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Original Article

Chronic *Toxoplasma gondii* Infection Potentiates Parkinson's Disease Course in Mice Model

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Abstract

Background: *Toxoplasma gondii* is a neuroinvasive protozoa pathogen that could manipulate its intermediate host's behavior. However, the possible link between *T. gondii* infection and the development of neurodegenerative disorders such as Parkinson's disease (PD) has been proposed, we tested the hypothesis that in chronic toxoplasmosis neuroinflammation, and molecular mediators potentiate behavioral-cognitive impairments in BALB/c mice with PD.

Methods: To establish chronic toxoplasmosis by Tehran strain, cysts of *T. gondii* were injected intraperitoneally into BALB/c mice in Kerman, Iran in 2019. To induce the PD model, mice (BALB/c) were treated with Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The behavioral experiments such as anxiety and motor coordination were performed using the Open field and Rotarod tests. Additionally, we investigated the contribution of *Toxoplasma*-induced neuroinflammation, and behavioral-cognitive impairments in the PD mice model.

Results: Chronic toxoplasmosis caused PD-like symptoms and induced various behavioral changes in infected BALB/c mice. In *T. gondii* infected+MPTP treated group, *T. gondii* infection could potentiate PD in infected mice receiving MPTP and caused remarkable dysfunction in motor coordination and change in anxiety and depression-like behaviors similar or more severe than PD group.

Conclusion: Chronic *T. gondii* infection exacerbates pathological progression of PD in BALB/c mice brain by promoting neuroinflammation, and behavioral changes establishing.

Introduction

Toxoplasma gondii, a pervasive coccidian parasite is an obligatory single-celled intracellular protozoan that can infect a wide range of warm-blooded animals as well as humans. Approximately 30%–80% of the world's human population harbor infection and due to the bulk of the burden disease, it was ranked as the second most important food-borne parasitic disease in Europe (1,2). *T. gondii* has a heteroxenous life cycle in which a member of the *Felidae* family (particularly cats) acts as a definitive host wherein sexual reproduction happens and oocysts shed in the feces result in parasite dissemination into the environment.

Human or rodents as intermediate hosts acquire infection via different transmission routes including ingesting tissue cysts/oocysts from undercooked meat, food, or drink contaminated water, organ transplantation and congenital transmission during pregnancy that is responsible for stillbirths, miscarriage and fetal damages (3). Due to this cunning neurotropic parasite's unique properties such as complicated life cycle, permanent high prevalence, it has been aptly given the auspicious moniker of one of the most successful protozoa in the world (4). Upon parasite life cycle in intermediate hosts, cyst stage forms in immune-privileged sites such as brain tissue.

Although the immune response is essentially required for parasite clearance in CNS, most inflammatory mediators could induce neuronal damage or even cell death (5). Apart from induced neuroinflammation that remains a core hypothesis supporting the link between toxoplasmosis and behavioral changes in the infected host, other functional changes in neuronal cells like dysregulation of neurotransmitters secretion, cellular signaling, receptors function and redox balance status are thought to underlie the pathophysiology of several neurodegenerative diseases in infected hosts (6,7).

As a lifelong neurodegenerative disease with exacerbations, PD is the second most age-related brain disease that affects more the 1% of the world population. It is characterized pathologically by progressive degeneration of dopaminergic neurons in substantia nigrapars compacta (SNpc) and clinically by sensorimotor deficits such as resting tremor, rigidity and bradykinesia and non-motor symptoms such as sleep abnormalities, cognitive deficits and impaired working memory. The genetic factors, oxidative stress, neuroinflammation, neurotransmitters level imbalance are likely major contributing factors in the pathogenesis of neurodegenerative diseases in general, and particularly PD (8).

So far, controversial conclusions of few seroepidemiological studies (*T. gondii*-specific antibody) investigating the link between toxoplasmosis and PD risk have been reported; by means that, some studies suggested that *T. gondii* could be a risk factor for PD (9,10) while others found no association between toxoplasmosis and PD (11,12).

Therefore, for the first time, the present study was designed to determine the effect of *T. gondii* chronic infection on the pathogenesis of PD in animal models. For this purpose, different aspects of PD pathogenesis profile such as behavioral changes, neuroinflammation status in brain tissue of established Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced PD mouse model (an accepted PD model) infected with Tehran strain of *T. gondii* were investigated.

