The Neuroprotective Effect of a Triazine Derivative in an Alzheimer's Rat Model

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Abstract- Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder. It is characterized by formation of amyloid plaques and neurofibrillary tangles in the brain, degeneration of the cholinergic neurons and neural cell death. This study was aimed to investigate the effect of a triazine derivative, C16H12Cl2N3S, on learning in an Alzheimer's rat model. Animals were divided into seven groups; each group contained seven animals. Control group: animals received no surgery and treatment; saline group: animals received normal saline after recovery; sham group: animals received 10% DMSO after recovery; STZ group (Alzheimer's model): animals received streptozotocin (STZ) in four and six days after recovery; T5, T10 and T15 groups: animals were treated with triazine derivative, C16H12Cl2N3S, at doses of 5, 10 and 15 μM, respectively. All drugs were injected intracerebroventricular. The spatial learning and histological assessment were performed in all groups. Animals in STZ group had more deficits in spatial learning than the control group in Morris water maze. C16H12Cl2N3S improved spatial learning significantly compared to STZ group. The CA1 pyramidal layer thicknesses in STZ group were reduced significantly compared to control group. C16H12Cl2N3S increased the CA1 pyramidal layer thickness in T15 group compared to STZ group. Current findings suggest C16H12Cl2N3S may have a protective effect on learning deficit and hippocampal structure in AD.

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Keywords: Triazine; Learning; Alzheimer's disease

Introduction

Alzheimer's disease (AD), the most common cause of senile dementia, is a progressive, degenerative disease of the central nervous system (1). The main abnormalities of AD are intracellular twisted strands of the tau protein (neurofibrillary tangles) and deposits of extracellular beta-amyloid (Aβ) protein (2). Accumulation of Aβ peptide causes an increase in the intracellular reactive oxygen species (ROS) and apoptotic cell death (3-8). Moreover, oxidative stress has been identified as a major agent in the pathogenesis of AD (9-11). Thus, antioxidants might reduce Aβ-induced neurotoxicity and cell death (12,13). Drugs used to treat AD, such as acetylcholinesterase inhibitors have side effects like hepatotoxicity and gastrointestinal disturbances, and are unable to treat AD completely (14-16). Therefore, developing a novel and effective drug, such as triazine is necessary for AD treatment. Recent studies revealed different derivatives of triazine

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including 1, 2, 3-triazine, 1, 2, 4-triazine and 1, 3, 5-triazine. These derivatives have different activities in inflammation ROS production, neural damage and cancer (17-24). Intraventricular administration of streptozotocin (STZ) in the brain is widely used as AD model in rats (25,26). Several studies showed various antioxidant agents prevent neural damages in different AD models (12,13). Most of the antioxidants are not permeable through the blood-brain barrier. 3-thiomethyl-5, 6-di-(4-chlorophenyl)-1, 2, 4-triazine ($C_{16}H_{12}Cl_2N_3S$), the most active triazine derivative, passes through the blood brain barrier. Moreover, in vitro studies have revealed the antioxidant and neuroprotective effect of $C_{16}H_{12}Cl_2N_3S$. Therefore, it seems to be a suitable antioxidant candidate for treatment of neuronal disorders (18). This study was aimed to investigate the effect of $C_{16}H_{12}Cl_2N_3S$ on spatial learning and structure of CA1 in an AD in vivo model.

Materials and Methods

Animals

A total of 49 male adult Wistar rats (200-260 g) were housed in the animal room with 24-26 ºC and 12 hours dark / light cycle (lights on from 06:00 to 18:00) with food and water ad libitum. The behavioral experiments were performed from 8 AM to 4 PM. All the experiments were carried out according to the protocol approved by the Animal Ethics Committee of Tehran University of Medical Science, Tehran, Iran.

Stereotaxic surgery

Animals were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) intraperitoneally, and their head were fixed into stereotaxic instrument (Stoelting Instruments, USA). Two stainless steel, 23-gauge guide cannulae were implanted in the lateral ventricles bilaterally. Stereotaxic coordinates were based on Paxinos and Watson atlas of the rat brain as following:

AP = -0.8 mm, ML = ±1.5 mm, and DV = -3 mm down from the skull surface (27). Animals were kept in cages for six days to recover.

