Original Article

Supplementation of Carvacrol Attenuates Hippocampal Tumor Necrosis Factor-Alpha Level, Oxidative Stress, and Learning and Memory Dysfunction in Lipopolysaccharide-Exposed Rats

Abstract

Background: Carvacrol is a natural phenolic monoterpene with anti-inflammatory and antioxidant bioactivities. Neuroinflammatory and oxidative stress responses play a crucial role in the pathogenesis of Alzheimer's disease. The present study examined the effect of carvacrol on brain tumor necrosis factor-alpha (TNF-α) level and oxidative stress as well as spatial learning and memory performances in lipopolysaccharide (LPS)-exposed rats. **Materials and Methods:** The rats were treated with either carvacrol (25 and 50 mg/kg) or Tween 80 for 2 weeks. Thereafter, LPS (1 mg/kg) or saline was intraperitoneally administered on days 15–19, 2 h before Morris water maze task, and treatments with carvacrol or Tween 80 were performed 30 min prior to behavioral testing. The level of TNF-α, lipid peroxidation, and total thiol concentration were measured in the hippocampus and cerebral cortex at the end of the experiment. **Results:** It was found that LPS-exposed rats exhibited spatial learning and memory dysfunction, which was accompanied by increased TNF-α level and lipid peroxidation, and decreased total thiol concentration in the hippocampus and/or cortex. Moreover, treatment with carvacrol at a dose of 25 mg/kg attenuated learning and memory impairments, decreased TNF-α and lipid peroxidation level in the hippocampus and cortex, and increased total thiol concentration in the cortex. **Conclusion:** Carvacrol exerts neuroprotective effects against LPS-induced spatial memory deficits through attenuating hippocampal TNF-α level and oxidative stress in rats.

Keywords: *Carvacrol, cytokine, lipopolysaccharide, memory, oxidative stress*

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Introduction

Prevalence of neurodegenerative diseases is increasing due to an enhanced global life expectancy.[1] Alzheimer's disease (AD) is the most common form of neurodegenerative disease, and contributes 60%–70% of all cases of dementia.[2] According to the World Health Organization, there are approximately 50 million people with dementia, and about 10 million new cases of dementia every year.[3]

Neurodegenerative processes occur in AD due to accumulation of amyloid plaques in the brain, which is regularly increased with age.[4] Oxidative stress resulting from plaque accumulation causes oxidative damage by overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and injury to the surrounding neurons.[5]

The AD brain also displays increased levels of pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-1 β (IL-1 β), IL-6, chemokines, prostaglandins, and nitric oxide (NO).[6] The release of pro-inflammatory molecules can cause synaptic dysfunction, neuronal death, and inhibition of neurogenesis.[7] The brain of an AD patient is damaged in the regions responsible for learning and memory process, such as hippocampus and frontal cortex, which is displayed by the cognitive deficits in AD.

Lipopolysaccharide (LPS) is the polysaccharide component of Gram-negative bacteria and plays the important role in pathogenesis of inflammatory responses and neuronal damage.[8] LPS binds to toll-like receptor 4 (TLR4) and activates the nuclear factor kappa B (NF-κB) signaling pathway.[9] Activated NF-κB upregulates the expression of inflammatory cytokines such as TNF-α, IL-1 β, NO, and prostaglandin E2.[9,10] LPS also causes

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oxidative stress,[11] beta-amyloid generation,[12] and apoptosis[13] in the hippocampus and produces cognitive impairments.

Recently, many nutraceuticals targeting neuroinflammatory and oxidative mediators have attracted consideration as an alternative for the prevention or treatment of neurodegenerative and neuroinflammatory diseases.[14,15]

Carvacrol (CAR, 2-methyl-5-isopropylphenol) is a monoterpene that has been related with neuroprotection in several neurological diseases, including cerebral ischemia,[16] traumatic brain injury,[17] and epilepsy.[18] Carvacrol possesses multiple biological activities, including antioxidant,[19] anti-inflammatory,[20] and anticancer[21] activities. In addition, this natural compound protects PC12 cells against Aβ25-35-induced cytotoxicity through inhibiting oxidative stress.[22] Carvacrol also exerts several actions on the neuronal system, including acetylcholinesterase inhibition.[23] In our previous study, we found memory-enhancing activity of carvacrol 3 weeks after single LPS administration; however, carvacrol had no effect on inflammatory and oxidative stress markers in the brain.[24] In the current study, we examined the effects of carvacrol on learning and memory performance and neuroinflammatory and oxidative stress responses following repeated exposure to LPS.

