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Article

Piracetam-induced neuroprotection in lipopolysaccharides-challenged EOC-20 cells and mouse brain via attenuating oxidative stress

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Therapeutically, piracetam has been used for decades as a cognitive enhancer for memory-related neuronal disorders. The present study aimed to investigate the neuroprotective potential of piracetam on lipopolysaccharides (LPS)-induced neuronal deficit using both *in-vitro* and *in-vivo* experimental models. For the *in-vitro* analysis, EOC-20 murine microglial cells were induced with a neuronal toxicity of 100 µg/ml of LPS, and the formation of intracellular reactive oxygen species (ROS) and nitric oxide (NO) productions were determined. For *in-vivo* neuroprotective analysis, groups of mice were treated orally with two doses of piracetam (200 and 400 mg/kg) for 30 days. Neuronal toxicity was induced by four intraperitoneal injections of LPS (250 µg/kg/day). The malondialdehyde (MDA) level was measured for oxidative stress, and catalase reduced glutathione (GSH), glutathione reductase (GRD), and superoxide dismutase (SOD) levels were determined as the antioxidant parameters. The result of the cell viability study was that pre-treatment with piracetam significantly protected the LPS-induced cell loss, and attenuated the ROS generation and NO production in LPS-induced EOC-20 cells. Moreover, the treatment of piracetam significantly reduced the MDA levels and improved catalase, GSH, GRD, and SOD activities in LPS-induced mice brains. The overall results from this study supported the neuroprotective effects of piracetam against LPS-induced neuronal toxicity.

Keywords: Neuroprotection. Piracetam. EOC-20 cells. Neuroinflammation. Antioxidant activity.

INTRODUCTION

Dysregulation of the inflammatory responses and chronic oxidative stress are widely reported to affect different brain regions and are also considered key factors in the development of numerous CNS-related disorders. In neurodegenerative processes, neuroinflammation and related oxidative stress are the primary causes of neuronal damage. The pathological progress of inflammation in the different brain areas has been reported in several neurodegenerative disorders like Alzheimer's disease (AD), multiple sclerosis, and Parkinson's disease (PD). Among these three major neurodegenerative disorders, AD is the most common. According to Alzheimer's Disease International, 47.47 million people were living with AD and dementia worldwide in 2015. It is estimated that the number will reach approximately 75.63 million in 2030, and it can further increase to 135.46 million in 2050. Also, the estimated total worldwide cost for AD alone was US$ 604 billion in 2010, which is equal to 1% of the world's gross domestic product (Prince, Guerchet, Prina, 2013). Therefore, research to improve treatment options and quality of care for neurodegenerative-related issues must be prioritized.

Not only neurodegenerative processes but also neuroinflammation is closely linked to other pathologies including stroke, trauma, and brain infections (Chen, Zhang, Huang, 2016). Additionally, the process of neuronal inflammation leads to an increase in the overproduction of reactive oxygen species (ROS) and reactive nitrogen

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species (RNS), which play a strong role in the progression of neurodegeneration. A wide range of proinflammatory mediators such as cytokines, ROS, complement factors, neurotoxic secretory products, free radical species, and nitric oxide (NO) are released from microglia cells due to local inflammation (Sastre, Klockgether, Heneka, 2006).

Lipopolysaccharide (LPS) is a pro-inflammatory endotoxin and isolated from the outer membrane of Gram-negative bacteria. LPS has been extensively used as a neuroinflammatory promoter in numerous *in vitro* and *in vivo* experimental models (Sheppard, Coleman, Durrant, 2019). The neuroinflammatory mechanisms of LPS relate to several molecular signaling pathways including induction of pro-inflammatory cytokines, prostaglandin E_2, chemokines, RNS, and oxygen-free radicals. It is known that the manifestation and progress of various neuronal and neurodegenerative disorders are aligned with the imbalance between inflammation and oxidative stress (Luca, Luca, Calandra, 2015). Overproduction of ROS is considered to play a key role in various biological alterations and also our brain is more vulnerable to oxidative stress.