Materials and Methods

Ethical approval

In the present study, all animal experimentation complied with regulatory standards in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Before starting this project, permission num-

ber IR.KMU.REC.1398.402 from the Ethical Review Board of Kerman University of Medical Sciences (Kerman, Iran) and the Kerman Neurosciences Research Center, Kerman, Iran was received.

Animal and chronic infection of toxoplasma

Thirty-five specific-pathogen-free (6-8 wk old) male BALB/c mice, weighing from 20-25 g delivered from the Animal Breeding Stock Facility of the Razi Institute of Iran (Karaj, Iran) in 2019. The mice were kept 7 per cage in the animal house of the Kerman Neurosciences Research Center under standard conditions including controlled temperature and ventilated environment,12:12 hr light-dark cycle and were sufficiently supplied with ration and water ad libitum. Tehran strain of *T. gondii* (type II) as a cystogenic strain gift from Prof. Keshavarz, Tehran University of Medical Sciences (Tehran, Iran), was applied to establishing chronic toxoplasmosis in mice. The brain tissue of previously infected BALB/c was homogenated with the saline solution, examined by light microscopy and cyst number was adjusted via hemocytometer (Neubauer slide) to 50 cysts per mL. Intraperitoneally, 0.5mL of prepared homogenate containing nearly 25 cysts was injected into each of male BALB/c mice (13).

Serological examination

The modified agglutination test (MAT) was used to confirm chronic toxoplasmosis in mice. The Anti-*Toxoplasma* IgG antibody of serum samples was measured 35 d post infection by a commercial kit (Toxoscreen DA, biomerieux®, Lyon, France). Accordingly, to the manufacturer's instructions, antibody titers of 1:20 or higher were considered positive.

Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment to induce Parkinson disease

The 7 Mice were randomly selected to developing Parkinsonism via MPTP (Sigma-Aldrich, St. Louis, MO, USA; dissolved in saline 0.9%) administration. According to the method described elsewhere with slight modification (14), we applied specific regiment injection as follows: Four doses of MPTP (20 mg/kg body weight) were injected intraperitoneally at 2-h intervals. This group of mice imported to our study as MPTP treatment after proving their health monitoring.

Experimental design

Overall, 35 healthy mice were split randomly into 5 groups (n=7 each) as follow: control group: (the uninfected), vehicle group: (administrated sterile saline intraperitoneally), *Toxoplasma* infection group: (infected with Tehran strain of *T. gondii*) MPTP treatment group: (received MPTP according to the above-mentioned paragraph) and *Toxoplasma* infected + MPTP treatment group: (infected mice that received MPTP 40 d post-infection). A specifically designed timeline (Fig. 1) was used in our investigation.

Behavioral assessments

All of the divided mice groups were tested for behavioral and cognitive changes. Blinding of the examiner was maintained until the end of all behavioral assessments. All of the behavioral experiments were performed in a sound-attenuated room under low-intensity light.