Materials and Methods

Animals

Adult male Wistar rats (weighing 200–250 g) procured from Royan Institute (Isfahan, Iran) were kept in an air-conditioned room at 22°C ± 2°C, under a 12-h light/ dark conditions. Food and water were freely available. All experimental protocols were approved by Ethics Committee for Animal Experimentation (IR.MUI.MED. REC.1398.570).

Experimental design

Rats were randomly classified into four groups (*n* = 8), including control group, LPS group, LPS + CAR25 group, and LPS + CAR50 group. Carvacrol-treated groups received carvacrol intraperitoneally at doses of 25 and 50 mg/kg every day.[25] Carvacrol (Sigma-Aldrich Co., USA) was emulsified with 1% Tween 80 and dissolved in normal saline. The control and LPS groups received 1% Tween 80 dissolved in saline at the same volume as the treated groups. Injection of carvacrol or 1% Tween was started 2 weeks before LPS injection and continued during LPS injections.[26]

LPS from *Escherichia coli* (Sigma-Aldrich Co., USA) was freshly prepared in saline and was injected intraperitoneally at a dose of 1 mg/kg, 2 h before behavioral testing[26] in the Morris water maze (MWM), which was performed on days 15–19. Treatments with carvacrol or 1% Tween 80 were performed 30 min prior to behavioral task. After

behavioral testing, rats were euthanized and their brains were immediately removed. The hippocampus and cerebral cortex were dissected, weighed, and homogenized with NaCl solution 10 times (w/v) for biochemical assessments. All chemicals for biochemical measurements (thiobarbituric acid reactive substances [TBARS] and total thiol concentration) were purchased from Merck Co. (Germany).

Morris water maze task

MWM is a task for evaluating spatial learning and memory in rodents that relies on distal cues to pass from start points around the perimeter of a swimming pool to locate a hidden escape platform.[27] The water pool was 1.5 m in diameter, filled with water at a temperature of 23 ± 1°C. A hidden platform (10 cm in diameter) was located in the southeast quadrant of the pool. In the spatial acquisition phase, the rats performed four 60s trials on each of the four consecutive days. A different starting point was used in each of the four trials. If the rat located the platform within 60s, it was allowed to stay on it for 30 s. Rats that could not find the platform for 60s were guided to the platform and stay on it for 30 s. A computer software was used to record the escape latency to find the platform. On the 5th day, a probe test was performed. The platform was removed and each rat was allowed to swim for 60s. The rat repeatedly passed through the quadrant where the platform was located and was tracked by a camera located above the pool. A computer software was used to calculate the time spent in the southeast quadrant.[28]

Cytokine level

The hippocampal and cortical homogenates were centrifuged at 3000 rpm for 5 min. Then, TNF-α level in the supernatants was measured by an enzyme-linked immunosorbent assay kit (eBioscience Co., USA). Data were presented as pg/ml.

Thiobarbituric acid reactive substances level

TBARS was measured to estimate lipid peroxidation levels in the hippocampus and cortex. To determine, trichloroacetic acid, thiobarbituric acid, and hydrochloric acid were added to the homogenate. The mixture was then incubated at 100°C for 45 min. After centrifuging at 1000 g for 10 min, the absorbance of samples was read at 535 nm. The level of TBARS was estimated by C (M) = $A/1.65 \times 10^5$.[29]

Total thiol concentration

2,2′-dinitro-5,5′-dithiodibenzoic acid was used as a reagent to estimate total thiol groups in the hippocampal and cortical homogenates according to the protocol described before.[29]

Statistical analysis

Data were presented as the mean ± standard error of the mean. The results were analyzed by two-way

repeated-measures ANOVA (for acquisition training over 4 days) and one-way ANOVA (biochemical data and probe trial) followed by Tukey's *post hoc* test. $P < 0.05$ was considered significant.

Results

Effects of carvacrol on spatial learning and memory deficits

Analyzing data with two-way repeated-measures ANOVA revealed that the escape latency to find the platform decreased over the course of the 4 learning days in all groups indicating spatial learning acquisition [F (3,81) = 50.79, $P < 0.001$, Figure 1a]. Data analysis also revealed that the rats in LPS group spent a longer time to find the platform compared to the control group [F (3,28) = 7.68, $P < 0.01$, Figure 1a and b], indicating impairment of spatial learning acquisition. Moreover, supplementation of carvacrol at a dose of 25 mg/kg decreased escape latency throughout all test days compared to LPS group [$P < 0.05$, Figure 1a and b].