Piracetam is the first nootropic drug group that was originally derived from the neurotransmitter gamma-aminobutyric acid (GABA), and it also belongs to a group of racetams. It is a wildly used drug for age-related cognitive disorders without causing any sedation or stimulation. Therapeutically, it is also used to treat a variety of disorders including vertigo, dyslexia, ischemia, cortical myoclonus, epilepsy, post-stroke aphasia, sickle cell anemia, and PD (Winblad, 2005; Verma *et al.*, 2018). At present, the exact mechanisms of this drug with relation to its effects are not fully understood, but some promising mechanisms have been reported to support its therapeutic functions. Piracetam acts as a continuous positive allosteric modulator on glutamate AMPA receptors and facilitates neuronal excitation (Ahmed, Oswal, 2010). Moreover, the administration of piracetam improves neurotransmitter acetylcholine (ACh) functions through the activation of muscarinic cholinergic receptors (Colucci *et al.*, 2012). Studies have supported the protective effects of piracetam on neuroinflammation and oxidative stress using various experimental models. In the rat hippocampus, piracetam treatment at 200 mg/kg resulted in a considerable reduction of LPS-induced IL-6 cytokine and amyloid-beta (A) levels. The same treatment was also found to provide considerable protection against LPS-induced mitochondrial dysfunctions and behavioral impairments (Tripathi, Paliwal, Krishnamurthy, 2017). Recently, the treatment of piracetam (600 mg/kg, p.o.) has been reported to protect against LPS-induced inflammatory responses by decreasing the expression of the proinflammatory cytokines TNF-α, IL-1β, and IFN-γ in the cortex, hippocampus, midbrain, and striatum areas of the brain in rats. Furthermore, it protects the neuron from oxidative stress-induced cellular damage, and mitochondrion-induced caspase-dependent as well as caspase-independent cellular death caused by LPS (Verma *et al.*, 2018). Based on the collective information, the present study aimed to evaluate the neuroprotective effects of piracetam against LPS-induced oxidative stress *in-vitro*, using EOC-20 microglia cells, and *in-vivo* using a mouse model.

MATERIAL AND METHODS

Cell culture

In the present study, EOC-20 murine microglial cells were used as a neuronal model. The cells were obtained from the *American Type Culture Collection* (*ATCC® CRL-2469™*) and cultivated in Dulbecco's modified Eagle medium (DMEM) accompanied with 10% fetal bovine serum, 1% penicillin-streptomycin and 20% leukocyte adhesion deficiency (LAD)-MAC condition media at 37 ^{o}C in a humidified incubator with 5% CO_2. Every two days, the media was replaced, and the cells were sub-cultured until they reached 60% confluence. At that point, the media was discarded and the cells were washed with phosphate buffer saline. Finally, a rubber policeman was used to detach the cells and the pellet was collected using centrifugation. Following that, according to the experimental design, the cells were plated at appropriate densities.

Cell viability assay

EOC-20 cells were seeded into 96-well plates at a density of 20,000 cells/ml and the cell viability was determined using an MTT [(3-(4, 5-dimethylthiazol-2-

yl)-2, 5-diphenyltetrazolium bromide)] reduction assay. This assay depends on the ability of a mitochondria dehydrogenase enzyme from viable cells to split the tetrazolium rings of the pale yellow MTT and the formation of dark blue formazan crystals (Limpeanchob *et al*., 2008). After incubating overnight, the cells were pre-treated with piracetam at concentrations of 100, 10, 1, 0.1, and 0.01 µg/ml at 37 °C for 24 h and further added to 100 µg/ml of LPS for 24 h. 50 µl of MTT solution (5 mg/ml) was added to each well and incubated at 37° C for 4 h. The purple formazan crystals formed by living cells were solubilized in dimethyl sulfoxide (DMSO) and the absorbance was determined colorimetrically at 570 nm using a microplate reader (BioTek Instruments, U.S.A). The results were expressed as the percentage of MTT reduction by assuming that the absorbance of untreated cells (control) was 100%.

Measurement of intracellular reactive oxygen species (ROS) generation

The production of ROS was evaluated using a 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is a fluorescent probe. DCFH-DA was hydrolyzed by intracellular esterases to non-fluorescent 2'7'-dichlorofluorescein (DCFH) and then converted to fluorescent 2'7'-dichlorofluorescein (DCF) in the presence of ROS (Rahim *et al*., 2020). EOC-20 cells were plated into a 96-well plate at a density of 20,000 cells/ml and incubated for 24 h before treatment. The cells were then pre-treated with piracetam at concentrations of 100, 10, 1, 0.1, and 0.01 µg/ml for 24 h before being induced with 100 µg/ml of LPS. After 24 h, DCFH-DA was added to each well and incubated for 30 min at 37 °C. The production of ROS was measured immediately using a microplate reader (BioTek Instruments, U.S.A.) at 485 nm excitation wavelength and 530 nm emission wavelength. The intensity of DCF fluorescence is proportional to the amount of ROS.

