

Research Paper

Involvement of Mice Hippocampus Brain-derived Neurotrophic Factor in Diazinon-induced Depressive Behavior in Mice



Mehdi Aliomrani¹, Azadeh Mesripour^{2*}, Tannaz Daneshseta¹

1. Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.
2. Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.



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* Corresponding author:

Azadeh Mesripour, Pharm. PhD.

Address: Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

E-mail: mesripour@pharm.mui.ac.ir

ABSTRACT

Background: Diazinon (Dzn), an Organophosphorus (OP) pesticide, is extensively used in agriculture. Acetylcholinesterase inhibition is linked to OP toxicity, and there are major mental health concerns associated with the use of pesticides. The objective of this study was to assess the depressive behavior in an animal model following their exposure to Dzn and the effect on the Brain-Derived Neurotrophic Factor (BDNF) as a critical neurotropic factor.

Methods: Male Swiss mice (N=42; 25±3g each) were used and their behaviors were examined on including the locomotor, Forced Swimming (FST), and Sucrose Preference (SP) tests. These tests were performed the day after a single daily Dzn administration by gavage (2.5-20 mg/kg). Specific animal groups were exposed to Dzn daily (2.5-10 mg/kg) for 14 days, and a test was performed on days 7 and 15.

Results: Following the acute exposure to Dzn, the animals' locomotor activity did not change significantly. During the FST, Dzn at 20 mg/kg significantly increased the animals' immobility time, indicating despair behavior. Imipramine, injected intraperitoneally at 10 mg/kg, did not cause the depressive behavior. The subacute exposure to Dzn induced less locomotor activity than that of the controls. The 7-day exposure to Dzn at 10 mg/kg significantly prolonged the immobility period compared to that of the controls. The 14-day Dzn exposure at 2.5, 5, or 10 mg/kg increased the immobility time significantly compared to that of the controls. None of the treatment groups showed SP, clearly showing animal anhedonia. The BDNF levels significantly decreased not only by subacute exposures to Dzn but also following a single exposure to this pesticide.

Conclusion: The acute and subacute exposure to Dzn induced depressive behavior and increased the BDNF levels in the hippocampus of Swiss male mice following exposure to Dzn at varying doses of 2.5, 5, or 10 mg/kg.

Keywords: Acetylcholinesterase, Brain-derived neurotrophic factor, Depression, Diazinon, Organophosphorus agents

Introduction



Organophosphorus (OP) pesticides are used most widely in agriculture. These toxic compounds function by inhibiting Acetylcholinesterase (AChE), leading to

an increase in Ach, which is an essential neurotransmitter in the nervous system. Some well-known pesticide varieties that belong to this family include Diazinon (Dzn), Malathion, and Phosmet [1]. An earlier cross-sectional study has demonstrated that chronic exposure to OP pesticides decreases the AChE activity and in-

creases the risk of adverse effects, such as muscle pain, headache, liver and renal dysfunctions in addition to increasing the lipid peroxidation levels in farmworkers [2]. There are major health concerns about the availability and use of pesticides, since exposure to OP agents may be associated with mental disorders [1]. It has also been shown that female farmers who are not certified OP users, have shown increased chances of developing depressive symptoms [3].

Although AchE inhibition is related to the primary toxic action of OP insecticides, oxidative stress may also be responsible the toxicity [4]. The neurotransmitter serotonin (5-HT) is known to be involved in the development of anxiety and mood disorder pathology [5]. Also, the neurotoxicity developed by OP pesticides is known to target the serotonin-linked systems [6]. Findings from an earlier study indicate that different OP agents have differing effects on neurotransmitter systems although they exhibit similar effects as AchE inhibitors [6]. An OP compound that is extensively used in agriculture as a soil pesticide is Dzn (O,O-diethyl-O-[2-isopropyl-6-methyl-4-pyrimidinyl]phosphorothioate) [7]. One major concern about Dzn is that it is absorbed by vegetables and fruits, and is potentially harmful when consumed by humans. Therefore, the European Union regulation has set the maximum residue limits of Dzn in fruits at 0.01 mg/kg [8].

