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ORIGINAL PAPER

Ellagic Acid Inhibits Neuroinflammation and Cognitive Impairment Induced by Lipopolysaccharides

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Abstract

Neuroinflammation is a predisposing factor for the development of cognitive impairment and dementia. Among the new molecules that are currently being studied, ellagic acid (EA) has stood out for its neuroprotective properties. The present study investigated the effects of ellagic acid in the object recognition test, oxidative stress, cholinergic neurotransmission, glial cell expression, and phosphorylated Tau protein expression. For this, 32 male Wistar rats received an intraperitoneal (IP) application of lipopolysaccharides (LPS) at a dose of 250 µg/kg or 0.9% saline solution (SAL) for 8 days. Two hours after the IP injections, the animals received 100 mg/kg of EA or SAL via intragastric gavage. Behavioral parameters (open field test and object recognition) were performed on days 5, 6, and 7 of the experimental periods. The results showed that the treatment with EA in the LPS group was able to inhibit cognitive impairment, modulate the immune system response by significantly reducing glial cell expression, attenuating phosphorylated Tau and oxidative damage with consequent improvement in the antioxidant system, as well as preventing the increase of acetylcholinesterase activity. Thus, the neuroprotective effects of EA and its therapeutic potential in cognitive disorders secondary to neuroinflammation were demonstrated.

Keywords Ellagic acid · Oxidative stress · Acetylcholinesterase · Microglia · Astrocytes · Rats

Introduction

Neuroinflammation is a characteristic of several neurological disorders, including Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, and acute traumatic brain injury [1–4]. Systemic administrations of lipopolysaccharides (LPS) have been described as experimental models that mimic the pathological disorders of these diseases, including AD-associated cholinergic neuronal degeneration. LPS can impair the consolidation of specific memory processes. Acute administration of LPS before training impairs the contextual fear conditioning test, a learning paradigm dependent on the hippocampus [5], while chronic LPS infusions affect spatial memory [6] and induce impairments in memory and learning analogous to cognitive impairment observed in AD [7]. In contrast, systemic administration of LPS results in damage to the hippocampus-dependent memory on object discrimination, but not on spatial memory [8].

Intraperitoneal (IP) injections of LPS cause cognitive impairment in laboratory animals through the activation of microglia, which stimulates the production of pro-inflammatory mediators. This mechanism is apparently due to the communication pathways between the immune system and the brain [9]. In response to the production of pro-inflammatory cytokines, several reactive oxygen species (ROS) are produced, which culminates in oxidative stress [10, 11]. Increased production of ROS promotes rapid changes in the

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antioxidant system, through the induction or depletion of cellular antioxidant reserves [12]. Also, excessive activation of the microglia perpetuates the inflammatory cycle [13], prolonging inflammation [14], which predisposes to the development of several neurodegenerative diseases [15], damage to the vascular endothelium, depletion of redox-glutathione, and mitochondrial respiratory dysfunction, which culminates in a reduction in the consumption of ATP and O_2 [16].

The tau protein (Tau) is related to several physiological processes in neurons. When hyperphosphorylated, Tau monomers detach from microtubules and tend to aggregate into neurofibrillary tangles. This process is observed in several neurodegenerative disorders, called tauopathies [17]. The neurodegenerative process in these diseases is characterized by an amyloid cascade with consequent formation of amyloid plaques, Tau phosphorylation, neuroinflammation, and neuronal death. It is believed that the formation of amyloid oligomer (A) is the first step towards neurodegeneration, initiating the amyloid cascade [18]. In a brain inflammatory microenvironment, the production of cytokines by microglia and astrocytes can potentiate the amyloid cascade, which demonstrates the relationship between tauopathies and neuroinflammation [19, 20].

Drugs for improving cognition such as memantine, aniracetam, piracetam and cholinesterase inhibitors such as galantamine are used to improve memory, mood, and behavior, but their side effects limit the use of these agents. Thus, other possibilities, including plant derivatives, have been considered and evaluated as therapeutic alternatives [21]. There are several evidences to support the potential of antioxidants in the prevention and treatment of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. Furthermore, evidences in the literature confirms the ability of components with antioxidant properties to protect neurons against the harmful effects of ROS, preventing, or delaying the development of neurodegenerative diseases [22, 23]. Among these antioxidants, ellagic acid (EA) stands out, which is relatively stable under physiological conditions in the stomach and can be a potential phytotherapeutic candidate for the development of neuroprotective drugs that can be administered orally. This antioxidant has multiple pharmacological properties that are useful in the treatment and maintenance of disorders of the central nervous system. It can regulate several molecular signaling pathways, in order to normalize mitochondrial dysfunctions that result in the generation of free radicals and thus attenuate neurodegeneration [24]. The antioxidant action of EA occurs due to its direct property of free radicals scavenging and potentiating endogenous antioxidants [10]. EA can protect the brain from inflammation through down-regulation of the expression of several pro-inflammatory cytokines (such as TNF-α) [11]. The suppression of microglial responses represents the therapeutic effect of EA in AD. Also, in vivo and in vitro studies have shown a reduction in the release of inflammatory cytokines by microglia and amyloid plaques induced by EA [25].

Thus, the present study aimed to evaluate the action of EA in the cerebral cortex and hippocampus by recognizing memory and oxidative stress parameters such as ROS, lipid peroxidation, protein carbonylation, and T-SHs and GSH levels in an experimental model of neuroinflammation induced by multiple applications of LPS in rats. The study also aimed to investigate the effect of EA on acetylcholinesterase (AChE) activity and expression of neural and phosphorylated proteins in this experimental model.

Materials and Methods

Animals

This work was approved by the Ethics Committee on the Use of Animals of the Federal University of Santa Maria under number 5580160118. Thirty-two male Wistar rats with 6 to 7 weeks old (200–230 g), from the Central Bioterium of the Federal University of Santa Maria, were used. Animals in this age group have been chosen as they are more anxious and show more exploratory behavior than rats aged 16 weeks (300–320 g) commonly used in several experimental models [26].

Four animals were housed per box with food and water available ad libitum. The rats were kept in an environment with controlled temperature and humidity (22–24 °C; 70% RH), light/dark cycle (7:00 a.m.–7:00 p.m.), and previously acclimated for 2 weeks. The animals were randomly divided into four groups, containing eight animals each: control (CTR + SAL), control treated with ellagic acid (CTRL + EA), lipopolysaccharide (LPS + SAL) and lipopolysaccharide treated with ellagic acid (LPS + EA). The animals in the LPS groups (LPS + SAL and LPS + EA) received, for eight consecutive days, a daily application (IP) of LPS at a dose of 250 µg/kg dissolved in 0.9% saline, while the control groups (CTRL + SAL and CTRL + EA) received only injections (IP) of 0.9% saline solution (SAL) in the same volume and period. One hour after the IP injections, the animals received via intragastric gavage (IG) EA at a dose of 100 mg/kg (CTRL + EA and LPS + EA) or 0.9% of saline in the same volume and route (CTRL + SAL and LPS + SAL). The animals were weighed daily to adjust the dose of the compounds to be used (Fig. 1).

Lipopolysaccharide

Systemic administration of LPS is a model widely used to induce neuroinflammation, as it results in increased levels

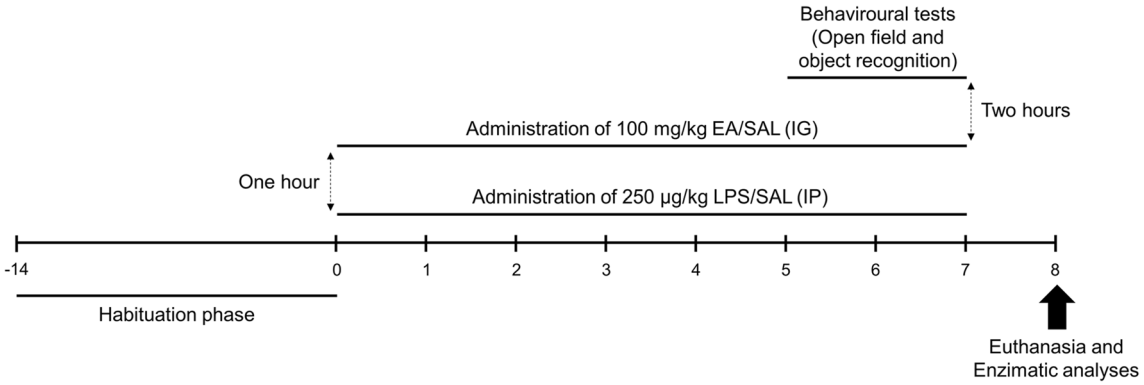

Fig. 1 Experimental protocol

of cerebral cytokines and activation of microglia [27, 28]. In this context, to induce the neuroinflammatory response, lipopolysaccharides from Escherichia coli (Sigma-Aldrich, O111-B4) diluted in saline and injected intraperitoneally at a dose of 250 µg/kg, once a day, for 8 days were used. This dose was selected according to previous studies [7, 29].