Determination of nitric oxide (NO) production

The level of NO was measured by using a Griess reagent. Before LPS induction, EOC-20 cells were seeded onto 24-well plates and treated for 24 h with piracetam at doses of 100, 10, 1, 0.1, and 0.01 µg/ml. Following the LPS toxicity, cell supernatants from each well were collected and mixed with the Griess reagent, which was then incubated at room temperature for 10 min. After that, the concentration of NO was determined by measuring the absorbance at 540 nm using a microplate reader (BioTek Instruments, U.S.A.).

Animals

The experiments were carried out using male, 8-12 week old Institute of Cancer Research (ICR) mice weighing about 25–35g, obtained from the animal facility of the Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University, Saudi Arabia. The animals were housed in polyacrylic cages and kept at room temperature. The relative humidity was retained at 45-65% with a controlled light-dark cycle. All the animals were allowed to access standard laboratory food and water *ad libitum*. Animals were housed in groups and acclimatized for at least five days before being used in the experiment. The experimental procedures were permitted by the ethical research committee, College of Pharmacy, Qassim University, Saudi Arabia (Reference Number: 2020-CP-16), and the animals were maintained according to the procedures from the National Research Council (U.S.A.) Guide for the Care and Use of Laboratory Animals.

Vehicle

Piracetam (200 and 400 mg/kg) was purchased from Toronto Research Chemicals (TRC), Canada. It was dissolved in 0.9% (w/v) normal saline and administered orally to the mice. LPS was procured from Sigma-Aldrich, U.S.A. It was dissolved (250 µg/kg) in 0.9% (w/v) normal saline and administered by intraperitoneal (i.p.) injection to the mice.

Drug administration

The mice were divided randomly into four groups (n=6), which were the control group, LPS group, and two treatment groups. For the control and LPS groups,

the animals were treated with the vehicle (0.9% w/v of normal saline). For the two treatment groups, piracetam (200 and 400 mg/kg, respectively) was orally administered to the animals for 30 days. The i.p. injection of LPS (250 µg/kg) was administrated daily for the last 4 days (27^{th}, 28^{th}, 29^{th}, and 30^{th} days) of treatment to all the groups except the control to induce neurotoxicity.

Collection of brain samples

At the end of the treatment (on the 30^{th} day), all the animals were sacrificed by cervical decapitation under light anesthesia using a combination of ketamine (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). Immediately after cervical decapitation, the whole brain was carefully isolated in cold PBS (pH 7.4). Then, it was homogenized using a glass WiseStir Homogenizer. The homogenized brain was then centrifuged at 4000 rpm for 10 min at 4 ^{0}C to collect the clear supernatant. The supernatant was kept at -80 ^{0}C for further biochemical analysis.

Measurement of antioxidants and malondialdehyde (MDA) levels in brain homogenate

The oxidative stress and antioxidant parameters were measured by following the ELISA methods. Antioxidant activities in the brain homogenate – catalase (CAT), reduced glutathione (GSH), glutathione reductase (GRD), and superoxide dismutase (SOD) – were evaluated by using corresponding biochemical ELISA assay kits from the Cayman Chemical Company (U.S.A.). Evaluation procedures and analysis of the listed antioxidants followed the manufacturer's protocol. Besides that, the MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation. The TBARs Assay kit from Cayman (U.S.A.) was used for the evaluation.

Statistical Analysis

Results from *in vitro* evaluations such as cell viability, ROS, and NO levels using EOS-20 cells were represented as mean ± standard deviation (SD) of three parallel measurements. Data from *in vivo* studies such as an evaluation of the oxidative and antioxidant parameters using a mouse model were presented as mean ± standard error means (SEM) of six animals. All the data were analyzed using a One-way ANOVA procedure for comparisons between all the treatments in both the *in vitro* and *in vivo* studies. A Tukey–Kramer post hoc test was done to calculate significance levels between the two results (Graph Pad version 9, GraphPad Software Inc., United States). Statistical significance was defined as a P-value of less than 0.05.

RESULTS

Pre-treatment with piracetam protected EOC-20 cells against LPS-induced cytotoxicity

The results of the experiment are shown in Figure 1. Exposure of EOC-20 cells with 100 µg/ml of LPS for 24 h resulted in 60% cell death as measured using the colorimetric MTT assay. The cultured EOC-20 cells with 24 h pre-treatment with respectively 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml of piracetam significantly ($P<0.01$; $P<0.001$; $P<0.001$; $P<0.001$; $P<0.001$, respectively) increased the cell survival as compared to those that were only LPS-induced. In addition, the percentages of cell viability were recorded as 68% (0.01 µg/ml), 76% (0.1 µg/ml), 83% (1 µg/ml), 89% (10 µg/ml), and 95% (10 µg/ml) with piracetam against LPS-induced cytotoxicity (100 µg/ml; 40%). Based on the results, the higher dosage of piracetam (100 µg/ml) showed better prevention of LPS-induced cell death. As a result, piracetam's effect was found to be dose-dependent.

