

Original Article

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The effect of azelaic acid on AlCl₃-induced neurocognitive impairments and molecular changes in the hippocampus of rats

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Abstract

Objectives: Cognitive function plays a pivotal role in assessing an individual's quality of life. This research aimed to investigate how azelaic acid (AzA), a natural dicarboxylic acid with antioxidant and anti-inflammatory properties, affects aluminium chloride (AlCl₃)-induced behavioural changes and biochemical alterations in the hippocampus of rats. **Methods:** Thirty-two male Wistar rats divided into four groups received distilled water, AzA 50 mg/kg, AlCl₃ 100 mg/kg and AzA plus AlCl₃, respectively, by oral gavage for 6 weeks. Behavioural changes were evaluated using open-field maze, elevated plus maze, novel object recognition (NOR), passive avoidance task, and Morris water maze (MWM) tests. Also, malondialdehyde (MDA), carbonyl protein, tumour necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), nuclear factor-kappa B (NF- κ B), C/EBP homologous protein (CHOP), glycogen synthase kinase-3 beta (GSK-3 β), brain-derived neurotrophic factor (BDNF) and acetylcholinesterase (AChE) activity were examined. **Results:** AzA significantly affected AlCl₃-provoked anxiety-like behaviours and learning and memory impairments. It also reduced the toxic effect of AlCl₃ on MDA, carbonyl protein, TNF- α , IL-1 β , NF- κ B and GSK-3 β status; however, its beneficial effects on AlCl₃-induced changes of CHOP, BDNF and AChE activity were not significant. **Conclusion:** These findings disclosed that AzA could improve behavioural and cognitive function and almost limit the oxidative stress and neuroinflammation caused by AlCl₃.

Significant outcomes

- Sub-chronic administration of azelaic acid mitigated anxiety-like behaviours and ameliorated learning and memory deficits induced by sub-chronic exposure to aluminium chloride in Wistar rats.
- Sub-chronic administration of azelaic acid ameliorated inflammation, oxidative stress and glycogen synthase kinase-3 beta in the hippocampus of Wistar rats after sub-chronic exposure to aluminium chloride.

Limitations

Despite extensive efforts to maintain cleanliness, silence and control of ventilation and light in the laboratory for experimental animals in accordance with standards, it was possible for these conditions to not be fully implemented throughout the entire procedure of the study which could potentially affect the behavioural changes of the animals.

Introduction

Cognitive performance is processed by the interaction between neural networks. As the most complicated brain function, it consists of executive function, social cognition, attention, learning, memory, language and verbal fluency, and perceptual-motor function (Harvey, 2019, Butterfield and Boyd-Kimball, 2019).

Dysfunction of cognitive performance is not described only as memory impairments but a decline in a range of cognitive abilities or general cognition. As outlined in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), neurocognitive disorders encompass delirium, mild neurocognitive disorder, and major neurocognitive disorder. Major neurocognitive disorder (previously categorised as dementia) is a severe condition in which the individual loses the ability to perform everyday activities independently. Mild cognitive impairment includes a noticeable change in cognition that does not interfere with the capability

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of independence in everyday activities, but it may progress to dementia or may not (Livingston *et al.*, 2020).

Cognitive deficits arise from various factors, including ageing and psychiatric disorders like schizophrenia, bipolar disorder, and major depressive disorder. Additionally, neurodegenerative diseases such as Alzheimer's and Parkinson's can result in cognitive decline due to alterations in neuroanatomical structures like the hippocampus and prefrontal cortex (McDonald, 2017).

Oxidative stress and neuroinflammation are components of neurodegeneration and cognitive decline. Even though, they have two different pathologies, oxidative stress and neuroinflammation are related and can stimulate one another. Oxidative stress refers to the imbalance of oxidation-reduction and failure of antioxidant system, leading to the aggregation of reactive species, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS). Although the reactive species are necessary for physiological functioning, their overproduction can cause macromolecular damage, resulting in disruption of signalling mechanisms and cell death. The inflammatory response of the central nervous system to neurological events is termed as neuroinflammation, which is a protective response at the beginning but can lead to the degeneration of neurones. Actually, neuroinflammatory cells produce reactive species, causing oxidative stress and, in turn, some reactive species induce the expression of pro-inflammatory genes (Teleanu *et al.*, 2022).

Heavy metals are remarkable neurodegeneration-provocative agents. Aluminium (Al) is one of the most distributed metals in the Earth's crust, which can be significantly neurotoxic. Although it has no physiological role in the body, the human body is widely exposed to Al compounds due to its extensive use in industry, medical and pharmaceutical products, antiperspirants, food additives, agriculture, and water treatment (Igbokwe *et al.*, 2019). Al can develop changes in blood-brain barrier (BBB), resulting in its accumulation in the brain (Wang, 2018). The neurodegenerative effect of Al is mainly utilised by stimulation of iron compounds to produce ROS, which leads to oxidative stress and inflammatory responses of the brain, leading to neuroinflammation. Also, Al can cause a disturbance in cholinergic function and neurotransmission, which is connected to cognition and behavioural responses (Colomina and Peris-Sampedro, 2017).

Azelaic acid (AzA) is a saturated dicarboxylic acid with chemical formula of $\text{HOOC}(\text{CH}_2)_7\text{COOH}$ found naturally in wheat, rye and barley (PubChem, 2004). This organic compound is used as a topical agent for treating mild to moderate acne. It has several biological activities, including anti-inflammatory and anti-oxidative effects (Sauer *et al.*, 2023) and showed remarkable neuroprotective effects particularly against Parkinson's disease (Sharma *et al.*, 2021, Gan *et al.*, 2011). A study on mice models of psoriasis showed that AzA could inhibit phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signalling pathway which is involved in cell death processes (Li *et al.*, 2022). Despite previous findings, AzA has never been studied for its potential protection against cognitive dysfunction. Thus, the current study evaluated the effect of AzA on aluminium chloride (AlCl_3)-induced behavioural changes and cognitive disorder in association with biochemical and molecular alterations in the hippocampus of rats.

Materials and methods

Chemicals

ELISA kits for measuring activity of GSK-3 β and BDNF were obtained from Millipore Corporation and Hangzhou

Eastbiopharm Co., respectively. Tripure Isolation Reagent and Expand Reverse Transcriptase were purchased from Roche Applied Sciences. The primers were synthesised and delivered by GenFanavar Ltd. For real-time PCR reaction, we used SYBR[®]Premix Ex Taq from Takara Bio Inc. AlCl_3 , AzA, acetylthiocholine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), thiobarbituric acid, dinitrophenylhydrazine and other chemicals were purchased from the Sigma Aldrich Co.

Experimental animals

In this study, a total of 32 male Wistar rats aged 3 months and weighing 200 – 250g were obtained from the Pasteur Institute of Iran. The animals were accommodated under standard laboratory conditions in standard cages ($45.5 \times 26.6 \times 18$ cm) (4 rats per cage) with $23 \pm 1^\circ\text{C}$ temperature and 12h light/dark cycle. All rats had free access to food and water and housed in a same room throughout the study. All the experiments were performed between 8:00 and 14:00 during the light cycle.

Experimental design

All rats were divided into four groups of eight each, and treated with chemicals by oral gavage for six weeks. The first group (control group) received distilled water (1 ml). The second group received 50 mg/kg/day AzA dissolved in distilled water. The third group was administered 100 mg/kg AlCl_3 suspended in distilled water. The fourth group was co-treated with the same dose of AzA and AlCl_3 as used in the second and third groups. The doses of AlCl_3 and AzA used in this study were selected based on previous research demonstrating their respective neurotoxic and neuroprotective effects. The dose of 100 mg/kg of AlCl_3 has been widely used in animal models to induce neurotoxicity and cognitive impairments, replicating conditions similar to those seen in neurodegenerative diseases like Alzheimer's (Zhang *et al.*, 2022). The 50 mg/kg dose of AzA was chosen based on its efficacy in previous studies where it exhibited significant neuroprotective effects without causing adverse reactions (Sharma *et al.*, 2021). The dual administration protocol, with a 2-hour interval between the gavage of AzA and AlCl_3 in the AzA+ AlCl_3 group, was implemented to minimise potential interactions in the gastrointestinal tract, thereby ensuring the bioavailability and effectiveness of both compounds.