Ellagic Acid

Ellagic acid (Sigma-Aldrich) was used in doses of 100 mg/kg, orally, once daily, one hour after application of LPS. The treatment lasted 8 days. The EA was suspended in saline and administered via gavage. The suspension was homogenized in a sonicator before each administration to obtain a homogeneous solution. This treatment protocol is based on previous studies with this polyphenol [30–38].

Open Field Test

This test was performed to identify changes in the locomotor and exploratory capacity of the animals, as previously described by [39] and was performed on day 5 (Fig. 1). The apparatus consists of a wooden box covered with waterproof material with dimensions 70 × 70 × 30 cm. The floor was divided into 16 squares measuring 12 × 12 cm each to assess the open field. The session lasted five minutes and was recorded for further processing by an automated activity monitoring system (AnyMaze, Stoelting, USA) to assess the total distance covered; mobile or immobile time; time in the central zones, walls or corners; and number of entrances or exits in the central zones, walls or corners.

Object Recognition Test

The object recognition task was used to study recognition memory in rats [40]. The animals were submitted to training on day 6 (Fig. 1), where they were individually placed in the open field containing two similar objects (A1 and A2) being allowed to explore them freely for 5 min. For the evaluation of short-term memory 2 h after the training session the animals were individually reintroduced into the open field, where one of the objects presented during training was replaced by a new object with different size and shape (A1 and B). To assess long-term memory the same procedure was performed 24 h after the training session, replacing object B with a new object of different size and shape (object C). This task consists of the spontaneous and differential exploration of familiar and new objects, and the recognition performance is derived from the time spent exploring the two stimuli. Exploration of objects was considered by animal's snout directing at a distance ≤ 2 cm from the object and sniffing or touching the object with the snout. Climbing or sitting on objects was not classified as exploratory behavior. The results were expressed as preference index (percentage of time = new object/[new object + family object] × 100) ± SEM, which evaluates the percentage of time exploring the new object, and total exploration time (total time = new object) + familiar object) ± SEM.

Brain Tissue Preparation

At the end of the behavioral assessments, the animals were euthanized by overdose of isoflurane. After opening the skull, the brain was removed and separated into the cerebral cortex and hippocampus and homogenized in a solution of 10 mM Tris–HCl (pH 7.4), under ice, in a proportion of 1:10 (weight/volume). After centrifugation, the aliquots resulting from the homogenates of the brain structures were used to determine the parameters of oxidative stress and acetylcholinesterase activity.

The protein of brain structures was previously determined through a range varying for each structure: cerebral cortex (0.7 mg/mL) and hippocampus (0.8 mg/mL), as determined by the Coomassie blue method [41].

Determination of Acetylcholinesterase Activity in the Brain

The AChE enzymatic activity was determined by the Ellman et al. [42] method as modified by Rocha et al. [43]. This method is based on formation of the yellow 5-thio-2-nitrobenzoic acid, which was measured spectrophotometrically at 412 nm for 2 min at 25 °C. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) and the AChE enzyme (40–50 µg of protein), which was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). The experiment was carried out in triplicate, and enzyme activity was expressed as µmol AcSCh/h/mg of protein.

Measurement of Reactive Oxygen Species (ERO)

The 2′,7′-Dichlorofluorescein fluorescence assay was used to measure the production of hydrogen peroxide and other reactive species [44]. 50 mL aliquots of the brain structure homogenate supernatant were added to a medium containing Tris–HCl buffer (0.01 mM, pH 7.4) and DCFH DA 2′,7′-Dichlorofluorescein-diacetate (1 mM). After adding DCFH-DA, the medium was incubated in the dark for 1 h until fluorescence measurement (excitation at 488 nm and emission at 525 nm, with both slit widths at 1.5 nM). Dichloro-oxidized fluorescein was determined using an oxidized dichlorofluorescein standard curve, and the results are expressed as DCFH-DA Fluorescence.

Thiobarbituric Acid Reactive Substances (TBARS) Measurement

The levels of thiobarbituric acid reactive substances (TBARS) were determined according to Jentzsch et al. [45] by measuring the concentration of malondialdehyde (MDA) as a product of lipid peroxidation through reaction with thiobarbituric acid (TBA). Briefly, the reaction mixture containing 200 µL of supernatant from the brain structure or standard homogenate (0.03mMMDA), 1 mL of 0.2 M orthophosphoric acid, and 250 µL thiobarbituric (0.1 M) was heated to 95 °C for 120 min. Absorbance was measured at 532 nm. Serum TBARS levels are expressed in nmol MDA/mg protein.

Protein Carbonyl Levels

Protein carbonyl was determined by the method of Levine et al. [46] and modified by Reznick, Packer [47] and Liebel et al. [48]. A medium containing 2,4-dinitrophenylhydrazine (DNPH) 10 mmol and hydrochloric acid (HCl) was added to the protein precipitate and incubated at room temperature for one h. During the incubation, samples of the supernatant from the brain structure homogenate were mixed vigorously every 15 min. Then, 500 µL of denaturation buffer (3% sodium dodecyl sulfate (SDS) plus 2000 µL of ethanol and 2000 µL of heptane were added. Resuspended in 1000 µL of denaturation buffer and placed in the maria for about 20 min (40 or 50 °C) until the pellets are dissolved. The reading was performed at 370 nm on the UV–VIS spectrophotometer. The results are expressed as nmol/mg of protein.

Determination of Total Thiols (T-SH) and Reduced Glutathione (GSH)

The total number of thiol groups was analyzed spectrophotometrically using the method of Ellman [49] and Boyne, Ellman [50], with some modifications. A 200 µL aliquot of the brain structures homogenate supernatant in a final volume of 900 µL of the solution was used for the reaction. The reaction product was measured at 412 nm after adding 50 µL of 10 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and it will be expressed as nmol of T-SH/mL of serum. GSH was measured spectrophotometrically with Ellman's reagent. An aliquot of 200 µL of serum in a final volume of 900 µL of the solution was used for the reaction. The reaction product was measured at 412 nm after adding 50 µL of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was added to calculate the content of non-protein thiol groups in samples and expressed as nmol of GSH serum/mL.

Flow Cytometry Analysis of Neural Marker Proteins and Phosphorylated Proteins

Flow cytometry experiments for measurement of p-Tau and Iba-1 were performed as previously described [51]. Briefly, cells from hippocampus were fixed for 10 min by adding 4% PFA. Primary staining was performed with monoclonal antibodies against the phosphorylated Tau (1:200; Sigma-Aldrich), glial fibrillary acidic protein (GFAP) (1:500; Sigma-Aldrich) and ionized calcium binding adaptor molecule 1 (Iba-1) (1:200; Wako) for 30 min followed by addition of secondary Alexa-Fluor-488 antibodies (1:500; Life Technologies). The measurements were performed on a Calibur Cytometer (BD Biosciences) and analyzed with Flowjo V10 software (*F*lowjo, Ashland, OR). The results are expressed as percentage (%) of positive cells.

Statistical Analysis

All data were analyzed using two-way ANOVA followed by Tukey's post hoc test in a statistical program (GraphPad

Prism 8). The data were expressed as mean ± SEM, and a statistically significant difference was considered $p < 0.05$.

