Toxicology Reports 8 (2021) 1280–1288

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

# Subchronic neurotoxicity of diazinon in albino mice: Impact of oxidative stress, AChE activity, and gene expression disturbances in the cerebral cortex and hippocampus on mood, spatial learning, and memory function

Asieh Karimani [a], Nasrin Ramezani [a], Amir Afkhami Goli [a], Mohammad Hossein Nazem Shirazi [b], Hosein Nourani [c], Amir Moghaddam Jafari [a,*]

[a] Department of Toxicology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Central Veterinary Laboratory, Veterinary Head Office of Khorasan Razavi, Mashhad, Iran
[c] Department of Pathology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

ARTICLE INFO

Handling Editor Dr. Aristides M. Tsatsakis

*Keywords:*
Diazinon
Neurodegenerative diseases
Cerebral cortex
Hippocampus
Spatial learning
Memory

ABSTRACT

Diazinon (DZN) with prominent neurotoxic effects perturbs CNS function via multiple mechanisms. This investigation intends to explore mood, spatial learning, and memory dysfunction, acetylcholine esterase (AChE) activity, and neurodegeneration-related gene expression in the cortex and hippocampus regions of mice exposed to DZN for 63 consecutive days (subchronic exposure). Adult male albino mice were orally given sublethal DZN ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg and $DZN_H$ = 10 mg/kg). All mice in the $DZN_H$ group died within 3 weeks postexposure. $DZN_L$ and $DZN_M$ caused body and brain weight loss ($p < 0.05$). Completing 9 weeks of DZN exposure, a marked decline in AChE activity and oxidative stress level was indicated in both brain regions ($p < 0.05$). Also, *synaptophysin*, *vesicular acetylcholine transferase*, and *glutamate decarboxylase* gene expressions were affected in both brain regions ($p < 0.05$). Furthermore, the present study revealed that DZN administration increased anxiety and depressive-like behaviors ($p < 0.0001$). Spatial learning and short- and long-memory were severely affected by $DZN_L$ and $DZN_M$ treatments ($p < 0.0001$). Taken together, subchronic exposure to low and medium doses of DZN can cause AChE inhibition, oxidative damage, and neurotransmitter disturbances in brain cells and induce neurodegeneration. These changes would impair mood, spatial learning, and memory function.

## 1. Introduction

The widespread use of pesticides has dramatically increased the consistent exposure of human populations to these chemicals [1–4]. There are gradually strengthening proofs that exposure to low-dose pesticides such as paraquat, pyrethroids, and organophosphates (OPs) for a long duration, potentially leads to neurological diseases [5], like Parkinson's disease (PD) and Alzheimer's disease (AD) [1,3]. PD and AD are progressive neurodegenerative disorders clinically characterized by motor symptoms (such as bradykinesia, rest tremor, and postural disturbances), and slow deterioration in cognition as well as progressive loss of memory [1].

Based on the World Health Organization (WHO) report, approximately a billion people are affected by neurodegenerative diseases around the globe [6]. The etiology of the majority of PD and AD cases is presumably due to some interactions between genetic and environmental factors. The role of chronic exposure to OP pesticides particularly diazinon (DZN), greatly used since organochlorine insecticides were banned, as a significant non-genetic contributor to the creation of neurodegenerative disorders has been specifically examined for PD and AD [1,7,8]. The initial mechanism of OPs toxicity is acetylcholine esterase (AChE) inhibition [5] which leads to muscarinic and nicotinic

*Abbreviations:* Ach, acetylcholine; AChE, acetylcholine esterase; AD, Alzheimer's disease; COX-2, cyclooxygenase-2; CX, cerebral cortex; DZN, diazinon; DZO, diazoxon; FRAP, ferric reducing antioxidant power; FST, forced swim test; GABA, ϒ-aminobutyric acid; *GAD65*, *glutamate decarboxylase 65*; HP, hippocampus; $LD_{50}$, lethal dose 50; MB, marble burying test; MDA, malondialdehyde; MWM, Morris water maze test; NOAEL, no-observed-adverse-effect level; Ops, organophosphates; PD, Parkinson's disease; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; RNS, reactive nitrogen species; SEM, standard error of the mean; *SYP*, *synaptophysin*; *VAChT*, *vesicular acetylcholine transferase*.

* Corresponding author at: Department of Basic Sciences, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
*E-mail address:* moghaddamjafari@um.ac.ir (A.M. Jafari).

https://doi.org/10.1016/j.toxrep.2021.06.017
Received 22 December 2020; Received in revised form 6 June 2021; Accepted 14 June 2021
Available online 19 June 2021

disorders [9]. However, there are a great number of reports about different cellular and molecular mechanisms which are deeply involved in subchronic and chronic toxic effects such as oxidative stress [10] and disturbances of neurotransmitter synthesis, degradation, and transport [11,12]. Besides, accumulating evidence has been obtained from human and animal models suggesting that oxidative stress played an essential role in the pathogenesis of both PD and AD [1,4,12]. Although the etiology of degenerative neurologic disorders is currently not clear, PD and AD are firmly associated with impairment in cholinergic transmission. Alterations of cholinergic and ϒ-aminobutyric acid (GABA)ergic neuronal activities in the cortical and hippocampal regions have been proposed as a major player in behavioral dysfunctions observed in neurodegenerative diseases. Consequently, it is assumed that PD and AD associate with up and down-regulation of genes related to neurotransmitter systems and signaling networks [13–19].

Despite a great number of investigations done around the neurodegeneration effects of DZN, the fundamental mechanisms of neurodegeneration induced by long-term exposure to this pesticide have not been entirely clarified. This study has been organized to elaborate on the significance of pesticide residue in the human diet in causing neurodegenerative diseases.

For the first time, the present study was designed to assess and compare the extent of cellular and molecular deteriorative effects of the different doses of 9-week exposure to DZN in the cerebral cortex (CX) and hippocampus (HP) of DZN-exposed mice. Therefore, the objectives of this investigation were to (1) determine the effect of different doses of DZN on the expression levels of three genes involved in neurodegeneration (2) evaluate behavioral changes induced by DZN (3) measure the extent of oxidative stress and AChE activity, and (4) calculate weight changes in the CX and HP.

## 2. Materials and methods

### 2.1. Animals

Animals used in this experiment were adult male albino mice (n = 40) in apparently good health, age 11 ± 1 weeks and weighing 27 ± 0.5 g, kept in individual polypropylene cages (55 × 35 × 30 cm) under standard laboratory conditions, acclimated for two weeks before experimental use (ambient temperature 22 ± 2 °C, relative humidity 50 %, 12/12 h dark/light cycle with lights turned on at 9:00 a.m.). The ethics committee of the institute approved the experimental protocol and all efforts were conducted to minimize animal suffering (the Animal Welfare Committee of the School of the Veterinary Medicine, Ferdowsi University of Mashhad, Iran).