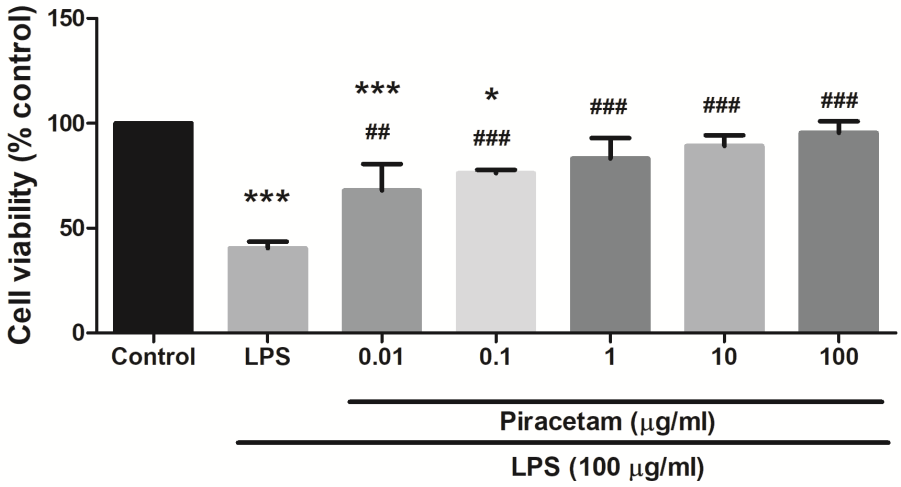

FIGURE 1 - Effect of piracetam on cell viability against LPS-induced cytotoxicity in EOC-20 cells. The viability of cells was assessed by MTT assay. The percentage of cell viability was relative to the untreated control cells. Each bar represents the mean ± SD of three independent experiments ($^{***}P<0.001$ and $^{*}P<0.05$ vs. control; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. LPS).

Pre-treatment with piracetam reduced the level of intracellular reactive oxygen species (ROS) and nitric oxide (NO) against LPS-induced oxidative stress in EOC-20 cells

The effect of pre-incubation of piracetam on LPS-induced ROS and NO productions in EOC-20 cells was evaluated (Figure 2). When EOC-20 cells were exposed to LPS, the intracellular ROS and NO levels were evaluated to demonstrate oxidative stress, which resulted in cell death. Exposure of EOC-20 cell culture with 100 μg/ml LPS alone significantly increased (P<0.001) the level of intracellular ROS and NO. Pre-treatment of EOC-20 cells with piracetam at concentrations of respectively 0.01 μg/ml, 1 μg/ml, 10 μg/ml, and 100 μg/ml significantly attenuated (P<0.05, P<0.01, P<0.01, and P<0.001, respectively) the level of intracellular ROS induced by 100 μg/ml of LPS (Figure 2A). Meanwhile, only the highest concentration of piracetam (100 μg/ml) significantly reduced (P<0.001) the level of NO as compared to cells that were only LPS-induced (Figure 2B). The results suggested that pre-treatment of piracetam significantly protects the LPS-induced oxidative stress using the EOC-20 cell line.

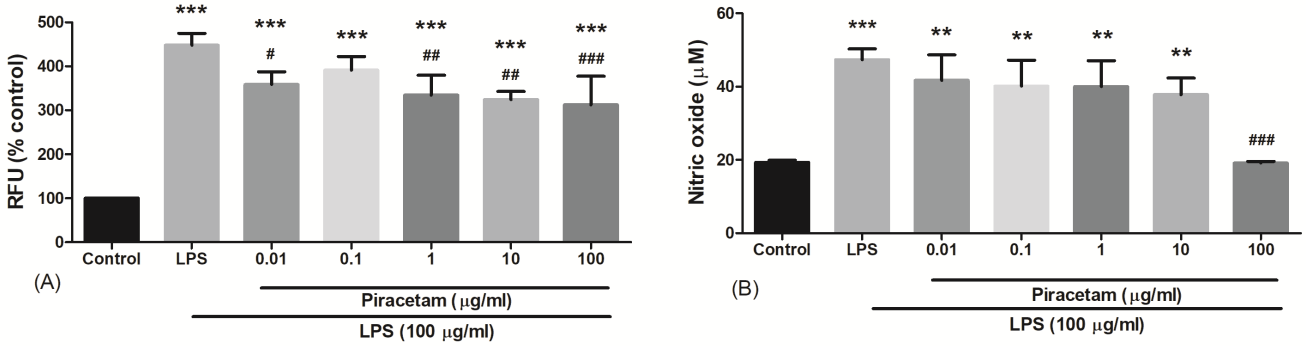

FIGURE 2 - Effect of piracetam on ROS and NO against LPS-induced in EOC-20 cells. (A) ROS level (RFU-Relative Fluorescence Units) was measured by the oxidation of DCFH-DA. (B) NO level was measured using a Griess reagent. Each bar represents the mean ± SD of three independent experiments ($^{**}P<0.01$ and $^{***}P<0.001$ vs. control; $^{\#}P< 0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs LPS).