In the probe trial, percentage of time spent in the target quadrant decreased in LPS group compared to control group [F (3,28) = 6.62, $P < 0.01$, Figure 1c]. In addition, CAR25 group showed a significant increase in percentage of time spent in the target quadrant [$P < 0.05$, Figure 1c] compared to LPS group.

Effects of carvacrol on tumor necrosis factor-alpha level

The levels of TNF-α in the hippocampus were increased in LPS group [$P < 0.05$, Figure 2]. Moreover, carvacrol at a dose of 25 mg/kg significantly decreased hippocampal TNF-α level compared to LPS group [$P < 0.05$, Figure 2].

There was no significant difference in TNF-α levels in the cortex of control and LPS-treated groups [Figure 2].

Effects of carvacrol on thiobarbituric acid reactive substances level

TBARS level was increased in the hippocampus ($P < 0.05$) and cortex ($P < 0.05$) in LPS group [Figure 3]. Moreover, carvacrol at doses of 25 and 50 mg/kg significantly decreased TBARS level in the hippocampus compared to LPS group [$P < 0.05$, Figure 3]. However, carvacrol at a dose of 25 mg/kg decreased cortical TBARS level [$P < 0.05$, Figure 3].

Effects of carvacrol on total thiol concentration

As shown in Figure 4, there was no significant difference in hippocampal total thiol concentration between experimental groups [Figure 4]. However, total thiol concentration was decreased in the cortex of LPS group compared to control group ($P < 0.05$), and carvacrol at a dose of 25 mg/kg significantly enhanced total thiol concentration compared to LPS group [$P < 0.01$, Figure 4].

Discussion

The present study demonstrated that repeated exposure of LPS led to the activation of inflammatory and oxidative stress pathways, and induced spatial memory deficits in rats. Moreover, carvacrol supplementation in diet at a dose of 25 mg/kg was effective for these damages.

Evidence has shown that neuroinflammation and oxidative stress could aggravate brain lesions and induce synaptic dysfunctions, neurodegeneration, and memory impairment.[30,31] LPS can specifically recognize and bind to TLR4 on the cell surface of microglia and astrocytes,
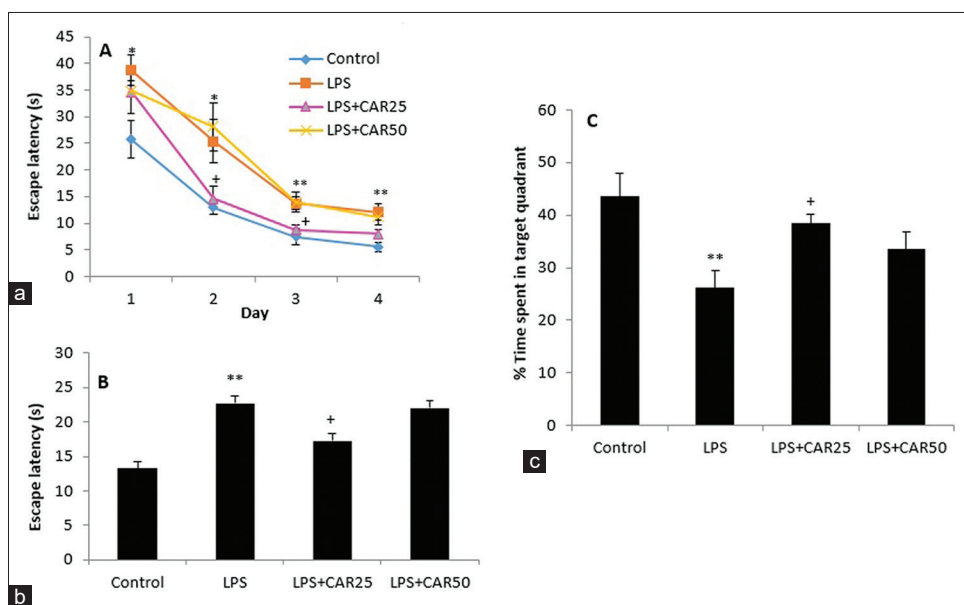