### 2.2. Experimental design

To induce neurodegeneration, DZN (99 %, Sigma-Aldrich) was administered daily in 3 doses for 9 weeks. All mice were randomly divided into the four following groups with 10 mice per group and administered orally through gavage, once a day, 9:00 a.m.: 1. Control (corn oil 100 % vehicle treated-group, 1 ml), 2. $DZN_L$ (low dose DZN-treated group, 0.1 mg/kg per day dissolved in 1 ml corn oil), 3. $DZN_M$ (medium dose DZN-treated group, 1 mg/kg per day dissolved in 1 ml corn oil), and 4. $DZN_H$ (high dose DZN-treated group, 10 mg/kg per day dissolved in 1 ml corn oil). DZN dosages were selected according to the previous studies [Based on the DZN no-observed-adverse-effect level (NOAEL) for subchronic toxicity, which are respectively 0.0008, 0.008, and 0.08 of lethal dose 50 ($LD_{50}$) and below the threshold to observe cholinergic crisis] [2]. Mice were monitored day by day for general behavior and incidence of signs of abnormalities and mortality.

### 2.3. Body and brain weights measurement and Tissue sampling

All mice were weighed at weekly intervals beginning 7 days after the first day until 63 days. For weighing, mice were put in cages without bedding on automatic balance (AND GX-600, Japan). Nine weeks post-exposure, all experimental mice were euthanized by a $CO_2$ gas chamber (allowed to remain there until respiration ceased). The brain was removed from the skull quickly (customarily, the brain was separated from the spinal cord just below the decussation of the pyramids), then weighed and dissected into cortical and hippocampal regions on an ice-cold surface after identifying locations with the help of mouse brain map (Allen Mouse Brain Atlas). The dissected brain regions were weighed, immersed in ice-cold saline (0.9 % $NaCl_2$). Tissues were homogenized at 0 °C in 0.05 M phosphate buffer (pH 7.7) for biochemical assays immediately.

### 2.4. Assessment of oxidative damage in CX and HP

Oxidative stress parameters including lipid peroxidation level and total antioxidant capacity were assessed in the two regions of the brain: the CX and HP, on day 63.

#### 2.4.1. Thiobarbituric acid reactive substance (TBARS) assay

Malondialdehyde (MDA), the end product of lipid peroxidation which reacts with thiobarbituric acid (TBA) leading to produces a red-colored complex, was used as an indicator of lipid peroxidation level in the cortical and hippocampal regions. In short, the mixture comprised of 0.2 ml of homogenized tissue, 1.3 ml of 0.2 M Tris-0.16 M KCl buffer (pH 7.4), and 1.5 ml of TBA reagent, was put in a boiling water bath for 10 min. After cooling, 3 ml of pyridine/n-butanol (3:1, v/v) and 1 ml of 1 M sodium hydroxide were supplemented and combined by robust shaking. The mixture was centrifuged at 3000 *g* for 10 min and the amount of MDA formed in the supernatant was measured by reading the absorbance at 548 nm using a universal spectrophotometer (Bio-Rad Lab, Inc, CA, USA). A blank was run simultaneously containing all reagents except the homogenized brain tissue. The nmol of MDA per ml was calculated using $1.56 \times 10^5$ as the extinction coefficient. The MDA result is expressed as nmol/mg protein [2,20].

#### 2.4.2. Total antioxidant capacity assay

Total antioxidant capacity was assayed in brain homogenates according to the ferric reducing antioxidant power (FRAP) assay. This method depends on the reduction of ferric tripyridyl-s-triazine ($Fe^{3+}$-TPTZ) at low pH. $Fe^{2+}$-TPTZ has an intensive blue color that can be monitored for up to 5 min at 593 nm by a spectrophotometer. Calculations were done by using a calibration curve of $FeSO_47H_2O$ (100−1000 mM) [2].

### 2.5. AChE enzymatic activity

AChE activity was assessed by an adjusted version of the colorimetric Ellman's method. To deposit the membrane and their bound AChE, aliquots of thawed cortical and hippocampal homogenates (prepared in triplicate) were centrifuged at 50,000 *g* for 120 min. The kinetic assay was initiated by supplementing 1 mM AChE to distinguish between specific and non-specific cholinesterase activities. AChE activity was delineated as the number of nmoles of acetylcholine (ACh) hydrolyzed per min at 22 °C [21].

### 2.6. RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To identify changes in genes involved in neurodegenerative disorders, we evaluated *synaptophysin (SYP)*, *vesicular acetylcholine transferase (VAChT)*, and *glutamic acid decarboxylase 65 ($GAD_{65}$)* expressions by real-time qRT-PCR analysis. All frozen homogenized samples (stored at −80 °C) taken from CX and HP were defrosted at room temperature and prepared in triplicate. In brief, total RNA was extracted using the High Pure RNA isolation kit (Roche, Germany) treated with DNase I (Roche,

Germany), and the first-stranded cDNA was synthesized using Transcriptor First Strand cDNA synthesis kit (Roche, Germany) following the manufacturer's guidelines. Spectrophotometrically, the quality, and quantity of isolated total RNA were assessed (Nanodrop 2000, Biotech, USA). RNA specimens with a ratio of $A_{260}/A_{280} \geq 2.0$ Nanodrop were used in this analysis. The sequences of the primers used are listed in Table 1.

cDNA was subsequently subjected to qPCR on a Corbett Rotor-Gene 6000 HRM real-time PCR machine (Corbett Life Science, Australia) using Power SYBER green PCR master mix (AB, USA). The main steps are elaborated in Table 2. After finishing the last cycle, size verification of the amplicons by agarose gel electrophoresis was used to confirm the specificity of the PCRs. Also, non-template reaction was assessed in reverse transcription negative samples and by melting-curve analysis. By using *β-actin* gene expression in the $2^{-\Delta\Delta CT}$ method, data of relative quantification of gene expression was normalized.

### 2.7. Behavioral tests

Behavioral tests were managed in a quiet room instrumented with a light-control system. Each day, at least 30 min before testing, mice were adapted to the conditions of the behavioral testing facility.

#### 2.7.1. Marble burying (MB) test

To examine DZN-induced anxiety, we employed the weekly MB test. Briefly, mice were placed at a standard polycarbonate rat cage (26 × 48 × 20 cm) with 20 fresh unscented glass (Ø1 cm) arranged (5 raw×4 marble) by impressing a template of parallel lines on the bedding (5 cm sawdust) surface. The dim red light was used to perform the experiments during the dark cycle. Thirty minutes were allocated for exploring the marble cage by each mouse. After returning the mouse to its cage, the marbles were counted by a blind observer unfamiliar with the design of the study. When two-thirds or more of the surface was covered with sawdust, marble was considered buried [22].

