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Original Article

L-Theanine Improves Locomotor Function in a Model of Multiple Sclerosis Mice

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Abstract

This study designed to investigate the protective effects of L-theanine on experimental Multiple sclerosis in mice. Frothy Male C57BL/6 mice were allocated into 4 experimental groups: control no treatment received a regular chew pellet, and the cuprizone (CPZ) group received a standard chew pellet containing 0.2% (w/w) CPZ. In group 3, mice were fed a regular diet and administered p.o. with L-theanine (50mg/kg). In group 4, mice received a diet containing CPZ and were administered p.o. with L-theanine (50mg/kg). Finally, reflexive motor behavior and serum antioxidant levels were determined. Based on findings, CPZ significantly decreased ambulation score, hind-limb suspension, front limb suspension, and grip strength (P<0.05). The CPZ + L-theanine reduced the adverse effect of the CPZ on ambulation score, hind-limb foot angle, surface righting, and negative geotaxis (P<0.05). The CPZ + L-theanine increased front and hind-limb suspension, grip strength, number of the cross, and duration of a stay on the rotarod compared to the control animal (P<0.05). CPZ administration significantly elevated serum malondialdehyde (MDA) while superoxide dismutase (SOD) and glutathione peroxidase (GPx) and total antioxidant status (TAS) levels decreased compared to control mice (P<0.05). The CPZ + L-theanine leads to the cessation of MDA production while increasing SOD, GPx, and TAS levels (P<0.05). These results suggested L-theanine has a protective effect against CPZ-induced MS in mice.

Keywords: Antioxidant, L-theanine, Multiple sclerosis, Mice

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease that leads to inflammation and demyelination of the central nervous system (1). In this study, the immune system attacks the myelin sheath of the neurons and leads to demyelinating and mitochondrial dysfunction. Cuprizone-induced (CPZ) experimental demyelination model is a valuable technique for investigating the etiology of the MS. Administration of the CPZ leads to myelin damage in numerous grey matter regions in the cortex, hippocampus, and cerebellum (2). Depression and anxiety are these patients' most reported psychiatric disorders (2). Also, physical, mental, cognitive, and motor activity are affected in MS patients (3). Oxidative stress plays a primary role in the development of MS. In biological systems, oxygen-derived free radicals (ROS) lead to oxidative stress-induced cell death, and administration of troxerutin enhances cell viability by increasing cellular antioxidant levels such as SOD, catalase (CAT), and GPx (4). One of the leading indicators in patients with MS is increased MDA production and decreased SOD, GPx, and catalase levels (5).

Because of the importance of inflammation and oxidative stress in the pathophysiology of MS, several antioxidants and anti-inflammatory agents are applied to prevent and treat MS (6). L-theanine (gamma-glutamyl diethylamide, biosynthesized from glutamine and ethylamide) is a naturally soluble amino acid in green tea and is responsible for its unique taste (7). L-theanine is a non-toxic and safe photogenic food additive, and its safety has been approved by FDA (8). L-theanine is famous for its antioxidant, hepatoprotective, antitumor, anti-aging, and neuroprotective activity (9). L-theanine decreases reactive oxygen species production (ROS) and attenuates lipid peroxidation. Also, it can reduce MDA production and increase CAT, SOD, and GPx levels (10). L-theanine can across brain-blood-barrier (BBB) L-theanine (1 and 4mg/kg) administered at 3, 12, and 24 h after reperfusion has a positive effect on cerebral ischemia-reperfusion injury in rats (11). Even though several types of research have been done on the beneficial activity of the L-theanine, there is no report on its possible effects on CPZ-induced demyelination. Thus, this study aimed to investigate the protective effects of L-theanine on experimental MS in mice.

2. Materials and Methods

2.1. Animals

Frothy Male C57BL/6 mice (4-6 weeks old; weighing 19 ± 2 g) were kept under laboratory conditions (temperature of 22 ± 2 ℃ and 12/h light/dark cycle) with adequate food and water in the Razi laboratory complex (Islamic Azad University, Science and Research Branch, Tehran, Iran). One week after acclimatization, mice were randomly allocated into 4 experimental groups (n=10).