Pre-treatment with piracetam attenuates oxidative stress and enriches the antioxidants in brain homogenates of LPS-challenged mice

The potential of piracetam for lipid peroxidation production was investigated in this study by measuring the level of malondialdehyde (MDA) in the mouse brain (Figure 3A). A significant increase ($P<0.001$) in the brain MDA level of LPS group (2.81 ± 0.36 μM) as compared with the control group (1.58 ± 0.04 μM) indicated the elevation of oxidative stress by LPS. However, the animals who received piracetam (200 and 400 mg/kg) had significantly ($P<0.01$) attenuated oxidative stress levels compared to the LPS group. The MDA levels in animals treated with 200 and 400 mg/kg of piracetam were 1.58 ± 0.12 μM, and 1.65 ± 0.08 μM, respectively.

Meanwhile, the activities of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and glutathione reductase (GRD) were measured to read the antioxidant capacity of piracetam in the mouse brain. For the catalase activity (Figure 3B), the LPS-induced group showed a significant decline (1.49 ± 0.06 nmol/min/ml; $P<0.01$) in CAT levels as compared with the control group (18.51 ± 1.84 nmol/min/ml). The groups of mice that were administered orally with the 200 and 400 mg/kg of piracetam significantly increased ($P<0.001$) CAT levels, which were about 23.94 ± 1.84 nmol/min/ml and 23.47 ± 2.21 nmol/min/ml as compared to the LPS-treated group. Besides, in terms of GSH (Figure 3C) activity, only the treatment of piracetam at 400 mg/kg considerably improved (106.2 ± 5.14 μM; $P<0.01$) the GSH activity in the mouse brain as compared to the LPS group (77.76 ± 3.59 μM). For the GRD activity (Figure 3D), the treatment of LPS expressed lower ($P<0.001$) GRD activity (60.33 ± 1.57 nmol/min/ml) when compared with the control group (99.12 ± 8.93 nmol/min/ml). Oral administration of 200 mg/kg (80.25 ± 1.191 nmol/min/ml) and 400 mg/kg (84.79 ± 2.97 nmol/min/ml) of piracetam showed significant increase ($P<0.05$, and $P<0.01$, respectively) in the levels of GRD compared with the LPS group. In SOD levels (Figure 3E), the LPS group showed a significant reduction (2.18 ± 0.17 U/ml, $P<0.01$) in SOD activity as compared with the control group (2.93 ± 0.07 U/ml). The group of mice treated with 400 mg/kg of piracetam showed an improvement (2.96 ± 0.16 U/ml; $P<0.01$) in SOD level as compared to the LPS group. Treatment with 200 mg/kg of piracetam did not lead to any significant difference in brain SOD level.