Behavioural tests

All behavioural examinations were performed in a noise and light-controlled room between 8 am to 1 p.m. Animals were given 1 h to adapt the testing environment before each test. A blinded observer recorded all the tests. Each testing apparatus was cleaned with 70% ethanol prior to the placement of each rat. Behavioural tests were conducted according to the following schedule:

- Day 1: Open field test (also served as the habituation session for the novel object recognition test).
- Day 2: Novel object learning session.
- Day 3: Novel object testing session and elevated plus maze (EPM) test.
- Day 4: Morris Water Maze (MWM) test for the first half of the rats.
- Day 5: MWM test for the second half of the rats, and shuttle box habituation and learning session for the first half of the rats.

- Day 6: Shuttle box testing session followed by anaesthesia and hippocampus extraction on the first half of the rats, shuttle box habituation and learning session for the second half of the rats.
- Day 7: Shuttle box testing session followed by anaesthesia and hippocampus extraction on the second half of the rats.

Open-field maze

Open-field maze (OFM) is widely used for testing animals' anxiety-like behaviours. The test was carried out on a wooden box with dimensions $60 \times 60 \times 50$ cm, black walls and a white floor divided by black lines to 16 squares. At the start of the test, each animal was being placed in the central zone of the chamber for a period of five min, and the level of anxiety were evaluated by variables including total time of activity, time spent in inner zone, number of entries to the inner zone and the frequency of rearing and grooming (Sadegzadeh *et al.*, 2020).

Elevated plus maze

The EPM is also one of the mostly conducted tests for assessment of anxiety-like behaviours. The apparatus was made up of wood with four '+' shaped arms and a central zone, placed about 50 cm above the floor. Two of the opposite arranged arms were enclosed with walls ($50 \times 10 \times 40$ cm) and an open top. The other pair of arms were open (50×10 cm) and equipped with 0.5×0.5 cm ledge to avoid rats from falling down. The test begins by placing an animal in the central zone, facing one of the open arms, and lasts for up to 5 minutes. Recorded factors include the time spent in the open arms (T_1), time spent in the closed arms (T_2), the frequency of entries into the open arms (F_1), and the frequency of entries into the closed arms (F_2). Anxiety levels are assessed by calculating the percentage of time spent in the open arms [$T_1/(T_1+T_2) \times 100$] and the percentage of entries into the open arms [$F_1/(F_1+F_2) \times 100$]. Additionally, the total number of entries into the arms serves as an indicator of locomotor activity (Sadegzadeh *et al.*, 2020).

Novel object recognition test

The novel object recognition (NOR) test is one of the most comparable recognition tests to those used in humans (Brodziak *et al.*, 2014). The test is completely based on rodents' natural curiosity for novel objects and the ability to recognise them without any primary reinforcement. The protocol of the test consists of three sessions: habituation, training and testing. During the habituation, each animal was placed in the open-field chamber to explore the environment without any objects in it for five min. After a 30-min interval, the training session was performed by placing the animal in the open-field box containing two identical objects for 10 min. After 24 hour, each rat was returned to the chamber and exposed to a familiar object and a novel one. The novel object was completely distinguishable for the animals, even though all of them were made up of the same material and were heavy enough for animals to displace. Each animal was allowed to explore the chamber for five min. The total time spent on investigating the novel object (T_1) and the total time of interaction with either object (T_i) in testing phase were calculated to determine the discrimination ratio ($100 \times T_1/T_i$) (Sadegzadeh *et al.*, 2020).

Morris water maze

The spatial and short-term learning and memory of the animals were assayed by the Morris water maze (MWM) test. The test occurred in a black pool with 160 cm diameter, 80 cm height and 40 cm depth. The pool was equally separated into four quadrants

(no.1– no.4). A rounded platform with 10 cm diameter was located in the second quadrant 1.5 cm below the water surface. The platform was black in order to be indistinguishable from the environment. Visual cues were fixed on the walls around the pool. A video camera connected to a computerised tracking system (Noldus Ethovision® system, version 5, USA) was set above the centre of the pool to record each session.

The MWM task was accomplished in two sessions of learning and testing. In the spatial learning phase, each animal was released into the pool from the 1st, 3rd and 4th quadrants and allowed to detect the platform during a 60s- interval in four training trials. Rats were given 60 second-rest between the trials. Each trial was repeated in three blocks with a 30-min interval between them. After detection of the platform, rats were permitted to rest on the platform for 30 s in order to analyse the visual signals before being put back in its cage. Each rat was dried with a towel right after returning to the cage and was allowed to rest for 30 s before the next trial. However, if a rat could not discover the platform within 60 s, the experimenter would put it on the platform. In this phase, the tracking system recorded the two parameters of latency and the distance travelled to find the platform. At the end, the average of the parameters in each trial for every block was reported. The testing phase was performed two hours after the end of the learning phase. To evaluate the spatial memory, the platform was removed from the pool and each rat was released into the water with the same order as in the previous phase. The time spent and the distance moved in the target quadrant and the number of crossings were used to measure spatial memory retention (Saadati *et al.*, 2015).

Passive avoidance task

Passive avoidance is a fear-motivated learning and memory test in which the animal learns NOT to enter an environment to avoid the punishment (electric foot shock) that was previously experienced, despite its natural photophobia. The test starts with two min of habituation in the light compartment (LC) of the light/dark chamber. After two min, each rat was put back in the LC for the training phase. In this phase the animals spent 20 s in the LC before opening the guillotine door between the two compartments. As soon as the rat entered into the dark compartment (DC) the door was closed and a 1.5 mA of foot shock was applied for 2 s. The testing trial was carried out 24 h after training. Each rat was placed in the LC of the apparatus in the same manner as the training session. The door was opened after a 20 s period and the animal was allowed to explore the apparatus for 300 s. During the testing trial, step through latency (STL), frequency of entries into the DC and total time spent in DC were measured as the index of learning and memory (Golitabari *et al.*, 2022).

Biochemical analyses

Following behavioural testing, rats were euthanized by decapitation under light anaesthesia, and their brains were rapidly removed and the hippocampus was dissected and homogenised for subsequent biochemical analyses.

Quantification of oxidative stress markers in the hippocampus

To detect the dimensions of oxidative stress, we measured the markers of lipid peroxidation and oxidative damage to proteins. Thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically as the indicator of lipid peroxidation products such as malondialdehyde (MDA). Results were expressed

as mg/g tissue as $\epsilon = 153 \text{ M}^{-1}\text{cm}^{-1}$. The oxidative damage to proteins was evaluated by quantification of carbonyl content based on the reaction with dinitrophenyl hydrazine. The carbonyl content (nmol/mg protein) was assessed using a molar extinction coefficient of $22,000 \text{ M}^{-1}\text{cm}^{-1}$ at 370 nm after subtraction of the blank absorbance (Mostafalou et al., 2015).

Investigation of gene expression changes by real-time PCR in the hippocampus

To assess changes in gene expression of nuclear factor-kappa B (NF- κ B), interleukin-1 beta (IL-1 β), tumour necrosis factor-alpha (TNF- α) and C/EBP homolouous protein (CHOP), real-time PCR was conducted following mRNA extraction and cDNA synthesis (Mousavi-Nasab et al., 2024). RNA was extracted from sample tissue solution using the BioFACT kit, and its concentration was determined using a Nano-Drop UV-vis Spectrophotometer (Thermo Fisher Scientific, CA). Subsequently, the RNA was reverse transcribed into cDNA using the BioFACT cDNA synthesis kit, with a concentration of $1 \mu\text{g}/\mu\text{l}$ RNA. Specific primers were designed and obtained from GenFanavaran Ltd. The real-time PCR reaction was performed using the SYBR green master mix. Cycle number (Ct) of each reaction was achieved from Light Cycler 96 (Roche Applied Sciences, USA). The values were normalised to GAPDH mRNA and the relative gene expression level was represented as $2^{-\Delta\Delta C_t}$. The following primers were used in this study: CHOP forward: CGGAGTGTACCCAGCACCATCA, CHOP reverse: CCCTCTCCTTTGGTCTACCCTCA; NF- κ B forward: TTCAACA TGGCAGACGACGA, NF- κ B reverse: AGGTATGGGCCA TCTGTTGA; IL-1 β forward: AGCCAGAGTCATTTCAG AGCAA, IL-1 β reverse: GTCCTTAGCCACTCCTTCTG; TNF- α forward: ACACACGAGACGCTGAAGTA, TNF- α reverse: TCCACTCAGGCATCGACATT; GAPDH forward: GTATGAC TCTACCCACGGCA, GAPDH reverse: AAGACGCCAGT AGACTCCAC.