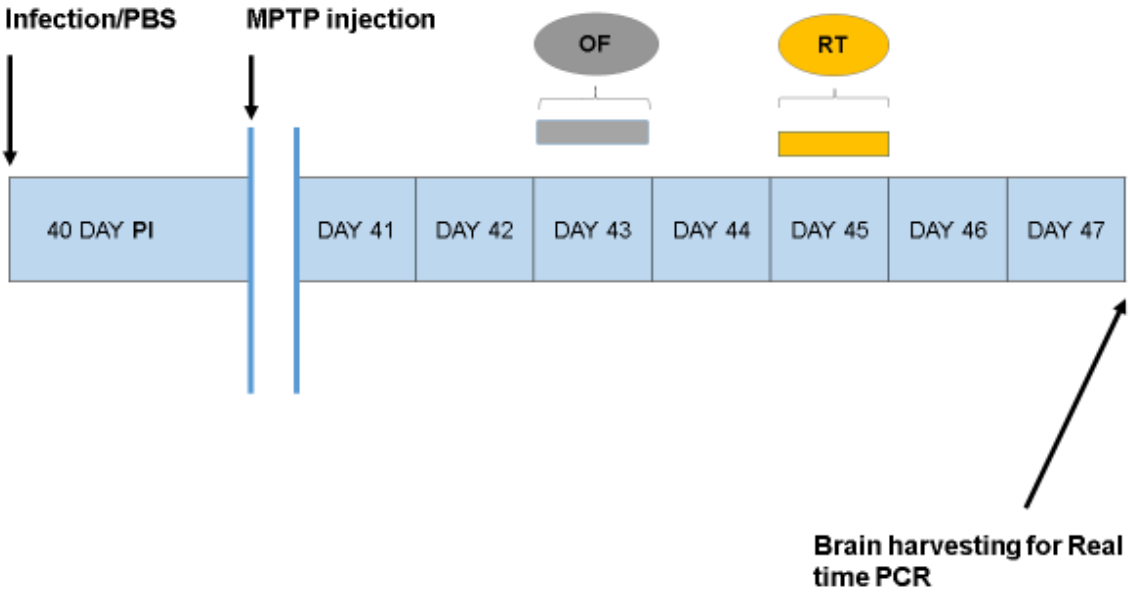

Fig. 1: The Timeline diagram for behavioral tasks, Real-time. OF: open field, RT: Rotarod

Open field activity

Exploratory activity, motor function (locomotor), and anxiety-like behavior determination of rodents has been validated previously by open field (OF) test. The OF apparatus is a cubic box with a Plexiglas arena (90×90×45 [H] cm) surrounded by transparent walls. Based on our previous procedure (15), each mouse was removed from the home cage by gently grasping its tail and released individually in the middle of the arena (facing the walls) for a single 5-min trial. The ambulatory movement was recorded by an automatic video camera fixed on the top of the apparatus (Noldus Ethovision system, version 7.1.). Intended indicators such as total traveled distance, immobility percentage and time spent in the central zone (as anxiety indicators) were collected.

Rotarod performance test

Motor coordination and balance skill of different mice groups were examined by an accelerating rotating rod. All mice were pretrained 1 day before the test trial. Each mouse completed three trails during 5 min cut-off and 30 min inter-trial rest on the accelerating rotarod system while its speed was set from 10 to 60 rpm. The average time of fall from the rotating rod was recorded as a coordination indicator (16).

Brain harvesting

The brain tissue was harvested after behavioral tests. In brief, mice were anesthetized with CO2 in a desiccator jar with low CO2 pressure-flow (13). After decapitation, whole-brain tissues were rapidly removed and then frozen in liquid nitrogen and stored at -80 °C for the investigation of cytokine expression.

Real-time polymerase chain reaction (PCR)

The major involving factor in PD and toxoplasmosis is neuroinflammation; thereby, we targeted mRNA expression level of some pro-inflammatory cytokines (IFN ɣ and inducible nitric oxide synthase (iNOS)) (17,18). The total RNA of half of the left hemisphere tissue was extracted using Trizol reagent (19) (Invitrogen, Life Technologies, Carlsbad, CA, USA). The purity and quantify of yielded RNA examined by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE) and A260/A280 ratio were calculated. The specifically designed oli-

gonucleotide primers of target genes are displayed in Table 1. The complementary DNA (cDNA) generation process was carried out by following the RT premix kit manufacturer's protocol (Intron, Sungnam, Korea).

Table 1: Sequence of designed primers for Real-time PCR

Template	*Forward and reverse sequences (5′-3′)*	*Product size (bp)*
iNOS	F- GTTCTCAGCCCAACAATACAAGA R- CAGAGGGGTAGGCTTGTCTC	288
IFN-γ	F-ATGAACGCTACACACTGCATC R-CCATCCTTTTGCCAGTTCCTC	182
GAPDH	F- AGCTTCGGCACATATTTCATCTG R- CGTTCACTCCCATGACAAACA	89

Real-Time PCR steps completed with amplification of obtained cDNA in an iQ5 real-time PCR detection system (Bio-Rad, Hercules, California) where SYBR green was used to detect target amplified cDNA. According to our previous effort, the applied Real-Time PCR program with slight modification was as follows: initial incubation at 95 °C for 2 min and 40 amplification cycles at 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 30 seconds. The iQTM5 optical system software (Bio-Rad) was applied to data analysis where duplicate PCR reactions were set for each gene. The comparative threshold cycle (Ct) method was chosen while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered as a reference gene. The upregulation or downregulation of mRNA level expressed as fold change, flowing mRNA normalization.

