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Endocrine-disrupting and cytotoxic potential of anticholinesterase insecticide, diazinon in reproductive toxicity of male mice

Reda H. ElMazoudy*, Azza A. Attia

Zoology Department, Faculty of Science, Alexandria University, Alexandria, Egypt

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ABSTRACT

We evaluated the effects of diazinon (2, 4.1 and 8.2 mg/kg bw/day for 4 weeks) in gonadotropins, testosterone and estrogen levels, whether the regulatory interactions in the hypothalamic–pituitary–testicular axis are modified by acetylcholinesterase inhibition and histopathological changes in adult mice testes. Diazinon at doses higher than 2 mg/kg bw/day resulted in decreased testis weight, inhibition in acetylcholinesterase activities, decrease in levels of luteinizing hormone and follicle stimulating hormone, following reduction in mating and fertility indices. Diazinon increased testosterone content in 4.1 mg/kg group, but decreased testosterone concentration in 8.2 mg/kg group. Diazinon increased estrogen, prolactine and decreased levels of acetylcholinesterase activities in 4.1 mg/kg group but levels of luteinizing hormone and follicle stimulating hormone remained unmodified. It may be simply postulated a scenario that acetylcholine in the cholinergic neurons has a potential threshold to perform a crucial part in the complex circuitry of neuroendocrine regulatory mechanisms. On overaccumulation, other neurotransmitters can be appropriately recruited to modulate the mechanisms of circuitry.

1. Introduction

Diazinon, CAS No. 333-41-5, is the common name of an organophosphorus pesticide (OP) used to control pest insects in soil, on ornamental plants, and on fruit and vegetable field crops. It was formerly as the active ingredient in household and garden products used to control pests such as flies, fleas and cockroaches. Diazinon is a synthetic chemical, it does not occur naturally in the environment [1]. It is considered such an endocrine disruptor because of its estrogenic and anti-androgenic activities [2,3] via stimulation of estrogen (ER) and androgen (AR) receptors expressions [4,5].

Exposure to diazinon induces several neurological and endocrine alterations in humans and different wildlife species [6]. At the central nervous system, diazinon modifies dopamine, norepinephrine and serotonin concentration in different brain regions [7], content of gonadotropin releasing hormone (GnRH) and its gene expression [8]. Among the endocrine effects, diazinon alters pituitary secretion increasing prolactin (PRL) and follicle stimulating hormone (FSH) content and decreasing plasmatic luteinizing hormone (LH) concentration [9] and testis activity, through several mechanisms such as induction of oxidative stress and inhibition of testosterone synthesis [10].

Little information is available on the endocrine effects of diazinon on prolactin in experimental animals. In agricultural workers, Recio et al. [11] demonstrated that OP exposure disrupts the hypothalamic–pituitary endocrine function and also that LH, FSH and PRL are the hormones most affected. Sarkar et al. [12] reported that sub-chronic oral administration of quinalphos (7–14 mg/kg/day for 15 days) to male rats resulted in increased serum concentrations of LH, FSH, PRL and testosterone without significant effects on dopamine, noradrinaline or serotonin levels in the hypothalamus or pituitary. Nag and Nandy [13] reported a significant inhibition of monoamine oxidase-A (MAO-A) and MAO-B, the two main dopamine degradative enzymes, in rat brain mitochondria exposed to OP and also that the reversibility of the effect was dependent on the OP used. Similarly, Choudhary et al. [14] showed that rats treated subcutaneously with the OP dichlorvos showed an increase in dopamine and norepinephrine levels indicating that the OP-induced decreased PRL resulted from dopaminergic inhibition. Therefore, these experimental studies demonstrate that OP exposure alters brain neurotransmitter levels and that the hypothalamic–pituitary axis is a direct target for OP toxicity in rodents. In adult male rats, OP exposure elevates plasma adrenocorticotropic hormone (ACTH) concentration in methamidophos treated rats [15]. These endocrinological facts may correlate with the observed changes in estrogen and progesterone receptors expression [16]. In addition, the biogenic amines regulate the

* Corresponding author. Tel.: +2 033925875/+2 034248913/+2 01273251813; fax: +2 033911794.
E-mail address: redaelmazoudy@yahoo.com (R.H. ElMazoudy).

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activity of the hypothalamic–pituitary–testicular pathway [17,18]. Whereas dopamine and serotonin inhibit LH and FSH secretion [19], norepinephrine stimulates gonadotropins release [20], and LH stimulates testosterone synthesis and secretion by the testes [21]. Furthermore, in the inhibitory neural pathway from the hypothalamus to testes that bypass the pituitary gland [17,18] the serotonin has a direct regulatory effect on steroidogenesis in testes [22]. PRL can modulate the activity of hypothalamic–pituitary–testicular pathway, modifying synthesis and/or secretion of biogenic amines at hypothalamic level [23], inhibiting gonadotropins secretion by the pituitary [24] and regulating testosterone testicular secretion [25].

Like other OP, diazinon is a potent inhibitor of acetylcholinesterase (AChE) activity and other serine hydrolases [26]. So, they can induce both acute toxicity and long-term neurological deficits [27]. Diazinon can have deleterious effects on the nervous system through a variety of mechanisms [28]; modify expression of neurotrophic factors [29]; and induce oxidative stress [30]. However, no evidences have provided for a cellular mechanism of diazinon action in the reproductive toxicity and the mechanisms of reproductive axis whether they are dependent on AChE inhibition.