Experimental protocol

Animals were divided into seven groups (seven in each group). Control group: animals in this group got no surgery and treatment; saline group: animals received normal saline after recovery; sham group: animals received 10% DMSO after recovery; STZ group (Alzheimer's model): animals received streptozotocin (3 mg/kg) in fourth and sixth days after recovery; T5, T10 and T15 groups: STZ was injected in days four and six and animals were treated with $C_{16}H_{12}Cl_2N_3S$ at doses of 5, 10 and 15 µM, respectively in 1st, 2nd, 3rd and 5th days after recovery.

All drugs were injected intracerebroventricular (i.c.v.) in a total volume of 10 µL in the rate of 1 µL/min. The 27-gauge injection needle was inserted into the guide cannula. The injection needle was attached to a 10 µL Hamilton syringe by a polyethylene tube.

Morris water maze test

Morris water maze consists of a circular water tank with 160 cm diameter and 60 cm height, filled with non-toxic water (25 ± 2 °C) to a depth of 25 cm. The pool was divided into four quadrants (North, South, East, and West) which were used as start points. An escape platform (10 cm in diameter) made of Plexiglass was placed in the middle of one of the randomly selected quadrants of the pool, 1 cm below the surface of water and kept in the same position throughout the entire experiment (north-west for this study).

Spatial learning of animals was tested 14 days after STZ infusion in Morris water maze. All animals in each group were tested (one animal at a time). Rats were trained for four days. Each animal was subjected to four consecutive trials on each day with an interval of 1 min. Each trial was started by placing the animal randomly in one of the four starting points. Animals were allowed to swim in the pool during a period of 90 second to find the hidden platform. If an animal did not find the hidden platform within this period, it was manually guided to the platform by the researcher. Then they were allowed to remain on the platform for 30 second (s). All trials were performed at about the same time in the morning (28).

Directions of the rats were recorded by a video camera located above the center of the maze that was linked to a computer. Spatial acquisition was evaluated by measuring escape latency (time to find the platform), traveled distance (path length to reach the platform), and swimming speed using the EthoVision tracking system (Noldus Information Technology, Wageningen, The Netherlands), as explained previously (29, 30). The data obtained from rats with visual impairment was excluded.

Histological assessment

After the behavioral test, animals were deeply anesthetized with chloral hydrate (350 mg/kg; Sigma-Aldrich) and perfused transcardially with 200 ml of saline followed by 600 ml of 4% paraformaldehyde (PFA) solution. The brains were removed and kept in

4% PFA for at least four days at 4 ºC and the serial (8 µm) coronal sections were prepared.

Some sections were selected by random systemic sampling from each animal and stained by cresyl violet. CA1 area was studied under a light microscope (BX51, Olympus, Japan) linked to a digital camera. Digital photographs were taken using a 40X objective lens (Olympus, Japan).

Drugs

The triazine derivative, 3-thiomethyl-5, 6-di-(4-chlorophenyl)-1, 2,4-triazine (C16H12Cl2N3S), was prepared in our laboratory as reported previously (18). The purification of the above compound was carried out using column chromatography. Crystallization from methanol- water gave the pure compound (Mp = 137-139 °C). STZ was purchased from Sigma–Aldrich, USA. DMSO was used as a vehicle for C16H12Cl2N3S dilution.

Statistical analysis

Analysis of variance (ANOVA) was used for comparison of behavioral and histological data. A Tukey multiple comparison post-test was performed to assess differences between groups. Significance was established when the probability values were less than or equal to 0.05.

Results

The effect of STZ on spatial learning

The spatial learning was analyzed in control, saline, sham, and STZ groups. There were no significant differences in escape latency and traveled distance parameters between the same training days in control, saline and sham groups ($p = 0.99$; Figures 1A and 1B). STZ injection significantly increased escape latency and traveled distance in the same training days compared to other groups (Table 1).