Brain-Derived Neurotrophic Factor (BDNF) is one of the neurotrophic proteins found abundantly in the Central Nervous System (CNS) [9]. A decline in the production of BDNF in the hippocampal neurons is associated with severe stress, impaired learning, and abnormal inspiration and mood [9]. Further, changes in the serotonin and Norepinephrine (NE) neurotransmitters in the hypothalamus are also linked to decreases in growth factors, such as BDNF and increases in inflammatory cytokines in the CNS [5, 10]. Probable effects of Dzn on the CNS neurotransmitters raise the occurrence of depressive symptoms that are not clear and need to be investigated when evaluating the neurotoxic aspects of OP chemicals.

Aim of the study: We investigated the effect of acute and subacute exposure of Dzn on the depressive behaviors in rodents. We also examined its effect on the brain BDNF levels in the hippocampus of the Swiss mice. This article reports the promising findings for the first time.

Materials and Methods

Animals: We used forty-two Swiss male mice weighing 25 ± 3 g each, aged 6-8 weeks old and kept under standard conditions of humidity, temperature and light/

dark cycle with free access to pellet food and filtered and disinfected drinking water. The animals were adapted to their experimental environment at the pharmacology laboratory 24hr before starting the study. The tests were performed in the morning up to 1 p.m. All of the experiments were performed according to the Care and Use of Laboratory Animals guidelines issued by National Ethics Committee of Iran (Code: IR.MUI.REC.1399.110). Changes in the mice's weight and food consumption were evaluated during the study to monitor the animals' health and well-being.

Chemicals: The two chemicals for this study were obtained from the following suppliers: Dzn from Kavosh Kimia, Iran batch no: 635485 (Tehran, Iran), and the reference antidepressant drug, imipramine hydrochloride, from Sigma-Aldrich (Delhi, India).

Study design: The 42 mice were randomly assigned to five groups of seven animals each (Flowchart 1). Four groups of mice were given a single dose of Dzn at 2.5, 5, 10 or 20 mg/kg by gavage feeding tube. One group received a dose of imipramine at 10 mg/kg (1ml/100g) by Intraperitoneal (IP) injection after exposure to Dzn at 20 mg/kg. The behavioral tests were performed on the next day to assess the acute toxicity of Dzn. To assess the subacute toxicity, other mice groups received Dzn at 2.5, 5 or 10 mg/kg once daily for 14 consecutive days. The behavioral tests were conducted in these groups on day 7 before the daily exposure to Dzn and again on day 15, after the last Dzn exposure. The control mice received water instead of Dzn. After the final behavioral assessment of acute and subacute toxicity, the mice were promptly decapitated and the whole brain was removed, weighed and the hippocampus was dissected, and kept on ice at -70°C until the biochemical assays were performed.

BDNF levels: Three Hippocampal regions from each group were homogenized in cold Ripa buffer containing Tris, NaCl, Triton-X 100, BSA, and PMSF at pH 7.4. The BDNF levels in each animal group was measured, using Picokine mice Enzyme-Linked Immunosorbent Assay (ELISA) kits (Toronto, Canada), based on the supplier's instructions. All samples were checked in triplicate test tubes. Initially, the samples' supernatants were separated from the homogenates by centrifugation (3000 RPM, 25 min, 4°C). One hundred μL of each supernatant was added to the ELISA plate based on the previously prepared plan. After 90 minutes of incubation and washing the wells, the biotinylated anti-mice BDNF was exposed to the samples for 30 minutes and incubated at 37°C . After the final washing with Phosphate-Buffered Saline (PBS), the colorogenic substrate (3,3',5,5'-tetra-

methylbenzidine, TMB) was added and the enzymatic reaction was allowed to develop for 30 minutes in dark. After adding the stop solution, the solution's color change intensity in each well was measured at 450nm, using a Synergy HTX microplate reader (BioTek, USA). The data were normalized based on the protein content and presented as picogram per milligram of the protein.

Locomotor activity test: The mice locomotor activity was measured at the beginning of the initial stage of the behavioral experiments by an open field apparatus (Borj Sanat, Iran) with red beams across its arena floor, dividing it into 15 specific zones. The mice were gently placed at the corner of the arena to explore the area for three minutes. The numbers of horizontal movement for each animal were counted automatically anytime the animals crossed the red beams, and stopped on hind-legs. The total activity for each mouse was the sum of these movements.