Results

LPS Promotes a Reduction in Body Weight After the First Application

To assess the systemic effects of LPS or EA, the bodyweight of the rats was measured. We found a statistically significant difference in body weight during the experimental period by both groups (F (3, 224) = 24.81, p < 0.0001) and days (F (3, 224) = 19.87, $p < 0.0001$), though the interaction between these terms was not significant (F (21, 224) = 0,8424, $p = 0{,}6655$). A slight reduction in mean body weight was observed in the groups that received IP injection of LPS (LPS + SAL) on the second day of the experimental period, as shown by a Tukey's test (Fig. 2), with a significant reduction in the bodyweight of the animals in the LPS + SAL group on days 3–5 when compared to the CTRL + SAL group. The animals in the present study showed a gradual increase in body weight during the experimental period. This fact was attributed to the growth phase of the animals.

LPS and EA Did Not Alter Locomotor Activity

In this experiment, the effects of repeated applications of LPS were evaluated, as well as the treatment with EA on the locomotor activity of the rats in an open field test, since the memory test can be affected by locomotor changes. There were no significant differences between groups in the total distance travelled; mobile or immobile time; time in the central zones, walls or corners; and number of entrances or exits in the central zones, walls or corners (Table 1 and Fig. 3) indicating that the compounds did not promote changes in the animals' locomotor activity and, therefore, the results observed in the memory recognition test are not related to locomotor impairment.

Ellagic Acid Reverses Cognitive Impairment Induced by LPS

Two-way ANOVA revealed a significant influence of both time and groups (treated and not treated) on object recognition index (Table 2). All groups, except LPS + SAL, learned the localization of the object A1, as evidenced by the longer time spent exploring the new objects (Fig. 4).

A significant reduction in the preference index of the new object was observed in 2 h (short term memory) and 24 h (long term memory) in the group that received multiple applications (IP) of LPS when compared to the control group. However, the group treated with 100 mg/kg of EA demonstrated a significant improvement in memory retention when compared to the LPS group in both short- and long-term memories, indicating that treatment with EA prevents cognitive impairments induced by LPS. Also, there was no significant difference between groups in the exploration time of both objects during the training phase, 2 h, and 24 h (Fig. 4).

EA Prevents LPS-Induced Increased AChE Activity

There was a significant influence of both control (SAL or LPS) and treatment (SAL or EA) and an interaction between these two terms on AChE activity (Table 3). Post-hoc Tuckey's shown a significative increase ($p < 0.05$) in AChE activity in the CO and HP in the LPS group when compared to the control group. In contrast, treatment with EA in the LPS group (LPS + EA) was able to prevent an increase in the activity of this enzyme (Fig. 5).

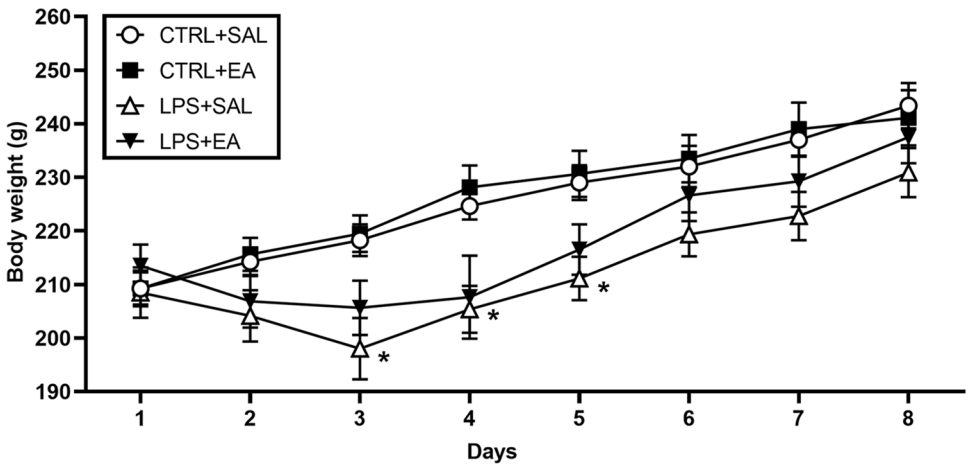

Fig. 2 Effect of multiple applications (IP) lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the weight of rats. The data were expressed as mean of the weights ± SEM. N = 8 animals/group. *Denotes significant difference of the LPS + SAL group compared to CTRL + SAL group. *$p < 0.05$

Table 1 Two-way ANOVA for the open field test

Locomotor ability	Effect	Df	*F* value	*p* value
Total distance travelled (m)	Control	1	0.1481	0.7033
	Treatment	1	0.002186	0.9630
	Control × treatment	1	0.1169	0.7350
Total time mobile (s)	Control	1	0.06356	0.8028
	Treatment	1	0.009537	0.9229
	Control × treatment	1	2.117	0.1568
Total time immobile (s)	Control	1	0.09282	0.7629
	Treatment	1	4.148e-005	0.9949
	Control × treatment	1	1.923	0.1765
Number of entries to the center zone	Control	1	0.2424	0.6263
	Treatment	1	0.004947	0.9444
	Control × treatment	1	0.8360	0.3683
Number of exits from the center zone	Control	1	0.2424	0.6263
	Treatment	1	0.004947	0.9444
	Control × treatment	1	0.8360	0.3683
Time in the center zone (s)	Control	1	0.8853	0.3548
	Treatment	1	0.06985	0.7935
	Control × treatment	1	0.8655	0.3602
Number of entries to the wall zone	Control	1	1.007	0.3243
	Treatment	1	1.252	0.2726
	Control × treatment	1	0.1206	0.7310
Number of exits from the wall zone	Control	1	1.172	0.2882
	Treatment	1	1.345	0.2559
	Control × treatment	1	0.07325	0.7886
Time in the wall zone (s)	Control	1	1.233	0.2762
	Treatment	1	0.3120	0.5809
	Control × treatment	1	1.265	0.2702
Number of entries to the corner zone	Control	1	0.5143	0.4792
	Treatment	1	1.025	0.3200
	Control × treatment	1	0.1139	0.7383
Number of exits from the corner zone	Control	1	0.2047	0.6545
	Treatment	1	1.233	0.2763
	Control × treatment	1	0.4330	0.5159
Time in the corner zone (s)	Control	1	0.9036	0.3500
	Treatment	1	0.03705	0.8487
	Control × treatment	1	0.006620	0.9357

The data correspond to the main effects and interaction between the factors "control" (saline or lipopolysaccharide) and "treatment" (saline or ellagic acid), as shown for 12 distinct locomotor ability parameters. There were 28 residual degree of freedom for each test. Results for post-hoc Tuckey's test are shown in Fig. 3

EA Prevents LPS-Induced Increased Oxidative Damage

Two-way ANOVA analysis of oxidative parameters of CO and HP showed a significant effect of of both control (SAL or LPS) and treatment (SAL or EA) on the levels of ROS, TBARS and protein carbonyl (Tables 4 and 5). Also, there was a significant increase in ROS levels in the CO (Fig. 6a) and HP (Fig. 6b) in the LPS group compared to group control. As a consequence of the increased production of these reactive species, it was also possible to observe a significant increase in lipid peroxidation, demonstrated by the high levels of TBARS (Fig. 6c and d), and protein damage, evidenced by the elevation of the protein carbonyl in CO and HP (Fig. 6e and f). On the other hand, compared to the LPS group, the treatment with EA (LPS + EA) was able to inhibit the oxidative damage caused by ROS in CO and HP, as evidenced by Figs. 6a-f.