Table 1. The mean ± S.E.M escape latency and traveled distance in every day of training in control, saline, sham, and STZ group

	Control	Saline	Sham	STZ
Escape latency (sec)				
Day 1	63.39±5.36	4497±7.70	36.78±5.83	71.51±4.95
Day 2	21.80±4.23	19.47±3.36	19.87±3.13	60.87±6.11
Day 3	17.30±3.09	11.91±3.36	20.51±3.55	42.45±7.14
Day 4	16.59±3.21	12.80±2.81	11.04±1.55	28.39±5.42
Travelled distance (cm)				
Day 1	712.25±122	1117.68±183	864.89±127	1780.08±160
Day 2	590.46±113	487.57±89.12	536.09±86.54	1640.91±174
Day 3	439.25±87.33	281.9±81.95	513.31±79.93	1171.92±183
Day 4	438.76±91.85	306.93±69.72	285.28±39.86	941.37±165

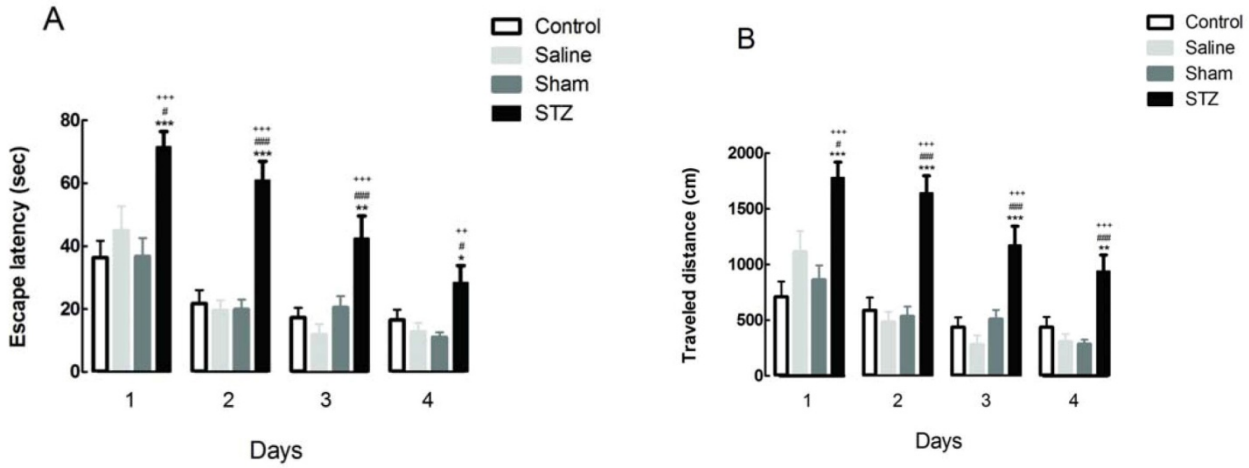


Figure 1. Morris water maze assessment in the same training days in control, saline, sham, and STZ groups. A) The bar graphs show the quantitative results (mean ± S.E.M) of escape latency in the same training days. Escape latency of STZ group was increased significantly in the same training days compared to control, saline, and sham groups. B) The bar graphs show the quantitative results (mean ± S.E.M) of traveled distance in the same training days. Traveled distance of STZ group was increased significantly in the same training days compared to control, saline, and sham groups. *, ** and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$. *, #, and + indicate comparing of STZ group with the control, saline, and sham groups, respectively

The average of escape latency and traveled distance in all trials during four training days were not significantly different among control, saline and sham groups (p = 0.96; Figures 2A and 2B). The mean average of escape latency and traveled distance in all trials during four training days in STZ group increased significantly compared to the other groups (p < 0.001). The mean escape latency was 23.02 ± 2.16 s in control group, 22.28 ± 2.76 s in saline group, 22.05 ± 2.06 s in sham group and 53.93 ± 3.39 s in STZ group (p < 0.001). The mean traveled distance was 602.47 ± 55.65 cm in the control group, 548.52 ± 68.20 cm in the saline group, 549.89 ± 47.73 cm in the sham group, and 1353.57 ± 89.83 cm in STZ group. The swimming speed in different experimental groups was not significantly different, indicated there was no motor activity disturbance in tested animals (p = 0.96; Figure 2C).