Figure 1: Effects of carvacrol on the performance of spatial memory acquisition phase in Morris water maze, (a) Escape latency during 4 days, (b) overall escape latency, (c) performance in probe trial. Data are shown as mean ± standard error of the mean. *$P < 0.05$, **$P < 0.01$ versus control group and + $P < 0.05$ versus lipopolysaccharide group
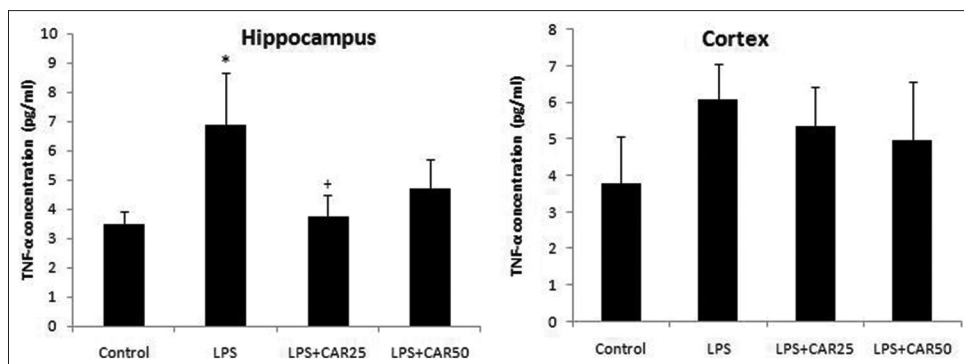
Figure 2: Effects of carvacrol on tumor necrosis factor-alpha level in the hippocampus and cerebral cortex of experimental groups. Data are shown as mean ± standard error of the mean. *$P < 0.05$ versus control group and + $P < 0.05$ versus lipopolysaccharide group

Figure 3: Effects of carvacrol on lipid peroxidation level (thiobarbituric acid reactive substances) in the hippocampus and cerebral cortex of experimental groups. Data are shown as mean ± standard error of the mean. *$P < 0.05$ versus control group and + $P < 0.05$ versus lipopolysaccharide group
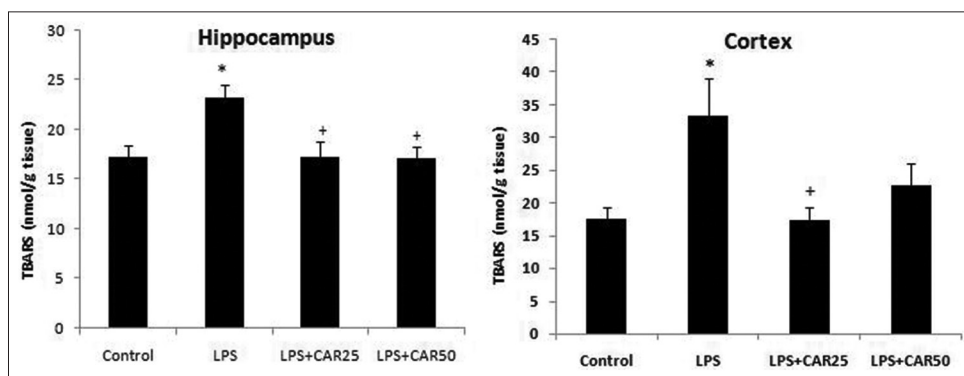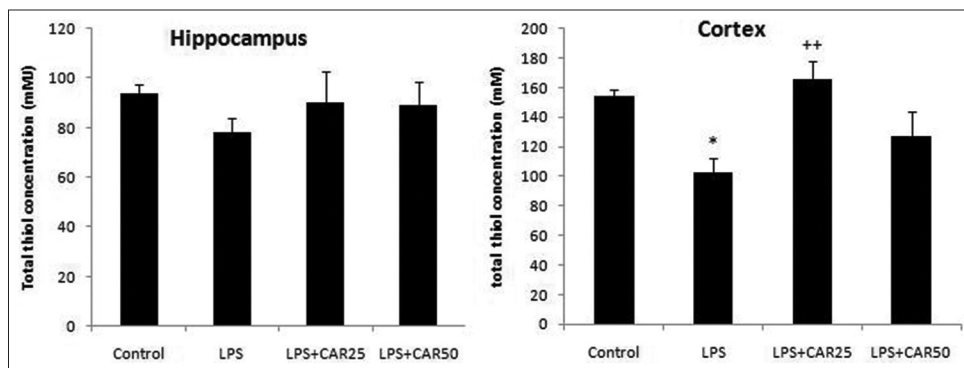

Figure 4: Effects of carvacrol on total thiol concentration in the hippocampus and cerebral cortex of experimental groups. Data are shown as mean ± standard error of the mean. *$P < 0.05$ versus control group and ++ $P < 0.01$ versus lipopolysaccharide group

further promotes the activation and translocation of NF-κB into the nucleus.[32] NF-κB is a key transcription factor mediating inflammatory responses through the induction of pro-inflammatory cytokines.[33] Through this, LPS induces the synthesis and release of pro-inflammatory cytokines, including TNF-α, IL-1 β, IL-6, IFN-γ, growth factors, and chemokines, resulting in impairment of learning and memory.[34,35] TNF-α is a pleiotropic cytokine which commonly elevated in AD.[36] It has been demonstrated that overexpression of TNF-α in neurons or glial cells impairs synaptic plasticity and learning and memory.[37] In addition, chronic LPS administration impairs learning and memory through TNF-α.[38]

