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Original Article

Acetylcholinesterase Inhibitor Improves Learning and Memory Impairment Induced by *Toxoplasma gondii* Infection

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Abstract

Background: Here, we established the mouse models of chronic toxoplasmosis by *T. gondii* Tehran strain to provide a good understanding about defining the possible association between *T. gondii* exposure and learning and memory impairments. Moreover, as secondary objective of the present study, we hypothesized whether administration of an acetylcholinesterase (AChE) inhibitor could reduce learning and memory impairments induced by *T. gondii* infection.

Methods: Twenty-four male BALB/c mice were used to establishment of latent toxoplasmosis. The animal model of *Toxoplasma* infection was established by the intraperitoneal inoculation of 20-25 tissue cysts from Tehran strain of *T. gondii*. Donepezil (2 mg/kg) an AChE inhibitor to treat Alzheimer disease was injected intraperitoneally once a day for two weeks starting from post-infection day 90. Morris water maze (MWM) task was used to assay spatial learning and short term spatial memory in all groups. One-way ANOVA with Tukey's post-hoc test was used to assess differences between experimental groups. $P<0.05$ was considered statistically significant.

Results: *Toxoplasma* infection impaired spatial leaning and short term spatial memory of the infected BALB/c mice, whereas donepezil, an AChE inhibitor, improved impairments induced by *Toxoplasma* infection.

Conclusion: *T. gondii* infection through increasing AChE reduces the level of Acetylcholine (Ach) and consequently affects learning and memory activity in infected hosts, whereas, donepezil as an AChE inhibitor improves these impairments by restoring ACh levels at synapses of neurons in brain.

Introduction

T*oxoplasma gondii*, as protozoan parasite, infects a broad spectrum of warm-blooded animals including humans. At present, approximately one-third of the world population has been infected with this parasite (1). *T. gondii* can cause infection in human via ingestion of tissue cysts or tachyzoites in raw or undercooked meat of infected animals, or ingestion of oocysts in the water or soil contaminated with feces of infected cats and congenitally (2). In human, *T. gondii* infection are normally asymptomatic or subclinical. However, the infection can cause serious symptoms including encephalitis, chorioretinitis, and systemic infections in immunocompromised individuals and abortion and still-birth in pregnant women (3, 4). Commonly, after infection with *T. gondii*, tachyzoites run away from the immune system, cause the establishment of tissue cysts containing bradyzoites in various organs particularly in the central nervous system (CNS). At the time of chronic infection in CNS, *T. gondii* cysts can affect different biological functions of neuronal cells such as neurotransmitter synthesis, synapse organization, and signal direction (5, 6). Moreover, the epidemiological studies in humans as well as experimental investigations in animals demonstrated that chronic toxoplasmosis might be caused several neurological and behavioral disorders (7-12).

Previous studies indicate a main role of acetylcholine (ACh) in the regulation of cognitive and behavioral functions (13). On the other hand, *T. gondii* infection enhances the activity of acetylcholinesterase (AChE) as a membrane-bound enzyme which hydrolyses ACh (14, 15). Donepezil hydrochloride is a well-known anti-dementia drug that increases acetylcholine levels via inhibition of AChE and improve learning and memory impairments (16).

In this study, we established the mouse models of chronic *T. gondii* infection by *T.*

gondii Tehran strain to provide a good understanding about defining the possible association between *T. gondii* exposure and learning and memory impairments. Moreover, as secondary objective of the current study, we hypothesized whether administration of an AChE inhibitor could reduce learning and memory impairments induced by *T. gondii* infection.

Materials and Methods

Ethical statement

The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kerman University of Medical Science (No.93/20) and Kerman Neurosciences Research Center, Kerman, Iran. Moreover, all efforts were made to minimize suffering.

Animals

Twenty-four male BALB/c mice (6–8 weeks old) weighing from 20 to 25 g to establish animal model of *T. gondii* were obtained from the Animal Breeding Stock Facility of Razi Institute of Iran (Karaj, Iran). Animals were housed in a colony room of Kerman Neurosciences Research Center with a 12:12 h light/dark cycle at $21 \pm 2^{\circ}\text{C}$ and were handled according to standard protocols for the use of laboratory animals. BALB/c mice were randomly divided to three experimental groups ($n=8$ per group) for the all assays: *T. gondii* infection, uninfected (control), and *T. gondii* + donepezil group.

