

## The Effect of Pentoxifylline on Passive Avoidance Learning and Expression of Tumor Necrosis Factor-Alpha and Caspase-3 in the Rat Hippocampus Following Lipopolysaccharide-Induced Inflammation

### Abstract

**Background:** Peripheral inflammation is effective in the development of neurodegenerative diseases. Pentoxifylline (PTX) has an inhibitory effect on inflammatory cytokines; therefore, we aimed to evaluate the effect of PTX on passive avoidance learning and the expression of tumor necrosis factor-alpha (TNF- $\alpha$ ) and *caspase-3* in the rat hippocampus, following systemic lipopolysaccharide (LPS) injection. **Materials and Methods:** Male Wistar rats were randomly divided into five groups: control, LPS, and LPS + PTX, receiving doses of 10, 25, and 50 mg/kg of PTX, respectively. The animals received daily injections of PTX (i.p.) 1 week before and 2 weeks after the LPS injection (5 mg/kg; i.p.). Learning and memory were evaluated by passive avoidance learning. Then, the expression of the associated genes was measured in the hippocampus. **Results:** The results showed that the peripheral LPS injection had no significant effect on learning and memory. PTX only with a dose of 10 mg/kg shows an improvement ( $P < 0.05$ ). Results from reverse transcription polymerase chain reaction showed that LPS had no significant effect on the expression of *caspase-3* and TNF- $\alpha$ . PTX with a dose of 10 mg/kg decreased the *caspase-3* expression in the LPS + PTX group ( $P < 0.001$ ), but the expression of both genes increased, using other concentrations. **Conclusions:** Findings showed that systemic application of LPS after 2 weeks had no effect on learning and memory and the expression of inflammatory genes in the hippocampus, but PTX led to an increase in the expression of these genes, which could be due to its direct effects or possible exacerbation of LPS effects.

**Keywords:** Hippocampus, inflammation, learning and memory, lipopolysaccharide, pentoxifylline

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### Introduction

Inflammation is a defensive response against harmful stimuli that induces defensive reactions in the body. Inflammation plays an important role in the development of pathological conditions in the central and peripheral nervous system,<sup>[1]</sup> and studies show that the main cause of the development and progression of many neurological diseases is the impotency of the regulatory system and the control of the inflammatory response in the brain.<sup>[2-4]</sup> Inflammation of the nervous system can be caused by the damage to the brain tissue itself or induced by the peripheral inflammation. It is determined by activating microglia, damaging the blood-brain barrier (BBB) and increasing its permeability, introducing peripheral immune cells into brain tissue, excessive production of cytokines, nitric oxide (NO),

reactive oxygen species (ROS), and prostaglandins, and ultimately by the damage and the death of the neurons.<sup>[5,6]</sup> Brain tissue is thought to be unaffected by systemic inflammatory processes due to the presence of a BBB, but studies have shown that stimulation and activation of the peripheral immune system have a powerful effect on brain tissue.<sup>[7]</sup>

In recent years, the use of vasodilators such as pentoxifylline (PTX) has been considered as one of the suitable and new strategies for protecting neurons (neuroprotective). The vasodilator effect of this drug is due to inhibition of phosphodiesterase enzyme and the increased concentration of cyclic adenosine monophosphate in the smooth muscle cells of the blood vessel wall.<sup>[8-10]</sup> Currently, various functional roles associated with this drug, including

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tumor necrosis factor-alpha (TNF- $\alpha$ ) inhibition, through reduced transcription of its gene, have been known to affect several stages of cytokine/chemokine pathways and anti-inflammatory properties.<sup>[8]</sup> As it is suggested that PTX probably has neuroprotective effects against inflammation, the purpose of this study was to evaluate the effects of PTX on learning and passive avoidance memory and changes in the gene expression of two cytokines affecting the apoptosis process, namely caspase-3 and TNF- $\alpha$ , by induction of peripheral inflammation, using systemic lipopolysaccharide (LPS) injection in rats.

## Materials and Methods

### Subjects

The experiments were carried out on male Wistar rats (200–250 g), housed under standard conditions of temperature (22  $\pm$  2°C) and light (12 h light-dark cycle), with free access to food and water. The Ethic Committee for Animal Experiments at Tehran University of Medical Sciences approved the study (Ethic registration code: IR.TUMS.VCR.REC.1396.3321), and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996.

