Original Article

Effect of Pentoxifylline on Apoptotic-Related Gene Expression Profile, Learning and Memory Impairment Induced by Systemic Lipopolysaccharide Administration in the Rat Hippocampus

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Abstract

Background: Inflammation is one of the effective factors, in the development of functional disorders of the nervous system. Pentoxifylline (PTX) has an inhibitory effect on inflammatory factors. Therefore the aim of this study was to evaluate the effect of PTX on learning, memory and expression of genes, involved in neuronal survival in the rat hippocampus, following systemic lipopolysaccharide (LPS) injection. **Methods:** Male rats were randomly divided into 5 groups of control, LPS and LPS + PTX, receiving doses of 10, 25 and 50 mg/kg of PTX, respectively. In LPS groups, LPS was injected (5 mg/kg; intraperitoneal), and after one week, rats received intraperitoneal PTX for 14 days, in the treatment groups. Learning and memory were evaluated by object location task (OLT) and novel object recognition (NOR). Then, the hippocampus was dissected in order to measure the expression of the associated genes. **Results:** The results showed that peripheral LPS injection caused significant damage ($P < 0.01$) to learning and memory with respect to controls, but PTX with doses of 10 and 50 mg/kg prevented these impairments. Results from reverse transcription polymerase chain reaction (RT-PCR) showed that LPS significantly increased the expression of *Bax* and *TNF-α* with respect to controls. PTX in the LPS + PTX group significantly increased the expression of *Bcl-2*, *BAD* and *Caspase-3*. **Conclusions:** Other than the increased *Bcl-2* expression, PTX had no significant effect on the expression of other genes, therefore further studies are needed to find out how PTX improves the learning and memory impairments, following the peripheral inflammation.

Keywords: *Hippocampus, inflammation, learning, lipopolysaccharides, memory, pentoxifylline*

Introduction

Inflammation of the nervous system is defined as the activity of the intrinsic immune system of the brain, in response to an inflammatory condition, characterized by a series of cellular and molecular changes in the brain which play an important role in the development of neurodegenerative diseases.[1] The cytokines released into the blood circulation, following a peripheral inflammation, pass through areas, lacking a blood-brain barrier (BBB), such as the ventricle, and enter the brain parenchyma through cytokines carriers.[2,3] Peripheral inflammation can increase the level of cytokines and pro-inflammatory mediators in the hippocampus. These cytokines have regulatory effects on vital physiological processes, such as learning and memory, but in pathologic conditions, high levels of cytokines lead to neurodegenerative diseases.[4]

PTX is a methyl-xanthine derivative with high permeability through the BBB that improves the blood flow to the extremities and small blood vessels. It induces its therapeutic effect by decreasing the blood concentration (viscosity), increasing the red blood cell flexibility, inhibiting platelet- activating factor (PAF) and reducing platelet aggregation. This drug is also introduced as a selective inhibitor of Tumor Necrosis Factor alpha (TNF-α).[5,6] Studies have shown that PTX is beneficial for the improvement of cognitive disorders, caused by cerebral ischemia, by increasing the blood flow and inducing an anti-inflammatory effect.[7] In addition, the anti-inflammatory effects of this drug and its beneficial effects, on the cognitive disorder caused by neurodegenerative disorders have been investigated.[5,7-10]

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TNF-α is one of the most important pro-inflammatory cytokines, produced mainly by activated macrophages, and bacterial lipopolysaccharide (LPS) is the most important inducer of TNF-α production.[11] When TNF-α binds to its type 1 receptor, it can induce inflammatory responses and cell death. Activation of caspase-8 in this pathway activates the caspases' cascade and eventually leads to cell death.[12,13] In addition, TNF-α can induce the release of reactive oxygen species (ROS), *Bcl-2* associated X protein (*Bax*), and cytochrome C from the mitochondria, which contribute to cell death by activating some caspases.[12]

The *BCL-2* protein as an anti-apoptotic molecule prevents the caspases' cascades through binding to channels, presented on the mitochondrial outer membrane, and eventually inhibits cytochrome C release.[14,15] Bax protein promotes apoptosis pathway and pro-apoptotic factors, such as *BAD*, increases mitochondrial membrane permeability and induces apoptosis.[14,16] Coupling of pro-apoptotic molecules with anti-apoptotic factors (*BAX* and *BCL-2*) counteracts their anti-apoptotic effect and, in sum, the ratio of inducers and inhibitors determines the survival of living cells.[17]

