ToxEHS

Prenatal Exposure to Diazinon Induced Developmental Impairments in Rat Offspring: Behavioral and Biochemical Aspects

Hakima Tayaa[1,2], Imed Eddine Bouhali[1] & Abdelkrim Tahraoui[1]

[1]Laboratory of Applied Neuro-endocrinology, Department of Biology, University of Badji Mokhtar, BP 12, 23000, Annaba, Algeria
[2]University Center of Mila, Department of Biology, Algeria
Correspondence and requests for materials should be addressed to H. Tayaa (tayaa.hakima@yahoo.fr, imedsami2009@hotmail.fr)

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Abstract

Prenatal acute and chronic exposure to organophosphorus pesticides may evoke physical and behavioral impairments in offspring development. In this report, pregnant Wistar rats were intraperitoneally treated with diazinon (DZN) (10 mg/kg b.w) from G7 to G14 of pregnancy. The animals (males and females) were randomly chosen from the litters. They were observed afterwards for physical (body weight change, incisor eruption and eye opening), neuromotor and behavioral development (surface righting reflex, palmar grasp reflex, negative geotaxis, forelimb support test, open field test and Morris water maze) and brain acetylcholinesterase activity was measured. The Results showed differences in physical and neuromotor parameters and brain acetylcholinesterase activity between treated and control groups. At the adolescence period, the open field test showed hyperactivity, as well as an enhancement of an exploratory state associated with anxiolytic-like effect. The Morris water maze showed an enhancement of spatial memory. We conclude that DZN at high doses was able to disrupt physical and neuromotor development in pups during lactation and also modify behavioral and cognitive state at adolescence period. This may have been linked with organophosphorus mediated inhibition of central nervous system acetylcholinesterase activity.

Keywords: Diazinon, Offspring, Prenatal, Developmental behaviour, Rat

Introduction

Organophosphorus compounds (OPs) are environmental contaminants widely used in pest control. OPs irreversibly inhibit acetylcholinesterase (AChE) activity in the central and peripheral nervous systems and lead to cholinergic synaptic hyperstimulation[1]. Particularly in developing countries, poor working conditions and lack of awareness of the potential hazards of OP's lead to acute and repeated chronic intoxication that evokes a consistent pattern of physical and neurobehavioral symptoms, such as depression, anxiety and cognitive impairments[2]. However, it is also emerging that trace environmental levels of OP's are acting as developmental neurotoxics, at human embryonic, foetal and neonatal stages of life, as well as during the early years of childhood[3-6]. Indeed, OP toxicity is believed to contribute to higher functional of central nervous system (CNS) disorders, such as behavioral abnormalities in infants, as well as other issues in older individuals such as anxiety and mood disorders[7,8]. Reports concerned with biomonitoring of OP exposure in inner cities and farming communities[9,10] have already led to regulatory restrictions regarding the residential use of certain agents (e.g. DZN, chlorpyrifos) and they have heightened concern over the potential cumulative neurotoxic effects of these agents on children[11,12].

Although the implications of trace environmental exposure to OP's have been intensively studied, there are also many documented cases of human exposure to high acute exposure to these agents; these include incidents where large amounts of OP's have been released into the environment, such as after the destruction of the Khamisiyah Munitions Facility in Iraq 1991 and the Tokyo subway sarin attack of 1995[13]. Those exposed to acutely high levels of OP agents in these incidents were found to suffer from neuropsychiatric disorders, delayed neuropathy and post-traumatic stress disorder symptoms[14,15]. In a cohort study in China, the high exposure of pregnant women to OPs affected neonatal neurobehavioral development of their offspring[16]. It is now understood that cholinergic systems play a crucial role in synaptogenesis, axonal growth and brain maturation during foetal development and such processes are integral to the normal course of behavioral and cognitive

development in postnatal life[17,18].

Rodent studies on the impact of chronic and high acute levels of OP's have confirmed that pre- or postnatal exposure to OPs evoke behavioral impairments in developing rat offspring, including alteration in motor function, learning and memory[10,19-21]. More detailed studies have revealed mechanisms of OP-mediated toxicity other than their impact on AChE; pre or postnatal exposure to chlorpyrifos affects various cellular processes (e.g., DNA replication, neuronal survival, glial cell proliferation), as well as noncholinergic biochemical pathways (e.g., serotoninergic synaptic functions, the adenylate cyclase system) leading to the behavioral abnormalities (e.g., locomotor skills, cognitive performance)[22,23]. Furthermore, postnatal exposure to the OP, DZN, altered emotional behavior in rats[24,25], whilst in mice, DZN exposure at 9 mg/kg daily during gestation caused significant motor impairment in the mice even in adulthood.