Statistical analysis

SPSS Statistics for Windows, ver. 17.0 (Inc., Chicago, IL, USA) and GraphPad Prism v8.0.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for data analysis. Differences between the experimental groups were examined with the one-way ANOVA followed by Tukey multiple test and the significance level was set at $P<0.05$.

Results

Behavioral and cognitive tests

OF test was carried out to survey locomotion and anxiety-like behaviors. A significant difference in travel distance in the OF paradigm was found in the *Toxoplasma* infected ($P<0.0005$), MPTP treated ($P<0.0001$) and *Toxoplasma* infected + MPTP treated ($P<0.0001$) groups as compared with the control group (Fig. 2A).

Both anxiety-like behaviors indexes including time spent in the inner zone and percentage immobility were affected by MPTP administration and toxoplasmosis; duration spent in the inner zone was decreased significantly in *Toxoplasma* infected ($P<0.05$), MPTP treated ($P<0.01$) and *Toxoplasma* infected + MPTP treated ($P<0.0001$) (Fig.2 B). A similar alternation regarding anxiety phenotype was observed. There was a significantly greater immobility percentage in the aforementioned groups and the same *P*-value was achieved (Fig. 2 C). There was no significant difference in all of OF test indexes between MPTP treated and *Toxoplasma* infected + MPTP treated groups.