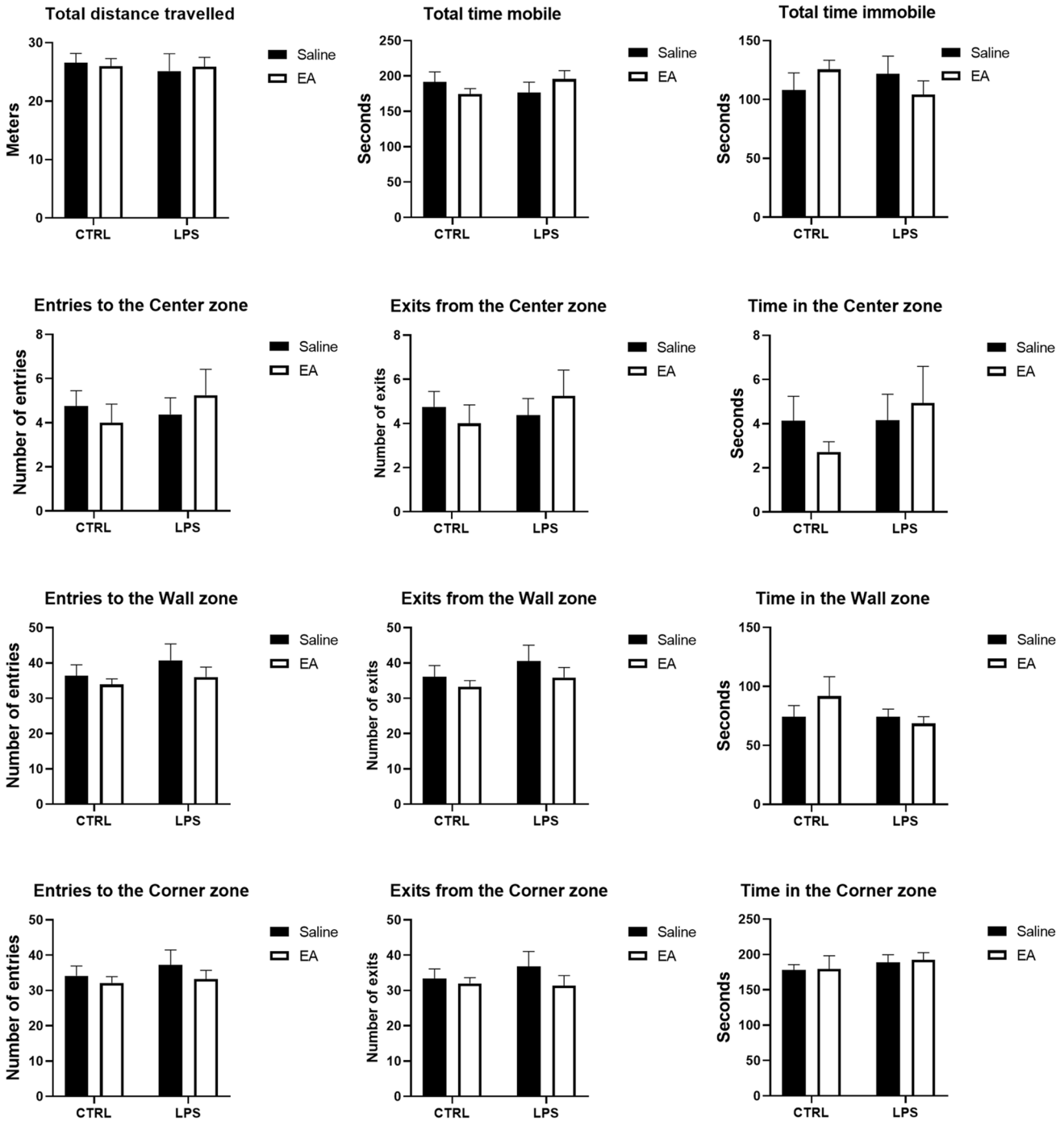

Fig. 3 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the locomotor activity of rats. The behavioral test was performed two hours after treatment (IG) with EA 100 mg/kg or saline, which occurred one hour after IP injection of LPS 250 µg/kg or saline. Data are expressed as mean ± SEM. N = 8 animals/group. There were no statistically significant differences ($p < 0.05$) between groups

EA Prevents LPS-Induced Depletion of Total (T-SH) and Non-protein (GSH) Thiols

Since we observed a reduction in the production of ROS and related damages after treatment with EA in the group that received LPS (LPS + EA), we evaluated the levels of antioxidants to better understand the mechanisms involved in the neuroprotection performed by EA. Thus, a significant reduction in the levels of T-SH and GSH was observed in CO and HP in the LPS + SAL group when compared

Table 2 Two-way ANOVA for the object recognition test

Memory task	Effect	Df	*F* value	*p* value
Object recognition index	Time	3	8.406	< 0.0001
	Groups	2	31.13	< 0.0001
	Time × groups	6	2.569	0.0247
Total exploration time (s)	Time	3	1.667	0.1803
	Groups	2	6.664	0.0021
	Time × groups	6	0.114	0.9949

The data correspond to the main effects and interaction between the factors "time" (0 h, 2 h and 24 h) and "groups" (lipopolysaccharide and saline treated or not with ellagic acid), as shown for 2 memory task parameters. There were 84 residual degree of freedom for each test. Results for post-hoc Tuckey's test are shown in Fig. 4

to the CTRL + SAL group. However, treatment with EA (LPS + EA) was able to prevent the reduction of T-SH and GSH in both brain structures when compared to the LPS + SAL group (Fig. 7). Also, it was observed influence of both control (SAL or LPS) and treatment (SAL or EA) on T-SH and GSH from cerebral cortex and hippocampus (Tables 4 and 5).

EA Inhibits LPS-Induced Neuroinflammation

There was a significant effect of both control (SAL or LPS) and treatment (SAL or EA) and an interaction between them in the percentage of Iba-1$^+$ and GFAP$^+$ cells (Table 6). Post-hoc Tuckey's shown significant increase ($p < 0.05$) was observed in the percentage of Iba-1$^+$ and GFAP$^+$ cells in the LPS + SAL group compared to the control group (CTRL + SAL) (Fig. 8). In contrast, the groups treated with EA (CTRL + EA and LPS + EA) had a low frequency of glial cells when compared to the LPS group (LPS + SAL), suggesting that this compound inhibits the neuroinflammatory process triggered by LPS.

EA Suppresses LPS-Induced Phosphorylation of Tau Protein (P-Tau)

In the present study, two-way ANOVA revealed a significant influence of both control (SAL or LPS) (F (1, 16) = 14.31, $p = 0.0016$) and treatment (SAL or EA) (F (1, 16) = 22.93, $p = 0.0002$) in the percentage of P-Tau$^+$ cells, though the interaction between these terms was not significant (F (1, 16) = 2.069, $p = 0.1696$). Post-hoc Tuckey's shown a significant reduction in the percentage of P-Tau$^+$ cells were observed in the groups treated with EA (CTRL + EA and LPS + EA), indicating a neuroprotective effect of this compound. Although there is no statistically significant difference between the control and untreated LPS groups (CTRL + SAL and LPS + SAL), there is an increase in the frequency of P-Tau$^+$ in the LPS + SAL group (Fig. 9).

Discussion

This study aimed to demonstrate the effects of EA on LPS-induced neuroinflammation through memory-related assessments, such as object recognition test and AChE activity. The percentage of Iba-1$^+$, GFAP$^+$, and p-Tau$^+$ cells was quantified to evaluate the neuroinflammatory effect, the redox profile was assessed by ROS generation, lipid peroxidation and protein carbonylation, as well as levels of non-enzymatic antioxidants. Also, the effects of multiple LPS applications on the animals' body weight and locomotor activity, assessed through the open field test, were evaluated. The results of this study demonstrated that EA was able to prevent cognitive impairment caused by multiple applications of LPS, as well as modulate the immune system response by significantly reducing the expression of glial cells, attenuating oxidative damage caused by the action of endotoxins.