Measurement of acetylcholinesterase activity in the hippocampus

A colorimetric kinetic method was used to determine acetylcholinesterase (AChE) activity in the hippocampus. A 96-well microtiter plate was filled with the samples, acetylthiocholine (ACh) as the substrate, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as the colorimetric reagent. The thiocholine, produced from the hydrolysis of the substrate by AChE, reacted with DTNB to yield a yellow-colored anion, 5-thio-2-nitrobenzoate (TNB). The rate of TNB formation was kinetically measured using an automated microtiter plate reader at 412 nm (Alivand et al., 2024, Mahdavi et al., 2024).

Measurement of glycogen synthase kinase-3beta (GSK-3 β) activity in the hippocampus

The activity of this serine/threonine kinase was assessed by using the STAR (Signal Transduction Assay Reaction) phospho-GSK-3 (Ser9) ELISA Kit, Catalog number 17-472, Millipore Corporation.

Measurement of brain-derived neurotrophic factor (BDNF) level in the hippocampus

The hippocampus samples were homogenated in lysis buffer and centrifuged at $12000g$ (at -4°C for 20 min). We used Rat BDNF ELISA Kit (Hangzhou Eastbiopharm Co., LTP) and followed the manufacturer instructions for assessing the brain derived neurotrophic factor (BDNF) protein level in the extracted supernatant.

Statistical analysis

The results were presented as mean \pm SEM. The normality of the data was assessed using the Shapiro-Wilk test. Statistical differences between groups were analysed using one-way ANOVA followed by LSD post-hoc test for behavioural tests and Tukey's post-hoc for biochemical results. The F -test results for each ANOVA analysis were reported. The p value < 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS version 25.0 (IBM Corp., USA).

Results

Open-field maze

As shown in the Fig. 1A, there was a significant decrease in the number of rearing in the AlCl_3 group compared to the control group [$F(3, 28) = 4.56, p < 0.05$]. AzA significantly increased the number of rearing in the AzA+ AlCl_3 in comparison to the AlCl_3 group ($p < 0.05$); however, when AzA group was compared to the control, the increment was not noticeable.

Also, the number of grooming was considerably increased in the AlCl_3 group compared to the control group [$F(3, 28) = 7.66, p < 0.01$]. The number of grooming of the AzA+ AlCl_3 group was not significantly different than either the control group or the AlCl_3 group. However, it was noticeably more than AzA group ($p < 0.05$). Though, AzA decreased the number of grooming in comparison to control group the change was not significant (Fig. 1B).

Analysis in the Fig. 1C shows a significant decrease in the number of entries to the inner zone when comparing the AlCl_3 group to the control group [$F(3, 28) = 5.63, p < 0.01$]. Conversely, in the AzA+ AlCl_3 group, there was a notable reduction in the number of entries to the central area compared to the AlCl_3 group ($p < 0.05$).

The Fig. 1D also shows that the AlCl_3 caused a significant decrease in the time spent in the centre zone compared to the control group [$F(3, 28) = 7.20, p < 0.01$]. However, in the AzA+ AlCl_3 group, this time was significantly longer than that of the AlCl_3 group ($p < 0.05$), although noticeably reduced compared to the AzA group ($p < 0.05$).

AlCl_3 also led to a remarkable decrease in the distance travelled comparing with control [$F(3, 28) = 7.34, p < 0.01$]. However, co-treatment with AzA was not able to increase this parameter in comparison with AlCl_3 group. The AzA+ AlCl_3 group also, showed a significant decrease in comparison to the AzA group ($p < 0.05$) (Fig. 1E).

Elevated plus maze

The Fig. 2A shows that the AlCl_3 caused a considerable decrease in time spent in the open arm in comparison with the control group [$F(3, 28) = 11.91, p < 0.001$] while this time was significantly longer in the AzA+ AlCl_3 group compared to the AlCl_3 group ($p < 0.05$) and the AzA group ($p < 0.05$). However, the AzA group indicated no remarkable change in comparison to the control group.

The number of entries to the open arm was considerably less in the AlCl_3 group compared to the control group [$F(3, 28) = 15.20, p < 0.001$] whereas the AzA+ AlCl_3 group had significantly more open arm entries compared to the AlCl_3 group following AzA intake ($p < 0.01$) (Fig. 2B).

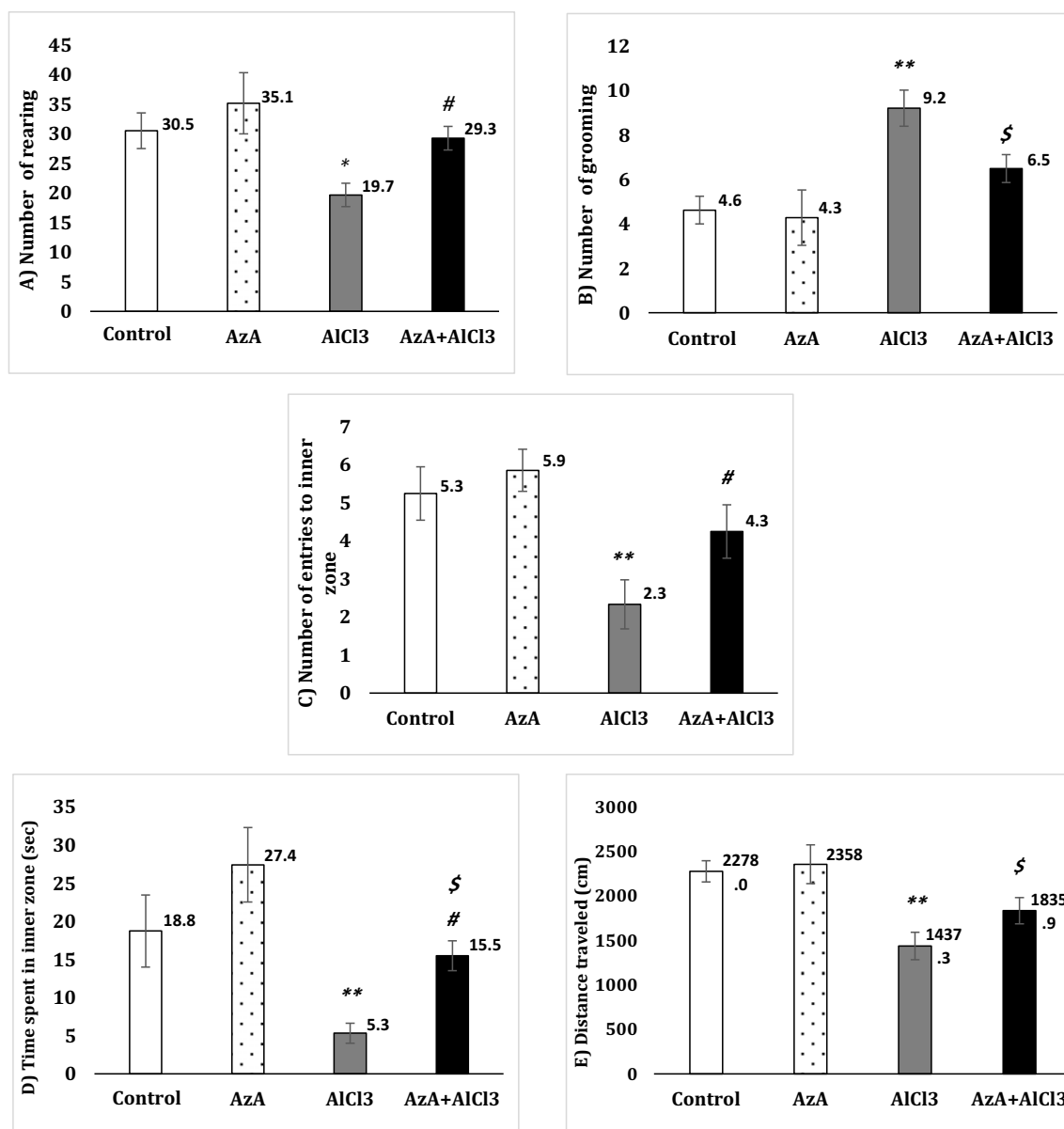


Figure 1. Effect of azelaic acid on the AlCl₃-provoked anxiety-like behaviours in the open-field test. The results were represented as mean±SEM (one-way ANOVA). (*) $p < 0.05$ and (**) $p < 0.01$ compared with the control group, (#) $p < 0.05$ compared with the AlCl₃ group and (\$) $p < 0.05$ compared with the AzA group. AzA: azelaic acid.