The majority of OP effects on developing rodents have used subtoxic or low doses, which are consistent with agricultural occupational exposure. The purpose of this study was to examine the effect of high doses of the DZN during the organogenesis period of pregnancy on brain AChE activity as well as the behavioral development of the progeny of Wistar rats.

Results

Delivery and Gestational Parameters

The treated group showed a significant decrease in the litter weight ($p < 0.001$) as compared to control group. No significant difference was noticed between both groups for the other parameters (Table 1). Notably, during treatment, dams showed slight signs of cholinergic toxicity.

Physical Development

On PND2, 5, 9, 12, 15 and 18, prenatal exposure to DZN decreased significantly ($p < 0.001$) the body weight (Figure 1) in male and female pups as compared to their controls sex counterparts. Three-factor ANOVA showed a significant effect of day, sex and treatment as well as of day × sex × treatment interaction for body weight [Day: $F = 13091.86$, $P = 0.001$; sex: $F = 1.70$, $P = 0.193$; treatment: $F = 2431.98$, $p = 0.001$; interaction: $F = 7.40$, $p = 0.001$].

Neonatal pups prenatally exposed to DZN showed a significant delay ($p < 0.001$) for both incisor eruption and opening eyes as compared to their control sex counterparts (Figure 3A and B). A two-way ANOVA test showed no interaction between sex and treatment for both paramerters.

Brain AChE Activity

On PND1, 3, 4, 9, 12, prenatal exposure to DZN inhibited significantly the acitivity of brain AChE (Figure 2) in male and female pups as compared with their controls same-sex counterparts. A three-factor ANOVA test showed no significative interaction between day, sex and treatment [Day: $F = 2516.18$, $P = 0.001$; sex: $F = 2.25$, $P = 0.139$; treatment: $F = 24433.33$, $P = 0.001$; interaction: $F = 1.75$, $P = 0.150$].

Table 1. Delivery and gestational parameters.

Parameters	Control group	Treated group
Litter size	11.75 ± 0.70	11 ± 0.75
Dead pups	0	0.25 ± 0.46
Living pups	11.75 ± 0.70	10.75 ± 0.70
Gestation length (days)	21.62 ± 0.74	22.12 ± 0.99
Sex ratio (%)	53.38 ± 8.25	54.08 ± 5.25
Litter weight (g)	87.35 ± 4.87	59.59 ± 2.79b***

b: treated group vs control group, n = 08 dams per group

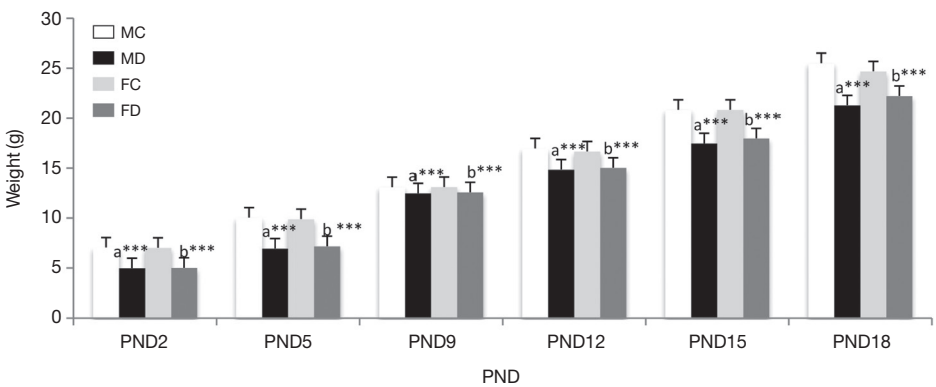

Figure 1. Effect of prenatal exposure to DZN on the body weight of the pups (male and female) on PND2, PND5, PND9, PND12, PND15 and PND18. Data are expressed as mean ± SE, n = 20 for each sex, a: MC (Male control) *vs* MD (Male DZN), b: FC (female controle) *vs* FD (female DZN). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