#### 2.7.2. Forced swim test (FST)

We used FST to assess depressive-like state weekly, whereupon a mouse was held by its tail, and gently placed in a plexiglass cylinder (20 width×30 height cm) filled with tap water (to the height of 15 cm, at 23 ± 1 °C) and then forced to swim. Swimming sessions were carried out in 6 min, however, generally, only the last 4 min of the test was analyzed since most mice were very energetic at the onset of the FST. During the behavioral examination, the time that each mouse spent mobile was recorded. The immobility time was calculated by deducting the sum of the mobility time from the 240 s of the test time. Immobility was explained as the period during which the mouse floats in the water with any movements necessary to keep its head above water, while active forward movements of the forepaw were recorded as mobility [23].

#### 2.7.3. Morris water maze test (MWM)

The MWM was conducted to assess the effects of low and medium doses of DZN on learning and short and long-term memory. MWM apparatus comprised of a white circular plastic pool (122 cm diameter and 58 cm deep) filled with water (to a depth of 30 cm) maintained at 23 ± 1 °C and made opaque with non-toxic white paint in which a circular platform (8 cm diameter) was submerged 1 cm below the surface of the water in one of the four equal quadrants of the pool. After introducing the mouse into the pool facing the wall, it could navigate for 60 s with visual cues to find the hidden platform. After finding the hidden platform, it remained on it for 15 s, ending that trial and the escape latency was recorded. If the mouse did not succeed to find the platform during the allotted time, it was directed to the platform and permitted to stay on it for 15 s and the escape latency was recorded 60 s. After performing each trial, mice were taken away from the pool, towel-dried, and sent back to their cage. Four trials were conducted during each session with an intertrial of 30 min and an intersession interval of 24 h. The acquisition sessions were expanded for 4 sequential days. On the fifth day, reference memory was assessed via a spatial probe trial to determine whether or not mice remembered the location of the removed platform within 30 s. Indications of such memory including time expended in the target quadrant and path length in non-target quadrants were recorded. Briefly, MWM tasks comprised of hidden-platform training and spatial probe trials at three periods, 1 week after low- and medium-dose DZN treatment, and then performed again 5 and 9 weeks post-exposure. Also, two additional probe trials, with the previous target quadrant, interspersed one day before the second and third acquisition training phases were done to determine the rate of memory consolidation [24]. The experimental chronology is shown in Fig. 1.

**Table 1**
The sense and antisense sequences of the primers used in quantitative reverse transcription-polymerase chain reaction (qRT-PCR) test for evaluating related gene expressions.

| Primers | Sense | Antisense |
|---|---|---|
| *SYP* | 5́-gtgccaacaagacggagagtgc-3́ | 5́-agggcccccatggagtagagga-3́ |
| *VAChT* | 5́-caggccatatcgttcactca-3́ | 5́-cggttcatcaagcagcacat-3́ |
| *GAD65* | 5́-ctggaaccaccgtgta-3́ | 5́-catcagtccctcctct-3́ |
| *β-actin* (housekeeping gene) | 5́-gtgctatgttgccctagcttcg-3́ | 5́-gatgccacaggattccataccc-3́ |

**Table 2**
The main steps of each polymerase chain reaction (PCR) cycle. Thirty-five cycles have been done at the end of the procedure.

| Steps of each cycle | Denaturation | annealing | extension |
|---|---|---|---|
| Temperature (°C) | 94 | 56 | 72 |
| Time (s) | 30 | 30 | 40 |

### 2.8. Statistical analysis

Data analysis was carried out using SPSS 11.5 for Windows (SPSS, Chicago, IL). All data are expressed as mean ± standard error of the mean (SEM). Differences between and within groups were analyzed using ANOVA (Analysis of Variance). Statistical analysis of weighing data obtained in the brain, CX, and HP was done by one-way ANOVA. A two-way analysis of variance was done to evaluate the significance of differences in gene expression levels, oxidative stress, AChE activity in the CX, and HP between the DZN-treated groups and the control group. Statistical analysis of body weight and behavioral tests with the time factor was carried out by repeated-measures ANOVA. To determine which one of the means was significantly different, the Tukey post hoc test was followed for multiple comparisons when the variances could be assumed homogenous and the Dunnett test when an assumption of homogeneity could not be made. Furthermore, multiple correlations among AChE activity, lipid peroxidation, mRNA levels, and behavioral tests were carried out. The level of significance was set at $p < 0.05$.

## 3. Results

After approximately 14 days, mice in the high-dose DZN group became depressed and anorectic and were slow to respond to tapping on the cage wall. Two mice were found dead on the morning observation. The mice (n = 8) that were still alive displayed subacute cholinergic manifestations within the next week. The diagnosis was typically based on the symptoms such as dyspnea, fasciculation, tremor, generalized seizure (clonic or tonic contractions of all limbs plus loss of posture whereas the mice fell onto their side or back). During the third week, all mice in the $DZN_H$ group died before study completion due to subacute cholinergic syndromes such as bradycardia, wheezing, and respiratory distress. At necropsy, no abnormalities were observed.

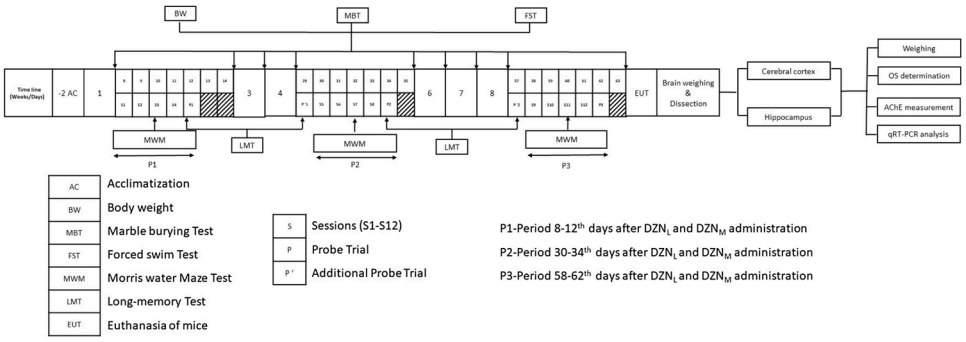


**Fig. 1.** Flowchart showing the experimental design. The timeline shows that this experiment lasted for a total of 63 days. The subjects were ascribed to four different groups: Control group (corn oil vehicle), $DZN_L$ group (0.1 mg/kg, low-dose diazinon), $DZN_M$ group (1 mg/kg, medium-dose diazinon), $DZN_H$ group (10 mg/kg, high-dose diazinon). The last day of each week on the timeline is specified to when the subjects were weighed and submitted to MB and FST tasks. P1, P2, and P3 periods corresponding to the interval when the subjects were submitted to the MWM task. On day 63, the subjects were euthanized and the brains were weighed and dissected to the cerebral cortex and hippocampus processing to analyze oxidative stress, AChE activity, and gene expression, except the mice of the $DZN_H$ group as they died until the end of the 3rd week postexposure due to the subacute cholinergic syndrome.