2.2. Study Protocol

The control group received a regular diet. In group 2, acute demyelination was induced by feeding mice with 0.2% (w/w) CPZ (Sigma Aldrich, St. Louis, MO, USA) mixed with ground chow for 5 weeks (1). In group 3, a regular diet was provided, and mice were administered daily p.o. with L-theanine (50 mg/kg) (Sigma Chemical Co., St Louis, MO, USA) for 5 weeks. In group 4, mice received a diet containing CPZ (0.2% w/w) for 5 weeks and were administered p.o. with L-theanine (50 mg/kg). Finally, reflexive motor behavior and serum antioxidant levels were determined.

2.3. Ambulation

Ambulation test as crawling behavior is used to determine the ability to walk following MS (12). Mice were placed in a transparent enclosure, visible from the top to all 4 sides. To motivate mice to walk, we used a gentle tail prod. The ambulation score was: 0= no movement, 1= crawling with asymmetric limb movement, 2= slow but symmetric limb movement, and 3= fast crawling/ walking. To eliminate learning, the test was performed in triplicate within 3 min (13).

2.4. Hind-Limb Foot Angle

Following the signs of MS, hind limb posture changes wherein walking. The hind limbs are positioned under the body. Therefore, the angle among the hind limbs in walking was less than the crawling position (13). A plain open field box with a camera above was used to record the mice's movements around the box. Recorded videos determined the foot angle. In recorded videos, a line was drawn from the end of the heel/shin to the tip of the toe. The measurements were recorded only in the mice performing a full stride in a straight line, and their feet were flat on the ground. No repeat-related learning was observed in this test (13).

2.5. Front‑Limb Suspension

This test was conducted on mice to hang onto the wire with both forepaws. After grasping the wire, mice were released, and the time needed to fall was recorded in seconds. To minimize testing errors, the test was performed in triplicate within 3 minutes (13).

2.6. Hind-Limb Suspension

The hind-limb suspension test was performed to determine mic strength and neuromuscular function. Mice were placed face down into the standard 50 mL conical laboratory tube. The mice's hind legs hung over the wire and were released, and the hind-limb posture score was recorded as score 0: constant clasping of the hind limb by holding onto the tube; score 1: weakness

was apparent, and the hind-limb were almost in a clasped position with the tail raised; score 2: hind-limb were close to each other and often touching; score 3: weakness was apparent, closer together and rarely touched each other; score: 4 normal hind limb separation with tail raised (13).

2.7. Surface Righting

The surface righting reflex is a motor ability of the mice to flip onto their feet from their supine position (14). This test was performed on a cotton sheet and kept in place for 5 seconds. Then, they were released, and the time needed to return to their prone position was recorded. As no-repeat-related learning was reported in this test, triplicate within 3 minutes was done (13).

2.8. Grip Strength

The test was conducted to determine the grip strength in which animals can grab onto a screen and generate a reading of the grip force. A 16 × 18 fiberglass screen was used in which the surface was rotated slowly from a horizontal to a vertical position to challenge the grasping of all four limbs (15). The hanging impulse, which indicated the force needed to resist gravity, was calculated per the below formula:

[weight (g) × latency to fall (s)] (16).

2.9. Negative Geotaxis

Mice were placed down on a 45° surface used as a slope. Then, they were released, and the time needed by mice to face the hill upward due to vestibular cues of gravity was documented (13).

2.10. Open Field Test (OFT)

The OFT was used to determine the possible effects of L-theanine on the locomotor and exploratory activities in mice. The test was done using a 45×45×30 cm^3 wooden box. The floor of the open field box was divided by masking tape markers into 9 squares. Each animal was placed individually at the center of the apparatus. Then, the number of segments crossed with the four paws was recorded for 6 min (17).

2.11. Rotarod Test

The accelerated rotarod test is a standard sensory-motor test to investigate animals' motor coordination and learning skills by measuring the mice's ability to stay and run on the accelerated rod. The test was done for 8 min with an acceleration of 0–20 rpm. The time was recorded when mice fell off the rod or started to rotate with the rotarod without running. After an initial training trial, mice were tested for 5 problems over two days. The recovery phase between practices was 10 minutes (18).