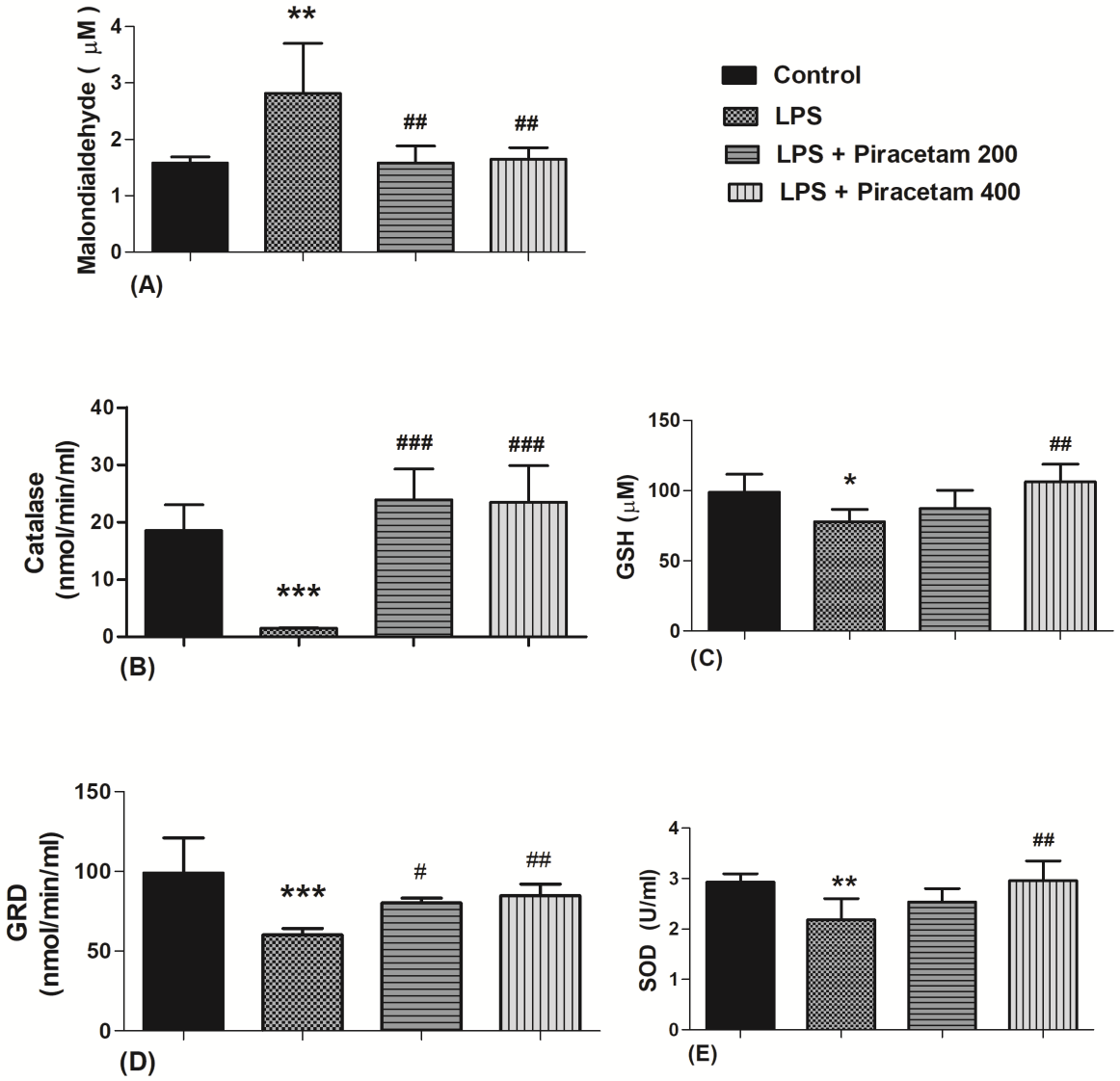

FIGURE 3 - Effect of piracetam on oxidative stress and antioxidants in brains of LPS-induced mice. The antioxidants and oxidative stress were measured with an ELISA kit, which examined: (A) MDA level in brain homogenate that reflected oxidative stress, (B) Catalase activity in brain homogenate, (C) Concentration of reduced glutathione (GSH) in brain homogenate, (D) Glutathione reductase (GRD) activity in brain homogenate, and (E) Superoxide dismutase (SOD) activity in brain homogenate. The values are mean ± SEM (n=6) (*P<0.05, **P<0.01 and ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS-induced group).

DISCUSSION

Neuroinflammation and oxidative stress are well-known contributors to the chronic neurodegenerative process in the brain. The event of increasing oxidative stress is correlated with excessive release of vulnerable reactive oxygen and nitrogen species (ROS and RNS), which result in neuronal damage and lead to neurodegenerative processes. In Alzheimer's disease (AD), the process of neuroinflammation is reported to result from the cytotoxicity of neurons that leads to AD lesions and majorly contributes to the development of neurodegeneration (Rahim *et al.*, 2020). Moreover, the production of inflammatory mediators during inflammation exerts adverse cellular effects, particularly through the excessive liberation of vulnerable oxidative

free radicals and depletion of antioxidant mechanisms (Fischer, Maier, 2015). Our present investigation evidenced the possible neuroprotective influence of piracetam against lipopolysaccharide (LPS)-induced neuroinflammation-related oxidative stress using both *in vitro* and *in vivo* experimental models.