### 3.1. Effects of $DZN_L$, $DZN_M$, and $DZN_H$ on the body, brain, and cortical and hippocampal weights

As shown in Fig. 2, significant differences in body weight were first evident at weeks 2, 3, and 6 in $DZN_L$ (10.92 %, $p < 0.05$), $DZN_M$ (10.88 %, $p < 0.05$), and $DZN_H$-treated (10.35 %, $p < 0.05$) groups compared to the control group, respectively. The decrease in body weight remained significantly different thereafter until the 9th week in $DZN_L$ (9.15 %, $p < 0.05$) and $DZN_M$ (22.70 %, $P < 0.05$) compared to control. Also, the $DZN_M$-treated mice showed more weight loss when compared to the $DZN_L$ group ($p < 0.05$). Furthermore, low and medium doses of DZN significantly reduced brain weight by as much as 10.53 % and 18.75 %, respectively. According to Tukey-Kramer analysis, brain weight was markedly diminished by $DZN_M$ compared to $DZN_L$ (8.22 %, $p < 0.05$). The relative brain-to-body weight ratio did not considerably differ among the groups ($p > 0.05$). Also, data analysis indicated a significant weight reduction by low and medium doses of DZN in the CX ($DZN_L$: 12.72 %, $p < 0.05$, and $DZN_M$: 22.44 %, $p < 0.05$) and HP ($DZN_L$: 7.99 %, $p < 0.05$, and $DZN_M$: 9.30 %, $p < 0.05$) compared to the control mice. No significant difference was observed in the HP weight between $DZN_L$ and $DZN_M$ groups. The relative weight of these regions to the brain did not change significantly ($p > 0.05$) (Table 3).

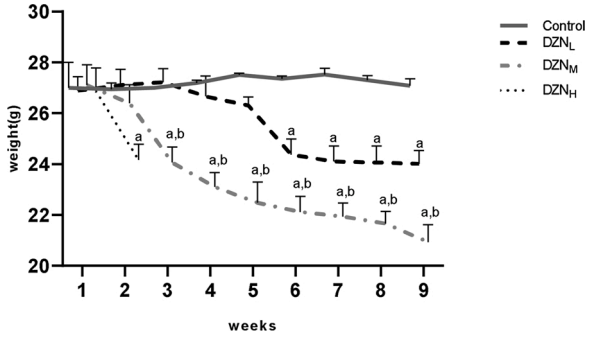


**Fig. 2.** Effect of diazinon ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg, and $DZN_H$ = 10 mg/kg) on the body weight alteration in mice. DZN in corn oil was given through gavage to mice for 9 weeks. Throughout 9 weeks, there was no significant change in body weight between control mice. There was a significant decrease in body weight after 2, 3, and 6 weeks of exposure with $DZN_L$, $DZN_M$, and $DZN_H$ treated groups compared to the control group. The reduction in body weight remained significant for $DZN_L$ and $DZN_M$ groups until the 9th week ($p < 0.05$). The $DNZ_M$-treated mice showed more weight loss when compared to the $DZN_L$ group. Data showed as mean ± SEM, a: comparison with Control group, b: comparison with $DZN_L$ group. $P < 0.05$, Tukey-Kramer test, n = 10.

### 3.2. Effects of $DZN_L$ and $DZN_M$, on oxidative stress parameters

#### 3.2.1. Effects of $DZN_L$ and $DZN_M$ on lipid peroxidation

As shown in Table 4, the results demonstrated DZN-induced oxidative damage in HP and CX. Following the administration of low and medium doses of DZN, there was a significant increase in lipid peroxidation in both regions of the brain particularly in the CX ($p < 0.05$). The Tukey-Kramer post hoc revealed that $DZN_M$-treated mice exhibited a significant increase in MDA levels when compared to the $DZN_L$ group.

#### 3.2.2. Effects of $DZN_L$ and $DZN_M$ on total antioxidant capacity

Antioxidant status was evaluated in the term of the ferric-reducing antioxidant power of both brain regions. Low and medium doses of DZN administration significantly depressed the FRAP values in the HP and CX regions ($p < 0.05$), but the depletion was more evident in the $DZN_M$-treated mice as compared to the $DZN_L$-treated group ($p < 0.05$) (Table 4).

### 3.3. Effects of $DZN_L$ and $DZN_M$ on AChE activity

As shown in Table 4, cortical and hippocampal AChE activity varied among different groups, with the $DZN_M$-treated mice having about 50 % and 25 % lesser activity, respectively as compared with the control group ($p < 0.05$). Also, in $DZN_L$-treated mice compared with the controls, mean AChE activity was markedly reduced in the CX (22 %) and HP (15 %) ($p < 0.05$). Mean AChE activity in $DZN_M$-treated mice was also significantly reduced compared with the $DZN_L$-treated group, 27 % in the CX and 10 % in the HP ($p < 0.05$).

### 3.4. Effects of $DZN_L$ and $DZN_M$ on gene expression

Analysis of the RT-PCR data revealed considerable changes in mRNA levels between DZN- treated and control mice. Decreased *SYP* expression was observed in the DZN groups compared to the controls in both brain regions. The decreasing rate of *SYP* mRNA expression was 2-fold higher in the CX than HP ($p < 0.05$). Low-dose DZN treatment elevated *VAChT* mRNA level by approximately 2.41 and 1.74-fold, in contrast, medium-dose DZN reduced mRNA level by 2.47 and 2.71-fold in the CX and HP compared to the control group, respectively ($p < 0.05$). Additionally, the cortical *GAD* gene expression was significantly decreased by 3-fold ($p < 0.05$) whereas hippocampal *GAD* significantly increased by 1.44-fold in $DZN_M$-treated compared to the control group ($p < 0.05$). However, the expression level of *GAD* did not change in $DZN_L$-treated mice compared to the control group ($p > 0.05$) (Fig. 3).