### Experimental design

The animals were randomly divided into five groups ( $n = 10$ ), including the control, the LPS, the LPS-PTX 10, 25, and 50 mg/kg. LPS was dissolved in saline and injected intraperitoneally (a single injection of 5 mg/kg; Sigma, St. Louis, USA).<sup>[11,12]</sup> In the PTX-treated groups, rats received daily injection of PTX (10, 25, or 50 mg/kg; i.p., dissolved in saline; Sigma, St. Louis, USA), 1 week before and 2 weeks after the injection of LPS. Animals in the control and LPS groups received the same volume of placebo. At the end of the treatment period, animals were subjected to behavioral studies and then thereafter were deeply anaesthetized with chloral hydrates (400 mg/kg, i.p.) and decapitated. Brains were rapidly removed, and instantly, the hippocampi were dissected in ice-cold artificial cerebrospinal fluid and deep-frozen in liquid nitrogen. Then, they stored at -80°C, until further studies.

### Passive avoidance learning test

Learning and memory performance were evaluated, using passive avoidance learning test. The apparatus consists of two separate chambers connected through a guillotine door. One chamber was illuminated, while the other was dark. The floor of both the chambers consists of steel grids, used to deliver electric shocks. In the acquisition trail, 4 h after the last PTX injection, each rat was placed in the illuminated chamber while its back was to the guillotine door. After 60 s of habituation, the guillotine

door separating the illuminated and dark chambers was opened, and the initial latency to enter the dark chamber was recorded. The guillotine door was closed immediately after the rat enters the dark chamber, and an electric foot shock (75 V, 0.5 mA, 50 Hz) was delivered to the floor grids for 2 s. Then, the rat was removed from the dark chamber and returned to its home cage. After 24 and 48 h, retention latency time to enter the dark chamber was taken in the same way as in the acquisition trail, but the foot shock was not delivered, and the latency time was recorded up to a maximum of 600 s.<sup>[13]</sup>

### Assessment of gene expression

Real-time reverse transcription polymerase chain reaction (PCR) was used to evaluate the expression of TNF- $\alpha$  and Caspase-3. Total RNA was extracted from the hippocampus, using the Biofact kit (Biofact, Korea), according to the manufacturer's instruction; initially, cells were lysed, using a chaotropic salt, then RNA was bound to the silica-based membranes, and washed with ethanol, containing wash buffer, and subsequently purified RNA eluted by RNase-free ddH<sub>2</sub>O. After isolation, the quality of messenger RNA (mRNA) was checked by gel electrophoresis, and RNA quantity was measured, using nanodrop (OD 260 nm and 280 nm). At the reverse transcription step, 5 ng of total RNA was used to synthesize the complementary DNA, using the Revert Aid First Strand cDNA Synthesis Kit and oligo (dT) primer (Biofact, Korea). Quantitative real-time PCR analyses were performed, using Real Q Plus 2x Master Mix Green with high ROXTM (Biofact, Korea) and StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Beta-actin (ACTB) was used as an endogenous control,<sup>[14]</sup> and samples were run in triplicate. Primers were designed, using AlleleID7.6. Table 1 shows the primer sequences. To determine the relative gene expression, the quantity of genes investigated in the present study was calculated as  $RQ = 2^{-\text{(target gene Ct} - \beta\text{-actin Ct)}}$ , where Ct represents the number of cycles, at which the output signal exceeds the threshold signal.<sup>[15,16]</sup>

### Statistical analysis

Data were analyzed using the SPSS Version 21 for Windows (IBM Corporation). Behavioral results were analyzed statistically, using Kruskal–Wallis

**Table 1: Primers used in real-time reverse transcription polymerase chain reaction experiments**

Name	Sequences (5' to 3')
ACTB-F	AGGCCCTCTGAACCCTAAG
ACTB-R	CCAGAGGCATACAGGGACAA
TNF $\alpha$ -F	ACGTCGTAGCAAACCACCAA
TNF $\alpha$ -R	CAAGGGCTCTTGATGGCAGA
Casp3-F	GAGACAGACAGTGGAACTGACGATG
Casp3-R	GGCGCAAAGTGACTGGATGA

ACTB was used as a housekeeping gene to compare the samples

test (nonparametric analysis of variance [ANOVA]) and Dunn's multiple comparisons for the posttest. Data from gene expression were analyzed statistically, using one-way ANOVA, followed by Turkey's test, and unpaired *t*-test for comparing the specific group. The significant level was defined as  $P < 0.05$ . The results are expressed as mean  $\pm$  standard error of mean.

## Results

The results of the behavioral study showed that the peripheral LPS injection after 2 weeks had no significant effect on the first time entering into the dark room, compared with the control group. PTX with moderate and high doses did not affect the time of first entering into the dark room but at a dose of 10 mg/kg in rats receiving PTX + LPS could significantly increase this period, 48 h after applying shock, compared to the LPS group ( $P < 0.05$ ) [Figure 1].