Locomotor activity was notably decreased in the both AlCl₃ [$F(3, 28) = 5.33, p < 0.01$] and AzA+AlCl₃ in comparison to the control group, and there was no significant difference between the AlCl₃ group and other three groups (Fig. 2C).

Novel object recognition test

The results revealed that AlCl₃ significantly decreased NOR in comparison to the control group [$F(3, 28) = 21.74, p < 0.001$] while AzA modified this cognitive disorder in the AzA+AlCl₃ group and considerably improved the recognition in comparison to the AlCl₃ group ($p < 0.01$). However co-treatment could not notably increase discrimination ratio compared to the control and AzA groups whose ratios were still significantly higher ($p < 0.01$) (Fig. 3).

Morris water maze

In the learning session of the test, no noticeable difference was detected in the path length between the groups in both Block 1 and Block 2; however, in Block3, the AlCl₃ group took a considerably longer path to reach the platform than the control group [$F(3, 28) = 12.33, p < 0.001$] but the AzA+AlCl₃ group showed a significantly better performance compared to the AlCl₃ group ($p < 0.001$) (Fig. 4A).

The time spent for finding the platform was analysed in each block. The data of block 1 detected no significant change between the groups. On the other hand, according to the data of block 2, the AlCl₃ group showed a noticeably longer period of time than the control group [$F(3, 28) = 3.17, p < 0.05$]. The other groups represented no significantly different performance compared to the control group. But, the AzA+AlCl₃ group showed significantly

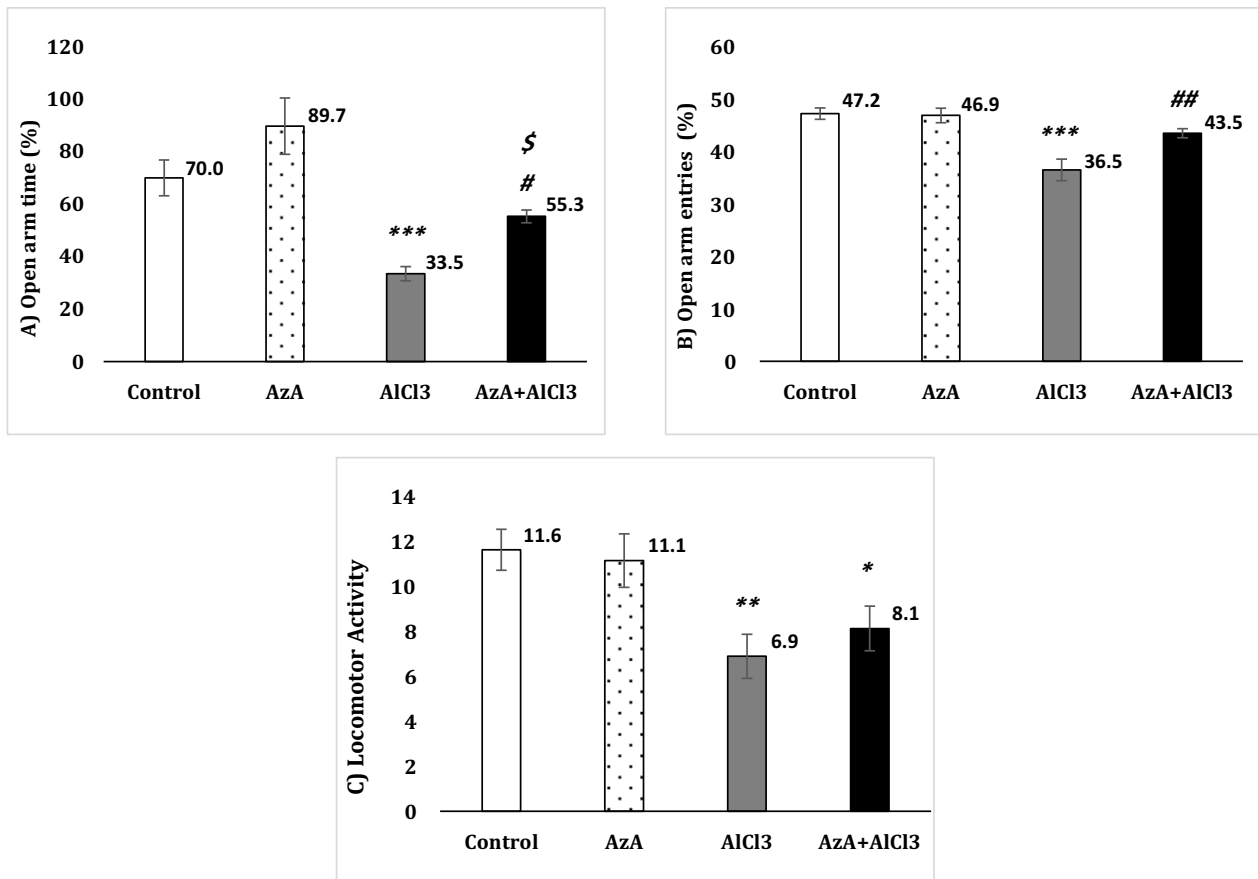


Figure 2. Effect of azelaic acid on the AlCl₃-provoked anxiety-like behaviours in the elevated plus maze test. The results were represented as mean±SEM (one-way ANOVA). (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ compared with the control group, (#) $p < 0.05$ and (##) $p < 0.01$ compared with the AlCl₃ group, (\$) $p < 0.05$ compared with the AzA group. AzA: azelaic acid.

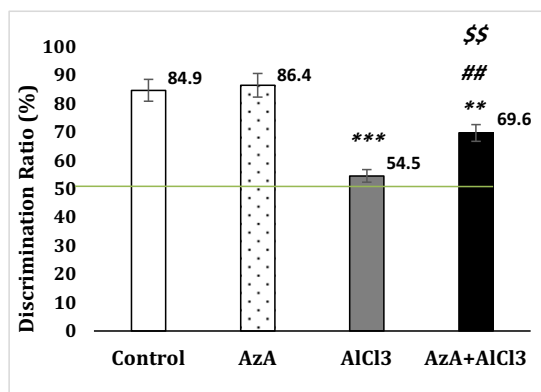


Figure 3. Effect of azelaic acid on the AlCl₃-provoked learning and memory changes in the novel object recognition test. The results were represented as mean±SEM (one-way ANOVA). (**) $p < 0.01$ and (***) $p < 0.001$ compared with the control group, (##) $p < 0.01$ compared with the AlCl₃ group and (\$\$) $p < 0.01$ compared with the AzA group. AzA: azelaic acid.

longer escape latency than the AzA group ($p < 0.05$). Compared to the control, the AlCl₃ group also had a significantly increased time spent to find the platform in block 3 [$F(3, 28) = 5.83$, $p < 0.01$] while the AzA+AlCl₃ group considerably spent less time than the AlCl₃ group ($p < 0.01$) (Fig. 4B).