**Table 3**
Evaluation of brain, cortex (CX), and hippocampus (HP) absolute and relative weights in diazinon-exposed mice ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg).

| Parameters | Brain weight | | CX weight | | HP weight | |
|---|---|---|---|---|---|---|
| Groups | Absolute(mg) | Relative (brain/body ratio) | Absolute(mg) | Relative (CX/Brain ratio) | Absolute(mg) | Relative (HP/Brain ratio) |
| Control | 382.08 ± 1.39 | 1.40 | 140.33 ± 4.32 | 36.72 | 35.26 ± 0.53 | 9.22 |
| $DZN_L$ | 341.81 ± 2.86[a] | 1.38 | 122.49 ± 6.02[a] | 35.83 | 32.44 ± 0.63[a] | 9.49 |
| $DZN_M$ | 310.42 ± 3.73[a,b] | 1.48 | 108.83 ± 4.48[a,b] | 35.05 | 31.98 ± 0.37[a] | 10.30 |

Diazinon in corn oil ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg) was given through gavage to mice for 9 weeks. Data showed as mean ± SEM. a: comparison with the Control group, b: comparison with $DZN_L$ group. $P < 0.05$, Tukey-Kramer test, n = 10.

**Table 4**
Effect of diazinon ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg), on acetylcholinesterase (AChE), malondialdehyde (MDA), and total antioxidant capacity (FRAP) in the cortex (CX) and hippocampus (HP).

| Parameters | AChE(nmol/min.mg protein) | | MDA(nmol/mg protein) | | FRAP($Fe^{2+}$nmol/mg protein) | |
|---|---|---|---|---|---|---|
| Groups | CX | HP | CX | HP | CX | HP |
| Control | 17.4 ± 0.7 | 23.5 ± 0.2 | 1.25 ± 0.23 | 0.68 ± 0.31 | 17.22 ± 2.45 | 13.75 ± 1.38 |
| $DZN_L$ | 13.6 ± 0.5[a] | 20.1 ± 0.7 [a] | 4.2 ± 0.54 [a] | 2.02 ± 0.26 [a] | 12.02 ± 2.12[a] | 9.81 ± 1.59[a] |
| $DZN_M$ | 8.9 ± 0.8[a,b] | 17.6 ± 0.3 [a,b] | 6.25 ± 0.33[a,b] | 3.12 ± 0.18 [a,b] | 8.52 ± 1.38[a,b] | 4.02 ± 0.88[a,b] |

Diazinon in corn oil ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg) was given through gavage to mice for 9 weeks. Data showed as mean ± SEM. a: comparison with the Control group, b: comparison with $DZN_L$ group. $P < 0.05$, Tukey-Kramer test, n = 10.

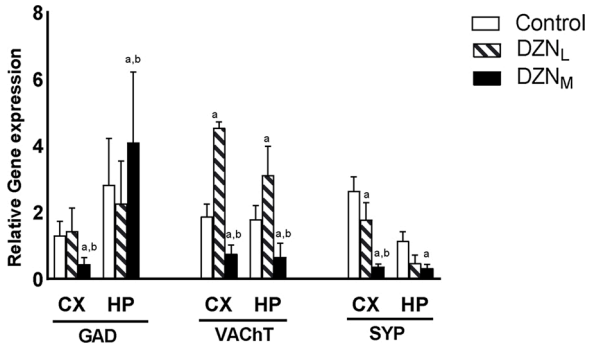


**Fig. 3.** Effect of diazinon ($DZN_L$ = 0.1 mg/kg and $DZN_M$ = 1 mg/kg) on gene expression. DZN in corn oil was given through gavage to mice for 9 weeks. The cortical (CX) *GAD* gene expression was significantly decreased ($p < 0.05$) whereas, hippocampal (HP) *GAD* significantly increased in $DZN_M$–treated mice compared to the control group ($p < 0.05$). DZN treatment with a low dose elevated the mRNA level of *VAChT* while medium-dose administration decreased it in both brain regions ($p < 0.05$). *SYP* expression was decreased in DZN groups compared with the control group in both brain regions ($p < 0.05$). Data showed as mean ± SEM, a: comparison with Control group, b: comparison with $DZN_L$ group. $P < 0.05$, Tukey-Kramer test, n = 10.

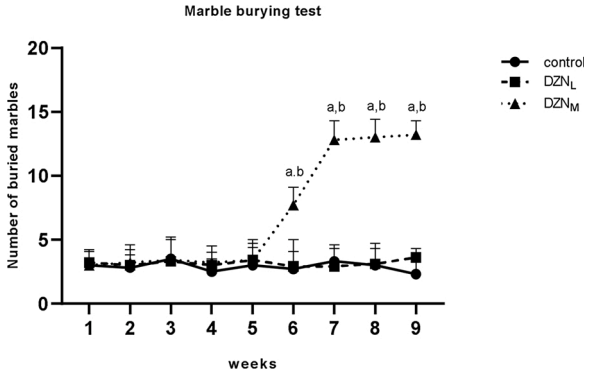


**Fig. 4.** Effect of diazinon ($DZN_L$ = 0.1 mg/kg, $DZN_M$ = 1 mg/kg) on anxiety-like behavior. DZN in corn oil was given through gavage to mice for 9 weeks. Digging behavior was evaluated during all these weeks. In $DZN_M$ mice, digging behavior has been increased from the 6th- to 9th-week postexposure, significantly in the last 3 weeks. The low dose of DZN did not affect the results. Data showed as mean ± SEM, a: comparison with Control group, b: comparison with $DZN_L$ group. $P < 0.001$, Tukey-Kramer test, n = 10.

### 3.5. Behavioral tests

#### 3.5.1. Effects of $DZN_L$ and $DZN_M$ on anxiety-like behaviors

Fig. 4 shows the total number of buried marbles in the control and DZN groups. One-way ANOVA revealed a significant effect of medium dose of DZN × time interaction ($p < 0.001$), indicated by an increase in digging behavior 6 to 9 weeks post-exposure. Tukey's posthoc test indicated the more anxiogenic effect of DZN during the last 3 weeks post-exposure. No anxiogenic symptom was observed when the $DZN_L$ group was compared with the control group.

#### 3.5.2. Effects of $DZN_L$ and $DZN_M$ on depressive-like behaviors

The FST was effective in evaluating the presence of depressive-like behavior in the DZN-treated groups. Four-week post-exposure, the mice exposed to the medium dose of DZN displayed a considerable increase in immobility when compared with the $DZN_L$ and control groups, while both DZN groups exhibited a significant decrease in mobility and increase in floating time in the following weeks (Fig. 5).

#### 3.5.3. Effects of $DZN_L$ and $DZN_M$ on learning and memory in the MWM test

The results of hidden-platform training evaluated by the MWM test in terms of latency time demonstrated that all mice did learn to navigate within the water maze to find the escape platform in the first period (1week post-exposure) (Fig. 6). Post hoc analysis revealed intrasession and interperiod within-group significant differences in all groups ($p < 0.01$), except the $DZN_M$-treated mice throughout the 9th week which had no significant difference in the intrasession latency time points (Fig. 6). The only exception their escape latencies decreased along sessions and periods ($p < 0.01$). These data. The results showed that latency times increased during Period 2 and Period 3 in the order of $DZN_M$> $DZN_L$> Control ($p < 0.01$), thus showing inferior performance to find the new platform position at 5th and 9th weeks post-exposure.