Forced Swimming Test (FST): Each mouse was placed in a 2-liter glass beaker filled with water (25°C) for 6 min, forcing it to swim. After two minutes of habituation period, the FST assessment was performed for four minutes. The immobility time during this test was identified as the despair behavior. This was considered when animals had no activity unless they kept their head above the water. The swim period was considered when the animals moved horizontally in the water involving two or four limbs. The climbing part was considered when the animals moved vertically by clinging on the sides of the beaker [11]. A camera mounted above the container recorded the experiment for later analyses. At the completion of this test, animals were dried in towels to prevent hypothermia and were returned to their cages.

Sucrose preference test: This test started from day-12 onward in the subacute groups on three successive days. Two sequential days were assigned for habituation and the last day was spent for completing the remaining assessments. On the first day, the mice had access to two bottles containing sucrose solution (2% w/v) and on the following day, one of the bottles was filled with water. On day-15, the bottles contained certain amounts of sucrose solution or tap water, and the amounts consumed from each bottle was measured after 24hr. The percentage of Sucrose Preference (SP), representing the animals' anhedonia as another criterion for depression, was determined based on the following formula: sucrose consumption/water plus sucrose consumption $\times 100$ [12].

Data processing and statistical analyses: The results are presented as group Means \pm SEM and evaluated by one-way Analysis of Variance (ANOVA), and Tukey's

post hoc test. The statistical data analyses were done, using Excel 2010 and the GraphPad Prism 8 (La Jolla, CA, USA) software programs. The $P < 0.05$ were considered statistically significant.

Results

Weight changes and food consumption: As it is shown in Figure 1a there was a 19% reduction in food consumption following 14 days of exposure to Dzn 5 and 10 ($P = 0.03$, vs control group). According to Figure 1b animals did not gain weight especially following exposure to Dzn10, therefore Dzn20 was not used for subacute exposure.

Effect of acute Dzn exposure on the mice depressive behavior: As reflected in Table 1, the total locomotor activity following exposure to varying doses of Dzn was not significantly different from that of the control group. The effect of acute Dzn administration on immobility periods during the FST is shown in Figure 2. The exposure to Dzn-20 significantly increased the immobility periods (161.4 \pm 4.93 sec. vs 120.0 \pm 6.64 sec. in the controls ($P < 0.001$). Imipramine prevented Dzn-20 immobility initiation and reduced immobility duration (93.67 \pm 6.83s vs Dzn- 20; $P < 0.001$). Table 1 also summarizes the swimming and climbing activities during the FST. The swimming activity of Dzn 10 and 20 was significantly less than that of the control group ($P < 0.05$). Except for Dzn-10 group that demonstrated slightly more climbing activity than that observed in the controls, the climbing activities for other Dzn doses were insignificantly lower than those noted in the controls.

Effect of subacute Dzn exposure on mice depressive behavior: As shown in Table 2, after 7 days of Dzn-5 ($P = 0.01$) and Dzn-10 ($P = 0.002$) exposure, the total activity count was considerably less than that noted in the control group. Also, after 14 days, the locomotor activities were less than those of controls for all Dzn doses; being significantly less for Dzn-2.5 ($P = 0.012$) and Dzn10 ($P = 0.042$). As reflected in Figure 3, the mice's immobility periods in FST increased dose-dependently after 7 and 14 days. The immobility periods after 7 days of exposure to Dzn-10 was significantly higher than that noted for the controls (173.4 \pm 3.63 sec. vs 127.6 \pm 3.30 sec.; $P < 0.001$). The mean immobility period for Dzn-10 was also significantly longer than that noted for Dzn-5 (156 \pm 6.63 sec.; $P = 0.045$) and Dzn-2.5 (152.0 \pm 3.51 sec.; $P = 0.01$). After 14 days of exposure to Dzn at 2.5, 5, or 10 mg/kg, the immobility periods were significantly longer than that of the controls (130.1 \pm 2.0 sec., $P < 0.001$). Immobility time for Dzn 5 (176.0 \pm 5.0 sec.) was significantly greater than

Table 1. Total locomotor activity, swimming, and climbing time during FST following diazinon acute exposure

Groups (n=7)	Total Activity (Unit)	FST	
		Swimming Time (Sec)	Climbing Time (Sec)
Control	174.9±15.35	83.5±7.75	36.5±13.79
Dzn-2/5mg/kg	127.8±15.53	87.0±6.35	13.0±7.65
Dzn-5 mg/kg	128.3±11.56	86.67±10.41	11.5±9.21
Dzn-10 mg/kg	149.7±12.43	56.33±4.22*	43.14±6.98
Dzn-20 mg/kg	164.6±26.59	60.29±5.47*	18.29±5.9
Dzn-20 mg/kg +Imp-10 mg/kg	208.5±8.15	108.3±5.86 ^{***}	38±11.14

Total activity in the locomotor test equals horizontal plus vertical exploration. Control animals; 0.9% saline. Results are presented as Mean±SEM and evaluated by ANOVA and Tukey's post hoc analysis. *P<0.05 compared to control group, ^{***}P<0.001 compared to Dzn-20. Dzn: Diazinon, Imp: Imipramine, FST: Forced Swimming Test.