The animals in the present study showed a reduction in body weight from the first application of LPS (LPS + SAL and LPS + EA), becoming significant ($p < 0.05$) on day 3 in the LPS + SAL group (Fig. 2). From the fourth day on, there was a gradual increase in the body weight of animals in the LPS groups (LPS + SAL and LPS + EA). Also, no statistically significant differences were observed in the open field test, performed on the 6th day of the experimental period. Corroborating with the results obtained by other authors [52], which performed an IP application of LPS (100 or 200 mg/kg) on days 1, 4, and 7 in female and male rats and evaluated locomotor activity, body weight, and hormone levels. The authors reported a reduction in locomotor activity and in the body weight of the animals after the first application of LPS. In contrast, there was a reduction in the deleterious behavioral effects of LPS after a second exposure to LPS in male and female rats, being more evident in females. After the third administration of LPS, no behavioral changes were observed. The authors attributed the findings to the mechanism of tolerance to LPS, which after multiple sublethal injections, results in less responsiveness to the compound and, consequently, higher survivability to the subsequent lethal dose of endotoxins. This low responsiveness has been called tolerance [53–55] and comprises an adaptation of the organism to limit excessive inflammation, trough less production of pro-inflammatory cytokines [55]. Consequently, there is a reduction in sickness behavior, since this mechanism is mediated mainly by the action of macrophages and cytokines on the periphery, as well as mechanisms of transduction of inflammation from the periphery to the brain [56]. Thus, it is suggested that the weight gain observed from the 3rd day of the experimental period is a consequence of the inhibition of sickness behavior, which

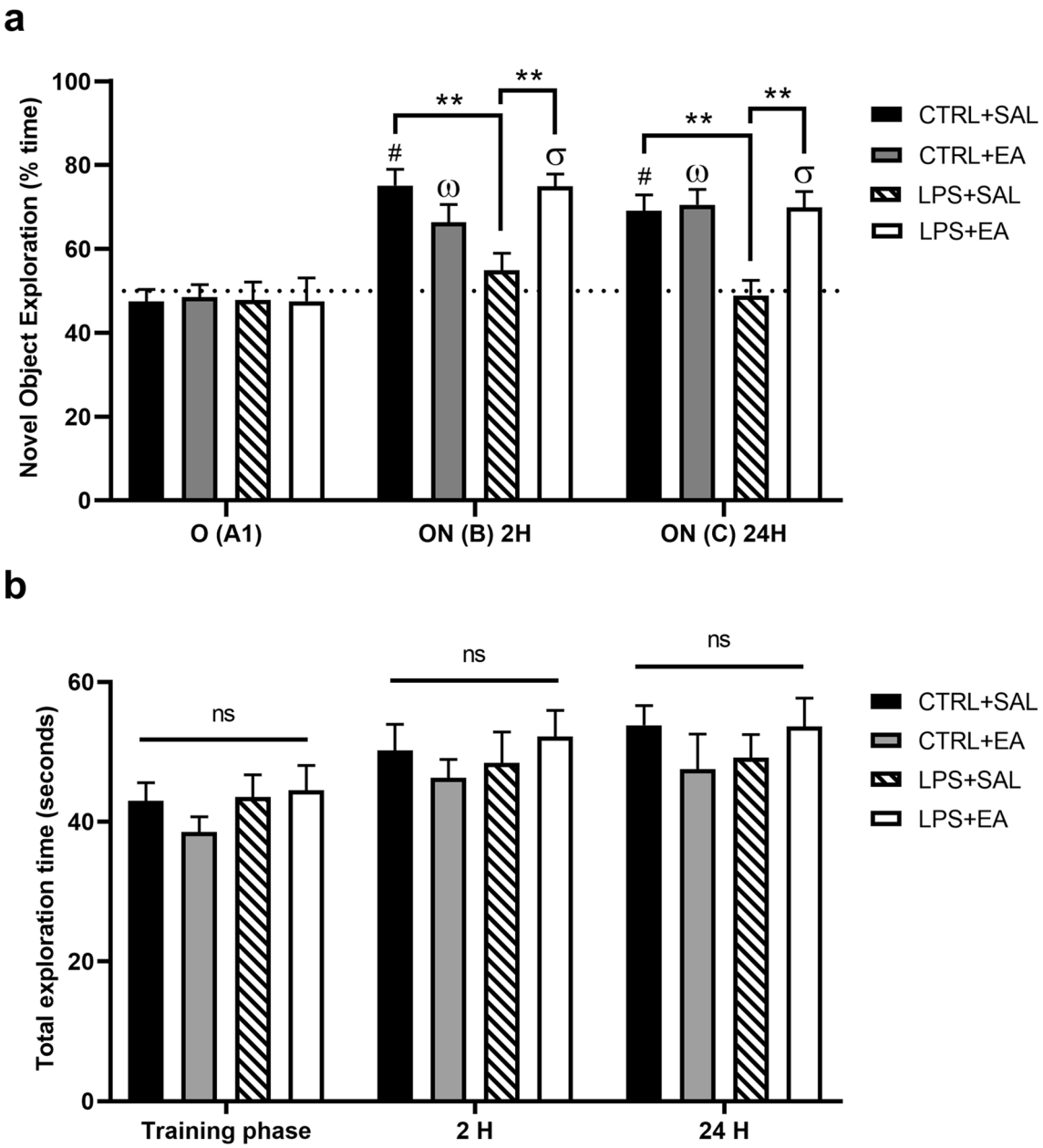

Fig. 4 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the short- and long-term memory of rats submitted to the memory recognition test. The results are expressed as % of the exploration time of the new object (percentage of time = new object/[new object + familiar object] × 100) ± SEM (**a**) and total exploration time of both objects (total time = new object + familiar object) ± SEM (**b**). N = 8 animals/group. Different symbols denote significant difference between groups. #: when compared to CTRL + SAL − O (A1), ω when compared to CTRL + EA − O (A1), σ when compared to LPS + EA − O (A1). ns $p > 0.05$, $^{**}p < 0.01$, $\#p < 0.05$, ω $p < 0.05$, σ $p < 0.05$

possibly resulted in higher food and water intake by the groups the groups that received multiple applications of LPS (IP) (LPS + SAL and LPS + EA). The same can be attributed to the absence of changes in the locomotor activity of the animals, evidenced by the open field test (Fig. 3).

Although the effect of tolerance to multiple IP applications of LPS has been well described in the literature [57], several authors have reported cognitive impairment [58–61] and elevation in pro-inflammatory cytokines in the central nervous system. Chen et al. [62] demonstrated, after multiple applications of LPS, that the expression of cytokines in response to this endotoxin can be regulated in different ways between the peripheral immune system and the CNS. The increase in the production of pro-inflammatory cytokines is associated with an increase in the activation of microglia

Table 3 Two-way ANOVA for the AChE activity

AChE activity	Effect	Df	*F* value	*p* value
Cortex	Control	1	7.916	0.0089
	Treatment	1	7.097	0.0127
	Control × treatment	1	3.024	0.0930
Hippocampus	Control	1	12.37	0.0015
	Treatment	1	3.987	0.0557
	Control × treatment	1	4.435	0.0443

The data correspond to the main effects and interaction between the factors "control" (saline or lipopolysaccharide) and "treatment" (saline or ellagic acid), as shown for 2 distinct cerebral structures. There were 28 residual degree of freedom for each test. Results for post-hoc Tuckey's test are shown in Fig. 5

AChE acetylcholinesterase

and astrocytes [63]. Usually, microglia cells act phagocyting dead cells and cellular debris to maintain CNS homeostasis, while astrocytes are responsible for preserving neurological function [64]. However, when stimulated in excess, microglia and astrocytes significantly increase neuroinflammation, resulting in pathogenesis by the secretion of several pro-inflammatory mediators [64–66].

In the present study, a significant increase in the percentage of positive glial cells (Iba-1$^+$ and GFAP$^+$) was observed in the LPS + SAL group (Fig. 8). These findings can be attributed to the action of LPS, a potent stimulator of microglia and astrocyte activation that can cause harmful neuroinflammatory responses through the production of TNF-α, IL-6, IL-1β, iNOS and COX-2 [67, 68]. In contrast, in the group treated with EA (LPS + EA), less expression of Iba-1$^+$ and GFAP$^+$ cells were observed. These results are in agreement with that described by other authors [25], who observed that the EA is able to inhibit microglial activation via attenuation of Nuclear factor of activated T-cells (NFAT) activity. Still, it is believed that polyphenols acts extracellularly by capturing cytokines to attenuate the stimulation of glial cells, thus exerting their anti-inflammatory function [69]. Thus, an anti-inflammatory effect of EA was observed, since this antioxidant reduced the expression of Iba-1$^+$ and GFAP$^+$ cells in the hippocampus of the LPS + EA group rats, which suggests that this compound can mitigate the deleterious effects observed in neurodegenerative disorders.