Considering that the previous studies have shown that PTX has anti-TNF-α effects (the dominant TNF-α inhibitor effect), in conjunction with the increase and improvement of blood supply,[5,6] therefore we have examined the *TNF-α* expression as a main pro-inflammatory cytokine in this respect. In addition, as we aimed to use this inflammatory model as a potential model for neurodegenerative diseases, we created a model of systemic inflammation by injection of LPS, in order to evaluate the effect of PTX on learning and memory and on the gene expression of the factors affecting cell survival, such as *BCL2, BAD, BAX* and *Caspase-3* in the rat hippocampus.

Methods

Subjects

The experiments were carried out on male Wistar rats (200-250 g), housed under standard conditions at ambient temperature (22 ± 2°C), and 12 h light-dark cycles, 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). 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. The animals were randomly divided into 5 groups (n = 10 in each experimental group), including the control, the LPS, the LPS + PTX 10 mg/kg, LPS + PTX 25 mg/kg, LPS + PTX 50 mg/kg, respectively.

LPS was dissolved in saline and injected intraperitoneally (5 mg/kg; Sigma, St. Louis, USA).[18] One week after the injection, the rats in the treatment groups started to receive daily injections of PTX 10, 25 or 50 mg/kg for 14 days (i.p., dissolved in saline; Sigma, St. Louis, USA).[8] 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 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 (145 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM Na_2HPO_4 at pH 7.4),[19] and deep-frozen in liquid nitrogen. They are then stored at -80°C until further studies.

Behavioral studies

The novel object recognition (NOR) and object location task (OLT) were used to assess learning and memory, and performed one time for each rat, at 5 min interval.[20] The device for recognizing new objects included a box of 60*60*60 cm, made of fiberglass, used to evaluate cognitive memory.[21] A camera was placed 2 m above the apparatus. This behavioral test has three phases of habituation, familiarization and testing. In the habituation phase, each animal is allowed to freely explore the environment, in the absence of objects. In the familiarization phase, each rat was placed in the open-field box, while the two identical objects (A1 and A2) were located opposite each other, at a distance of 8 cm from the wall of the device. Each rat was given 5 minutes, to be acquainted with the objects. Then, at the test phase, in the OLT test, one of the objects (A2) was moved from its previous position. In the NOR behavioral test, one of two objects (A2) was replaced with a new object. The diagnostic index (DI) was calculated as the ratio of the time that the rat spent on the new object (in NOR) or the dislocated object (in OLT), to the total time that each rat spent for the two objects in percent (DI = N/(N + F) × 100; where N is the time, spent to explore the new or dislocated object, and F is the time spent to explore the old or unchanged object).[22]

Assessment of gene expression

Real-time polymerase chain reaction (PCR) was used to evaluate the expression of *Bax, BAD, Bcl-2, TNF-α* and *Caspae-3*. Total RNA was extracted from 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 was eluted by RNase-free ddH_2O. After isolation, the quality of messenger RNA (mRNA) was checked by gel electrophoresis, and RNA quantity was measured, using a nanodrop (OD260 nm and 280 nm). At the reverse transcription step, 5 ng of total RNA was used to synthesize the complementary DNA, using the RevertAid First Strand cDNA Synthesis Kit and oligo (dT) primer (Biofact, Korea). Quantitative real-time PCR analyses were performed, using the RealQ Plus 2x

Master Mix Green with high ROX™ (Biofact, Korea) and Step One Plus Real-Time PCR System (Applied Biosystems, USA). Beta-actin (ACTB) was used as an endogenous control,[23] and samples were run in triplicate. Primers were designed, using Allele ID7.6. Table 1 shows the primer sequences. To determine the relative gene expression, genes investigated in the present study were calculated as RQ = $2^{-(\text{target gene Ct} - \beta\text{-actin Ct})}$, where Ct represents the first cycle, at which the output signal exceeds the threshold signal.[24,25]

Statistical analysis

Data was analyzed using the SPSS 21 for Windows, following normality tests by the one-way Analysis of Variance (ANOVA) for any probable differences between the groups. If there was a difference, Tukey's post-test, as well as unpaired *t*-test were used for comparing the specific groups. The significant level was defined as $P < 0.05$. Results are expressed as mean ± standard error of the mean (SEM).