Parasite

In the present study, to establish the chronic toxoplasmosis, the Tehran strain of *T. gondii* was kindly provided by Prof. Keshavarz of

Tehran University of Medical Sciences (Tehran, Iran). It was maintained by intraperitoneal inoculation of cysts (15-20 cysts) from brain tissue of infected BALB/c mice after 3 months. Cysts for the infection of BALB/c mice were isolated from the brain tissue of infected mice and the number of cysts was counted under a microscopy with a 10× objective.

Animal model of *Toxoplasma* infection

The animal model of *Toxoplasma* infection was established as described previously elsewhere (17). Brain homogenized suspension in saline was prepared from the mice infected with the tissue cysts of *T. gondii* three months earlier. Then, 0.5 ml of the brain suspension containing 20-25 tissue cysts was inoculated intraperitoneally to each of 8 male BALB/c mice. After 2 months, all the mice were tested for anti-*T. gondii* antibodies by serological tests.

Serological tests

In order to confirm *Toxoplasma* infection in tested mice, collected serum samples were examined for anti-*T. gondii* IgG antibody via the modified agglutination test (MAT) using a commercial kit (Toxoscreen DA, Biom'erieux, Lyon, France) in accordance with the manufacturer's instructions, and starting at a 1/20 dilution (18). Sera showing an agglutination titer of 1/20 or higher were considered positive and were end-titrated using 2-fold dilutions.

Treatment with AChE inhibitor

Donepezil hydrochloride (2 mg/kg, Sigma-Aldrich, USA) was dissolved in saline and was injected intraperitoneally once a day for two weeks starting from post-infection day 90.

Morris water maze (MWM) test

The MWM task was used to assay spatial learning and memory (19). The MWM consisted of a black circular swimming pool, painted with nontoxic materials black circular

pool, 160 cm diameter, 80 cm height-filled with water maintained at room temperature to a depth of 40 cm. The pool was geographically divided into four quadrants of equal size and starting points were designated at each quadrant as N, S, E, and W. A square platform (10 cm diameter) was hidden just below (1.5 cm) the surface of the water in the center of the northeast quadrant. The experiments were carried out in a dimly light room with various and fixed extra maze geometric images (e.g., circles, squares or triangles) attached at different points on the walls around the maze. Performances were recorded by a smart video tracing system (Noldus Ethovision® system, version 5, USA) and animals could be traced on the screen of a computer.

Spatial learning

In the spatial acquisition phase, the mice were allowed to find a submerged hidden platform during a 60-second-interval in four training trials (inter-trial interval = 60 s) repeated in three blocks (inter-block interval = 30 min). After finding the platform, the animals were allowed to rest on the platform for 20–30 s. The mice were dried with a towel and returned to their cages. After 20 to 30 s of rest, they were once again put in the chamber for the next trial. When mice did not find the platform within 60 s, the experimenter would put it on the platform. On each trial, mice were randomly released into the water from one of the four quadrants of the maze with their faces toward the wall of the quadrant where they were released. Each mouse had 4 different releasing points. Parameters such as latency and the traveled distance to find the platform were recorded in each trial.

Short-term spatial memory

Two hours after the acquisition phase, a probe test was performed to evaluate spatial memory retention. For the probe test, the platform was removed and each mouse was allowed to swim for 60 s. The time and dis-

tance spent in the target quadrant (quadrant 4) were analyzed as a measure of spatial memory retention.

Latency to visible platform and swimming speed

Following the probe trial, mice had to complete a visible platform test to determine any possibility of *Toxoplasma* infection and A β_{1-42} model interference with sensory and motor coordination or motivation. In this test, the ability of animals to escape to a visible platform was evaluated (the platform was raised 2 cm above the water level and was visible with aluminum foil).

Statistical analysis

Obtained results are expressed as the mean \pm SEM. Data analysis was carried out by using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with Tukey's post-hoc test was used to assess differences between experimental groups (20). In addition, $P < 0.05$ was considered statistically significant.

Results

Establishment of *T. gondii* infection

MAT showed that all of mice infected with *T. gondii* tissue cysts were seropositive for anti-*T. gondii* IgG antibody with agglutination titers of $>1/20$. Fig. 1 shows tissue cysts of *T. gondii* Tehran strain isolated from brain of infected mice.