Microglia cells are considered as a primary immune cell in the brain that mediates inflammation-related neuronal damage (Garden, Möller, 2006). In the present *in vitro* evaluation, EOC-20 microglial cells were used to study the potential of piracetam on cell viability, and intracellular production of ROS as well as NO against LPS-induced neuronal toxicity. Originally, the microglial cells are derived from the resident macrophages and performed surveillance functions in response to a wide range of invading pathogens. Microglia initiate innate immune responses, characterized by the production of cytokines and chemokines, upregulation of cell surface molecules, and expansion of local immune responses (Kielian, Mayes, Kielian, 2002). Furthermore, microglial cell functions are directly controlled by neuron-microglia interactions. Neurons can control the microglial activities via various neurotransmitters and direct cell-to-cell interactions. On the other hand, the expression of specific receptor numbers allows microglia to respond to the neurotransmitter signals from neurons (Cui *et al.*, 2018). In parallel, the scientific reports also strongly suggest that the regulation of microglia function could be a successful future therapeutic target for the prevention of various neurological dysfunctions associated with chronic diseases (Garden, Möller, 2006; Bureta *et al.*, 2019). In the current study, the effects of piracetam on cell viability, intracellular ROS generation, and NO production in EOC-20 microglial cells against LPS-induced neurotoxicity are explained through its neuroprotective potential.

Before the evaluation of neuroprotection, the cytotoxicity potential of piracetam alone was confirmed on EOC-20 cells using its serial concentrations such as 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml. The percentages of cell viability were recorded as 87%, 81%, 78%, 73%, and 56% at concentrations of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml, respectively. The IC_{50} value (half-maximal inhibitory concentration) of piracetam on EOC-20 microglial cells was not found until the maximum concentration of 100 µg/ml. For further cell viability studies and neuroprotective evaluations against LPS toxicity, the same concentration ranges of piracetam were used. Interestingly, pre-treatment with all the selected doses of piracetam resulted in a significant improvement in EOC-20 cells viability and protected the cells from LPS-induced neuronal toxicity. Piracetam could protect the EOC-20 cells in a dose-dependent manner. LPS derived from Gram-negative bacteria is known as endotoxin, and there is a great interest in inducing various experimental models that are related to neurodegenerative diseases including AD and PD (Sharifi, Hoda, Noor, 2010; Sheppard, Coleman, Durrant, 2019). Regarding LPS-induced neuronal vulnerability, the exact mechanism is unknown. However, reports from *in vitro* studies highlight that LPS can increase apoptosis in neurons, astrocytes, and microglial cells (Niewiarowska-Sendo *et al.*, 2015). Additionally, it was reported that exposure to LPS with neuronal inflammation released several pro-inflammatory cytokines including TNF-α and IL-1β, and induced oxidative stress, with the generation of ROS as well as NOS (Shi *et al.*, 2016; Stansley, Post, Hensley, 2012).

Elevation of ROS, malondialdehyde (MDA), nitric oxide (NO), and inducible nitric oxide synthase (iNOS) levels were reported as key factors contributing to oxidative damage in the body (Bian *et al.*, 2016). It is known that abundant increases in ROS production lead to oxidative stress, which could extensively involve neuronal degeneration in AD and PD. Moreover, higher ROS levels of neurons result in neuronal toxicity by damaging the cellular lipid, nucleic acids, proteins, and mitochondria. In fact, higher cytosol ROS levels could also release further ROS through triggering the neighboring mitochondria and leading to significant neuronal damage (Mohsenzadegan, Mirshafiey, 2012). In microglial cells, activation of LPS triggers oxidative stress-induced mediators including ROS and NO. These might be actively involved in generating neuroinflammatory mediators and eventually inducing neurodegeneration (Sheppard, Coleman, Durrant, 2019). Similar to other microglial cells, EOC cells are able to release many mediators such as inflammatory cytokines and reactive species when induced by a stimulating agent. Particularly, LPS-induced NO production is regulated by a

higher activity of inducible nitric oxide synthase (iNOS and NOS-II) and superoxide anion (O_2•) through stimulation of NADPH oxidase complexes (Boje, Arora, 1992). Using the EOC-20 cells, our study found that the LPS (100 µg/ml) stimulation initiated a significant elevation of ROS and NO productions as compared to non-treated control cells. However, the elevation of the ROS levels by LPS was significantly attenuated by pre-incubating the cells with piracetam at 0.01 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml. Furthermore, the exposure of piracetam at a higher concentration of 100 µg/ml significantly reduced the NO production in LPS-induced EOC-20 cells. These obtained data support the neuroprotective effect of piracetam against LPS-induced neuroinflammatory-related oxidative stress in EOC-20 cells.