In probe trials managed 24 h post-acquisition semesters (probes 1, 2, and 3), control mice showed a significant preference for the target quadrant compared to DZN-treated mice with more path length in the non-target quadrant and less time in the target quadrant. Each probe trial was analyzed to determine whether mice within DZN-treated groups demonstrated a significantly lower performance for the target quadrant relative to the other three quadrants, therefore indicating they

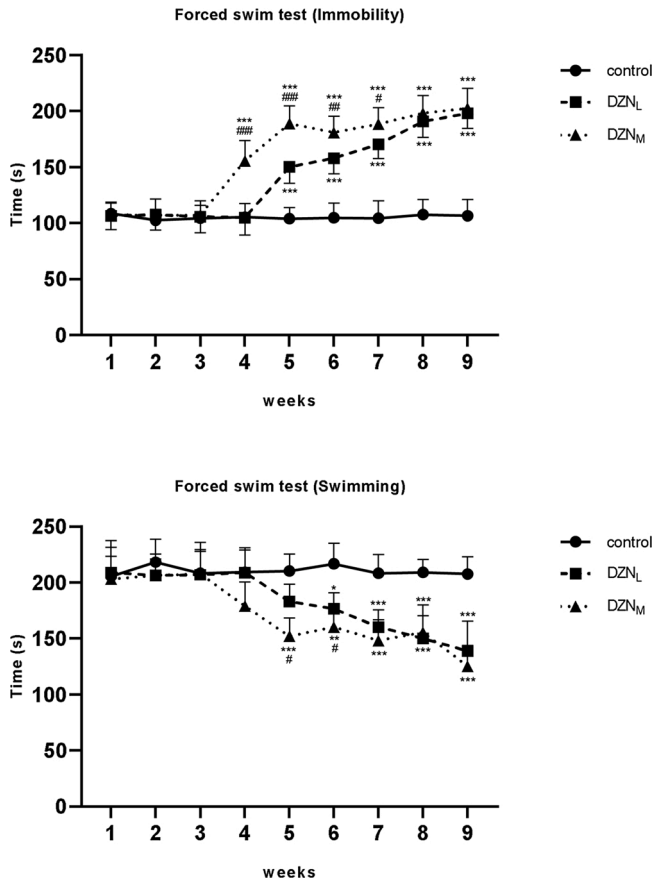


**Fig. 5.** Effect of diazinon ($DZN_L = 0.1$ mg/kg, $DZN_M = 1$ mg/kg) on depressive-like behavior. DZN in corn oil was given through gavage to mice for 9 weeks. The forced swim test was carried out weekly. At 4 weeks postexposure in $DZN_M$ mice, a significant increase in immobility was shown when compared with $DZN_L$ and control groups. Throughout the next weeks both DZN groups exhibited a significant decrease in mobility (upper image) and an increase in floating time (lower image) ($p < 0.01$). Data showed as mean ± SEM. *, **, ***: comparison with Control group $P < 0.01$, 0.001, and 0.0001, respectively. #, ##, ###: comparison with $DZN_L$ group $P < 0.01$, 0.001, and 0.0001, respectively, Tukey-Kramer test, $n = 10$.

had not effectively remembered in which quadrant the learned target platform had previously been located. The result of probe trial 1 demonstrated the equivalence between DZN groups compared with the control one ($p < 0.01$). However, DZN-treated groups showed lower short-term memory performance after 9 weeks post-exposure indicating the destructive effect of prolonged DZN administration ($p < 0.01$).

The results of additional probe trials ($P_1$ and $P_2$) shown in Fig. 6, allow us to analyze the effects of low- and medium-dose DZN treatment on long-term memory. Tested in $P_1$ and $P_2$ conducted at 5th- and 9th-week post-exposure (4week after the $P_1$ and $P_2$, $p < 0.01$), neither $DZN_L$- nor $DZN_M$-treated mice were able to effectively recall the target quadrant when compared with the control group. Also, a significant reduction in the time spent in the target quadrant among groups as $DZN_M$>$DZN_L$&Control in $P_1$ and $DZN_M$>$DZN_L$>Control in $P_2$ ($p < 0.01$) shows that medium-dose DZN could impair long-term memory function.

## 4. Discussion

Despite the toxic cholinergic effects of DZN, there are now influential pieces of evidence that non-cholinergic mechanisms might be associated with the harmful consequences from long-term exposure with low doses of DZN, such as attentional and cognitive impairments [25]. DZN and its oxygen metabolite diazoxon (DZO) induce oxidative stress through the