Dzn 2.5 (160.3±3.67 sec., P=0.0264) and the value for Dzn-10 (179.9±3.02 sec.) was higher than for Dzn-2.5 (P=0.003). The swimming and climbing periods during FST, as summarized in Table 2, show lower values following subacute exposure to Dzn than that of the control group. After 14 days, the swimming periods of Dzn-5, and 10 were significantly shorter than that of the controls. The depressive effect of Dzn was also reflected by the SP results, clearly showing that the animal experienced anhedonia. The SP was 63.63% for the controls and following exposure to Dzn at 2.5, 5 or 10 mg/kg, the SP decreased to 43.46%, 37.60%, 26.04%, respectively.

Brain BDNF levels: As shown in Figure 4, following a single Dzn exposure the BDNF levels were significantly lower compared to that of the control group (821.6±68.20 pg/mg). Administering imipramine following exposure to Dzn-20 (502.1±22.4 pg/mg), almost doubled the BDNF level (1046±116.3 pg/mg, vs Dzn 20, P<0.001).

After 14 days of exposure to Dzn-2.5 (525.2±21.2 pg/mg, P=0.014), Dzn-5 (484.9±8.6 pg/mg, P=0.005), and Dzn-10 (564.6±8.5 pg/mg, P=0.024), the BDNF levels were significantly lower than that noted in the control group.

Discussion

In this study, we discovered that observed that exposure to Dzn caused depressive behavior in mice not only after multiple exposures but also due to a single dose of this drug. The BDNF level in the hippocampus was lower than normally expected concentration following both acute and subacute exposures to Dzn. During the subacute exposure, signs of anhedonia, i.e., inability to feel pleasure, were evident in mice, suggesting the sense of despair which is another form of depression. Also, some behavioral and physical characteristics were observed in mice that were indicative of acetyl cholinesterase inhibition most likely by Dzn. These include reduced food and

Table 2. Total locomotor activity, swimming, and climbing time during FST following diazinon subacute exposure

Group (n=7)	Total Activity (Unit)		FST			
			Swimming Time (sec)		Climbing Time (sec)	
	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15
Control	214.4±13.4	183.7±15.4	81.29±10.1	91±8.7	31.14±10.36	18.86±9.1
Dzn-2.5 mg/kg	176±12.5	122.5±14.1*	87±3.27	79±3.44	1±0.68*	1.17±0.54
Dzn-5 mg/kg	146.7±17.8*	150.7±10.3	78.67±4.9	59.83±3.62*	5.33±2.78	4.17±2.66
Dzn-10 mg/kg	135.9±15.2**	135.1±13.5*	59±4.48 [#]	52.43±3.4 ^{****}	7.57±3.23	7.57±3.57

Total activity in the locomotor test equals horizontal plus vertical exploration. Control animals; 0.9% saline. Results are presented as Mean±SEM and evaluated by ANOVA and Tukey's post hoc analysis. *P<0.05 compared to control group, [#]P<0.05 compared to Dzn-2.5; Dzn: Diazinon, FST: Forced Swimming Test.