The present *in vitro* results were further validated using an *in vivo* experimental model to evaluate malondialdehyde (MDA) levels for the oxidative stress, and the levels of catalase (CAT), reduced glutathione (GSH), glutathione reductase (GRD), and superoxide dismutase (SOD) as the antioxidant parameters in LPS-challenged mice brain homogenate. Reportedly, LPS acts as a neurotoxin that binds to toll-like receptor-4 and induced neuroinflammatory mediators like cytokines and chemokine expressions in the brain (Lee *et al.*, 2019). An early report highlighted that LPS-induced acute inflammation results in oxidative stress, blood-brain barrier disruption, and neuronal loss in the hippocampus and cerebellum in adult as well as aged rats (Aslankoc *et al.*, 2018; Garcia *et al.*, 2018). Furthermore, it was reported that intraperitoneal (i.p.) injection of LPS elevated the MDA level, i.e. oxidative stress, and reduced glutathione peroxidase (GPx) activity and GSH levels in the cerebellum (Garcia *et al.*, 2018). In another study, the intracerebroventricular (ICV) microinjection of LPS in mice was found to cause a significant decrease in SOD activity and an increase of MDA level (Zhao *et al.*, 2017). These results strongly support the present finding of i.p. injection of LPS (250 µg/kg) for 4 days, presented with various changes that increased MDA level and decreased CAT, GSH, GRD, and SOD levels in the mouse brain. However, thirty days of continuous oral piracetam administration ameliorated LPS-induced changes in the mouse brain. Similarly, it has been reported that a continuous 9-day administration of piracetam (200 mg/kg, i.p.) attenuated LPS by ICV infusion-induced hippocampal mitochondrial oxidative stress, by significantly reducing LPO as well as nitrate levels and increasing SOD activity in rats (Tripathi, Paliwal, Krishnamurthy, 2017). Furthermore, in the spinal cord ischemia/reperfusion injured rabbit experimental model, treatment with piracetam (250 mg/kg, i.p.) resulted in a significant reduction of MDA levels and elevation of GP_X activity in isolated spinal tissue (Kalkan *et al.*, 2011).

The main cause of oxidative stress in the body is an imbalance between the production of free radicals and the ability to scavenge them. In particular, brain tissue contains a high level of polyunsaturated fatty acids and is also more susceptible to oxidative damage due to its high level of oxygen content and low capability in antioxidant protection (Moreira *et al.*, 2005). Commonly, excessive production of free radicals leads to the elevation of LPO levels that could alter the functions of cellular protein and nucleic acid, resulting in cell death. Following this, an elevated level of the MDA, an end product of LPO, is a key marker for oxidative stress (Tripathi, Paliwal, Krishnamurthy, 2017). The present study demonstrated that pre-treatment with both doses of piracetam (200 and 400 mg/kg, p.o.) reduced the oxidative stress in brain tissue by a significant reduction in MDA level in the LPS-induced mouse brain.

Furthermore, the antioxidant defense system is essential to remove the overproduction of free radicals in oxidative stress. In oxygen metabolism, the superoxide anion radical is a vulnerable by-product, which is catalyzed into hydrogen peroxide (H_2O_2) by an antioxidant enzyme, SOD. The high level of H_2O_2 in brain tissue could also induce polyunsaturated fatty acids oxidation and increase LPO as well as MDA levels (Xu *et al.*, 2014). On the other hand, the production of H_2O_2 is detoxified to H_2O and O_2 by CAT or GPx activities. Moreover, in peroxidases activity, GSH is utilized as an electron donor (Ahmad, 1995). In brain tissue, the GSH redox cycle plays a major role in the detoxification of cellular free radicals. In the presence of GPx, GSH detoxifies the ROS molecules, including H_2O_2, from the conversion of superoxide anion (O2-·) by SOD. During this detoxification reaction, GSH is converted to an

oxidized form, GSSG, and then reduced back to GSH by the enzymatic reaction of glutathione reductase (GRD) with the utilization of NADPH (Simpson, Pase, Stough, 2015). Our results highlight that pre-administration of piracetam markedly increases activities of antioxidants such as SOD, CAT, GRD, and GSH, and also reduces MDA levels in the brains of LPS-induced mice.

CONCLUSION

Collectively, our results, using *in-v*itro and *in-vivo* experimental models, have highlighted the neuroprotective effects of piracetam by ameliorating the oxidative vulnerability to LPS. Pre-treatment with piracetam attenuated LPS-induced oxidative stress by significantly reducing the intracellular ROS and NO levels in microglial EOC-20 cells. Also, the treatment significantly improved the cell viability against LPS toxicity. In addition, a continuous 30-day pre-treatment of piracetam in groups of mice supported their antioxidant potential by causing a significant increase in catalase, reduced glutathione, glutathione reductase, and superoxide dismutase activities in brain tissues. Furthermore, the treatment of piracetam reduced the malondialdehyde levels in the LPS-induced mouse brain. These findings suggest that piracetam might be a useful drug for the prevention of neuroinflammatory associated oxidative stress in AD management.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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