Results

Effect of LPS and PTX on NOR and OLT

In the NOR test, LPS caused a significant decrease in the DI, compared to the control group (34.58 ± 6.2 and 59.77 ± 6.26 percent, respectively; $P < 0.01$; Figure 1). PTX, only with doses of 10 and 50 mg/kg (72.79 ± 5.12 and 78.43 ± 2.62 percent, respectively) increased the DI significantly, with respect to the LPS group [$P < 0.001$; Figure 1], and with a dose of 50 mg/kg with respect to the control group [$P < 0.05$; Figure 1].

The DI in the OLT test, was unaffected by LPS and there was no significant difference between the control and LPS groups. PTX with a dose of 25 mg/kg decreased the DI (18.68 ± 8.29 percent) with respect to both the control and LPS groups [54.91 ± 6.98 and 50.14 ± 4.62 percent, respectively; $P < 0.01$; Figure 2]. However, PTX with a dose of 50 mg/kg increased the DI (71.33 ± 3.28 percent) with respect to the control and LPS groups [$P < 0.05$ and $P < 0.01$, respectively; Figure 2].

Table 1: Primers used in real-time PCR experiments

Genes	Sequences (5′ to 3′)
ACTB-F	AGGCCCCTCTGAACCCTAAG
ACTB-R	CCAGAGGCATACAGGGACAA
Bcl2-F	TAACGGAGGCTGGGATGC
Bcl2-R	TGAGCAGCGTCTTCAGAGA
Bax-F	GAGGCAGCGGCAGTGATG
Bax-R	TCCTGGATGAAACCCTGTAGCA
BAD-F	GACCAGCAGCCCAGAGTAT
BAD-R	CGCCTCCATGATGACTGTTATTG
TNFα-F	ACGTCGTAGCAAACCACCAA
TNFα-R	CAAGGGCTCTTGATGGCAGA
Casp3-F	GAGACAGACAGTGGAACTGACGATG
Casp3-R	GGCGCAAAGTGACTGGATGA

ACTB was used as a housekeeping gene to compare the samples

Effect of LPS and PTX on relative gene expression

As shown in Figure 3, in comparison to the control group, LPS significantly increased the expression

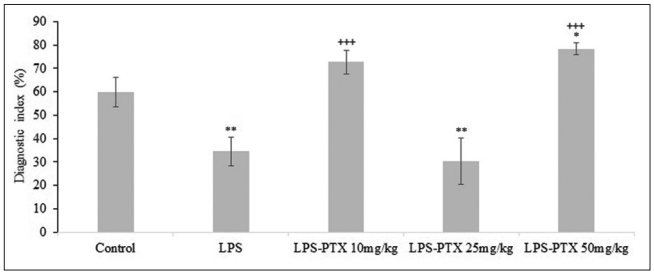

Figure 1: The effect of lipopolysaccharide (LPS) and pentoxifylline (PTX) on the diagnostic index (DI) in the Novel Object Recognition (NOR) Behavioral Test. Data are expressed as mean ± SEM (*n* = 10).*$P < 0.05$ and **$P < 0.01$ with respect to the control group;+++ $P < 0.001$ with respect to the LPS group (*n* = 10)

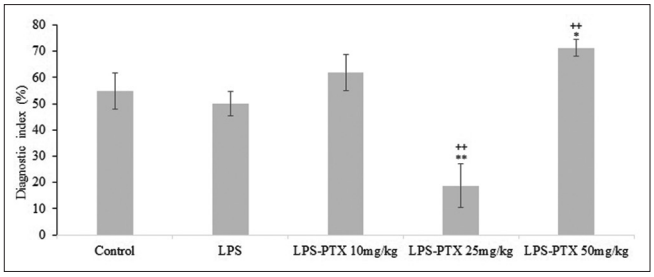

Figure 2: The effect of lipopolysaccharide (LPS) and pentoxifylline (PTX) on the diagnostic index (DI) in the object location task (OLT) behavioral test. Data are expressed as mean ± SEM (*n* = 10).*$P < 0.05$ and **$P < 0.01$ with respect to the control group;+++$P < 0.01$ with respect to the LPS group (*n* = 10)