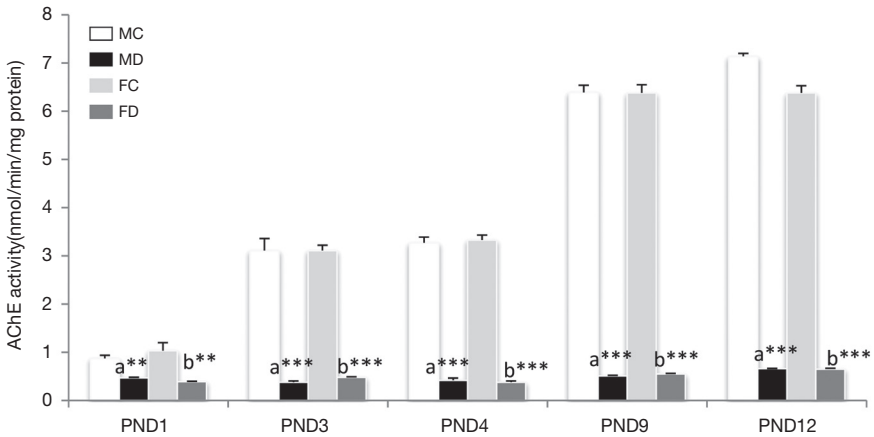

Figure 2. Effect of prenatal exposure of DZN on the brain acetylcholinesterase activity of the pups (male and female) on PND1, PND3, PND4, PND9, PND12. Data are expressed as mean ± SE, n = 05 for each sex, a: MC (Male control) *vs* MD (Male DZN), b: FC (female controle) *vs* FD (female DZN). (*p < 0.05, **p < 0.01, ***p < 0.001).

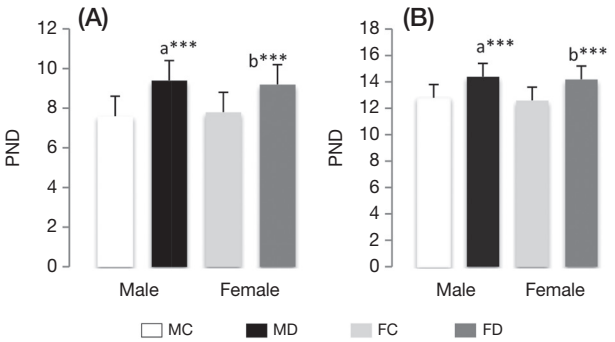

Figure 3. (A) Effects of prenatal exposure to DZN on pups incisor eruption (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (B) Effects of prenatal exposure to DZN on pups eyes opening (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC).

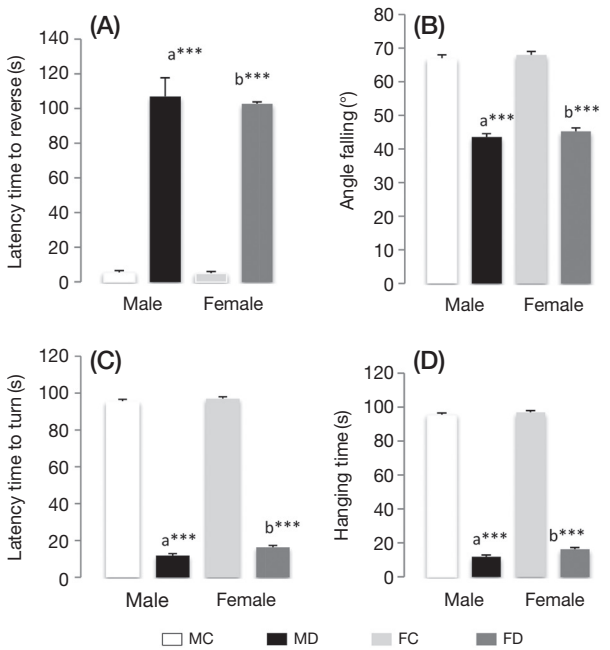

Figure 4. (A) Effects of prenatal exposure to DZN on the latency time to reverse in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (B) Effects of prenatal exposure to DZN on pups falling angle (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (C) Effects of prenatal exposure to DZN on latency time to turn in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (D) Effects of prenatal exposure to DZN on pups hanging time (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC).