As previously described, the activation of microglia and astrocytes results in the cerebral release of cytokines. These pro-inflammatory interleukins directly affect neuronal function, such as long-term potentiation (LTP), glutamate release, AMPA receptor trafficking, and activation of cell-signaling pathways [70–72], which are related to synaptic plasticity and neurotransmission. Therefore, there may be impairment of neuronal processes related to cognition.

In the present study, the animals in the LPS + SAL group showed significantly lower performance in object recognition in the short- and long-term memory tests when compared to the other groups (Fig. 4). This cognitive impairment is due to the high density of receptors for cytokines in the hippocampus, particularly in the dentate gyrus [73], indicating that this structure may be particularly vulnerable during neuroinflammation [8]. Consequently, the administration of immunogenic stimuli, such as LPS, can compromise hippocampus-dependent memory and learning processes [74]. In contrast, there was a protective effect of EA in the short and long-term memory test, in which the LPS + EA group had a significantly higher performance than the LPS + SAL group. Several authors have reported the beneficial effects of EA on memory in models of cognitive impairment [30, 38, 75, 76], which occurs from the action of this antioxidant at the molecular level through the attenuation of oxidative

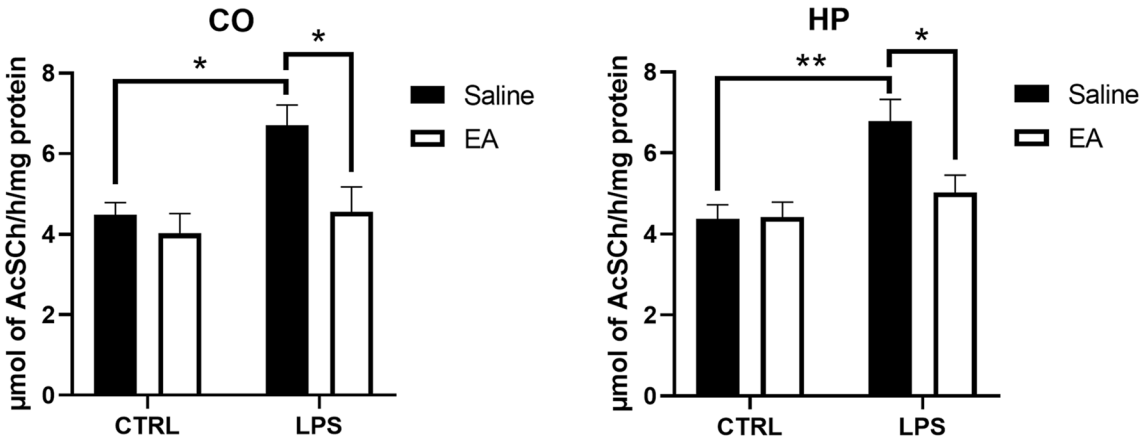

Fig. 5 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the activity of acetylcholinesterase (AChE) in the cerebral cortex (CO) and hippocampus (HP) of rats. Data are expressed as mean ± SEM. N = 8 animals/group. *Denotes significant difference between groups. *$p < 0.05$, **$p < 0.01$

Table 4 Two-way ANOVA for oxidative parameters of cerebral cortex (CO The data correspond to the main effects and interaction between the factors "control" (saline or lipopolysaccharide) and "treatment" (saline or ellagic acid), as shown for five distinct oxidative parameters

CO oxidative parameters	Effect	Df	*F* value	*p* value
ROS	Control	1	6.597	0.0158
	Treatment	1	10.33	0.0033
	Control × treatment	1	3.177	0.0855
TBARS	Control	1	40.47	< 0.0001
	Treatment	1	14.76	0.0006
	Control × treatment	1	5.011	0.0333
Carbonyl	Control	1	9.658	0.0043
	Treatment	1	12.79	0.0013
	Control × treatment	1	12.75	0.0013
T-SH	Control	1	3.974	0.0560
	Treatment	1	5.958	0.0212
	Control × treatment	1	4.829	0.0364
GSH	Control	1	3.617	0.0675
	Treatment	1	3.201	0.0844
	Control × treatment	1	8.731	0.0063

There were 28 residual degree of freedom for each test. Results for post-hoc Tuckey's test are shown in Figs. 6 and 7

ROS reactive oxygen species, *TBARS* lipid peroxidation, *Carbonyl* protein carbonylation, *T-SH* total thiols, *GSH* non-protein thiols

Table 5 Two-way ANOVA for oxidative parameters of cerebral hippocampus (HP)

HP oxidative parameters	Effect	Df	*F* value	*p* value
ROS	Control	1	14.51	0.0007
	Treatment	1	11.72	0.0019
	Control × treatment	1	7.206	0.0121
TBARS	Control	1	8.837	0.0060
	Treatment	1	12.80	0.0013
	Control × treatment	1	1.985	0.1699
Carbonyl	Control	1	75.69	< 0.0001
	Treatment	1	36.00	< 0.0001
	Control × treatment	1	51.38	< 0.0001
T-SH	Control	1	2.945	0.0972
	Treatment	1	7.380	0.0112
	Control × treatment	1	5.069	0.0324
GSH	Control	1	10.38	0.0032
	Treatment	1	6.300	0.0181

The data correspond to the main effects and interaction between the factors "control" (saline or lipopolysaccharide) and "treatment" (saline or ellagic acid), as shown for five distinct oxidative parameters. There were 28 residual degree of freedom for each test. Results for post-hoc Tuckey's test are shown in Figs. 6 and 7

ROS reactive oxygen species, *TBARS* lipid peroxidation, *Carbonyl* protein carbonylation, *T-SH* total thiols, *GSH* non-protein thiols

stress, reduced AChE activity and modulation of the pathway of nuclear factor kappa B (NF-κB), nuclear factor erythroid 2–related factor 2 (Nfr2) and Toll-like receptor (TLR4) signaling, which are related to the neuroinflammation mechanism induced by LPS. This endotoxin binds to TLR4 on the surface of the microglia. It activates several transduction pathways, which result in the activation of NF-κB, which will mediate the production of pro-inflammatory cytokines, chemokines and inducible enzymes, such as inducible synthase oxide (iNOS) and COX-2, culminating in neuroinflammation [21, 77], as observed by the increased expression of positive glial cells (Iba-1$^+$ and GFAP$^+$) in the LPS + SAL group. These findings demonstrate the potential of EA to reverse cognitive impairments secondary to neuroinflammatory processes. This hypothesis is supported by the reduction in the expression of positive glial cells observed in the LPS + EA group observed in the present study and improved performance in the object recognition test compared to the untreated group (LPS + SAL).

Also, the cognitive impairment produced by systemic administration of LPS may be involved with the dysregulation of the cholinergic system, evidenced by the reduction in levels of acetylcholine (Ach), a neurotransmitter involved in the processes of memory and learning [78, 79]. Previous studies have shown that LPS causes depletion in brain ACh levels as a consequence of inducing AChE activity [12, 79, 80], which degrades ACh. Also, the expression of AChE increases in response to IL-1 [81] and oxidative stress [82, 83] induced by LPS. This pattern was observed in the present study, in which the animals that received LPS (LPS + SAL) showed a significant increase in AChE activity compared to the animals in the control group (CTRL + SAL) (Fig. 5). In contrast, the increased AChE activity was prevented in animals treated with EA (LPS + EA). It is believed that this prevention occurs through changes in the gene expression profile involved in the synthesis of AChE [84]. These results corroborate with previous studies [84, 85]. Thus, it is suggested that the improvement in cognitive performance may also be related to the reduced activity of AChE in the LPS + EA group compared to the LPS + SAL group since the reduction in the activity of this enzyme promotes an increase in the concentration of ACh. This hypothesis is supported by studies that have observed that AChE inhibition promotes learning and memory improvement in animals [84, 86].