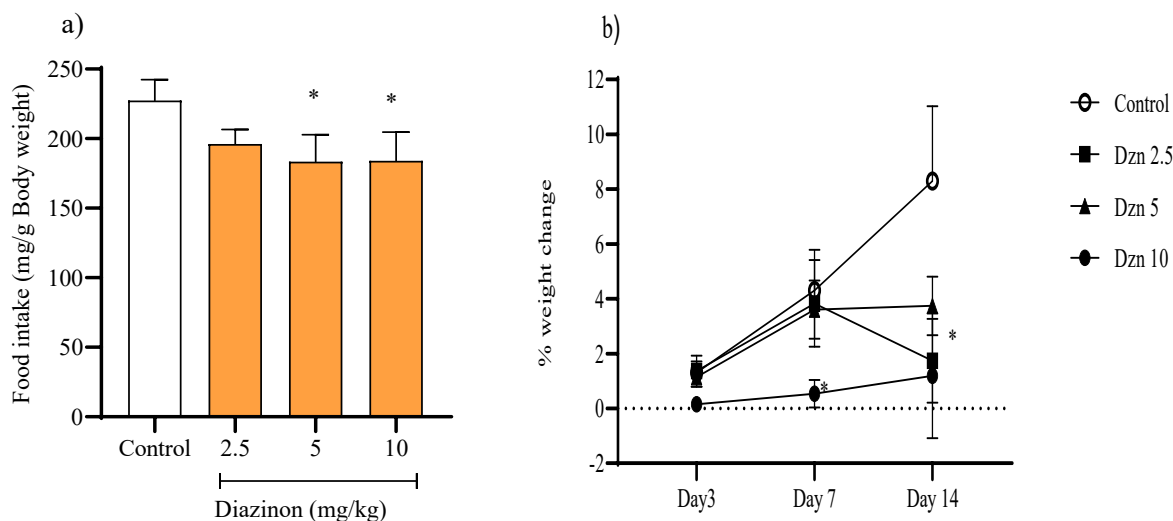


Figure 1. Effect of Dzn sub-acute exposure on food consumption (a) and weight changes (b)

Control animals; 0.9% saline. Results are presented as group (n=7) Mean±SEM and evaluated by ANOVA and Tukey's post hoc analysis. *P<0.05 compared to control group.

water intakes, weight loss, and low defecation frequency in the mice that were treated with

Diazinon exposure dose-dependently augmented immobility periods of the animals during FST, further suggesting the sense of despair in the mice. This side effect was reversed after imipramine administration, which is a tricyclic antidepressant drug. This finding was in agreement with the results reported previously for phosalone

pesticide (an OP ester) makes animals exhibit despair behavior after a single exposure or after one-week of exposure. These changes lasted for a week after the drug exposures ended [13]. Exposure of female Wistar rats to malathion for three days has also remarkably increased their immobility period during FST [14]. Depending on the route of exposure and the bioavailability, OP compounds enter the blood following their absorption. Because of their lipophilic property, OP agents are usually

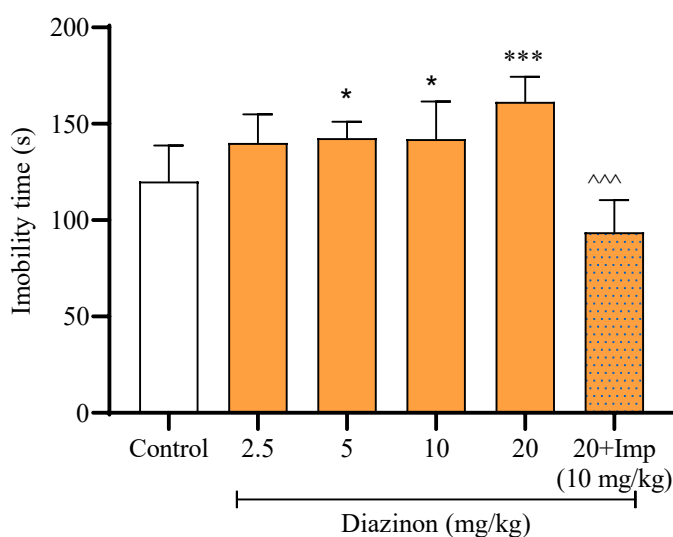


Figure 2. Effect of diazinon acute exposure on immobility time during FST

Control animals; 0.9% saline. Results are presented as group (n=7) Mean±SEM and evaluated by ANOVA and Tukey's post hoc analysis. *P< 0.05, ***P<0.001 compared to control group, ^^^P<0.001 compared to Dzn 20. Imp: Imipramine.