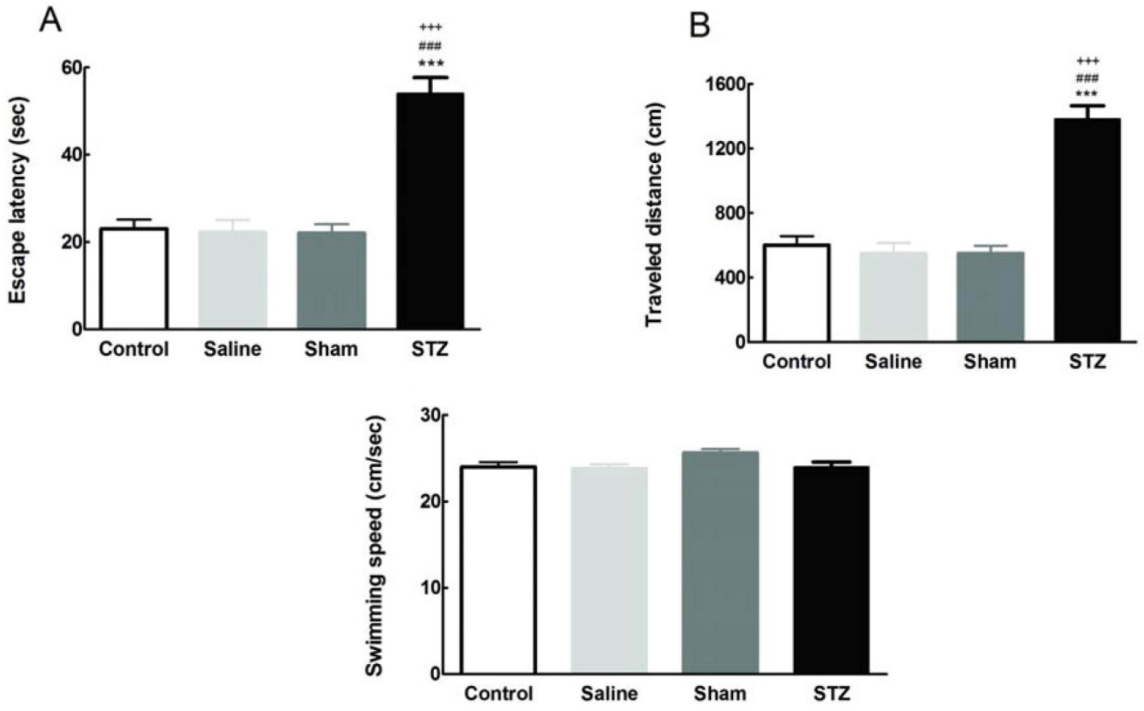


Figure 2. The average of escape latency, traveled distance and swimming speed in all trials during four training days in control, saline, sham, and STZ groups. A) The bar graph shows the quantitative results (mean ± S.E.M) of escape latency. The mean escape latency in STZ group was increased significantly compared to the other groups. B) The bar graph shows the quantitative results (mean ± S.E.M) of traveled distance. The mean traveled distance in STZ group was increased significantly compared to the other groups ($^{***}P < 0.001$). Swimming speed was not significantly different between all groups. *, ** and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$. *, #, and + indicate comparing of STZ group with the control, saline, and sham groups, respectively

The effect of triazine on spatial learning

The effect of triazine on escape latency and travel distance in the same training days of different groups were illustrated in figures 3A and 3B, respectively. Escape latency in the first training day decreased significantly in T5 and T15 groups compared to STZ group ($P < 0.05$, $P < 0.01$). Escape latency in the second training day decreased in all treated groups compared to STZ group ($P < 0.001$). In the third training day, escape latency decreased in T10 and T15 groups compared to STZ group ($P < 0.05$, $P < 0.01$).

Traveled distance in the first training day decreased in T5 and T15 groups compared to STZ group ($P < 0.01$, $P < 0.001$). Traveled distance in the second training day decreased in all treated groups compared to STZ group ($P < 0.001$). In the third training day, traveled distance decreased in T10 and T15 groups compared to STZ group ($P < 0.05$, $P < 0.001$). In the fourth training day, traveled distance decreased in T15 group compared to STZ group ($P < 0.05$; Table 2).