2.12. Antioxidant Activity

After determining the behavioral tests, blood samples were taken from each cardiac mouse, and serum MDA, SOD, GPx, and TAS were determined using Zell Bio GmbH (Germany) assay kits.

3. Results

As shown in figure 1, the Supplementation of L-theanine significantly amplified the ambulation score in CPZ-induced MS compared to the control group (P<0.05). CPZ significantly reduced ambulation scores compared to the control mice (P<0.05). Co-administration of the CPZ + L-theanine significantly reduced the adverse effect of the CPZ on ambulation score (P<0.05).

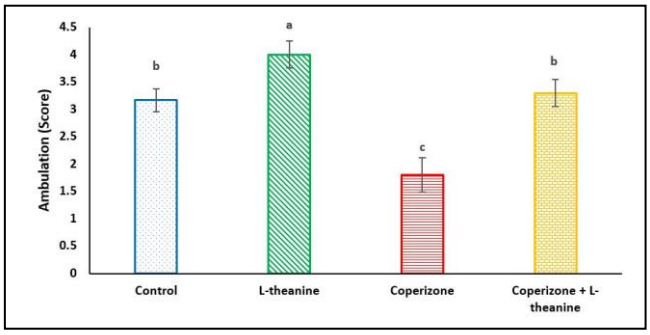

Figure 1. Effects of cuprizone, L-theanine and their combination on ambulation score in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; P≤0.05)

Based on figure 2, L-theanine significantly reduced hind-limb foot angle compared to the control mice (P<0.05). Hind-limb foot angle significantly enlarged following CPZ-treated mice compared to control mice (P<0.05). CPZ + L-theanine significantly reduced the revered adverse effect of the CPZ (P<0.05).

According to figure 3, L-theanine significantly increased hind-limb suspension compared to mice (P<0.05). Hind-limb suspension significantly reduced the in CPZ-treated mice (P<0.05). Co-administration of the CPZ + L-theanine significantly decreased the adverse impact of the CPZ (P<0.05).

Based on figure 4, L-theanine significantly decreased surface righting compared to the control group (P<0.05). CPZ significantly increased surface righting (P<0.05). Co-administration of the CPZ + L-theanine significantly reversed the adverse effect of the CPZ on surface righting (P<0.05).

L-theanine significantly increased grip strength compared to control mice (P<0.05). Administration of the CPZ significantly decreased grip strength compared to control mice (P<0.05). CPZ + L-theanine improved grip strength (P<0.05) (Figure 5).

As presented in figure 6, L-theanine significantly increased front limb suspension compared to the control animal (P<0.05). Front limb suspension significantly decreased in mice that received CPZ compared to the control group (P<0.05). Co-administration of the CPZ + L-theanine significantly decreased the adverse effect of the CPZ (P<0.05).

In this study, supplementation of the L-theanine significantly decreased the negative geotaxis of the control mice (P<0.05). Negative geotaxis significantly increased in CPZ-treated mice (P<0.05). CPZ + L-theanine improved the negative geotaxis compared to the control group (P<0.05) (Figure 7).

As seen in figure 8, supplementation of the L-theanine did not affect the number of the cross in OFT compared to the control animal (P>0.05). CPZ significantly decreased the number of crosses in the OFT than in the control group (P<0.05). CPZ + L-theanine decreased the adverse effect of the CPZ on a number of the cross in the OFT than in the control group (P<0.05).

According to figure 9, supplementation of the L-theanine significantly increased the duration of stay on the rotarod compared with the control group (P<0.05). CPZ significantly decreased the duration of stay on the rotarod compared to the control mice (P<0.05). Co-administration of the CPZ + L-theanine decreased the adverse impact of the CPZ on the duration of a stay on the rotarod (P<0.05).

According to figure 10, L-theanine decreased serum MDA than to control mice (P<0.05). CPZ administration significantly elevated serum MDA compared to control mice (P<0.05). Co-administration of the CPZ + L-theanine significantly reduced CPZ-induced elevation in the MDA production (P<0.05).