Several authors have documented the relationship between oxidative stress and inflammation. Inflammation induces oxidative stress and DNA damage, which triggers an exacerbated production of ROS by microglia and macrophages. Damage from oxidative stress, such as oxidized proteins, glycated products, and lipid peroxidation, results in neuronal degeneration frequently reported in brain disorders [87]. Cells damaged by oxidative damage produce a large number of inflammatory mediators that promote the aging

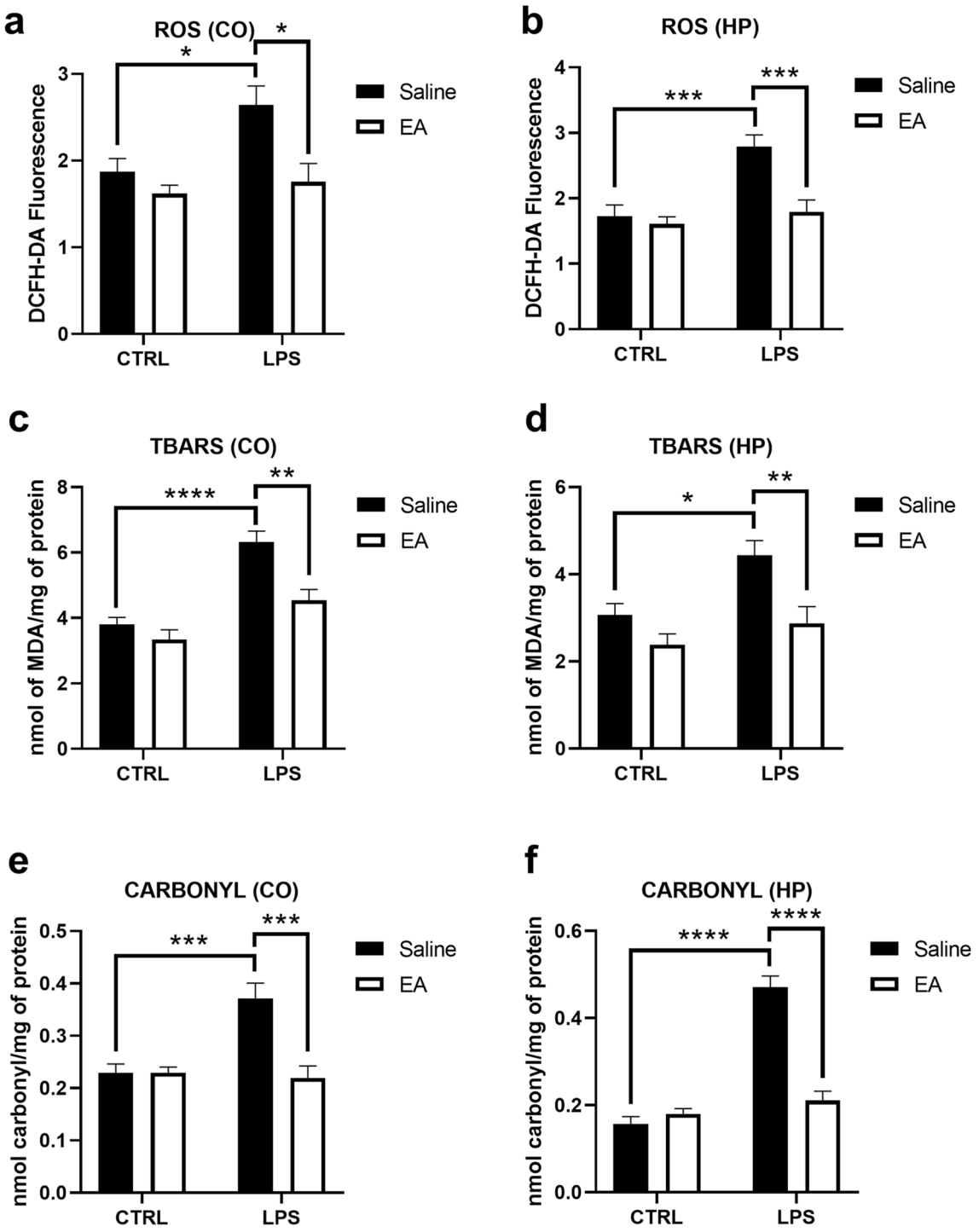

Fig. 6 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the levels of reactive oxygen species (ROS), lipid peroxidation (TBARS) and protein carbonylation (carbonyl) in the cerebral cortex (CO) and hippocampus (HP) of rats. Data are expressed as mean ± SEM. N = 8 animals/group. *Denotes significant difference between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

of the microglia [88]. In addition to the oxidative damage of ROS in macromolecules, these reactive species can also trigger inflammatory responses by stimulating several genes that regulate the inflammatory signaling cascade. Acute and chronic inflammation and aging processes are the primary triggers for excessive ROS production.

We observed significantly high levels of ROS, TBARS, and protein carbonylation (carbonyl) in the cerebral cortex

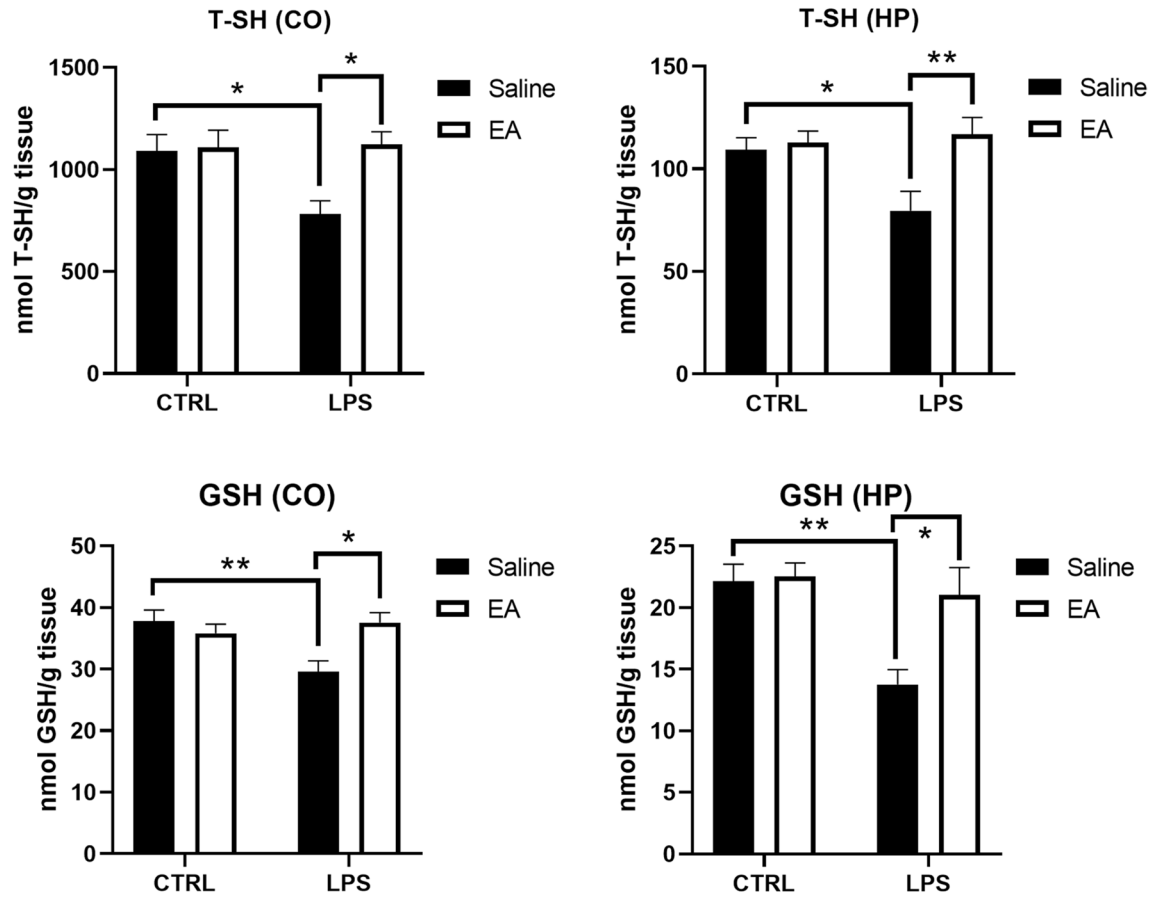

Fig. 7 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the levels of total thiols (T-SH) and non-protein thiols (GSH) in the cerebral cortex (CO) and hippocampus (HP) of rats. Data are expressed as mean ± SEM. N = 8 animals/group. *Denotes significant difference between groups. $*p < 0.05$, $**p < 0.01$