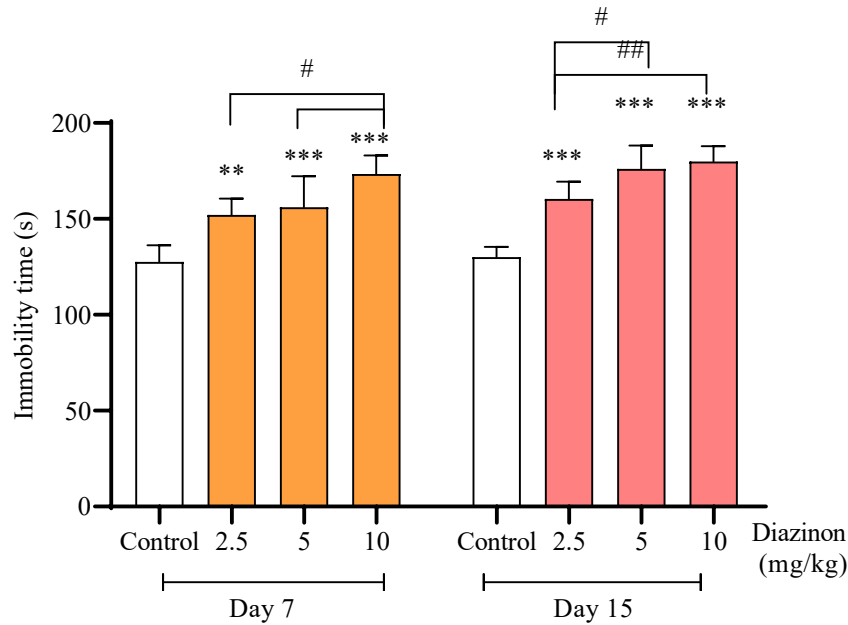


Figure 3. Effect of diazinon subacute exposure on immobility time during FST

Control animals; 0.9% saline. Results are presented as group (n=7) Mean±SEM and evaluated by ANOVA and Tukey’s post hoc analysis. **P<0.01, ***P<0.001 compared to control group, #P<0.05, ##P<0.01 as shown in the figure.

distributed in the body, particularly within fatty tissues [15]. The mechanism of toxicity and pharmacological target of OP pesticides arise by the inhibition of AChE. After this inhibition, ACh accumulates in synapses,

which stimulates the cholinergic receptors, leading to muscarinic, nicotinic and CNS manifestations [15].

As has been shown earlier, the depressive effect that arises from exposure to Phosalone is due to its inhibi-

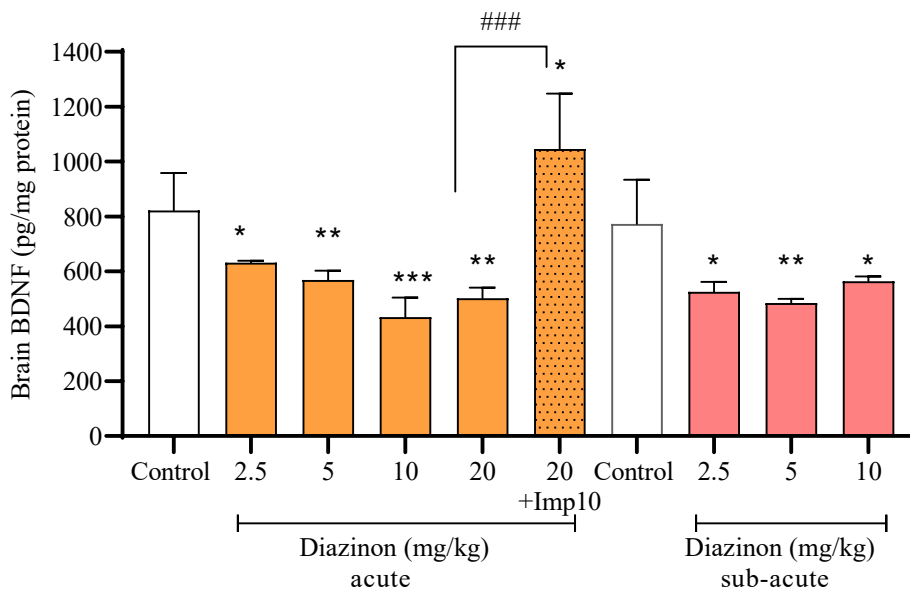


Figure 4. Normalized brain derived BDNF level. Control animals; 0.9% saline

Results are presented as group (n=5) Mean±SEM and evaluated by ANOVA and Tukey’s post hoc analysis. *P<0.05, **P<0.01 compared with the relevant acute or sub-acute control group, ###P<0.001 as shown in the figure.

tion of muscarinic Ach receptors, causing despair behavior, similar to what occurs following scopolamine administration, which is also a muscarinic Ach receptor antagonist [13]. In addition, the inhibition of AChE, the BDNF levels in the hippocampus decreased significantly following a single dose of Dzn at all of the tested doses (2.5-20 mg/kg) in the mice. The decreased levels of BDNF could be a good reason for the development of depressive behavior in the mice.