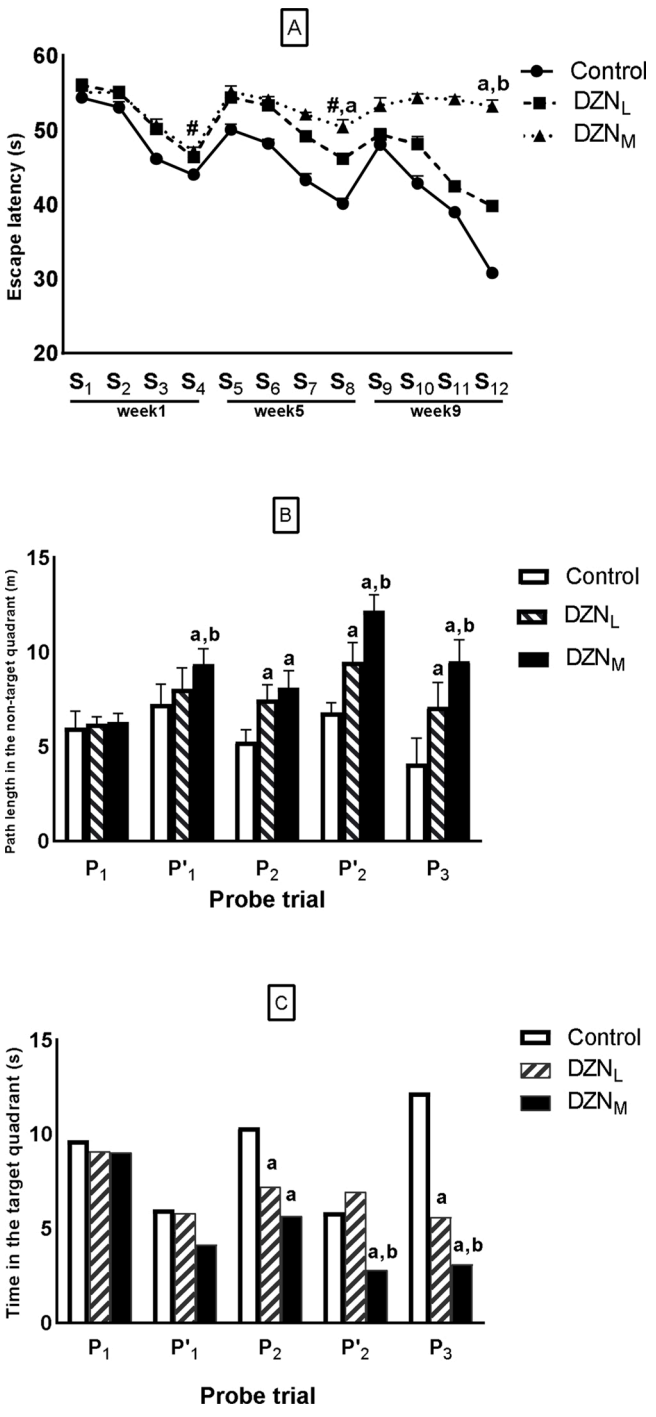


**Fig. 6.** Effect of diazinon ($DZN_L = 0.1$ mg/kg, $DZN_M = 1$ mg/kg) on learning and memory. DZN in corn oil was given through gavage to mice for 9 weeks. Morris water maze test was done in weeks 1, 5, and 9. Escape latencies decreased along with sessions and periods ($p < 0.01$) (A). Learning capacity did not differ among different groups 1-week postexposure (Period 1) ($p < 0.01$), but latency time increased during Period 2 (5th-week post-exposure) and Period 3 (9th weeks post-exposure) in the order of $DZN_M$> $DZN_L$> Control ($p < 0.01$) (B). The results of $P_1$ and $P_2$ revealed a significant reduction in time spent in the target quadrant among groups as $DZN_M$> $DZN_L$&Control ($p < 0.01$). Data showed as mean ± SEM, a: comparison with Control group, b: comparison with $DZN_L$ group. $P < 0.01$, Tukey-Kramer test, $n = 10$.

overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and emptying antioxidant capacity [2,26]. Various factors including the high rate of oxidative metabolic activity, the abundance of oxidizable polyunsaturated fatty acids, the high levels of redox-active metals (iron and copper), and restricted cell replenishment made the brain a particularly vulnerable organ to oxidative damage [27–31]. In this study, the evaluation of oxidative stress parameters was carried out in cortical and hippocampal regions which are believed to be involved in learning and memory [31]. Our results indicated that DZN at low and medium doses increases MDA levels in both CX and HP [31]. Besides elevation in MDA production, the decrement of total antioxidant capacity due to DZN exposure confirms that DZN can make the brain defenseless to oxidative stress damage [2,30]. Interestingly, the activity of antioxidative power in the CX was higher than HP. It is assumed that a greater redox imbalance that occurs in the CX leads to the enhanced compensatory mechanism [30]. Regional differences following DZN-induced oxidative stress in the brain could be explained by the fact that neuron density in the CX is higher than HP. Neurons are particularly sensitive to oxidation. One other point is the ratio of astrocytes to neurons which is lower in the CX than HP. Astrocytes contain higher levels of endogenous antioxidants made them more resistant to oxidative stress than other neural cell types, so the lower ratio of astrocytes to neurons in the CX plays a main role in increasing susceptibility to oxidative stress in this region [32,33]. The second potential explanation for regional differences in the effects of DZN on oxidative stress in the brain may be related to the impacts of DZN on AChE [34]. Despite the higher density of ACh neurons in HP than CX, the activity of AChE was discovered to be dropped in the CX and to a lesser extent in HP in DZN-treated mice which made CX more susceptible to oxidative stress. The action of various brain areas was increased with subsequent ATP and creatine phosphate reduction in the neurons due to long-term inhibition of AChE. AChE inhibition also damaged oxidative phosphorylation followed by neuronal $Ca^{2+}$ influx and activation of nNOS, connected with oxidative and nitrozative injury of the neurons [1,35]. Although the role of AChE in the pathogenesis of neurodegeneration is still not completely understood [36], mounting reports explained reduced activity of AChE in several brain disorders, including neurodegenerative disorders [21,37–39].

Our finding determined that at the end of the 9th week, DZN-treated mice lost body weight comparing with control mice, respectively. It was deliberated that reduced food and water intake on account of losing appetite may be associated with brain and plasma cholinesterase inhibition. Furthermore, metabolic disturbances due to Ops exposure lead to increased degradation of lipids and protein catabolism. The significant weight loss could be related to the fact that lack of nutrients which causes further metabolic stress, maybe ended up in brain dysfunction [40]. Contradictory, only a few studies have unexpectedly observed OP-exposed mice tend to gain weight, but it should be noticed that it was more related to visceral lipid deposition [41]. Besides, our findings showed a reduction in absolute brain weight at the end of the investigation. It has been widely discovered that aging causes macroscopic atrophy leading to volume and weight loss of the brain. The aging brain exhibits various changes some associated with neurodegenerative diseases [42]. Brain changes do not take place to the same extent in all regions. For instance, AD predominantly affects the CX, and PD predominantly involves the basal ganglia [43]. These are in agreement with our findings that the weights of the CX and HP were significantly reduced, although the CX was most affected. It might be related to neuronal loss in the CX and HP. AD and PD are neuropathologically characterized by neuronal loss in CX and HP, respectively [44]. In this regard, our histopathological examination of CX and HP in DZN-treated mice showed mild vascular congestion and ischemic neuronal changes such as shrinkage of neuronal cell bodies, more eosinophilic cytoplasm, reduction of nucleus size, and pyknosis. No infiltration of inflammatory cells was seen in the brain sections. According to the histopathologic findings, AChE activity, oxidative stress determinants, and weight changes, the CX and HP seem to be degenerated in DZN-treated mice.

OPs also alter the expression of genes related to synthesis, storage, and degradation of neurotransmitters systems such as cholinergic, GABAergic, serotonergic, glutaminergic, and endocannabinoids [4]. For this purpose, we assessed gene expression changes associated with neurodegeneration including *VAChT*, *GAD*, and *SYP* following subchronic low-level exposure to DZN. We observed that genes associated with neurodegenerative disorders were significantly affected by DZN exposure in both the CX and HP. Vesicular ACh transporter encoded by the *VAChT* gene is a neurotransmitter transporter accountable for loading ACh into secretory organelles in neurons and making ACH attainable for secretion. Alterations in the ACh innervation, synthesis, breakdown, and reuptake that cause cholinergic metabolism malfunction have been reported in AD [16,45]. In this study, after low-dose DZN treatment, *VAChT* expression was upregulated, while in the medium dose, the gene expression was downregulated in the CX and HP. It is speculated that the upregulation of *VAChT* may be related to a compensatory mechanism due to the lack of ACh release. In $DZN_M$-treated mice, it is postulated that cholinergic neuronal and synaptic loss mediated by excessive oxidative damage leads to transcriptional attenuation [46].