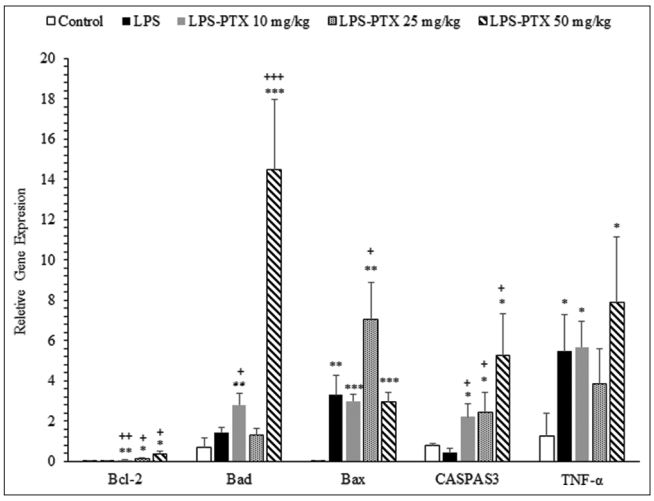

Figure 3: The effect of LPS and PTX on the relative gene expression of *Bax, BAD, Bcl-2, TNF-α,* and *Caspase 3,* in the rat hippocampus. The extent of expression was measured by RT-PCR. The mRNA expression data was normalized to the Beta-actin (ACTB) signal. Fold changes relative to the control are presented. Mean ± SEM 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 LPS group (*n* = 10)

of *Bax* (0.04 ± 0.008 and 3.29 ± 0.97, respectively; $P < 0.01$) and *TNF-α* (1.28 ± 1.12 and 5.45 ± 1.84, respectively; $P < 0.05$). The *Bax* expression was increased by PTX with a dose of 25 mg/kg (7.06 ± 1.82; $P < 0.05$), compared to the LPS group. PTX with all doses (10, 25 and 50 mg/kg) increased the expression of *Bcl-2 (*0.071 ± 0.01, 0.119 ± 0.046 and 0.344 ± 0.153, respectively; $P < 0.01$, $P < 0.01$ and $P < 0.01$, respectively) and *Caspase-3 (*2.22 ± 0.62, 2.45 ± 0.98 and 5.28 ± 2.05, respectively; $P < 0.05$) in the LPS + PTX rats, with respect to the control (0.028 ± 0.008 and 0.77 ± 0.11, respectively), and LPS (0.026 ± 0.006 and 0.43 ± 0.24, respectively) groups. PTX with doses of 10 and 50 mg/kg increased the *BAD* expression (2.8 ± 0.56 and 14.49 ± 3.47, respectively), compared to the control (0.71 ± 0.44; $P < 0.01$ and $P < 0.001$, respectively) and the LPS group (1.44 ± 0.23; $P < 0.05$ and $P < 0.001$, respectively).

Discussion

The present data showed that peripheral inflammation by intraperitoneal injection of LPS can damage learning and memory. However, PTX could partially prevent these destructive effects.

Inflammation is the physiological response of the immune system against harmful stimuli, which has now been considered as a major contributor to the pathophysiology of neurological diseases, such as Alzheimer's disease.[26-28] LPS is a bacterial endotoxin and a strong cytokine inducer, which, in systemic injection, induces significant releases of pre-inflammatory factors.[29] It has been demonstrated that in severe peripheral LPS-induced inflammation,[30] both LPS and inflammatory factors can damage the BBB, and facilitate the entry of the cytokines to the central nervous system (CNS). These lead to the neuronal inflammation and probably result in neurodegenerative diseases.[31] LPS by activating microglia and macrophages stimulates the release of inflammatory cytokines, such as TNFα, Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6) and other mediators such as nitric oxide (NO), prostaglandin E2 (PGE2) and reactive oxygen species (ROS).[32,33]