Anxiety and mood disorders are known to be associated with the modifications of serotonin system in humans [5]. As it has been established previously, selective serotonin reuptake inhibitor antidepressants, such as fluoxetine, decrease the immobility period in mice while increasing their swimming time [16]. Further, antidepressants that influence the catecholamine system mainly increase the climbing time in rats and other experimental animals [16]. In the current study, we observed a dose-dependent decline in the swimming time of rats after acute exposure to Dzn. This suggests that the drug may have a role in serotonin system. In 2016, Judge and colleagues reported an 80% decline in raphe nuclei serotonin (5-HT1A) receptor sensitivity after exposing male Lister rats to a low dose of Dzn [17].

In the current study, the immobility period in rats increased on FST following subacute exposure to Dzn. Also, anhedonia was manifest in the rats during the SP test. In addition, Dzn prevented weight gain and reduced food intake in rats. Due to the potential effect of subacute Dzn-10 exposure in preventing animals from gaining weight, we did not treat the animals with multiple doses of Dzn-20.

Further, exposure to malathion has been shown to prevent weight gain and reduce food intake in rats compared to those of the controls [18]. This could be due to its antagonistic effect on the muscarinic Ach receptors in the gastrointestinal smooth muscles [19]. In this study, the hippocampal BDNF level was lower than that of the controls after 14 days of exposure Dzn. Although the rats' locomotor activity declined following the subacute exposure to Dzn, the anhedonic behavior and the decreased BDNF levels imply the development of despair behavior during FST.

Previously, after the rats received a single IP injection of Dzn they were sacrificed on the following day (18±1h) for the electrophysiologic experiments, which subsequently demonstrated that the increased release of serotonin might have been due to the down-regulation of 5-HT1A autoreceptors [17]. That could be another reason that exposure to OP induced depressive behavior

in mice. Similar to BDNF, modifications in 5-HT signal transduction have shown to influence neurogenesis and synaptic plasticity in the hippocampus [20, 21]. The activation of 5-HT1A autoreceptors has a vital role in stimulating neurogenesis in the hippocampus, an essential role for the therapeutic efficacy of antidepressant drugs [20, 21]. Interestingly, it is suggested that BDNF stimulates the differentiation and function of serotonin neurons [22]. Further, serotonin also exerts much control over BDNF expression [23]. Therefore, depressive behavior caused by Dzn is likely to be linked to a reduction in BDNF and probably to its interactions with the serotonergic system.

Conclusions

This study demonstrated that acute or subacute exposure to Dzn could induce depressive effects in Swiss male mice. Based on the findings, we also conclude that the BDNF production in the hippocampus has a critical role in the neurobehavioral changes induced by Dzn. We speculate that in addition to the AChE inhibition, the Dzn toxicity also influenced interactions between 5-HT and BDNF. Lastly, the findings of this study raised serious concerns about the use of Dzn in agriculture.

Limitations of the study: A drawback of our study was that the neurotransmitters were not evaluated; however, since there was an obvious decline in the swimming time, we assume that serotonin was most likely involved in the development of the observed depressive behavior [16]. This has been established before through observing that AChE inhibition by Dzn occurred the most in the dorsal raphe nucleus, that is the main origin serotonin-secreting neurons in the brain [20].

Recommendations for future studies: Investigations on the Dzn exposure could be conducted in rodents as a broad model for the study of depression in animals, by which the BDNF level in the hippocampus may be reduced. Evaluation of the neurobehavioral changes in mice, and the associated molecular and genetic factors are recommended following chronic exposure to Dzn.

Ethical Considerations

Compliance with ethical guidelines

Compliance with ethical guidelines of The National Ethical Committee of Iran (Code: IR.MUI.REC.1399.110)

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Authors' contributions

Conceptualization, methodology, funding acquisition and study supervision were provided by AM & MA. Investigations and the writing of the initial drafts of the manuscript were done fairly equally by the contribution of all authors. Writing of the final draft, review and editing were performed by all authors supervised by AM.

Conflict of interest

The authors declare no conflict or competing interests with any internal or external entities in conducting this original research.

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