Neuromotor Development

Prenatal exposure to DZN significantly ($p < 0.001$) increased the latency time to regain the prone position and decrease falling angle to grasp in both treated sex as compared to their controls sex counterparts (Figure 4A and B). The two-way ANOVA showed a significant effect of treatment and sex, as well as of interaction between sex and treatment for both reflexes [Latency time to reverse; treatment: $F = 9286.15$, $P = 0.001$; sex: $F = 12.79$, $P = 0.001$; interaction: $F = 9.61$, $P = 0.003$], [Angle falling; treatment: $F = 80.26$, $P = 0.001$; sex: $F = 80.26$, $P = 0.001$; interaction: $F = 107.32$, $P = 0.001$].

In addition, prenatal exposure to DZN decreased significantly ($p < 0.001$) latency time to turn, hanging and falling in both treated sexes when compared to their control sex counterparts (Figure 4C and D). The two-way ANOVA showed a significant effect of treatment and sex, as well as of treatment and sex interaction for the latency time to turn, however, for hanging time, a significant effect only for treatment, but no in-

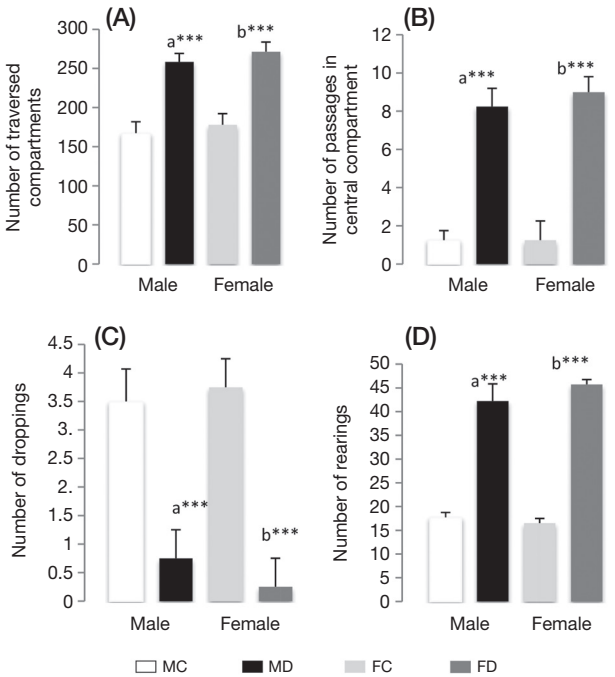

Figure 5. (A) Effects of prenatal exposure to DZN on number of traversed cases in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (B) Effects of prenatal exposure to DZN on number of passages in central case in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (C) Effects of prenatal exposure to DZN on number of droppings in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC). (D) Effects of prenatal exposure to DZN on rearing number in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC).

teraction treatment × sexe [Latency time to turn; treatment: F = 11316.01, P = 0.001; sex: F = 10.16, P = 0.002; interaction: F = 7.65, P = 0.007], [Hanging time; treatment: F = 2527.74, P = 0.001; sex: F = 3.20, P = 0.07; interaction: F = 0.87, P = 0.345].

Neurobehavioral and Cognitive Development

In relation to the Open Field test, prenatal exposure to DZN increased significantly ($p < 0.001$) the number of compartments traversed in both treated sexes compared to their respective controls (Figure 5A). Moreover, the number of passages in central compartment was significantly increased ($p < 0.001$) in both treated sexes as compared to their control counterparts group (Figure 5B). The number of droppings also decreased significantly ($p < 0.001$) in both treated sexes (Figure 5C) compared with control. Finally, the number of incidences of rearing increased significantly ($p < 0.001$) in both sexes as compared to controls sex counterparts group (Figure 5D). However, there was a significant interaction between sex and treatment in the following parameters presented respectively [Number of cases traversed; treatment: F = 85298.68, P = 0.001; sex: F = 1547.53, P = 0.001; interaction: F = 26.68, P = 0.001], [Number of passages in central case; treatment: F = 614.29, P = 0,001; sex: F = 1.59, P = 0.232; interaction: F = 1.59, P = 0.232], [Number of droppings; treatment: F = 123.43, P = 0.000; sex: F = 0.00, P = 1.000; interaction: F = 0.86, P = 0.373], [Number of rearing; treatment: F = 10667.31, P = 0.000; sex: F = 18.69, P = 0.001; interaction: F = 83.31, P = 0.373].