The mean average of escape latency and traveled distance in all trials during four training days were significantly reduced in T5, T10 and T15 groups compared to STZ group ($P<0.001$; Figures 4A and 4B).

Table 2. The mean ± S.E.M escape latency and traveled distance in every day of training in STZ, T5, T10, and T15 group

	STZ	T 5	T 10
Escape latency (sec)			
Day 1	71.15±4.95	45.53±6.68	55.55±7.71
Day 2	60.87±6.11	29.17±4.14	28.05±7.06
Day 3	42.45±7.14	31.85±4.66	20.60±3.70
Day 4	28.39±5.42	31.10±5.92	19.39±4.22
Traveled distance (cm)			
Day 1	1780.08±160	1041.58±154	1304.56±168
Day 2	1640.91±174	845.13±135	742.94±184
Day 3	1171.92±183	796.22±129	590.74±93.40
Day 4	941.37±165	734.62±141	579.39±127

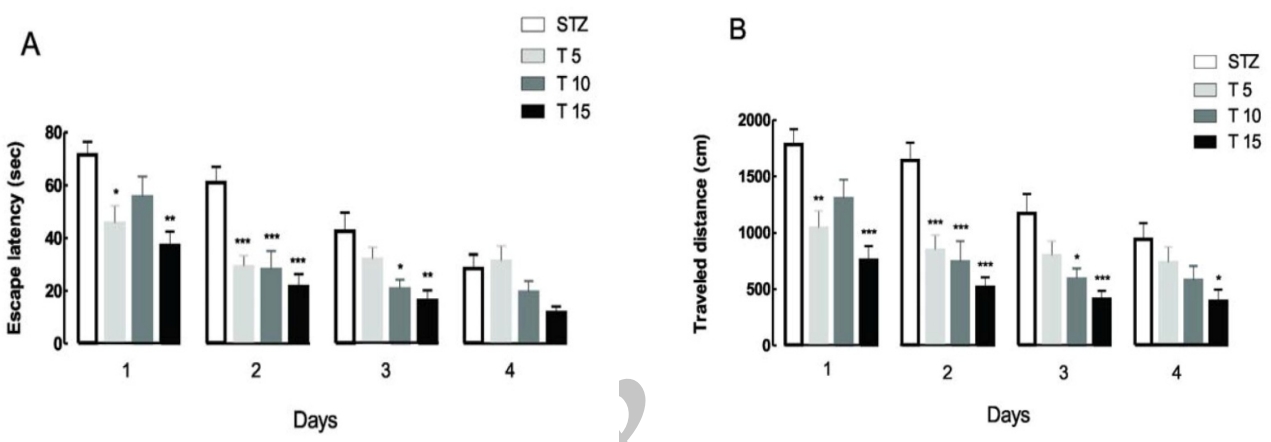
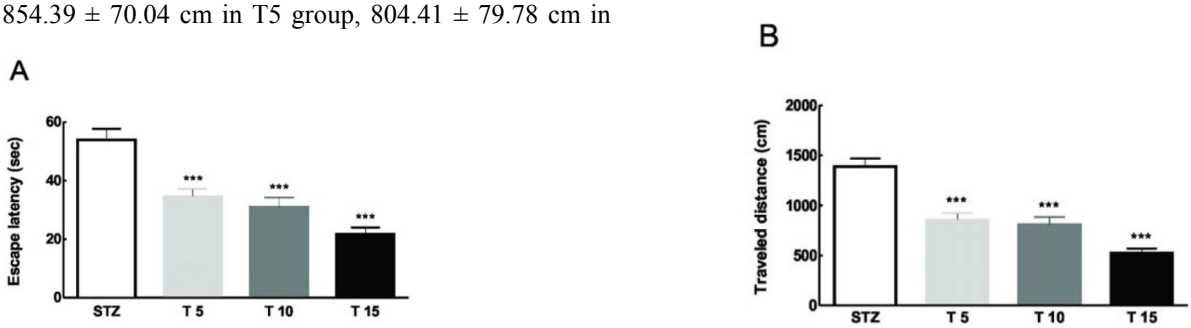