*SYP*, a major integral membrane protein of the synaptic vesicle, is got involved with neurotransmitter release and synaptic vesicle cycle. *SYP* is closely related to reorganization and strengthening synapses [14]. We found that *SYP* transcripts severely decreased in both brain regions. The CX was more affected in $DZN_M$-treated mice. In other words, it has been deduced that there had been regional gene expression within the brain. Synaptic degeneration might be triggered by the down-regulation of the *SYP* gene to irreversible stages associated with marked synapse loss [47]. Earlier researchers used electron microscopy and biochemical analyses for evaluating *SYP* in AD biopsies and autopsies. Their findings prove that synaptic loss in the CX and HP is an early event [14].

GABAergic interneurons play a key role in the circuity of the CX and HP. *GAD* is the synthetic enzyme for the inhibitory neurotransmitter GABA and its presence is a reliable and specific marker for GABA neurons. GABA is an initial inhibitor transmitter in mammalian CNS which is critically important in the responsiveness and synchronization of cortical neurons to subcortical networks [48]. Our result in the medium-dose DZN reveals that *GAD* genes were down-regulated in the CX, whereas up-regulated in the HP. However, the expression levels of the low-dose group were unchanged compared with control mice in both brain regions. We identified significant regional differential *GAD* expression in both defined brain regions due to DZN exposure compared with the control group. The role of GABAergic dysfunction in AD and PD is a poorly understood and contentious issue. The findings of these studies are difficult to interpret due to their often–contradictory. Moreover, an antemortem agonal state like acidic conditions may substantially affect *GAD* activity. Some research exhibited that GABAergic neurotransmission is depressed in the prefrontal CX in PD [49]. While recent studies in PD cases and MPTP-treated primates demonstrated an increased *GAD* mRNA in GABAergic neurons of the basal ganglia [17]. Despite the controversies, alterations in GABAergic pathways such as GABA levels, GABA receptor distribution, GABA transporters, and *GAD* expression can be broadly observed in neurodegenerative disorders [15,18].

OPs exposure has been also linked with neurobehavioral disorders, such as mood changes, memory disturbances, learning deficits, cognitive impairment, and even neuropsychiatric changes like mania which are clinically characterized by AD and PD diseases [1,3,50]. The mechanism of neurobehavioral symptoms following long-term low-level exposure is not yet clearly identified. Probably, in OPs exposure the excess ACh merely disturbs the neurotransmitter systems balance in cortical and subcortical regions or alters the ACh feedback loop [1,25]. Another mechanism that may be hypothesized to explain neurological sequelae is attributed to imbalances between provocative and suppressive neurons in different areas of the brain [50]. Significant alterations in behavior

and memory were noted in DZN-treated mice using behavioral tests. The result obtained from the MB test revealed that subchronic exposure to medium-dose DZN accentuated anxiety-like behavior observed by increased digging. These findings were parallel to previous reports about the association of OPs exposure and anxiogenesis [1,3]. However, questions regarding the specificity of MB as an indicator of anxiety alone have been raised due to studies illustrating a reduction in the number of buried marbles following OPs exposure [3]. The neuronal circuity of this behavior has not been elucidated. The HP, nucleus accumbens, and dorsal raphe nucleus are likely to be important since lesions in these areas produce anxiety [51]. The induced anxiety by OPs was attributed to cholinergic neuronal overstimulation due to AChE inhibition as well as secondary serotonergic and GABAergic neuronal damage induced by oxidative stress implicated in many neurological disorders [52,53]. Moreover, our result revealed that DZN-treated mice exhibited meaningful depression-like behavior. The outcomes were in accord with other researchers who showed that chlorpyrifos, DZN, and methamidophos at neonatal age and adulthood exhibited a depression-like phenotype [1,3]. Depression is not only a homogenous disorder but a complex phenomenon. Many interactions within brain systems need to take into account for the understanding of the pathophysiology of depression. Doubtlessly, a dysfunction in the monoaminergic system is a cornerstone mechanism in depression. Also, under pathological conditions in the CX and HP, increased cyclooxygenase-2 (COX-2) related to inflammation produces toxic amounts of peroxides and ROS that may additionally contribute to oxidative damage [54]. Depression is also associated with oxidative stress by carbonylation of protein components of cytoskeleton which leads to disruption of cytoskeleton morphology and consequently dendritic regression in the HP, one of the prominent features of depression [1,29,52]. Our data support a link between oxidative stress and depressive-like behavior in DZN-treated mice. In the present study, DZN-treated mice demonstrated significant impairment of spatial learning and memory in the MWM test. In light of the close relationship between HP and learning and spatial navigation, it is deduced that the DZN-induced oxidative stress in HP may underlie the impairment in learning and memory tasks [24]. It has also been proposed that the proliferation of neural connections by increasing dendrite and axon branching in the CX may correlate with learning and intelligence. The reduction in synaptic vesicle proteins due to *SYP* gene expression changes is likely related to cognitive disorders similar to AD and PD, gives rise to vesicle trafficking disturbances [30,51,55].

It can be concluded that the subchronic administration of 0.1 and 1 mg/kg DZN impairs cognitive function induced by neuronal damage in HP and CX. This effect might be mediated by oxidative stress [10,56], AChE inhibition [5] as well as regional differential *SYP*, *VAChT*, and *GAD* gene expression. Also, these results may contribute towards a better understanding of the underlying mechanisms of neurodegeneration induced by long-term exposure to OPs, elucidating the role of pesticide residue in the human diet in predisposition to neurodegenerative diseases. Further studies are required to more fully characterize whole-brain genomic changes after DZN exposure. Moreover, additional experiments with different types of OPs and different time points would be advantageous in creating toxic footprints of all types of OPs.

## Authors' contributions

- Asieh karimani, designing the study, writer of the primary draft of the manuscript, and following up the procedure, analyzing the data, approving the final version of the manuscript.
- Nasrin Ramezani, interpreted of data, revising the intellectual content, approving the final version of the manuscript.
- Amir Afkhami Goli, interpretation and analysis of data, approving the final version of the manuscript.
- Mohammad Hossein Nazem Shirazi, gathering data, interpretation of data, approving the final version of the manuscript.
- Hosein Nourani, data analysis, approving the final version of the manuscript.
- AmirMoghaddam Jafari, primary idea, designing the study, writer of the primary draft of the manuscript and following up the procedure, revising the intellectual content, approving the final version of the manuscript.

## Funding

This work was supported by the veterinary faculty, the Vice-Chancellor of Research, Ferdowsi University of Mashhad, and the Veterinary Head Office of Khorasan Razavi, Mashhad, Iran.

## Declaration of Competing Interest

The authors report no declarations of interest.

## Acknowledgment

The authors appreciate Afsaneh Moghaddam Jafari for her help in the study.

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