Spatial learning

Distance traveled to reach the platform was significantly increased in the *Toxoplasma* infection group ($P < 0.05$) compared to the control group, indicating an impaired learning in *Toxoplasma* infected mice. Treatment with donepezil reduced the effect of *T. gondii* infection; the distance traveled to reach the platform was reduced in the *T. gondii* + donepezil

mice in comparison with *Toxoplasma* infected mice (Fig. 2A).

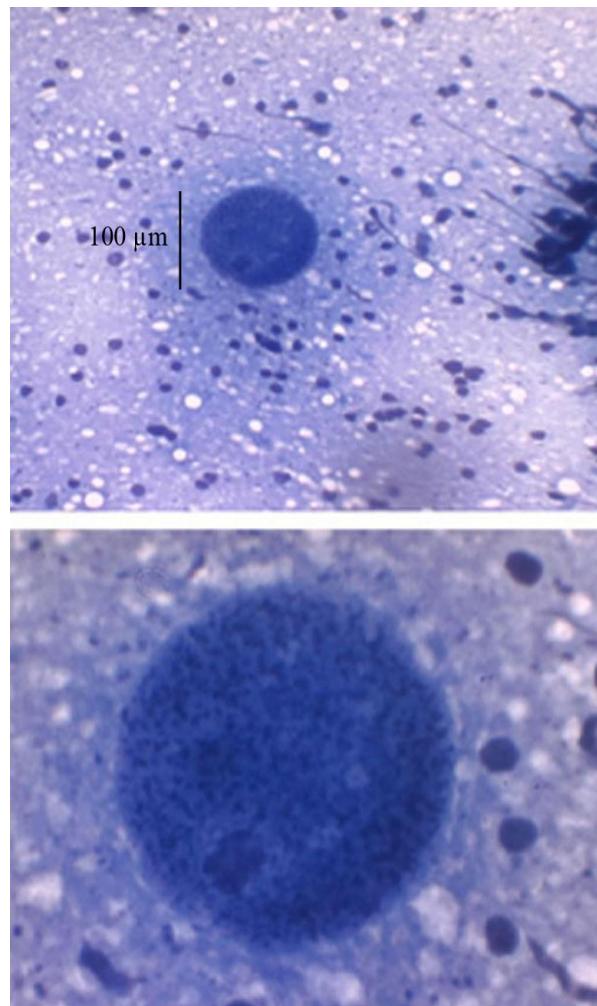


Fig. 1: Tissue cysts of *T. gondii* Tehran strain In brain crushed smear of a mouse stained by Giemsa stain (at $\times 100$ and $\times 400$ magnification)

As shown in Figure 2B, the escape latency of *Toxoplasma* group significantly ($P < 0.01$) increased in compared to the control group, whereas in the *T. gondii* infected mice treated with donepezil the escape latency significantly decreased ($P < 0.05$) compared to *Toxoplasma* group. Moreover, analysis of ANOVA indicated that there was no significant difference in the swimming speed among the all tested groups (Fig. 1C).