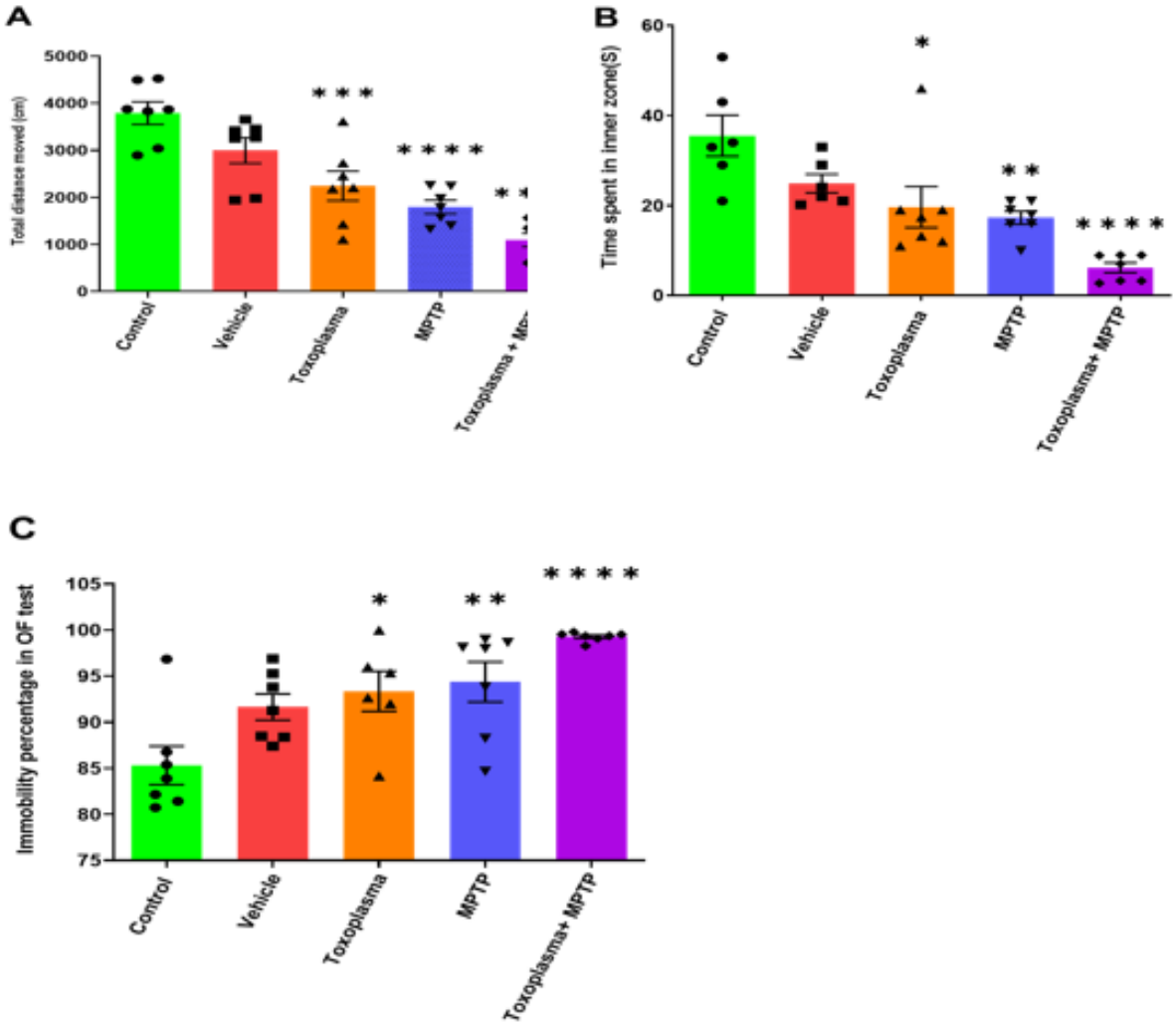

Fig. 2: The locomotion and anxiety-like behaviors change in the open field test. Total distance moved(A), time spent in inner zone (B), immobility percentage (C) in different experimental groups. One-way ANOVA followed by Tukey's multiple comparison test was used for data analysis. Data are expressed as means ± SEM (**** P<0.0001, *** P<0.0005, ** P<0.01, * P<0.05 compared to control group. P<0.05 considered as significant

Rotarod test

A significant reduction of time spent on the rotating rod was detected in *Toxoplasma* infected (P<0.0005), MPTP treatment (P<0.0001), and *Toxoplasma* infected+MPTP treatment (P<0.0001) groups was detected as compared to the control group. As Fig. 3 demonstrates less sustaining balance was observed in mice simultaneously infected with *T.gondii* and treated with MPTP (*Toxoplasma* infected +MPTP treated, P<0.01) versus mice that only received MPTP.

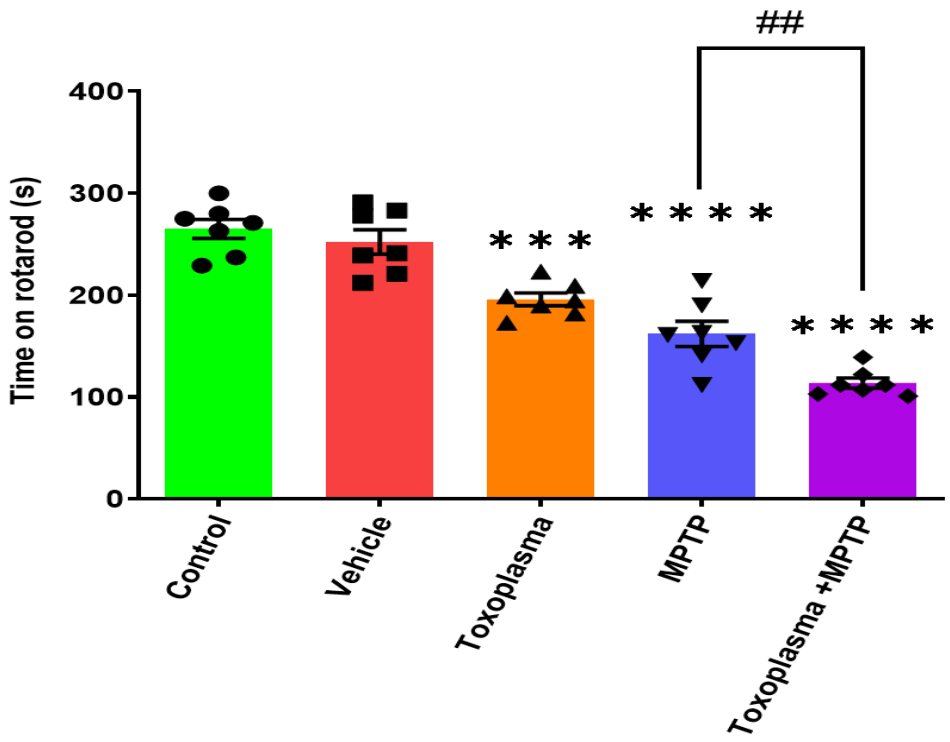

Fig. 3: The latencies to fall in the rotarod test of different experimental groups. One-way ANOVA followed by Tukey's multiple comparison test was used for data analysis. Data are expressed as means ± SEM (**** P<0.0001, *** P<0.0005 compared to control group, $^{\#\#}P$<0.01 compared to MPTP treated group). P<0.05 considered as significant