Based on figure 11, supplementation of the L-theanine enhanced serum SOD activity (P<0.05). CPZ significantly decreased serum SOD activity compared to control mice (P<0.05). Co-administration of the CPZ + L-theanine decreased the adverse effect of the CPZ on serum SOD (P<0.05).

As seen in figure 12, L-theanine significantly improved serum GPx activity compared to the control mice (P<0.05). CPZ significantly decreased serum GPx activity compared with control mice (P<0.05). Co-administration of the CPZ + L-theanine reduced the adverse effect of the CPZ on serum GPx (P<0.05).

In this study, L-theanine significantly increased serum TAS levels compared to control mice (P<0.05). CPZ significantly decreased serum TAS compared to the control group (P<0.05). Co-administration of the CPZ + L-theanine decreased CPZ-induced elevation on TAS (P<0.05) (Figure 13).

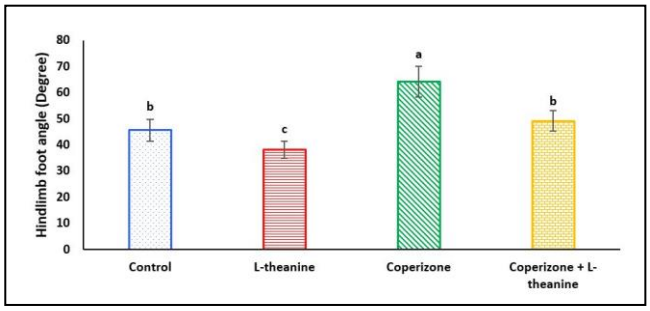

Figure 2. Effects of cuprizone, L-theanine and their combination on hind-limb foot angle in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-b; $P \leq 0.05$)

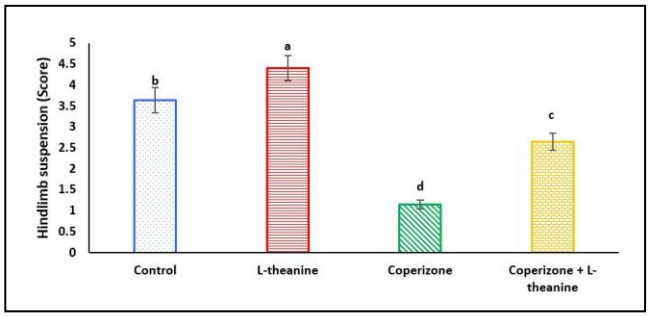

Figure 3. Effects of cuprizone, L-theanine and their combination on hind-limb suspension in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

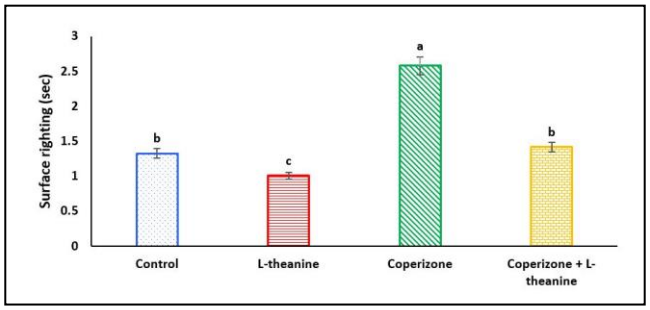

Figure 4. Effects of cuprizone, L-theanine and their combination on surface righting in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

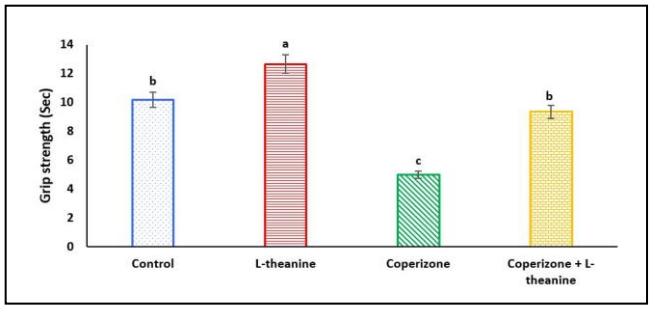

Figure 5. Effects of cuprizone, L-theanine and their combination on grip strength in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