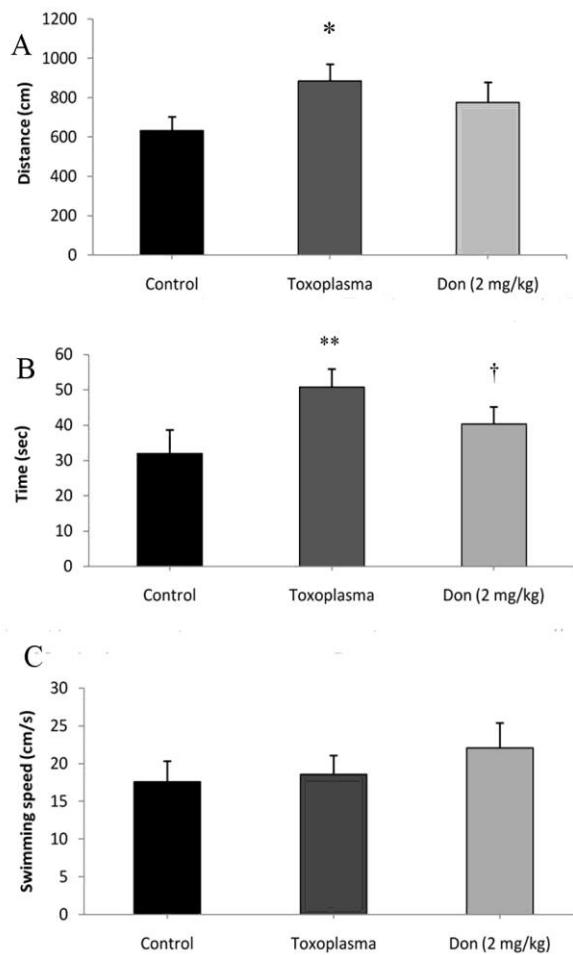


Fig. 2: Impaired learning observed in the *Toxoplasma* group compared to the control groups in Morris water maze task. Increased distance (A) and time spent (B) to reach the hidden platform were observed in the *Toxoplasma* group in compared with control group; while they were significantly decreased in mice treated by donepezil. There was no significant alteration in swimming speed of mice in all groups (C). * $P < 0.05$, ** $P < 0.01$, indicating the significant differences with the control group. † $P < 0.05$ compared with the *T. gondii* group

Short-term spatial memory

To assess short-term spatial memory retention, 2 h after the spatial learning phase, a probe test was carried out. The obtained results included the mean percentage (%) for time, distance (travel) and the number of crossing in the target quadrant.

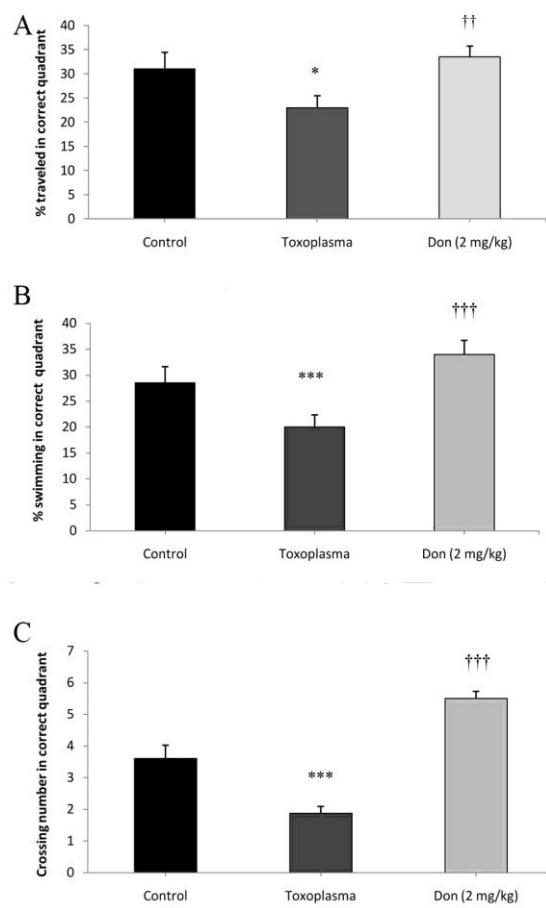


Fig. 3: The effects of *Toxoplasma gondii* infection and donepezil on spatial short term memory. The distance (A) and time (B) in the target quadrant decreased significantly in the *Toxoplasma* group compared to the control group. The number of crossing from the platform region was also significantly decreased in the *Toxoplasma* group compared to the control group. The distance, time, and the number of crossing were significantly increased in infected mice treated by donepezil.* $P < 0.05$, *** $P < 0.001$ indicating the significant differences with the control group. †† $p < 0.01$ and ††† $P < 0.001$ compared with the *T. gondii* group

The findings revealed that the mice in the *Toxoplasma* group significantly ($P < 0.05$ for distance and $P < 0.001$ for time in the target quadrant) spent less distance and time in the target quadrant compared to the control groups (Figs. 3 A, B), which indicating short term memory impairment in the *T. gondii* in-

fected mice. Obtained findings also revealed that treatment with donepezil counteracted the effect of *T. gondii* infection, because the *Toxoplasma* + donepezil mice spent more distance and time in the target quadrant compared to the *Toxoplasma* group ($P < 0.01$ for distance and $P < 0.001$ for time in the target quadrant). Analysis of ANOVA also showed that crossing number was significantly different in the *Toxoplasma* groups ($P < 0.001$) in comparison with control group (Fig. 3C), while in the *T. gondii* infected mice treated with

donepezil the crossing number was significantly ($P < 0.001$) increased compared to *Toxoplasma* group.