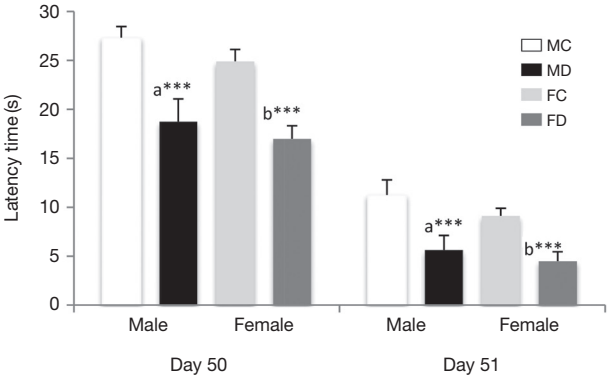

Figure 6. Effects of prenatal exposure to DZN on latency time in pups (Data are expressed as mean ± SE, n = 20 for each sex a: MD *vs* MC; b: FD *vs* FC).

In relation to Morris test. Latency time decreased significantly ($p < 0.001$) on day 50 & 51 in both treated sexes as compared to their controls sex counterparts (Figure 6). Three-way ANOVA showed no significant interaction between day, sex and treatment for latency time, however, a significant effect of day, treatment and sex was recorded [Day: F = 1137.61, P = 0.001; sex: F = 146.33, P = 0.001; treatment: F = 1085.89, P = 0.001; interaction: F = 2.03, P = 0.101].

Discussion

In this report, repeated administration of DZN at 10 mg/kg promoted alterations in physical and behavioral development. For the first time, our study highlights the outcomes of high doses of DZN on developing rat progeny. During lactation, pups prenatally exposed to DZN grew slowly in comparison with expected body weights, which was indicative of DZN induced toxicity in pups through *in utero* exposure. Together with the ability of OPs to cross the placenta, poor clearance by immature foetal detoxifying enzymes promotes the teratogenic effects of these agents[17,26]. In a previous report, a decreased body weight in early postnatal life was seen in

rat pups prenatally exposed to repeatedly low dose of chlorpyrifos-ethyl which returned to natural levels during lactation; this recovery may due to the low toxicity of treatment[27]. Our findings in rats of OP-induced body weight reduction are consistent with a cohort study, in which the newborns of pregnant women exposed to high level of OPs exhibited a reduction in body weigh and length[26].

Regarding other aspects of physical and reflex development, our findings revealed that DZN delayed significantly both eye opening and incisor eruption. In addition, prenatal exposure to DZN modified neuromotor development and postural adjustment in offspring, as well as surface righting reflex, palmar grasp reflex, negative geotaxis and the forelimb support test. These findings showed that DZN disrupts physical and neuromotor development. Several reports have indicated that the impact of OP agents on CNS development and function is linked with the inhibition of CNS AChE. The activity of the cholinergic system is vital to normal behavior and muscular function[28]. Animals exposed pre or postanatally to AChE inhibitors during the period of active synaptogenesis are thought to be particularly vulnerable to develop several behavioral impairments, including motor development and coordination deficits[29]. Indeed, gestational exposure to a single high dose of chlorpyrifos altered muscular maturation in pups, which was revealed by the righting reflex test[30]. Our biochemical estimation showed that DZN caused a drastic inhibition of the activity of brain AChE in suckling rats. This impact on brain AChE was also seen in rat pups prenatally exposed to chlorpyrifos and they also exhibited delayed physical and reflex responses[30]. Taken together, these studies underline DZN's effects on developmentally crucial neural processes of brain maturation and development, which are likely to be strongly linked with its impact on cholinergic function in specific regions of the developing CNS[12]. In addition, the cholinergic deficit appears to be sustained through to adulthood and is manifested in modified behavioral responses[31].

Other behavioral impacts of DZN were explored in our study. During the adolescence period, prenatal exposure to DZN increased locomotor activity and exploratory behavior in the Open Field test; this test is used to measure exploratory behavior and activity in rodents. Behaviors such as thigmotaxis (staying close to the walls of the field) and defecation are cues related to anxiety or fear, while time spent in the centre of the field and high locomotor activity indicate an exploratory behavior[32]. Our results showed an increase in the number of traversed compartments, number of rearings and the number of passages through the central compartment. The decrease in the number of droppings could be interpreted as a reduction in anxiety levels, which suggested that DZN treatment may have anxiolytic-like behavior effect on the animals. To our knowledge, little has been reported on the cognitive effects on adolescent rats prenatally exposed to DZN. In general, the effects of OP's on higher CNS functions have been somewhat variable: anxiolytic responses have been linked with exposure to OP's and was ascribed to a toxic consequence of CNS AChE inhibition[33,34]. Prenatal exposure to chlorpyrifos elicited hyperactivity in adolescent rat[10,35], whilst prenatal exposure to dichlorvos decreased locomotor activity in the Open Field Test[19]. Gupta *et al*.[36] and Bird *et al*.[37] also found a decrease in exploratory and motor activities after prenatal exposure to methyl-parathion and dichlorvos, respectively. In man, studies on the CHAMACOS cohort suggested that prenatal exposure to OP induced hyperactivity in children[38].