Three parameters were considered in the testing session: the time spent in target zone, number of crossing and the distance in

target zone. The AlCl₃ group crossed the target zone remarkably in less times than the control group [$F(3, 28) = 9.13$, $p < 0.001$]. The increase in the number of crossing of the AzA+AlCl₃ group was not significant compared to the AlCl₃ group and the crossing number of the AzA+AlCl₃ group still was considerably less than the control group ($p < 0.05$) (Fig. 4C).

The time spent in target zone showed better results. The AlCl₃ group spent significantly less time in the target zone compared to the control group [$F(3, 28) = 17.05$, $p < 0.001$], while there was a significant change between the AlCl₃ and the AzA+AlCl₃ groups ($p < 0.001$) (Fig. 4D).

The distance in target zone was considerably reduced in the AlCl₃ group compared to the control group [$F(3, 28) = 26.50$, $p < 0.001$]. However, an absolute significant change were observed when comparing the AzA+AlCl₃ and AlCl₃ groups ($p < 0.001$). Yet, the AzA+AlCl₃ group had moved less distance in the target zone than the control group ($p < 0.05$) (Fig. 4E).

Passive avoidance task

Analyses of STL indicated a remarkable decline of this parameter in the AlCl₃ group compared to the control group [$F(3, 28) = 17.65$, $p < 0.001$]. Also, AzA noticeably increased STL in the AzA+AlCl₃ group ($p < 0.001$). However this increase was not enough to raise STL to the level of the control group ($p < 0.05$) (Fig. 5A).

The number of entries to the DC did not show a significant change between AzA and control groups, while AlCl₃ group

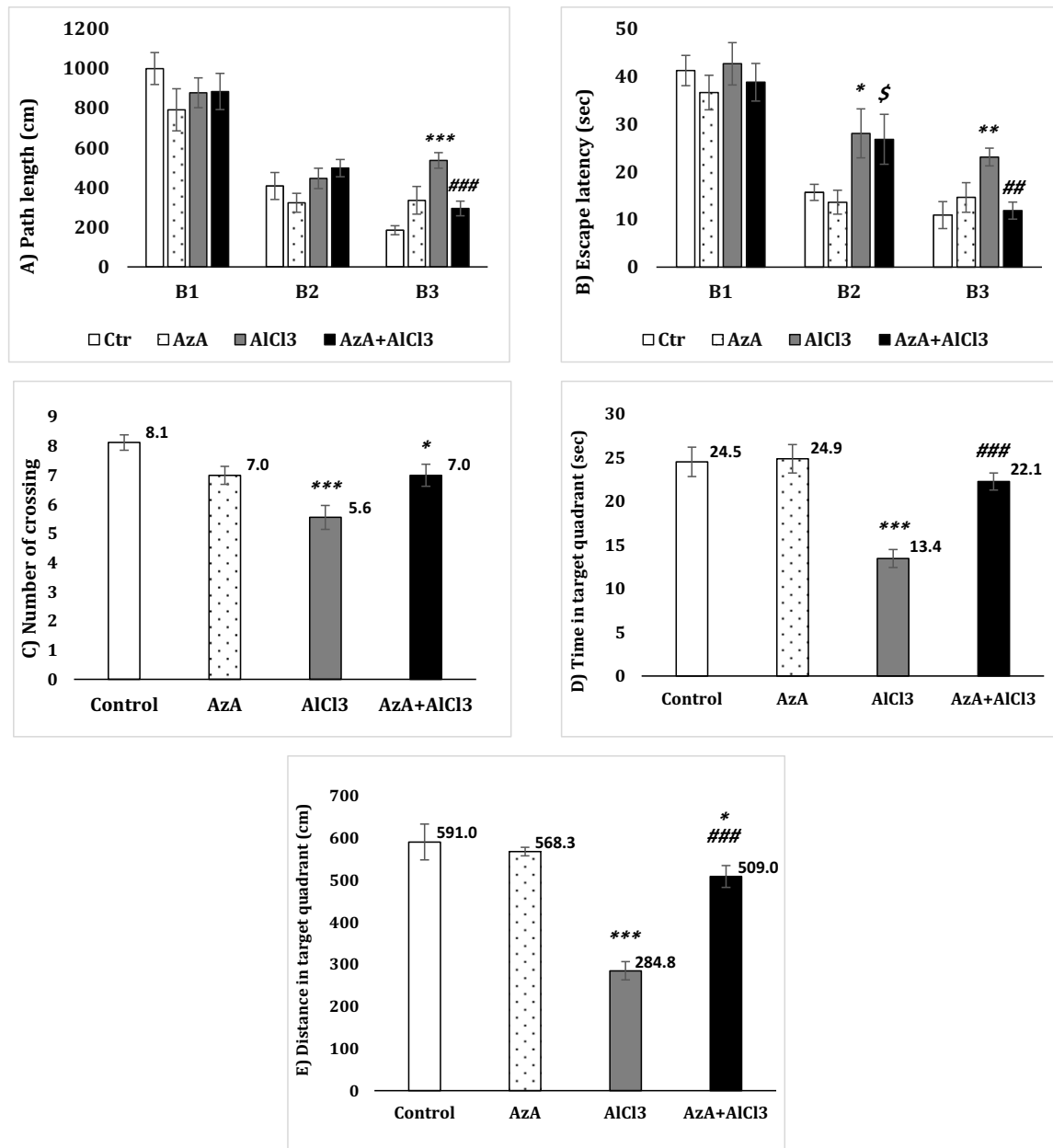


Figure 4. Effect of azelaic acid on the AlCl₃-provoked memory impairment in the morris water maze test. The results were represented as mean±SEM (one-way ANOVA). (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ compared with the control group, (##) $p < 0.01$ and (###) $p < 0.001$ compared with the AlCl₃ group, (\$) $p < 0.05$ compared with the AzA group. AzA: azelaic acid, B1: block1, B2: block2, B3: block3.

entered the DC remarkably more often than the control group [$F(3, 28) = 11.03$, $p < 0.001$]. AzA+AlCl₃ group had significantly less number of entries to this area than the AlCl₃ group ($p < 0.01$), however (Fig. 5B).

Also, AlCl₃ group spent considerably more time in the DC than the control and AzA groups [$F(3, 28) = 15.56$, $p < 0.001$]. The group AzA+AlCl₃ also spent significantly less time in this area than the AlCl₃ group ($p < 0.001$) (Fig. 5C).

Oxidative stress markers in the hippocampus

As shown in the Fig. 6A, significantly higher levels of TBARS was found in the AlCl₃ group than the control group [$F(3, 28) = 8.55$, $p < 0.001$]. The AzA+AlCl₃ group had significantly lower status of

TBARS than the AlCl₃ group ($p < 0.05$). However, there was no significant change between the control, AzA and AzA+AlCl₃ groups.

There was a notable increase in carbonyl content in the AlCl₃ group compared to the control group [$F(3, 28) = 6.80$, $p < 0.01$]. Notably, the AzA+AlCl₃ group exhibited significantly lower carbonyl levels than the AlCl₃ group ($p < 0.05$). However, no considerable change was observed between the AzA and control groups (Fig. 6B).