Gene expression in Real-time PCR

The mRNA expression level in various experimental groups was assessed via Real-time PCR. Comparison of obtained results demonstrated that fold changes levels of iNOS and also IFN-γ significantly (P<0.05) increased in *Toxoplasma* infected, MPTP treated and *Toxoplasma* infected+MPTP treatment in comparison with the control and vehicle groups (Fig. 4 A,B). The maximum fold changes were recorded in *Toxoplasma* infected + MPTP treated groups for mentioned genes. For all the above genes, a significant difference was found between MPTP treated and *Toxoplasma* infected. In this context, fold changes level for these genes was significantly up-regulated in the *Toxoplasma* infected + MPTP treated group compared with MPTP treated group. Result details and relevant P-value for each gene in different groups were described in the caption of Fig.4. In general, fold change levels for these studied genes demonstrated that *Toxoplasma* could interfere with gene expression either in *Toxoplasma* infected or in *Toxoplasma* infected +MPTP treated groups. In Real-time data, a significant difference between control and vehicle groups was not achieved.

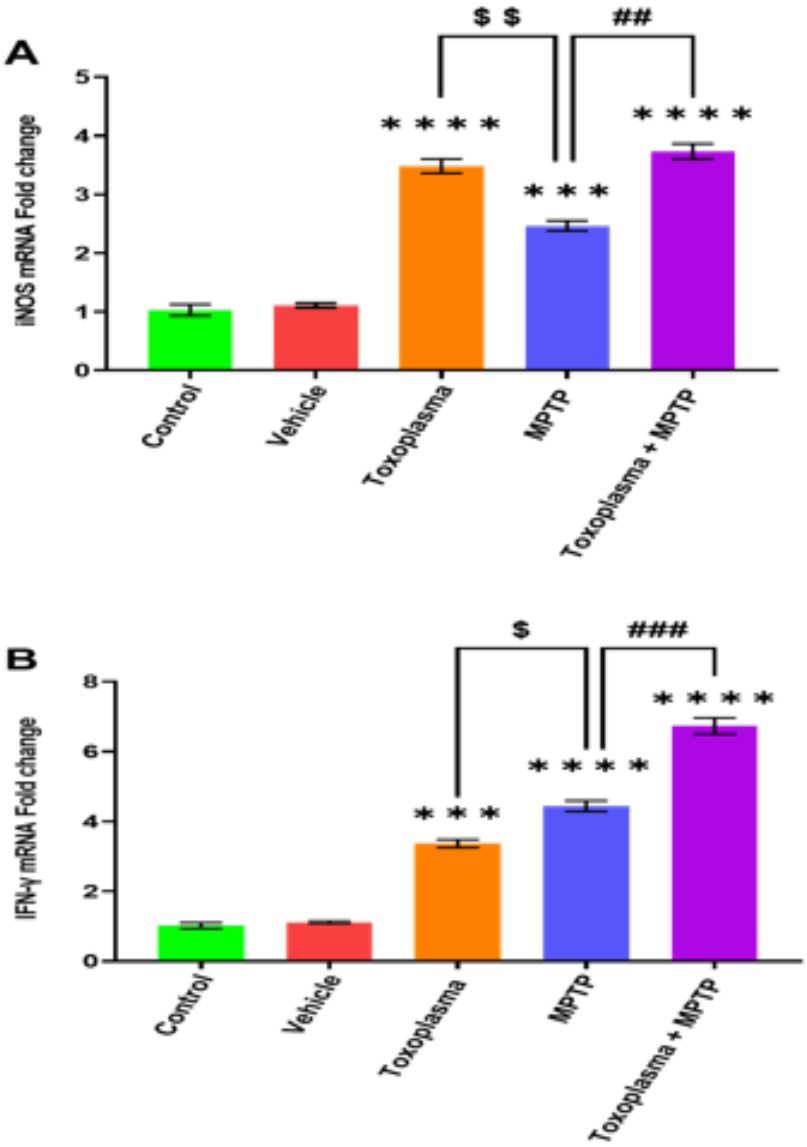

Fig. 4: The results of different gene expressions by Real time PCR. Total RNA obtained from pooled 7 brain mice tissue in different groups. The one-way ANOVA test was used and the results are statistically different from each other. Data are expressed as mean ±SD of duplicate assays and normalized to GABDH and expressed as fold change.**(A)** Fold change of iNOS (****P< 0.0001 for *Toxoplasma* infected, ***P = 0.0009 for MPTP treatment, ****P< 0.0001 for *Toxoplasma* infected + MPTP treatment groups when compared to control group, $^{\#\#}P$ = 0.0015 compared to MPTP treated and $^{\$\$}P$ = 0.0042 compared to *Toxoplasma* infected group).**(B)** Fold change of IFN-γ (***P =0.0004 for *Toxoplasma* infected, ****P<0.0001 for MPTP treatment, ****P<0.0001 for *Toxoplasma* infected + MPTP treatment groups when compared to control group, $^{\#\#\#}P$ = 0.0005 compared to MPTP treated and $^{\$}P$ = 0.0154compared to *Toxoplasma* infected group). P<0.05 considered as significant

Discussion

Over the last decade, several lines of toxoplasmosis serological evidence illustrate that there has been a possible correlation between toxoplasmosis and many neuropsychiatric diseases such as PD, AD, schizophrenia (20); In this context, several attempts have been made

to answer the basic questions related to the underlying mechanism responsible for behavioral changes upon *Toxoplasma* infection (10,21). For the first time, we evaluated some behavioral changes, immune responses on different animal model groups.

Here, exploratory and anxiety behavior was assessed using the OF paradigm. Consistent with the previous reports, our data confirmed that infected mice showed anxiety in the OF test (6,22). Serotonin has been documented to have a main role in the pathogenesis of depression. Perturbation of serotonin secretion upon toxoplasmosis has been reported (23). Additionally, *T. gondii* leads to a release GABA and GABAergic dysfunction that is known as a signature of depression (24).