and hippocampus in the LPS + SAL group compared to the CTRL + SAL group (Fig. 6). Studies have shown that LPS activates astrocytes and microglia that secrete gliotransmitters, such as glutamate and adenosine triphosphate (ATP), which play the role of substrate for the production of extracellular adenosine and neurotoxic molecules, such as free radicals [89, 90], which justifies the results found by our group, since there was an increase in the expression of positive glial cells in the LPS + SAL group as previously described. Furthermore, there was a depletion of the intracellular antioxidant system, demonstrated by the significant reduction in the levels of GSH and T-SH in the cerebral cortex and hippocampus of the LPS + SAL group compared to the CTRL + SAL group (Fig. 7). These results suggest exhaustion of the antioxidant system, due to the progression of the inflammatory reaction, which may contribute to the neurodegeneration process [91]. In contrast, the EA promoted a reduction in oxidative parameters (ROS, TBARS, and carbonyl) in the cerebral cortex and hippocampus (Fig. 6) through its antioxidant action, which occurs due to its direct property of free radical scavenging [10]. The hydroxyl group and the lactone ring present in the EA directly detoxify superoxide, hydroxyl radicals,

Table 6 Two-way ANOVA for the neuroinflammation

Neuroinflammation	Effect	Df	*F* value	*p* value
$GFAP^+$ cells	Control	1	7.916	0.0089
	Treatment	1	7.097	0.0127
	Control × treatment	1	3.024	0.0930
Iba-1^+ cells	Control	1	12.37	0.0015
	Treatment	1	3.987	0.0557
	Control × treatment	1	4.435	0.0443

The data correspond to the main effects and interaction between the factors "control" (saline or lipopolysaccharide) and "treatment" (saline or ellagic acid), as shown for 2 distinct neuroinflammatory markers. There were 16 residual degree of freedom for each test. Results for post-hoc Tuckey's test are shown in Fig. 8

$GFAP^+$ cells, glial fibrillary acidic protein positive cells; Iba-1^+ cells, ionized calcium binding adaptor molecule 1 positive cells

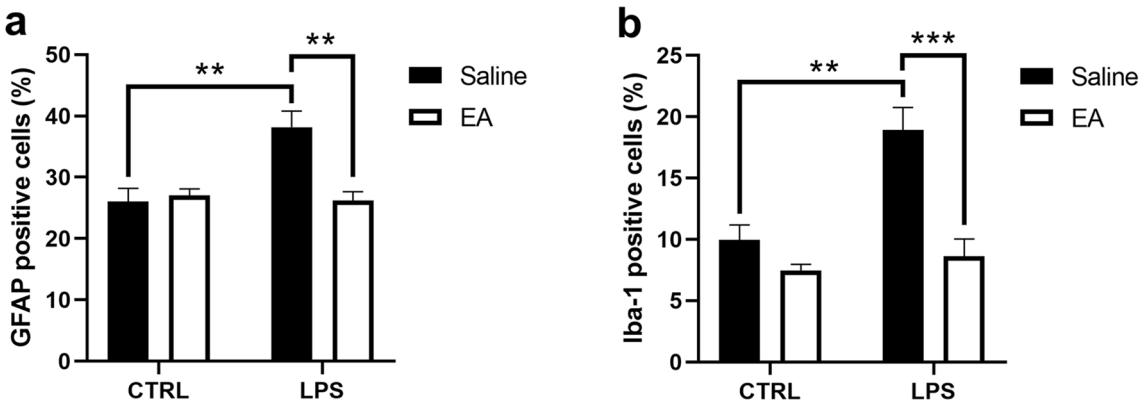

Fig. 8 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the expression of positive GFAP (**a**) and positive Iba-1 cells (**b**) in the hippocampus (HP) of rats. Data are expressed as mean ± SEM. N = 5 animals/group. *Denotes significant difference between groups. $^{**}p < 0.01$, $^{**}p < 0.001$

hydrogen peroxide, and peroxynitrite [92]. Furthermore, this compound has a potentiation effect of endogenous antioxidants such as GSH, SOD, catalase, glutathione reductase and glutathione peroxidase [10], which can be evidenced by the significant increase in the levels of GSH and T-SH in the cerebral cortex and hippocampus in the LPS + EA group compared to the LPS + SAL group (Fig. 7). Herewith, we can relate the neuroprotective effects of EA to its anti-inflammatory potential by reducing the expression of positive glial cells and its antioxidant properties, as evidenced by the increase in the antioxidant system and consequent reduction in the generation of ROS and its by-products.

A recent study has shown that synaptic pathologies and microgliosis may be the initial manifestations of neurodegeneration related to tauopathies. Furthermore, the authors observed that the prominent activation of the microglia precedes the formation of neurofibrillary tangles, and the immunosuppression of the animals reduced the pathology related to Tau and increased the life expectancy of the animals. The causal relationship between Tau phosphorylation and neuronal dysfunction is not well established, but there are two main hypotheses: the loss of function may be caused by a reduction in the binding of Tau to microtubules (MT), resulting in destabilization of MT and transport disruption axonal; Hyperphosphorylated Tau results in aggregation and toxic effects on neuronal cells. Studies in transgenic mices have indicated that neuronal loss and impairment in memory are associated with the presence of soluble and highly phosphorylated Tau (oligomers), and suppression of its expression causes improved memory and increased number of synaptic connections [93–95]. Thus, it was concluded that neuroinflammation is related to the early progression of tauopathies.

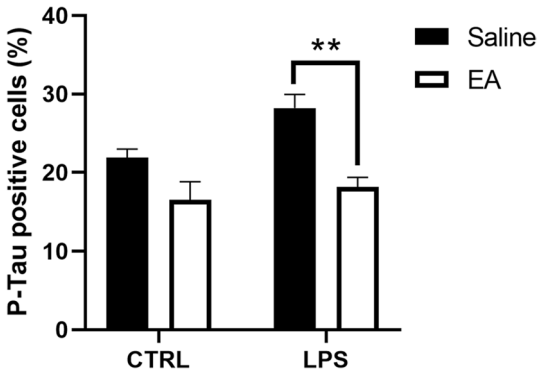

Fig. 9 Effect of multiple applications (IP) of lipopolysaccharide (LPS) 250 µg/kg or saline (SAL) and treatment (IG) with ellagic acid (EA) 100 mg/kg or SAL on the expression of positive P-Tau cells in the hippocampus of rats. Data are expressed as mean ± SEM. N = 5 animals/group. *Denotes significant difference between groups. $^{**}p < 0.01$

In this context, in the present study, a significant reduction in the percentage of p-Tau^{+} cells were observed in the group LPS + EA when compared to the LPS + SAL group (Fig. 9). Zhong et al. [96] demonstrated that the potential of EA to inhibit hyperphosphorylation of Tau is related to the reduction in the activity of glycogen synthase kinase 3β (GSK3β), which is involved in the phosphorylation of Tau. However, the authors point out that several other kinases may be involved in this mechanism. These results demonstrate the potential of EA to reduce the deleterious effects caused by the hyperphosphorylation of Tau, which includes the formation of neurofibrillary tangles with consequent cognitive impairment.

The results of this study demonstrated that EA was able to prevent cognitive impairment caused by multiple applications of LPS, as well as, modulate the immune system response by significantly reducing the expression of glial cells and phosphorylated Tau, attenuating oxidative damage

caused by the action of endotoxins and prevent the increase in AChE activity. Thus, this study demonstrated the beneficial effects of EA on memory, neuroinflammation, and restoring redox balance. These effects are the consequence of the anti-inflammatory and antioxidant action of this compound. With these results, the therapeutic potential of EA in cognitive disorders secondary to neuroinflammation was demonstrated.

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