It has been demonstrated that OP pesticides may produce neurobehavioral damage during development[39,40]. Moreover, behavior and cognitive changes such as mood disorders and memory dysfunction following prenatal exposure to OP have been related to the AChE inhibition during development[10], although OP-mediated toxicity on non cholinergic pathways is also likely. Regarding the Morris Water Maze results in this report, latency times in reaching the platform decreased significantly on both days of experiment, mainly on day 51. Latency time is considered as an index to assess spatial memory function, suggesting that DZN actually enhances the spatial memory. This is in broad agreement with studies showing the enhancement of reference and working memory in rats postnatally exposed to chlorpyrifos and methyl-parathion; this was accounted for by OP-mediated impact on cholinergic and non-cholinergic systems[41]. Furthermore, rats prenatally exposed to a subtoxic dose of chlorpyrifos-ethyl exhibited apparent competence for both reference and working memory; the authors of the study suggest that this may have occurred due to the OP-induced deficit in cholinergic system being offset by adaptive changes in neuronal plasticity[42].

In summary, we have demonstrated that exposure to DZN at high levels during the organogenesis period delayed physical and neuromotor development in the rat, which was associated with a marked and sustained inhibition of brain acetylcholinesterase activity. This in turn, may have contributed to a modified behavioral and emotional state at the adolescence stage of the animals.

Materials and Methods

Animals and Housing Conditions

Three-month-old male and virgin female Wistar rats weighing 220.10 ± 10.02 g were purchased from the

Pasteur Institute (Algiers, Algeria) and randomly housed in large polyethylene cages (04 rats per cage for each sex) at standard facility conditions of temperature (25.10 ± 2.10°C), humidity (65.20 ± 5.05%) and 12 h light/12 h dark regime. Rats were supplied with commercial standard rat chow and tap water *ad libitum*.

Mating

Two weeks later, twenty six female rats of the same weight (approximately 250.20 ± 5.10 g) were individually housed in appropriate polyethylene cages and subjected to the first vaginal smears to determine estrus cycle phases based on a standard cytological analysis[43]. Each proestrus female was mated overnight with one sexually experienced male. In the morning, the presence of sperm plugs in the second vaginal smears determined the first day of conception (GD1). Each pregnant female was then singly housed in its home cage for the entire experimental period. From 26 dams, the progeny of 16 dams was used to assess the physical, neuromotor and behavioral development, and the others 10 dams, their progeny was used to assess the activity of brain acetylcholiesterase.

DZN Administration

On GD7, the female rats were divided into two groups: One group (DZN group, n = 13) was treated intraperitoneally once daily from GD7 to GD14 with 10 mg/kg of body weight of DZN (60% EC, Vapco, Jordan). The second group (control group, n = 13) was identically treated with the DZN vehicle (1 mL/kg of olive oil). The dose and route of administration were selected from works in our laboratory. LD_{50} of i.p DZN in rat is 65 mg/kg[44,45].

Gestational and Delivery Parameters

All the 16 dams were allowed to litter normally and nurture their offspring. Parturition was considered to be postnatal day 0 (PND0). On PND1, the pups were weighed and the gestational length for each dam was registered. Litters were rapidly checked for the number of born pups, dead pups, living pups and sex ratio [i.e. (The number of males/the total number of pups) × 100].