As shown in Figure 2, after a 14-day period of LPS injection, no change in the gene expression of caspase-3 and TNF- $\alpha$  was observed. However, PTX at doses of 25 and 50 mg/kg significantly increased the expression of caspase-3, in comparison with the control group ( $P < 0.01$  and  $P < 0.05$ , respectively) and LPS ( $P < 0.05$ ), but at 10 mg/kg dose, the caspase-3 expression was decreased, in comparison with the control group ( $P < 0.001$ ) and LPS ( $P < 0.001$ ).

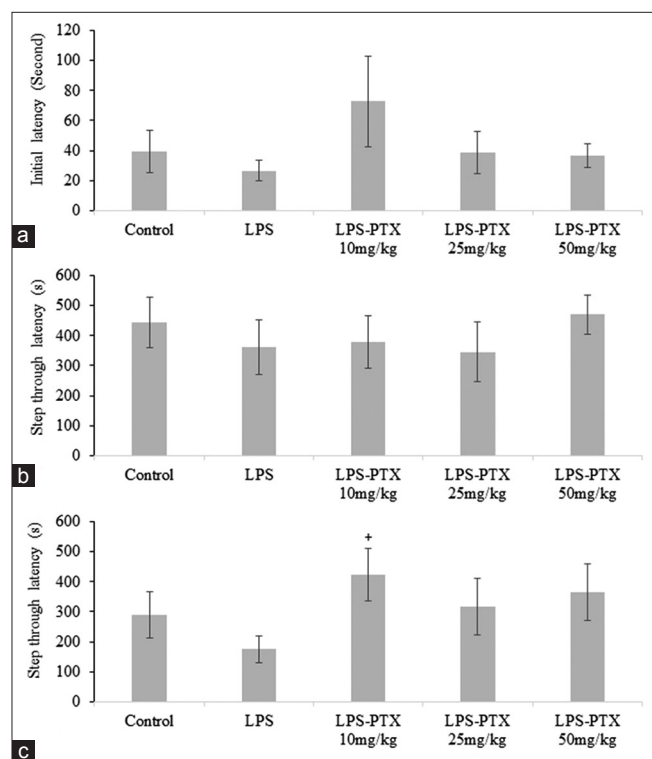


Figure 1: Effects of lipopolysaccharide and pentoxifylline on passive avoidance learning. (a) Initial latency before and (b) step through latency 24 h and (c) 48 h after the foot shock. Data are expressed as mean  $\pm$  standard error of mean. \* $P < 0.05$  with respect to the lipopolysaccharide group ( $n = 10$ )

PTX with all three doses (10, 25, 50 mg/kg) significantly increased the TNF- $\alpha$  expression, in comparison with the LPS group ( $P < 0.01$ ,  $P < 0.05$ , and  $P < 0.05$ , respectively) and with doses (10 and 25 mg/kg), compared with the control group ( $P < 0.01$  and  $P < 0.05$ , respectively).

## Discussion

The results showed that the systemic injection of LPS did not affect passive avoidance learning after 2 weeks. PTX at the dose of 10 mg/kg could somehow have beneficial effects, but not at higher doses.

In this study, it was observed that LPS injection had no significant effect on the expression of genes such as caspase-3 and TNF- $\alpha$ , in the hippocampus. Studies have been shown that the systemic use of LPS can produce peripheral inflammatory responses and increase the proinflammatory cytokines and the activation of peripheral macrophages.<sup>[12,17]</sup> Peripheral inflammation caused by LPS can cause damage to the BBB,<sup>[18]</sup> and following damage to the BBB, peripheral macrophages enter the brain tissue and accelerate the process of inflammation in the brain.<sup>[5,6]</sup> Effects of LPS on BBB transport are dependent on the injection pattern that is applied and the timing of the study. Cytokines and chemokines show distinct profiles following an inflammatory reaction,<sup>[19]</sup> so, the timing of the study must also be taken into consideration. It has been shown that the use of LPS induces a transient increase of cytokines in the blood circulation and central nervous system, which is