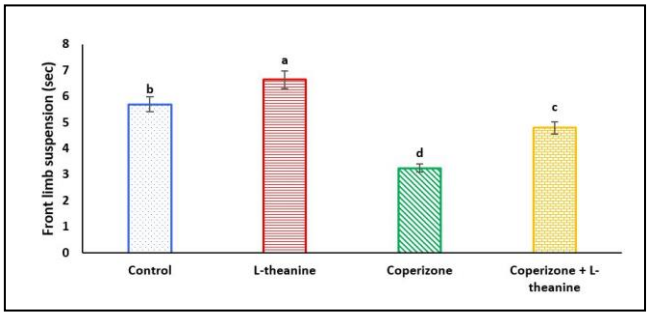

Figure 6. Effects of cuprizone, L-theanine and their combination on front limb suspension in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-d; $P \leq 0.05$)

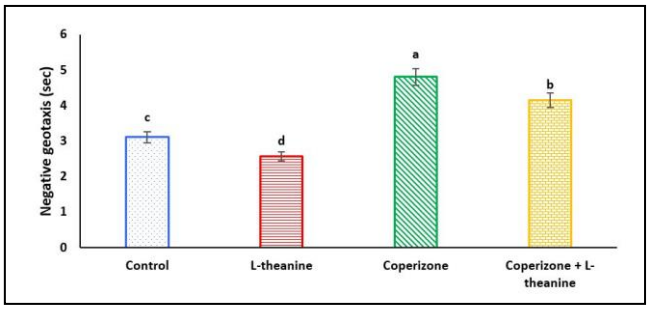

Figure 7. Effects of cuprizone, L-theanine and their combination on negative geotaxis in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-d; $P \leq 0.05$)

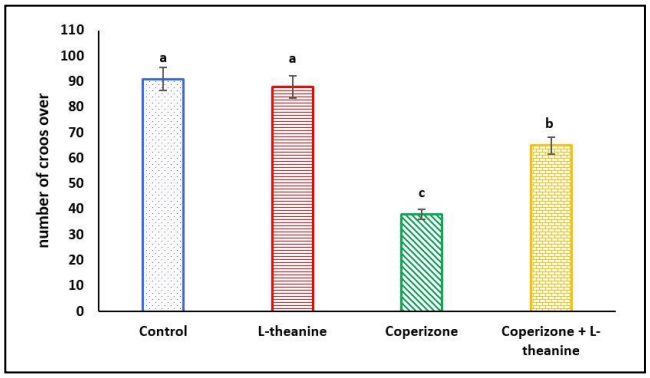

Figure 8. Effects of cuprizone, L-theanine and their combination on number of cross on open field test (OFT) in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-d; $P \leq 0.05$)

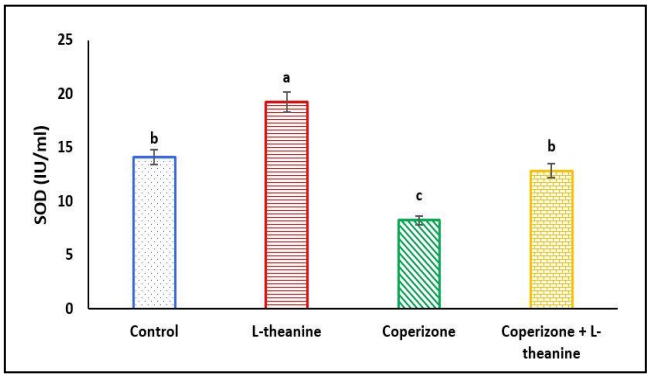

Figure 11. Effects of cuprizone, L-theanine and their combination on serum Superoxide dismutase in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