In the present study, LPS significantly increased the expression of *TNF-α* and *Bax* genes. TNF-α is mainly produced by activated tissue macrophages and LPS induces its production.[34] When TNF-α attaches to its type 1 receptor (TNFα-R1), this connection can activate the cell death pathway by activating Caspase-8 and triggering caspase cascades.[35] TNF-α can also release ROS, Bax protein and Cytochrome-C from mitochondria, which induces cell death by activating some other caspases. It has been shown that by modifying the mitochondria membrane, Bax can create holes in this membrane and release the cytochrome C.[36]

We found that PTX could not improve the LPS-induced increase in the expression of *TNF-α* and *Bax* genes. In addition, it increased the expression of *BAD* and *Caspase-3* in LPS-treated rats.

Previous studies showed that administration of PTX before induction of peripheral inflammation can prevent the deleterious effects of LPS on learning, memory and expression of pro-inflammatory *caspase-3* and *TNF-α* genes.[37] Studies have shown that PTX can reduce cytokines and specifically inhibit TNF-α.[38] In a study that was conducted on PC12 cells, the results showed that LPS increased the protein expression of Caspase-3 and BAD in these cells, but PTX significantly prevented this increase.[5] However, it is demonstrated that PTX, through reducing the expression of fibroblast growth factor (FGF) and insulin-like growth factors (IGFs), activates the Bax pathway, and subsequently facilitates the initiation of the cell death pathway.[39] Also, it has been shown that PTX had no effect on the reduced serum IL-10 level that was induced by the injection of LPS.[40]

Unlike previous studies that showed a decrease in TNF-α following treatment with PTX, in this study, PTX had no desirable effect on the increased levels of *TNF-α*expression in the hippocampus, following the LPS-induced inflammation. Since PTX has been administered in the groups, with LPS-induced inflammation, these results are not the absolute effects on PTX, and its effect is largely influenced by inflammation. However, the *Bcl-2* expression level was significantly increased in the LPS group. Although, in the expression of genes in the hippocampus, there was a contradictory effect but in the behavioral study, PTX could improve the damage to learning and memory induced by inflammation.

It is important to note that the expression of each gene is not necessarily associated with the production of protein,[41] therefore, further studies are needed to explore the exact effects of PTX. In the present study, some contradictions may be due to the high dose of LPS and the initiation of the treatment by PTX, after 7 days of LPS injection and the establishment of its effects. Because of the effect of PTX on the enhancement of blood flow to the brain, it is possible that this increase, in a situation that the levels of inflammatory factors are high in the peripheral circulation, and the BBB is damaged by LPS leads to the entry of more inflammatory factors into the CNS. However, in our behavioral study, significant effects of PTX were observed on the improvement of damaged learning and memory associated with LPS injection.

In our study, the *BCL-2* expression was significantly increased by PTX in the LPS groups. Bcl-2 protein as an anti-apoptotic factor, which can have a significant effect on the modulation of other pro-apoptotic members of the Bcl-2 family.[42] Therefore some helpful effects of PTX can be attributed to the increased expression of Bcl-2 in the hippocampus.

In our behavioral study, we observed that the effects of PTX with the dose of 25 mg/kg were different from doses of 10 and 50 mg/kg. Thus, it is possible that in dose-response function, the response to this drug in inflammation is a U-shaped curve and may be different mechanisms are involved at different doses. The same results were also observed in the gene expression. Similarly, previous studies have shown different results of different doses of PTX.[10] The suggestion is that the dose-dependent effects of this drug can be a potential consequence of the activation of various signaling pathways. It is likely that the anti-inflammatory or vasodilator effects of the drug vary in different doses, leading to different outcomes.

Conclusions

The results of this study indicate that peripheral inflammation can impair learning and memory, and these impacts are probably a result of the affected hippocampus. PTX, in a non-dose dependent manner, could alleviate the behavioral impairments. However, other than the increased *Bcl-2* expression, the significant effects of PTX on the expression of other genes were not observed. Further studies are therefore needed to find out how PTX changes the impacts of peripheral inflammation in the CNS.

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Conflicts of interest

There are no conflicts of interest.

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