Latency to visible platform and swimming speed

As shown in Table 1 mice in all groups had a similar escape latency and swimming speed in the MWM test, which indicating no significant differences between the groups in visual and motor functions.

Table 1: Comparisons of swimming speed and latency to escape onto the visible platform in Morris water maze among groups using one-way analysis of variance (ANOVA) (the differences were not significant). Data are means \pm S.E.M. (8 mice/group)

Group	Swimming speed (cm/s)	Escape latency (s)
Control	20.4 ± 3.6	18.8 ± 2.4
<i>Toxoplasma</i>	19.4 ± 1.8	20.1 ± 3.6
<i>Toxoplasma</i> +Don*	23.1 ± 2.3	21.2 ± 2.81

*Donepezil 2 mg/kg/day/2w

Discussion

This study aimed to investigate the possible association between *T. gondii* exposure and learning and memory impairments, and also assessment of administration of donepezil, an AChE inhibitor to improve learning and memory impairments induced by *T. gondii* infection. *T. gondii* as a neurotropic protozoan parasite is considered as one of the most successful pathogens globally (21). Currently, there are several reports concerning the link of *T. gondii* infection with increased risk of some neurological disorders such as schizophrenia, personality disorders, obsessive-compulsive disorder, and Parkinson's disease (7-12, 22).

We found that *T. gondii* infected mice represent remarkable impairments in the spatial learning and short time memory in MWM test compared to control group. Consistent with our results, Zhou et al. have reported that chronic toxoplasmosis induced by *T. gondii* Prugniaud strain impaired learning and memory functions in Kunming mice (23). In

the infected rats, chronic toxoplasmosis lead to some neurocognitive disorders particularly memory impairment (24). In contrast, in latent toxoplasmosis, *T. gondii* in the CNS restrain neuronal degeneration as well as improves learning and memory disorders in mice with Alzheimer's disease (25). This difference in the reported impacts of *T. gondii* infection on cognitive functions can be attributed to several factors including rodent species, route of infection, parasite strain, and dosage (24, 26, 27).

Changes in behavior observed during latent *T. gondii* infection can be the consequence of a range of indirect and/or direct effects. Whereas, indirect effects can involve immune response to chronic toxoplasmosis (28); the direct effects are probably due to the mechanical presence of the *T. gondii* in the brain or their effects or products on neuronal cells biology (29). Regarding direct effects of chronic toxoplasmosis, infection by *T. gondii* is able to affect cholinesterase activity and enhance the AChE levels in brain of infected mice (14, 15). ACh play a main role in the regulation of

learning and memory functions (13). Today, the effective strategies to treat neurocognitive impairments designed to ameliorate the ACh activity through increase of level of Ach level via synthesis promoters as well as inhibitors of its metabolizing enzyme. Among the different approaches tried, the inhibition of AChE is the most successful one (30). To date, AChE inhibitors are widely used to improve the cognitive impairments such as learning and memory ones in patients with Alzheimer's disease through enhance acetylcholine levels at synapses where the neurotransmitter has been evacuated in order to degeneration of neuronal cells (31).

Consistent with previous investigations, the obtained findings of the present study revealed a protective role for donepezil as an AChE inhibitor on learning and memory alterations induced by *T. gondii*. Donepezil at the dose of 2 mg/kg once a day for five consecutive days for two weeks improve spatial learning and memory deficits following traumatic brain injury independent of its effects on neurogenesis (16). Moreover, after treatment with donepezil a considerable improvement of memory as well as a dose-dependent depletion of amyloid- β (A β) was observed in brain of hAPP/PS1 mice (31). In gerbils, administration donepezil at the dose of 5 mg/kg for 21 consecutive days reduced hippocampal neurodegeneration and cognitive impairments after global cerebral ischemia (32). Our findings also exhibited that in MWM test donepezil resulted in an increase in swimming speed in treated mice. This suggests that one of the beneficial effects of donepezil on improvement of learning and memory in *T. gondii* infected mice which might be through improvement in motor activity as described elsewhere (16).

Conclusion

T. gondii infection through increasing AChE reduces the level of ACh and consequently

affects learning and memory activity in infected hosts, whereas, donepezil as an AChE inhibitor improves these impairments by restoring ACh levels at synapses of neurons in brain. However, further studies are required to elucidate these mechanisms and other possible ones.

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