Figure 3. The effect of triazine on spatial learning in the same training days. A) The bar graphs show the quantitative results (mean ± S.E.M) of escape latency in the same training days. Escape latency in the first day of training decreased significantly in T5 and T15 groups compared to STZ group. Escape latency in the second day of training decreased in all of the treated groups compared to STZ group. In the third day of training, escape latency decreased in T10 and T15 groups compared to STZ group. There were no significant differences between all treated groups and STZ group in the fourth day of training. B) The bar graphs show the quantitative results (mean ± S.E.M) of traveled distance in the same training days. Traveled distance in the first day of training decreased in T5 and T15 groups compared to STZ group. Traveled distance in the second day of training decreased in all of the treated groups compared to STZ group. In the third day of training, traveled distance decreased in T10 and T15 groups compared to STZ group. In the fourth day of training, traveled distance decreased in T15 group compared to STZ group *, ** and *** indicate $P < 0.05$, $p < 0.01$, and $P < 0.001$.

The mean escape latency time was 53.93 ± 3.39 s in STZ group, 34.41 ± 2.76 s in T5 group, 30.89 ± 3.34 s in T10 group, and 21.67±2.27 s in T15 group. The mean traveled distance was 1383.57 ± 89.83 cm in STZ group, 854.39 ± 70.04 cm in T5 group, 804.41 ± 79.78 cm in T10 group and 520.37 ± 55.41 cm in T15 group. The mean swimming speed was not different among all tested groups, indicated there was no motor activity disturbances in tested animals ($P = 0.96$; Figure 4C).



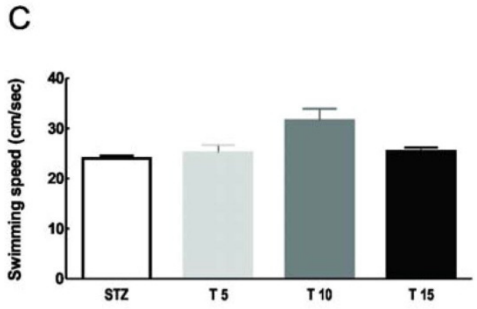


Figure 4. The effect of triazine on the average of escape latency, traveled distance and swimming speed in all trials during four training days. A) The bar graph shows the quantitative results (mean ± S.E.M) of escape latency. The mean escape latency reduced significantly in T5, T10 and T15 groups compared to STZ group. B) The bar graph shows the quantitative results (mean ± S.E.M) of traveled distance. The mean traveled distance reduced significantly in T5, T10 and T15 groups compared to STZ group. *, ** and *** indicate $P<0.05$, $P < 0.01$, and $P<0.001$. Swimming speed was not significantly different among any groups

Histological results

To analyze the histological results, we measured the hippocampal CA1 pyramidal layer thickness in all tested groups. The CA1 pyramidal layer thickness in the STZ group was significantly less than the control group ($P < 0.001$). The mean CA1 pyramidal layer thicknesses were 654.81 ± 27.01 µm in the control group and 396.32 ± 37.63 µm in STZ group ($P < 0.001$). The mean CA1 pyramidal layer thickness in the T15 group was 629.44 ± 57.80 µm which shows a significant difference to STZ group ($P < 0.01$, Figures 5A and 5B).