MPTP may lead to cognitive deficits and increased anxiety behaviors. Moreover, in the current study and consistent with other studies, destructive effects of MPTP in cognitive and locomotor activity were observed (25).

In the present study, employing, OF and rotarod, we demonstrated that in the *Toxoplasma* infected + MPTP treated group cognitive function and motor activities were more impaired than either *Toxoplasma* infected and MPTP treated groups alone.

Behavioral changes during chronic toxoplasmosis could be results of direct factors due to the recruitment of parasite cysts or its effector molecules in the brain and immunological response, redox imbalance and neurotransmitter level changes were proposed as possible indirect factors (21).

Our data provided a good snapshot of immune response status and approved that the mRNA level of iNOS and IFN-γ in both *Toxoplasma* infected and MPTP treated groups significantly increased as compared with the control group. Moreover, our results placed more emphasis on the role of *T. gondii* in worsening the PD course. In line with our result, during toxoplasmosis caused by various strains, enhancement of inflammatory cytokines in the brain or other intended tissues was observed (13,26,27). Inconsistent with our data, previous in vivo\in vitro investigation approved that during latent toxoplasmosis not only neuroinflammatory response reduced but also the neuroprotective effect of *T. gondii* due to suppressive mediators like IL-10 and TGF-β by which prevent neuron degeneration, onset, or progress wide spectrum of neurodegenerative like AD disease was observed (28-31).

The relation between IFN-γ and NO production was subjected to series research. Briefly, these studies acclaim that NO production control by three nitric oxide synthase (NOS) isoforms (eNOS, nNOS and iNOS); while NOS itself regulation depends on IFN-γ signaling. eNOS and nNOS activation have a neuroprotection effect but iNOS function led to a neurotoxic effect on the brain. iNOS augment in the animal model of PD and up-regulation of all isoforms of NOS upon toxoplasmosis, strongly suggest that NO as an antiparasitic mediator and as an essential neurotransmitter could determine the level of toxoplasmosis or PD pathogenesis (32–34).

The immune response is the leading cause of neurotransmitter level changes. Inflammatory mediators could induce neuron apoptosis that leads to neurotransmitters dysregulation in synaptic junctions. Parasite cysts are principally observed in a limited number of neuronal cells; this fact advocates the possibility that parasite-secreted mediators would exert global changes in neuronal function (23,35).

Conclusion

T. gondii chronic infection results in behavioral disorders that could be due to changes in immune responses and dysregulations of other parameters. Moreover, toxoplasmosis could worsen PD course in behavioral deficits, immune response in the PD mice model and further researches is needed in future studies.

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Conflict of interest

The authors declare that there is no conflict of interest.

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