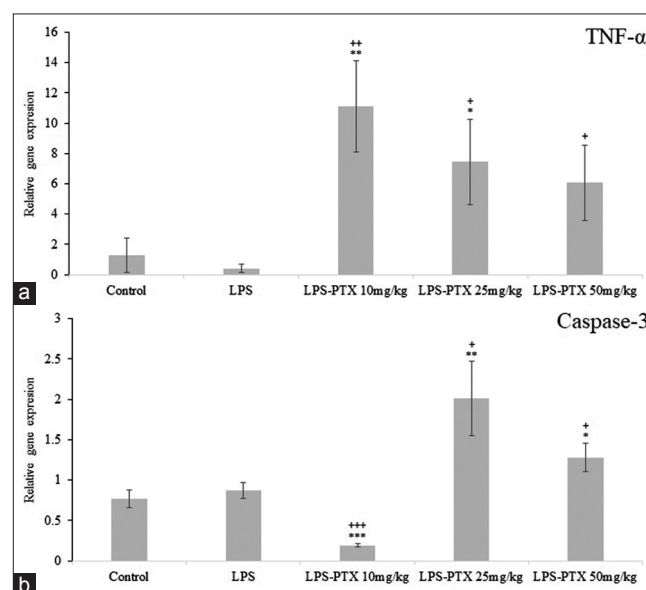


Figure 2: Effects of lipopolysaccharide and pentoxifylline on relative gene expression of tumor necrosis factor-alpha (a) and caspase-3 (b) in rat hippocampus. The extent of expression was measured by real-time reverse transcription polymerase chain reaction. The messenger RNA expression data were normalized to the beta-actin (ACTB) signal. Fold changes relative to the control are presented. Mean  $\pm$  standard error of mean values of experiments are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  with respect to the control group; \* $P < 0.05$  and \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  with respect to the lipopolysaccharide group ( $n = 10$ )

dose dependent,<sup>[20]</sup> and it has been reported that the level of serum cytokines involved in responding to the acute phase of inflammation (interleukin [IL]-1  $\beta$ , IL-6, and TNF- $\alpha$ ) increases within 2 h after the injection of LPS, and within 24 h of infusion to its basal level.<sup>[21]</sup> Furthermore, in this study, it is possible that in the first stages of inflammation, the expression of the genes increased, and then, the production of protein increased, and after 2 weeks, due to negative feedback, the expression of the genes reduced to the initial level. Therefore, the expression of these proteins should be considered.

LPS increases the release of NO, ROS and cytokines and prostaglandin E2<sup>[18,22,23]</sup> and initiates inflammatory cascades in the brain tissue with the activation of microglia.<sup>[24]</sup> The TNF- $\alpha$  is one of the main cytokines that induces neuroinflammation and neurodegeneration and has regulatory and intervention effects in several cellular processes, such as inflammation and cell death.<sup>[25-27]</sup> Caspases, the most active member of the apoptosis family, play an important role in inducing and exacerbating apoptosis mechanisms. These enzymes coordinate the apoptosis pathway and by parsing their own substrates play an important role in promoting cell death.<sup>[28,29]</sup> Caspase-3 is an executable caspase that is activated in the next steps by initiating caspases, such as caspase-7, and triggering caspase cascades.<sup>[28]</sup> LPS stimulates the production of TNF  $\alpha$ , triggering this cell death cycle. When these receptors are attached to their ligand, TNF- $\alpha$ , the pro-caspase-8 is converted to caspase-8 (active form), and then, caspase-3 gets activated and promotes cell death.

Based on the results of the present study, injection of LPS after 2 weeks did not affect learning and avoidance memory, although some studies have shown that intraperitoneal injection of LPS has caused cognitive impairment.<sup>[30,31]</sup> Various results have reported the effects of cytokines on learning and memory. Several studies have shown that acquisition of the Morris water maze and consolidation of contextual are disrupted during neuroinflammation,<sup>[32-34]</sup> and there is also evidence that cytokines can even facilitate learning and memory.<sup>[35,36]</sup> It has been reported that male rats who received systemic LPS exhibited intact performance in tasks that do not require hippocampal pattern separation processes, novel object recognition, and spatial memory in the water maze.<sup>[37]</sup> Memory retrieval in activities that require the hippocampal pattern separation has been severely degraded, while there has been any gross effects on exploratory activity or motivation.<sup>[37]</sup>

Our results show that except for the 10 mg/kg dose of PTX, which reduces the expression of the caspase-3, following the pretreatment with PTX, the rate of expression of these inflammatory factors has increased dramatically, perhaps the most important justification for this is the increased blood flow, due to the vasodilation effects created by PTX. However, according to previous studies, it was

expected that PTX inhibits or reduces the production of these inflammatory factors, due to its anti-inflammatory effects.<sup>[9,38]</sup> It seems that the use of PTX in inflammatory processes, despite its anti-inflammatory effects, is a double-edged sword due to its vasodilating effects and can increase the amount of tissue damage if not used with proper dosage and time.

## Conclusions

Results of this study showed that systemic application of LPS after 2 weeks had no significant effects on learning and memory and the expression of inflammatory genes in the hippocampus. However, PTX increased the expression of these genes in the LPS groups. These increases could be due to its direct effects on the hippocampus or possible exacerbation of LPS effects. There is a possibility that PTX through vasodilation increased the flow of inflammatory factors in the brain vessels. Overall, the results of this study indicate that peripheral inflammation in the short term does not affect brain function. However, further studies are needed in this regard due to contrasting results in different doses.

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## Conflicts of interest

There are no conflicts of interest.

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