our study and Wei et al. research – e.g. type of animals, the dose of LPS, doses, the number of RA injections, time of the study, sampling (whole brain/hippocampus), and measured parameters (iNOS mRNA expression vs. NOx content) – we think that RA dose (40-80 mg/kg vs. 2.5-5 mg/kg) is one of the most possible reasons explaining this discrepancy.

Furthermore, we investigated the effects of RA on the antioxidant capacity of the hippocampus by determining the SOD and MDA. Our results indicated that RA in a dose-dependent manner could increase hippocampal

SOD activity and decrease MDA accumulation as a marker of membrane lipid peroxidation. These findings substantiate previous findings in the literature. Although no study has investigated the effect of RA on hippocampal oxidative markers in the LPS-induced NI model, there are several *in vivo* and *in vitro* studies that show RA could increase antioxidant enzymes expression of catalase (CAT), glutathione peroxidase (GPx), and SOD while decreasing the levels of lipo-peroxidation markers such as MDA [34-38].

3.2.1. Limitations

Like other studies, this research has some limitations. This work was a preliminary study to evaluate the possible antioxidant potential of systemic administration of RA on hippocampal oxidative damage. Accordingly, only three oxidative stress markers were evaluated. Thus, further studies are needed to evaluate more oxidative stress markers and investigate the probable cross-talk between oxidative stress cascade and inflammation pathways through them RA modulating the NI.

4. Conclusion

The results demonstrated that systemic administration of RA could affect oxidative stress markers of the CNS, particularly the hippocampus. As oxidative stress plays a key role in the activation and intensification of NF-κB and NLRP3 inflammasome pathways, we can put forward the hypothesis that these effects might be partially due to RA antioxidant potential as well.

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Figures:

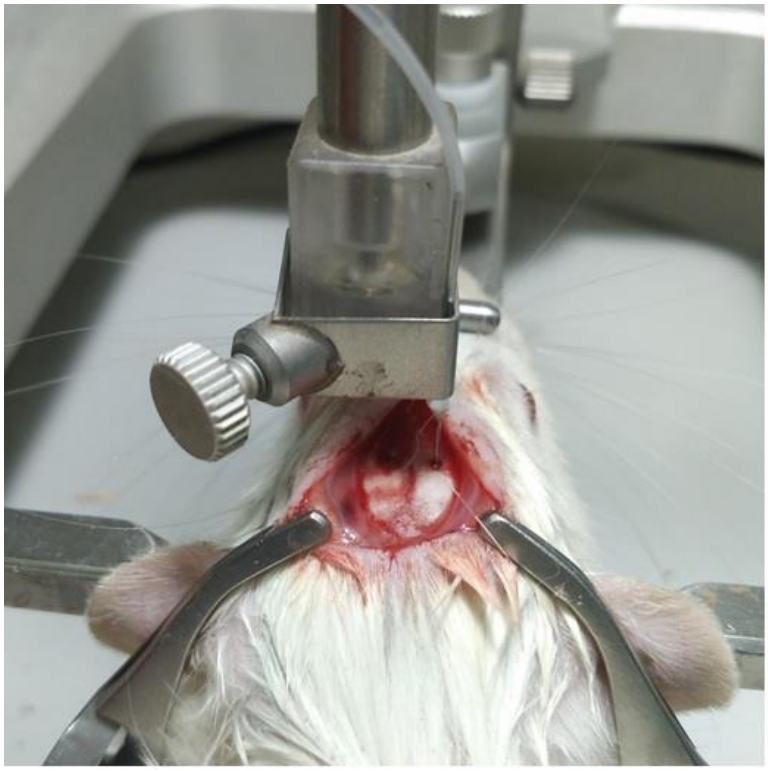

Figure 1. Injection of lipopolysaccharide (LPS) into lateral ventricles using stereotactic apparatus with the infusion rate of 1µL/min.