Gene expression changes in the hippocampus

The evaluation of TNF- α gene expression revealed no remarkable change between the control group and the AzA group, while it was significantly increased in the AlCl₃ group compared to the control

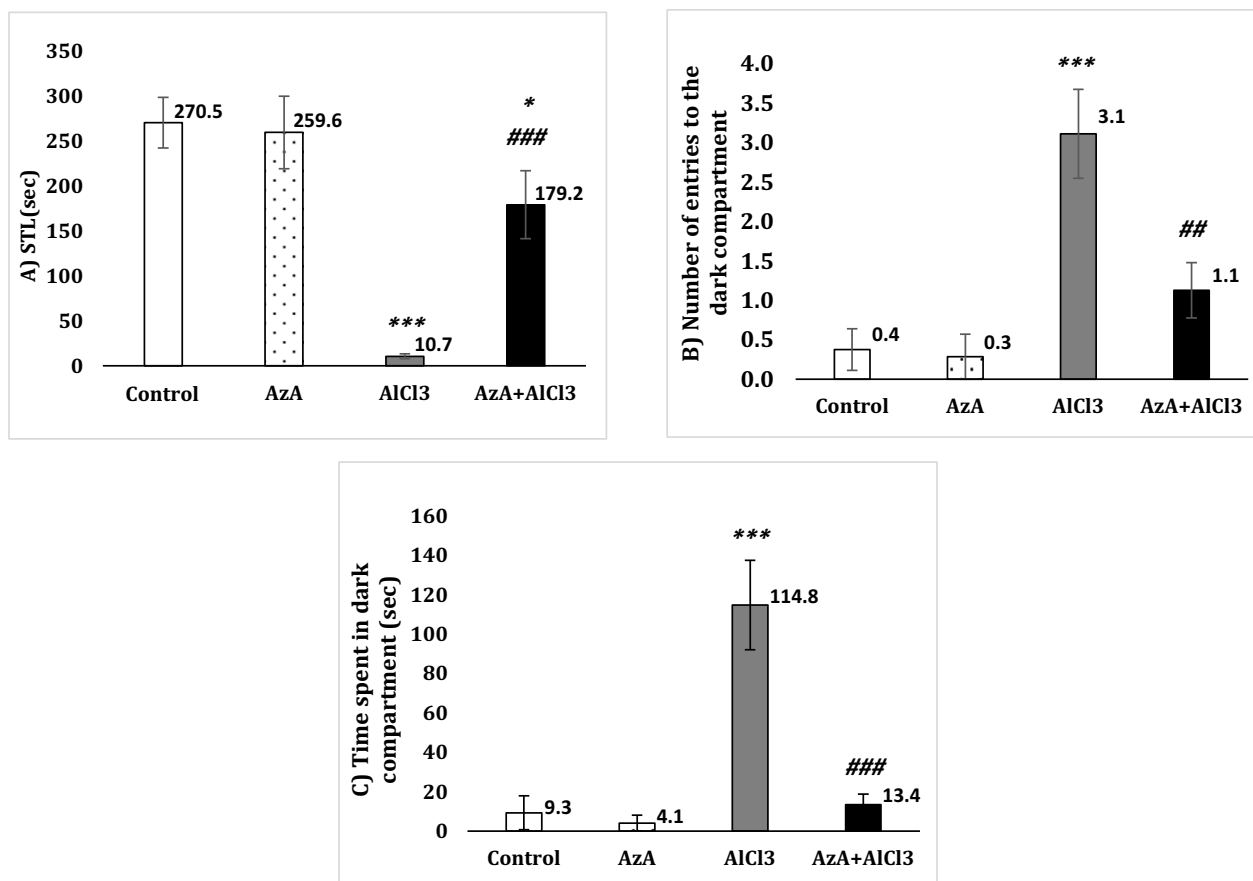


Figure 5. Effect of azelaic acid on the AlCl₃-provoked behavioural changes in the passive avoidance learning and memory test. The results were represented as mean±SEM (one-way ANOVA). (*) $p < 0.05$ and (***) $p < 0.001$ compared with the control group, (##) $p < 0.01$ and (###) $p < 0.001$ compared with the AlCl₃ group. AzA: azelaic acid, STL: step through latency.

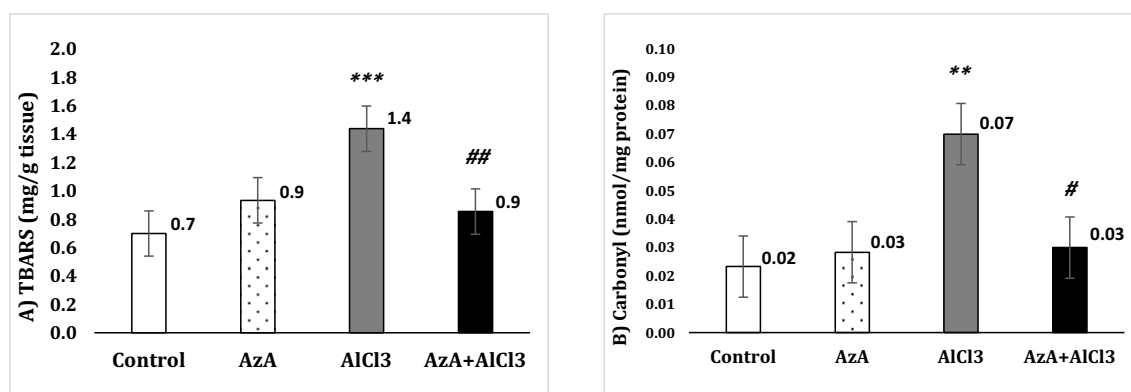


Figure 6. Effect of azelaic acid on AlCl₃-induced oxidative stress in the hippocampus. The results were represented as mean±SEM (one-way ANOVA). (**) $p < 0.01$ and (***) $p < 0.001$ compared with the control group, (#) $p < 0.05$ and (##) $p < 0.01$ compared with the AlCl₃ group. AzA: azelaic acid, TBARS: thiobarbituric acid reactive substances.

group [$F(3, 28) = 23.82$, $p < 0.001$]. Although, the AzA+AlCl₃ group was observed to have noticeably less levels of expression than the AlCl₃ group ($p < 0.01$), it still showed significantly increased levels compared to the control group ($p < 0.05$) and the AzA group ($p < 0.05$) (Fig. 7A).

Though, the expression of IL-1 β was not notably different in control, AzA, and AzA+AlCl₃ groups, it was considerably increased in the AlCl₃ group when compared to the control

[$F(3, 28) = 12.72$, $p < 0.001$], AzA, and AzA+AlCl₃ ($p < 0.05$) groups (Fig. 7B).

When compared to the control group, AlCl₃ intake caused a remarkable promotion of NF- κ B gene expression [$F(3, 28) = 9.73$, $p < 0.001$]; nevertheless, AzA significantly reduced it in AzA+AlCl₃ group ($p < 0.01$). Meanwhile, no significant change was demonstrated between the control group and the groups with AzA resorption (Fig. 7C).