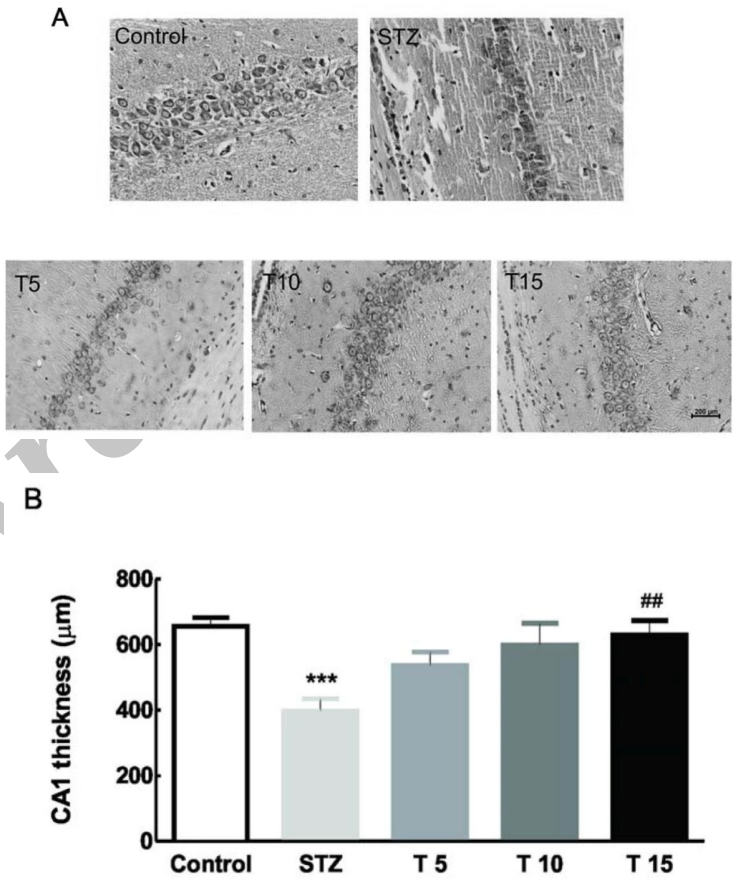


Figure 5. Analysis of the CA1 pyramidal layer thickness. A) Photomicrographs of the CA1 pyramidal layer thickness. B) The bar graph shows the quantitative results (mean ± S.E.M) of the CA1 pyramidal layer thickness. The CA1 pyramidal layer thickness in STZ group was significantly less than the control group ($^{***}P < 0.001$). The CA1 pyramidal layer thickness in the T15 group was higher compared to STZ group ($^{**}P < 0.01$)

Discussion

The results of this study indicate that C16H12Cl2N3S reduced the escape latency and traveled distance parameters in Morris water maze in the animal model of AD. These results indicate that C16H12Cl2N3S can improve spatial learning in this experimental model. Moreover, it prevents the thickness reduction of CA1 pyramidal cell layer. This finding along with the previous in vitro studies revealed the neuroprotective effect of triazine derivatives in the experimental model (18-22).

STZ administration in rats induces oxidative stress in the brain, Aβ plaques aggregation, Tau protein hyperphosphorylation, neuroinflammation, and apoptosis. All these pathological changes impair memory and learning in the AD animal models (26,31,32). Confirming these findings, current data shows that STZ injection causes defect of spatial memory.

Oxidative stress is the most important hypothesis involved in the pathophysiology of AD (33). Excess free radicals of oxygen lead to cellular damage, a progressive cognition and memory loss (34-36). The brain is highly sensitive to the harmful effect of oxidative stress, moreover the antioxidants play a crucial role to remove these toxic agents (36). C16H12Cl2N3S working as free radical scavenger inhibits free radical products (18).

Aβ plaque is the proteolytic product of the amyloid precursor protein (34). Aβ can generate the ROS especially hydrogen peroxide, which may cause cell death in the neuronal cultures and toxicity in hippocampal neurons (37,38). The reciprocal effects of oxidative stress and Aβ aggregation intensify this effect (22,39-41).

It has been shown that ROS modulators, such as vitamin E, melatonin, and estrogens, reduce cellular injury and neurotoxicity during Aβ exposure (42). Antioxidants and neuroprotective agents decrease the risk of memory deficit and AD progression (43,44). Current findings indicate that C16H12Cl2N3S improve spatial learning and prevents the thickness reduction of CA1 pyramidal cell layer in AD model. This effect might be due to antioxidative characteristic of this agent. C16H12Cl2N3S as a potent antioxidant might inhibit free radical products (37) and disperse Aβ plaques (20,33). C16H12Cl2N3S can be a suitable therapeutic candidate for neural disorders such as AD, therefore, should be considered in the experimental and clinical trials.

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