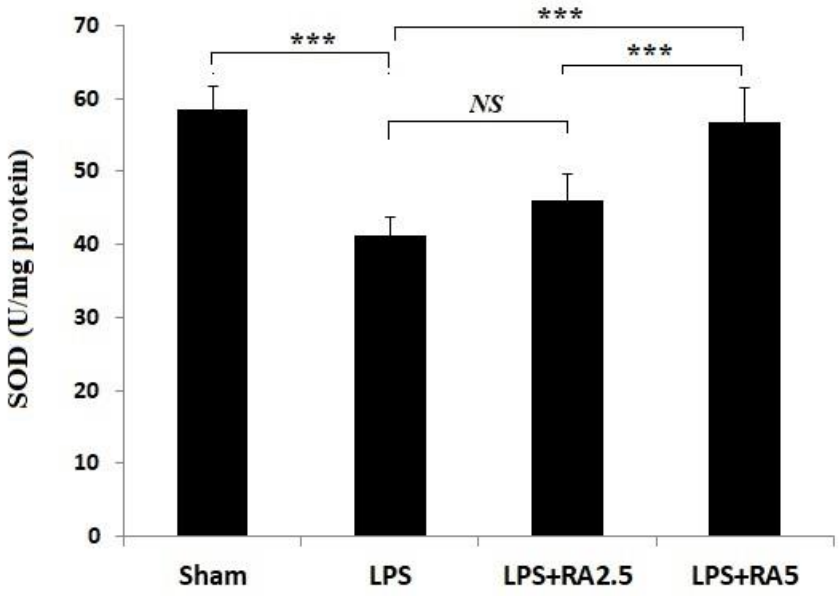

Figure 2. Effects of Rosmarinic acid (RA) on hippocampal superoxide dismutase (SOD) activity. Sham: control rats received vehicle; LPS: rats received lipopolysaccharide (LPS); LPS+RA2.5 and LPS+RA5: the LPS injected rats received RA at doses of 2.5mg/kg and 5mg/kg, respectively. Values are presented as mean±SD (n=6). *** $p<0.001$, NS: not significant.

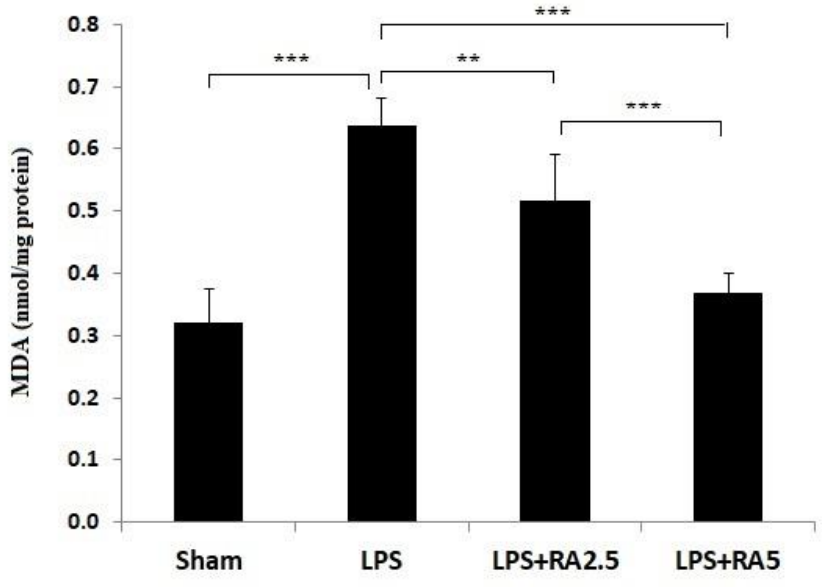

Figure 3. Effects of Rosmarinic acid (RA) on hippocampal malondialdehyde (MDA). Sham: control rats received vehicle; LPS: rats received lipopolysaccharide (LPS); LPS+RA2.5 and LPS+RA5: the LPS injected rats received RA at doses of 2.5mg/kg and 5mg/kg, respectively. Values are presented as mean±SD (n=6). ** p<0.01; *** p<0.001.

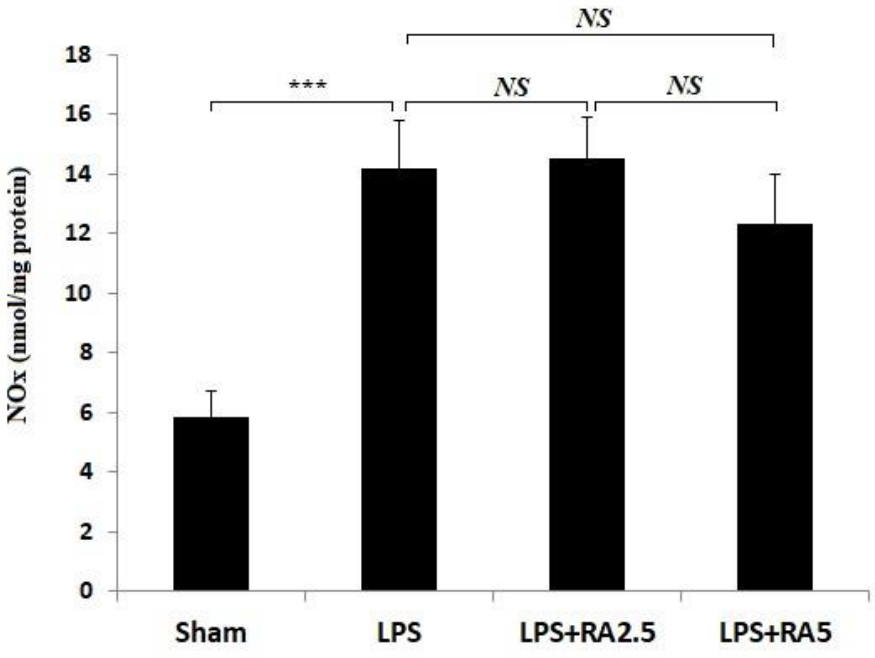

Figure 4. Effects of Rosmarinic acid (RA) on hippocampal nitric oxide content (NOx). Sham: control rats received vehicle; LPS: rats received lipopolysaccharide (LPS); LPS+RA2.5 and LPS+RA5: the LPS injected rats received RA at doses of 2.5mg/kg and 5mg/kg, respectively. Values are presented as mean±SD (n=6). ** p<0.01; *** p<0.001; NS: not significant.

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