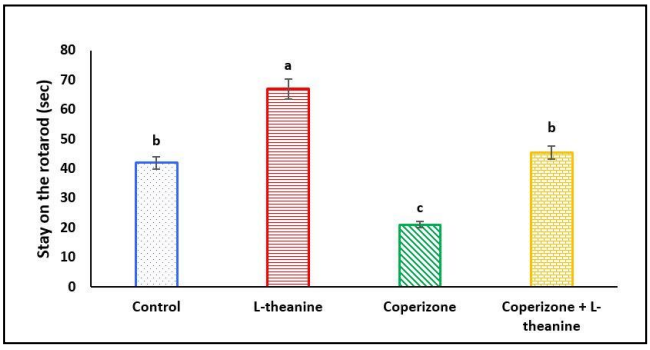

Figure 9. Effects of cuprizone, L-theanine and their combination on stay on the rotarod in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

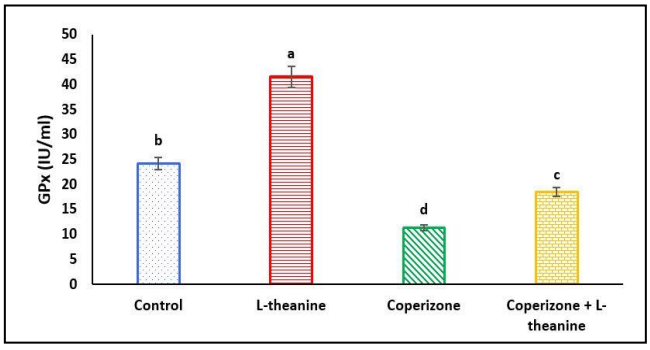

Figure 12. Effects of cuprizone, L-theanine and their combination on serum Glutathione peroxidase in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

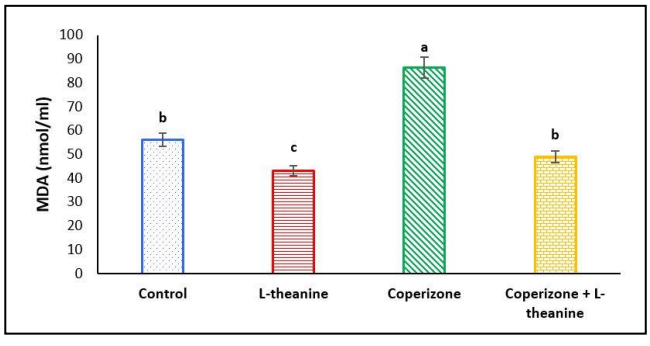

Figure 10. Effects of cuprizone, L-theanine and their combination on serum Malondialdehyde in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

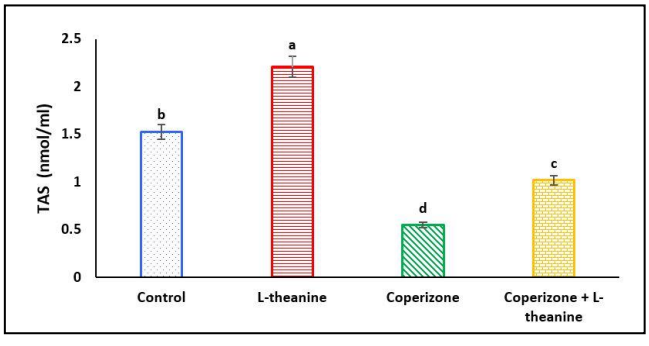

Figure 13. Effects of cuprizone, L-theanine and their combination on total antioxidant status in Cuprizone-induced model of multiple sclerosis mice (n=10). There are significant differences between groups with different superscripts (a-c; $P \leq 0.05$)