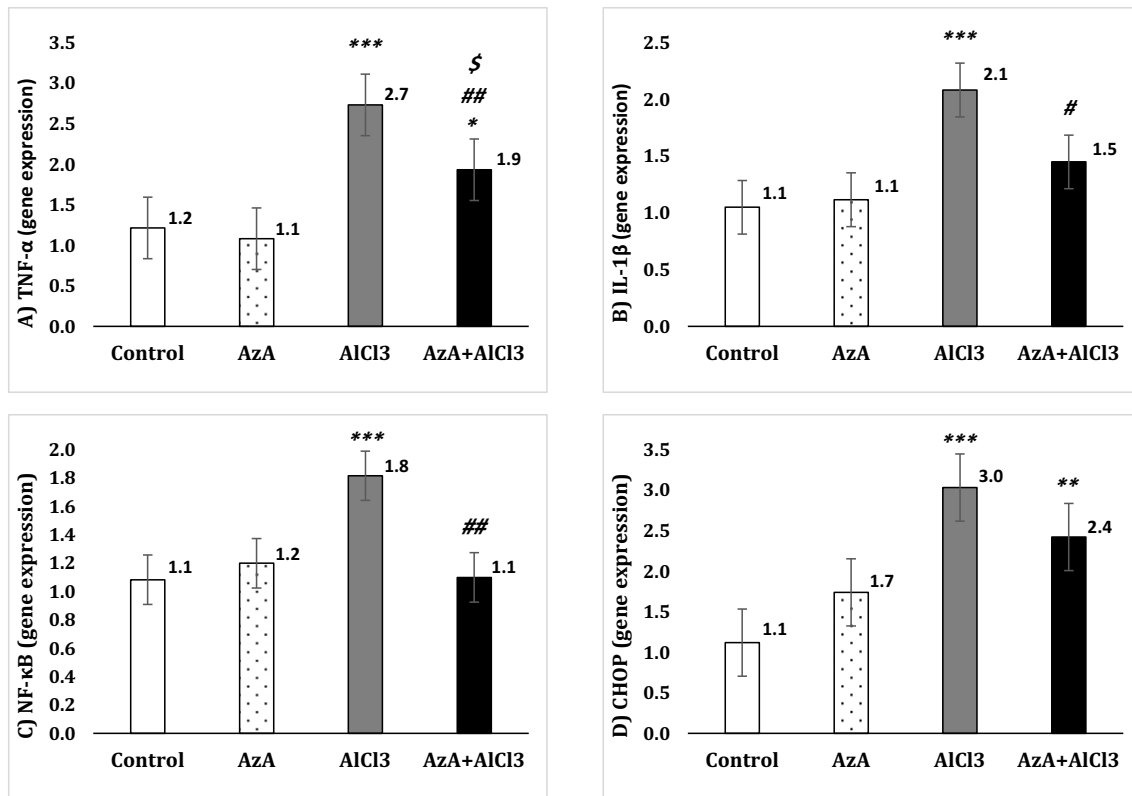


Figure 7. Effect of azelaic acid on the AlCl₃-induced neuroinflammation in the hippocampus. The results were represented as mean±SEM (one-way ANOVA). (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ compared with the control group, (#) $p < 0.05$ and (##) $p < 0.01$ compared with the AlCl₃ group and (\$) $p < 0.05$ compared with the AzA group. AzA: azelaic acid, TNF-α: tumor necrosis factor-α, IL-1β: Interleukin-1β, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, CHOP: C/EBP homologous protein.

The results also demonstrated a significant induction of CHOP gene expression in the AlCl₃ group when compared to the control group [$F(3, 28) = 12.68$, $p < 0.001$]. Despite the reduction that AzA induced in the expression of this gene in the AzA+AlCl₃ group, it was considerably more than the control group ($p < 0.01$) and statistically not very different than neither the AlCl₃ group nor the AzA group (Fig. 7D).

AChE activity in the hippocampus

The evaluation of hippocampus samples showed noticeably boosted AChE activity in the AlCl₃-administered group compared to the control group [$F(3, 28) = 6.14$, $p < 0.05$]. Although AzA decreased the effect of AlCl₃ in the AzA+AlCl₃ group and even reduced AChE activity in the AzA group in comparison to the control, both changes were not significant (Fig. 8A).

GSK-3β activity in the hippocampus

Signal transduction assay reaction detected considerably increased activity of GSK-3β in the AlCl₃ group in comparison to the control group [$F(3, 28) = 15.11$, $p < 0.001$] and the AzA group. Also, AzA significantly decreased the GSK-3β activity in the AzA+AlCl₃ group compared to the AlCl₃ group ($p < 0.01$), the enzyme activity level in this group was not notably different than the AzA group unlike the control group ($p < 0.05$) (Fig. 8B).

BDNF levels in the hippocampus

The BDNF content of AlCl₃ group was considerably decreased compared to the control group [$F(3, 28) = 10.12$, $p < 0.05$] and the

AzA group, even though this decrement was not significant compared to AzA+AlCl₃ group. However, despite the increased level of BDNF, the AzA group exhibited no significant change compared to the control group and the AzA+AlCl₃ group (Fig. 8C).

Discussion

Cognition is described as brain processes such as learning, memory, and executive functioning. It is operated by the frontal lobe, hippocampus, and other structures of the CNS (Harvey, 2019). Impairment of cognitive function takes place following damage to neuronal tissue and leads to the disruption of the individual's performances in society and personal life. The excessive production of ROS and RNS, as well as inflammatory mediators in the CNS, damage to the cell signalling pathways particularly PI3K/Akt/mTOR and synapses are among the main causes of cognitive deficits (Das *et al.*, 2023, Gutierrez and Limon, 2022).

Present study investigated protective effects of AzA against AlCl₃-induced behavioural and cognitive deficits in male Wistar rats. AlCl₃ could impair the NOR, MWM learning and memory, passive avoidance task, and cause significant provocation of anxiety-like behaviours in the EPM and OFM tests. Administration of the AlCl₃ also, causes reduction in BDNF levels of hippocampus and intensification of AChE activity, GSK-3β, oxidative stress and neuroinflammatory biomarkers including protein carbonyl, MDA, TNF-α, IL-1, CHOP and NF-κB.

Al can simply cross the BBB via specific receptors with high affinity for transferrin, and accumulate in all the regions of the

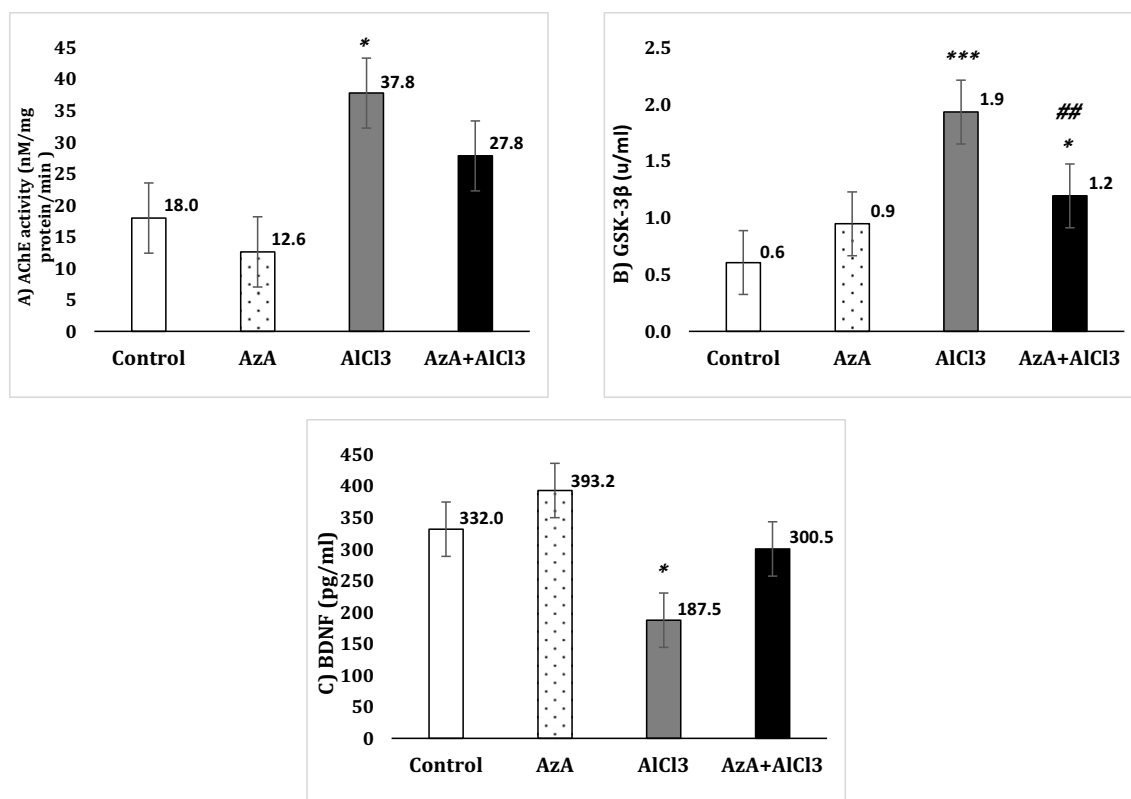


Figure 8. Effect of azelaic acid on AlCl₃-induced changes of acetylcholinesterase activity, glycogen synthase kinase-3beta and brain derived neurotrophic factor in the hippocampus. The results were represented as mean±SEM (one-way ANOVA). (*) $p < 0.05$ and (***) $p < 0.001$ compared with the control group, (##) $p < 0.01$ compared with the AlCl₃ group. AzA: azelaic acid, AChE: acetylcholinesterase, GSK-3β: glycogen synthase kinase-3beta, BDNF: brain-derived neurotrophic factor.

brain such as hippocampus (Exley, 2016, Skalny *et al.*, 2021). Persistent Al exposure is followed by the reduction of dopamine level and purkinje cells that cause cholinergic disorder (Singla and Dhawan, 2017). Meantime, this metal can inhibit catalase, glutathione and glutathione S-transferase, increase nitrite and provoke the AChE activity leading to excessive reduction of ACh in the brain (Abu-Taweel and Al-Mutary, 2021, Khan *et al.*, 2013). On the other hand, AlCl₃ significantly increases the concentration of proteins such as Aβ₁₋₄₂ (Zhang *et al.*, 2022). Al accumulation also forces mitochondria to release cytochrome *c* and eventually the extreme production of ROS and RNS resulting in the activation of innate immune system of the brain (Abbas *et al.*, 2022, Maksoud *et al.*, 2020, Arab-Nozari *et al.*, 2019). The immune system mediates the release of factors such as TNF-α, IL-1, CHOP and NF-κB and the degeneration of the neuronal cells. In agreement with the results of the previous researches, AlCl₃-intoxicated rats in our study showed significantly higher AChE activity, inflammatory and oxidative stress factors in the hippocampus compared to the control group.