Physical and Neuromotor Development

The progeny of 16 dams (DZN = 8, Control = 8) were used to assess the physical and neuromotor development. Depending on litter size at birth, two or three male-female pairs from each litter (DZN = 20, control = 20, for each sex) weighed at PND2, PND5, PND9, PND12, PND15 and PND18. The following physical parameters were assessed in males and females of each litter: *Incisor eruption* (observation of superior and inferior teeth, checking between PND8 and PND11) and *eye opening* (opening of both eyes, checking between PND12 and PND16. The following reflexes were assessed in males and females of each litter: the *surface righting reflex* was assessed on PND 3. When the pup is placed in a supine position, it seeks to return to prone position. The measured variable is the required time for the pup to regain the prone position (neuro-muscular maturation measurement). The *palmar grasp reflex* was assessed on PND4, when the animal is placed on a wire plate and the pup must grip to not fall when the plate is rotated. The measured variable is the obtained angle compared with the horizontal line when the pup falls (grasp reflex measurement). The *negative geotaxis test* was carried out on PND9, when the animal is placed "upside down" on a 20° inclined plane, the pup rat turns over to find itself "head upwards". The measured variable is the time necessary for the animal to carry out a complete half-turn of 180° (measurement of equilibration, maturation of the cerebellum and semicircular canals of the inner ear). The *forelimb support test* was assessed on PND12, where the forepaws of the pup rat are put in contact with a tight wire suspended 40 cm above the ground. This stimulation triggers a grip reflex and the animal hangs on to the wire. The measured variable is the hanging time (muscular force and "fatigability" measurement). Pups were observed daily between 9:00 and 9:30 a.m. and removed from mothers for observation and then immediately returned to their home cage. The appearance day of each of the above parameters was calculated. Data were analysed considering the litter as the smallest unit. Weight measures, physical parameters and reflex tests were performed according to applied ethology[46].

Brain AChE Activity

The activity of brain AchE was estimated in neaonatal rats at PND1, PND3, PND4, PND9, PND12 which are paralleled with the period of assessment of reflexes response. The activity of AChE assay was estimated according to Ellman *et al*.[47]. At each PND, one male-female pair from each litter (5 litter per group; Control = 5; DZN = 5) was removed and decapitated, The brains were dissected out, washed with cold saline and immediately stored at −80°C. Brain samples (1 : 10, w/v) were homogenized in phosphate buffer (0.1 mol/L, pH 7.4). The homogenate was centrifuged at 10000 g for 15 min and aliquots of supernatant were separated and used for AChE estimation. the assay mixture contained 500 µL sodium phosphate buffer (pH 8), 40 µL of (5,5′-dithiobis-(2-nitrobenzoic acid) DTNB, 40 µL of (acetylthiocoline) Asch and 20 µL supernatant. The change in absorbance was measured spectrophotometrically immediately at 410 nm. The enzymatic activity in supernatant was expressed as nmol/min/mg protein.

Behavior and Cognitive Development

Locomotors, Exploratory Activity and Emotional State in Open-field Test (PND45)

The test was performed according to applied ethology[46]. The device consists of an arena (Ø50 cm) divided into nine compartments; one central and eight peripherals. This test allows the evaluation, on 5 minute test session, of the animal's locomotor activity, exploratory behavior and emotional state. The studied variables are the number of crossed compartments (locomotor activity) and rearings (vertical exploratory activity), the number of droppings and the number of passages through the central compartment of the device (anxiety criteria). The parameters were recorded with camera video and counted visually.

Morris Water Maze: Spatial Memory (PND50 and 51)

The test was performed according to applied ethology[46]. When it is placed in a water-filled circular tank (Ø150 cm), divided into four equal quadrants, The water was made opaque using nontoxic black ink and maintained at 25 ± 1°C. The rat swims and seeks to escape from the aversive aquatic environment. The first test session (Day 50) comprises 5 trials, during which the animal learns how to locate the site of a platform immersed 2 mm below the water surface and to take refuge there. During the first two trials (test familiarization trials), the platform is placed against the basin wall. For the other sessions, it is placed 10 cm distant from the wall. The second test session (Day 51) comprises two trials, during which the platform is placed 10 cm distant from the basin wall. This test allows evaluating spatial memory. The studied variable is the latency time to take refuge onto the platform. The parameters were recorded with camera video and counted visually using stopwatch.

Statistical Analysis

All results were expressed as the means ± standard error of the mean (SEM). Two-factor ANOVA was used to analyse the data of the control and treated groups based on sex and treatment as factors. Three-factor ANOVA was used to analyse the data of control and experimental groups based on sex, treatment and time as factors. We used an ANOVA followed by the Tukey post hoc test to compare data presenting interaction. To analyse data without interaction, Student's T test was used to compare groups. Results were considered significant for $P < 0.05$.

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