4. Discussion

Multiple sclerosis is a neurodegenerative disease of the CNS that happens because of pathophysiolgical condition between immune and nervous systems and oxidative status. However, several investugations have been done to achieve goals in the prevention and treatment of MS, and its occurrence is increasing worldwide. Thus, animal and CPZ models are preferred for studying demyelination MS pathogenesis (19). CPZ inhibits copper ions and causes oxidative stress, oligodendrocyte apoptosis, and demyelination. Thus, CPZ creates lesions like pattern 3 of MS, and demyelination begins in the first week of poisoning and, after 5 weeks, happens in the brain. Thus, in this method, MS does not happen via the immune system. In this study standard chew pellet containing 0.2% (w/w) CPZ was provided to the mice, and after 5 weeks, the reflexive motor behavior tests were done. Based on findings, CPZ decreased ambulation score, hind-limb suspension, front limb suspension, and grip strength. The battery of reflexes testing method is used for neurodevelopmental of the nervous system. These tests include limb grasping and placing, cliff avoidance, righting, accelerated righting, gait, auditory startle, posture, and eye-opening (20). Demyelination leads to abnormal reflexes and progressive disabilities. Thus, a battery of reflexes is a definitive test to determine motor skills in the model of MS (21). A study reported that administration of the solid lipid nanoparticles loaded with dimethyl fumarate improved neurological deficit score, grasping ability, forelimb strength, and motor function in CPZ-induced demyelination rats (22). So, here we used a battery reflex test on the model of CPZ-induced demyelination.

CPZ administration significantly elevated serum MDA while SOD, GPx, and TAS levels decreased compared to control mice. ROS include hydrogen peroxide and unstable free radicals with unpaired electrons in their outer orbits (23). Excess ROS generation or decreased antioxidant defenses (22, 23). Plant extract and bioactive compounds have an antioxidant effect by decreasing oxidative stress markers with a concomitant increase in SOD, CAT, and GPx activity (24). In this study, co-administration of the CPZ + L-theanine reduced the adverse effect of the CPZ on ambulation score, hind-limb foot angle, surface righting, and negative geotaxis. Compared to the control animal, the co-administration of the CPZ + L-theanine increased front and hind-limb suspension, grip strength, number of the cross, and duration of a stay on the rotarod. Co-administration of the CPZ + L-theanine significantly reduced CPZ-induced elevation in the MDA production while increasing SOD, GPx, and TAS levels. Recently, Suleghani, Asghari (25) reported L-theanine (200 and 400 mg/kg) decreased MDA and increased tissue SOD and GPx activity in an experimental model of testicular ischemia/reperfusion injury in the rat. L-theanine increases the synthesis of nerve growth factors and neurotransmitters in infant rats (26). Also, L-theanine supplementation has neuroprotective activity in the model of stroke rats (27). L-theanine has antipsychotic activity in patients with schizophrenia (28). L-theanine has ant-depressive activity, decreasing immobility time in forced swimming test mice (29). Chronic administration of the L-theanine during stress-induced cognitive impairment alters catecholamines and neurogenesis in the hippocampus (30). L-theanine modulates the activity of neurotransmitters such as serotonin, dopamine, and GABA in the CNS, but the mechanism of action is not determined (31).

Perhaps, some protective effects of the L-theanine against CPZ-induced MS are related to its antioxidant properties. Despite the direct mechanism for how L-theanine improves antioxidant activity during MS, it seems crocin increases the expression of brain-derived neurotrophic factor and vascular endothelial growth factor in the rat hippocampus (32). The decreased levels of the antioxidant enzymes MS lesions is associated with neuroinflammation. On the other hand, applying bioactive components decreases the activation of immune-regulatory transcription factors like

interleukin and tumor necrosis factor. Also, the activity of SOD and GPx decreased in CD_4^+T cells in patients with MS (33).

However, based on the current study's limitations, we were unable to determine neurotransmitter levels or antioxidant activity in the brain tissue and the possible effects of the L-theanine on inflammatory mediators of the experimental CPZ-induced MS model mice. These results suggested L-theanine has a protective effect against CPZ-induced MS in mice. However, there was no report to compare these findings with it. However, further investigation is needed to determine its active constituents and precise mode of action.

Authors' Contribution

Design, Proofing the Paper: Sh. H.
Experimental Procedure, Draft of Paper: Sh. Kh.
Advisor: S. H.

Ethics

All the procedures were approved by the ethics committee of the Islamic Azad University, Science and Research Branch, Tehran, Iran (IR.IAU.SRB.REC.1401.103; 2022.05.31).

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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