BDNF has been known to have an essential role in the regulation of synaptic plasticity and cognitive processes so that higher levels of BDNF slows down the rate of cognitive decline. Reduced BDNF levels are frequently associated with neurodegenerative conditions, such as Alzheimer's disease, where they correlate with cognitive deficits. AlCl₃ exposure has been shown to decrease BDNF expression in the brain, contributing to cognitive impairments observed in animal models. Our findings confirm that AlCl₃ significantly reduces BDNF levels in the

hippocampus, consistent with previous studies (Kasbe *et al.*, 2015, Abbas *et al.*, 2022, Zhang *et al.*, 2022).

In addition to oxidative stress and neuroinflammation, Al would cause neuronal cell death through other mechanisms such as the activation of PI3K/Akt/mTOR signalling pathway (Skalny *et al.*, 2021, Shang *et al.*, 2020). PI3K/Akt/mTOR stimulates the expression of pro-apoptotic factors such as GSK-3β. GSK-3β is a key regulator in several cellular processes, including inflammation, apoptosis, and neuronal plasticity. Its dysregulation, often characterised by hyperactivity, is implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease. AlCl₃ exposure is known to increase GSK-3β activity, leading to the enhanced phosphorylation, decrease in glycogen concentration and ultimately apoptosis and neurodegeneration (Hamdan *et al.*, 2022). In this study, we observed a significant increase in GSK-3β activity in the hippocampus following AlCl₃ exposure, which aligns with previous findings that link GSK-3β hyperactivity to neurodegenerative processes.

The explained changes in the mentioned biochemical processes supported behavioural deficits of AlCl₃-exposed animals indicated by memory impairments (Kasbe *et al.*, 2015, Al-Amin *et al.*, 2016) and anxiety-like behaviours (Singla and Dhawan, 2017, Abu-Taweel and Al-Mutary, 2021). In the EPM test, the administration of AlCl₃ decreased the number of entries to the open arm, the time spent in the open arm, and locomotor activity. In the OFM test, AlCl₃ administration decreased grooming number, distance moved, number of entries to the inner zone of the OFM chamber, and time spent in that area and additionally, it increased the

rearing number of the rats. These results collectively indicate an exacerbation of anxiety in the AlCl_3 group.

The findings from the passive avoidance test revealed that the AlCl_3 group exhibited significantly less STL, spent more time in the DC, and had more entries to the DC compared to the control group. Furthermore, in both the learning and testing sessions of the MWM test, the AlCl_3 group performed significantly worse than the control group. Additionally, their discrimination ratio in the NOR test was significantly decreased compared to the control group. The findings of these tests are evidence of the damaging effects of AlCl_3 on the brain and cognitive functioning.

Using antioxidants is one of the strategies to prevent or treat oxidative stress-related diseases (Lee *et al.*, 2020). AzA has been proved to have anti-inflammatory and antioxidant effects (Sieber and Hegel, 2014, Sauer *et al.*, 2023). There is a report indicating neuroprotective effects of AzA against SH-SY5Y-JNK3 cell apoptosis (Gan *et al.*, 2011). This compound also reduced neurodegeneration in animal models of Parkinson's disease (Sharma *et al.*, 2021). Meanwhile, recent investigations suggested the possible inhibitory effect of AzA on PI3K/Akt/mTOR Signaling Pathway (Li *et al.*, 2022). Based on these evidences, the effect of this organic compound on AlCl_3 stimulated cognitive dysfunction was evaluated through animal behaviour and memory tests and biochemical assessment of hippocampus.

We observed that, in comparison to the AlCl_3 group, the presence of AzA in the AzA+ AlCl_3 group led to significant improvements in various behavioural tests. AzA+ AlCl_3 group showed enhanced performance, specifically, in the NOR test, passive avoidance test, and MWM test. These results indicate that AzA may effectively counteract the learning and memory deficits induced by Al, as demonstrated in the AzA+ AlCl_3 -treated rats.

The AzA+ AlCl_3 group exhibited significantly lower levels of anxiety compared to the AlCl_3 group. This was evidenced by their increased entries to the open arm, extended time spent in the open arm, and higher locomotor activity in the EPM test. Additionally, this group displayed a higher grooming number and covered a longer distance in the OFM test. Furthermore, in the OFM test, there was an increase in the number of entries to the centre zone and time spent there, while the rearing number decreased following AzA+ AlCl_3 administration, when compared to the AlCl_3 group.

AzA also could bring back the oxidation-reduction balance, as indicated by a significant decrease in TBARS and protein carbonyl content in the AzA+ AlCl_3 group compared to the AlCl_3 group. These findings suggest that the neuroprotective effects of AzA may involve its role in managing oxidative stress and influencing downstream signalling networks like the PI3K/Akt pathway. By potentially inhibiting this pathway, AzA could reduce the activity of GSK-3 β pro-apoptotic factor in the hippocampus of AzA+ AlCl_3 group compared to the rats only treated with AlCl_3 , thereby protecting against neuronal cell death. AzA+ AlCl_3 group had lower hippocampal inflammatory factors including TNF- α , IL-1 β and NF- κ B in comparison to the AlCl_3 group. However, the decrease in the CHOP levels in the AzA+ AlCl_3 group was NOT noticeable when compared to the AlCl_3 -administered group. Based on the statistics of the AzA+ AlCl_3 group, it appears that AzA was not able to effectively suppress the additive effect of AlCl_3 on the AChE activity. However, AzA did manage to decrease the AChE activity of the AzA+ AlCl_3 group to a level where there was no significant difference observed when compared to the control group and the AzA group either. The non-significant change in AChE activity suggests that while AzA can counteract some of the

neurotoxic effects of AlCl_3 , it may not be sufficient to completely reverse cholinergic dysfunction at the dose used in this study. This finding raises the possibility that higher doses of AzA or combination therapies may be required to achieve more robust neuroprotection.

Behavioural improvements in the learning and memory tests are likely underpinned by the observed molecular changes, such as the reduction in GSK-3 β activity and the partial restoration of BDNF levels in the hippocampus of AzA+ AlCl_3 -treated rats in comparison to the AlCl_3 group. The increase in BDNF, although NOT statistically significant, may still contribute to the enhanced synaptic plasticity and cognitive function observed in the AzA+ AlCl_3 group, as BDNF is a key regulator of learning and memory processes.

Conclusion

Based on the data obtained from our research, AzA indeed could alleviate AlCl_3 -induced behavioural alterations in rats. AzA reduced most of the oxidative and inflammatory disturbance induced by AlCl_3 . Findings of this study highlight the potential effect of AzA against neurocognitive impairments, but the sufficiency of these effects remained a question we couldn't confidently answer. Although AzA could ameliorate cognitive functioning and limit the oxidative stress and neuroinflammation caused by AlCl_3 , further research is required in the future.

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Competing interests. The authors declare that they have no conflicts of interest.

Animal welfare ethical statement. The authors assert that all procedures contributing to this work comply with the ethical standards of the Medical Research in Iran and institutional guides on the care and use of laboratory animals. All the experiments were performed in accordance with the animal guidelines, and the experimental methods were also approved by the Research Ethics Committee of Ardabil University of Medical Science (No. IR.ARUMS.AEC.1401.021).

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