The present study showed that LPS induced an increase in TNF-α level in the hippocampus. Meanwhile, carvacrol reduced TNF-α level in the hippocampus and attenuated LPS-induced memory impairment. Accordingly, the positive influence of carvacrol on LPS-induced memory impairment in the present study could be partly due to its anti-inflammatory activity. Other studies have also indicated the anti-inflammatory activity of carvacrol. For instance, it has been reported that carvacrol decreases LPS-induced production of TNF-α and IL-1 β by activated macrophages.[39] Carvacrol was also shown to inhibit the inflammatory response through inhibition of the

NF-kB signaling pathway in a rat model of focal cerebral ischemia.[20] Recently, a few studies have evaluated the effect of carvacrol on LPS-induced neuroinflammation in rats. A recent study reported that daily administration of carvacrol at a dose of 100 mg/kg attenuated the levels of IL-1 β, TNF-α, and cyclooxygenase-2 in the brain, 28 days after a single injection of LPS into the lateral ventricle of rats.[40] In line with our study, it was also shown that treatment with carvacrol reduced IL-6 and NO metabolites in the brain.[41] Altogether, this study is reporting the beneficial effect of carvacrol at a dose of 25 mg/kg on LPS-induced spatial memory deficits through attenuating hippocampal TNF-α level and oxidative stress in rats.

Oxidative stress, mainly involving ROS and RNS, is always intertwined with inflammation. ROS and RNS are toxic at certain levels due to reactions with proteins, lipids, and nucleic acids.[42] Brain oxidative damage is also considered an important mechanism for LPS-induced memory deficits.[15,26] In the present study, we observed that memory impairment caused by LPS was accompanied by brain tissue oxidative stress. LPS resulted in significantly increased levels of TBARS in the hippocampus and cerebral cortex, and decreased levels of total thiol concentration in the cortex, which were blocked by carvacrol. Therefore, the beneficial effect of carvacrol on memory deficits induced by LPS could also be partly due to its antioxidant activity. The results are consistent with the previous reports that carvacrol exhibits potent antioxidant properties.[19,43] For instance, it has been reported that carvacrol attenuates the cognitive dysfunction, oxidative stress, and apoptosis of the mice treated with ethanol.[19] In addition, carvacrol protects against cerebral ischemia injury of mice through decreased malondialdehyde levels and increased superoxide dismutase activity in ischemic cortical tissues.[20] Recently, Naeem *et al* (2021) reported that carvacrol at a dose of 20 mg/kg improves LPS-provoked anxiety and depression through activating the endogenous antioxidant Nrf2, which further regulated the antioxidant levels and eventually attenuated LPS-induced neuroinflammation and neurodegeneration.[44]

It should be noted here that our findings showed that a low dose of carvacrol (25 mg/kg) is more effective than a high dose (50 mg/kg) in attenuating TNF-α level and oxidative stress as well as improving memory deficits in LPS-exposed rats. Previous studies have also demonstrated that carvacrol at a dose of 20 mg/kg had more benefits than 50 mg/kg in improving LPS-induced anxiety and depressive-like behavior[44] or 6-OHDA-induced memory deficits.[45] It is unclear why carvacrol has a reverse dose–response effect, but one possibility is that carvacrol at high doses may exert prooxidant effects. Several evidences support that high doses of polyphenols can potentially cause adverse effects through prooxidant properties.[46]

Several studies have shown a relation between memory deficits and cholinergic dysfunction following LPS injection.[47] It was shown that systemic LPS impairs memory function through increase in acetylcholinesterase activity[47] and reduction of acetylcholine.[48] It has been reported that the carvacrol improves scopolamine-induced memory deficits in rats.[49] Moreover, the acetylcholinesterase inhibitory activity of carvacrol has been shown in several studies.[23] Thus, the impact of carvacrol on improvement of memory in LPS-treated rats could also be attributed to its anticholinesterase activity.

Conclusion

In summary, our results demonstrated that carvacrol at a dose of 25 mg/kg exerts neuroprotective effects against LPS-induced spatial memory deficits most likely through its antioxidant and anti-inflammatory activities. It is promising that carvacrol can be used as an adjunct therapy in neurodegenerative diseases such as AD. Further research needs to be carried out to distinguish the exact pathways responsible for the neuroprotective effects of carvacrol.

Conflicts of interest

There are no conflicts of interest.

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