In rats, exposure to diazinon results in reduction in reproductive organs weights and spermatozoa parameters with increase in sperm death and abnormalities [31]. Pina-Guzman et al. [32] demonstrated that diazinon can affect late steps of spermatogenic maturation in mice causing damaged DNA and reduced chromatin in spermatogonia and spermatids. Additionally, diazinon has been described to disrupt reproductive potency [33] and alters semen quality [34]. Epidemiologic studies have suggested associations between OP exposure and reproductive disorders (infertility, birth defects, pregnancy outcomes) [35], fertility index, intrauterine fetal death and gestational period [36].

Thus the present study was addressed to (1) evaluate neurotoxic effects induced by diazinon exposure on reproductive axis (hypothalamus–pituitary–gonads) due to acetylcholinesterase inhibition; (2) analyze the possible differential diazinon effects on pituitary and steroidogenic hormones titer as being targets for disruption; and (3) elucidate whether the regulatory interactions in the hypothalamic–pituitary–testicular axis are modified by acetylcholinesterase inhibition and histopathological changes in adult mice testes.

2. Materials and methods

2.1. Insecticide qualification and test

Diazinon (98% purity) was purchased from Egychem for chemicals company, Egypt. It was emulsified in distillated water at different doses and administrated orally. Stability of the preparation was demonstrated over 7 days at room temperature; according to the guidelines of EPA [37]; dosing formulations were freshly prepared four times during the administration period.

2.2. Animals and dosage exposures

Twelve weeks of age (30 ± 5 g) sexually mature male mice of CD-1 (ICR) strain were obtained from High Institute of Public Health, Alexandria University, Alexandria, Egypt. Mice were acclimatized for at least two weeks prior to the experimental assignment to ambient conditions (room temperature $23 \pm 2\,^{\circ}C$, relative humidity $50 \pm 10\%$, and 12 h light–dark cycle). They were fed with a commercial pellet diet (El-Kahira Company for oils and soap, Cairo, Egypt) composed of protein, carbohydrates, fibers, vitamins, minerals and a small amount of fat and tap water ad libitum. Males were assigned and grouped according to approximately equal mean body weight (bw). Among the four groups (25 male/each), one was considered as control and received distilled water by gavage. The other three groups were given diazinon regularly at 9.00 am by gavage at the dose levels of 2.0, 4.1 and 8.2 mg/kg bw/day for four consecutive weeks. Dose concentrations were adjusted to a mean body weight (10 ml/kg bw). The doses were chosen based on studies reported by Bruce et al. [38] and correspond, respectively, to approximately 1/40, 1/20 and 1/10 of LD_{50} value obtained for mice (82 mg/kg bw).

2.3. Male inspection

Clinical signs of males were evaluated daily throughout the experimental period for toxicological indices. Symptoms of toxicity were observed and recorded according to the toxicological signs of organophosphorous insecticides [39].

2.4. Mating and fertility indices

After 28th day, each treated male mouse, with continuation of daily doses, was co-housed with unexposed proven fertility, sexually receptive, female mice (one to one/box). They were left together for 10 days during which two estrus cycles should have elapsed [40] following which they were separated. The females were examined daily for the presence of the vaginal plug as a criterion of successful insemination that was considered the day zero of gestation. Each plug-positive female was caged individually. Then mating and fertility indices were recorded.

2.5. Body and reproductive organs weights

Initial (weight of start point) and final (weight of end point) body weights were recorded. On day after fecundity test, male mice from each group were slightly anesthetized with ether and killed by decapitation. The reproductive organs were stripped from fatty tissues and blood vessels, blotted, and their absolute weights were determined. Clinical signs of body and reproductive organs were evaluated for toxicological criteria. To normalize the data for statistical analysis, organ weights were expressed per 100 gram body weight.

2.6. Plasma acetylcholinesterase activity (AChE)

The enzymatic plasma acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) activity was determined following Ellman et al. [41].

2.7. Evaluations of reproductive hormones in plasma

Immediately after the animals reached the mating period peripheral blood samples were harvested from the retro-orbital plexus vein via glass capillary according to Schalm [42] on heparinized tubes. The plasma was separated by centrifugation and stored at $-20\,^{\circ}C$ for subsequent hormone assays [43]. Blood plasma preparations were used for measurement of the pituitary hormones, follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactine (PRL). The concentrations of each were measured by commercial enzyme-linked immunosorbent assays (ELISA) purchased from ALPCO Diagnostics, Salem, NH (03079). Whereas steroid hormones, testosterone and estradiol were measured by radioimmunoassay, RIA, using commercial kits (Biodiagnostic, Giza, Egypt) as per manufacturer's instructions [44]. The limit of testosterone detection was 0.06 ng/ml and the assay coefficient of variation was 4.5%. The antibody used for testosterone, RIA, had low cross reactivity to dihydrotestosterone (DHT) (4%) and other androgens (less than 0.01%). The sensitivity of the

estradiol assay was 15 pg/ml and the intra- and inter-assay coefficients of variation were 4.3% and 5.6%, respectively. Testosterone secretion has a circadian rhythm with higher levels in the morning than evening. Therefore, blood samples for testosterone measurements should be drawn in the morning (9:00-10:00 am) [45].

2.8. Evaluation of sperm characteristics

2.8.1. Sperm collection

After the sexual cohabitation and fecundity test period, the laparotomy was conducted for all exposed male mice following weight and anesthesia with diethyl ether. Testes and epididymides were carefully excised. The sperm count was assessed from right cauda epididymides while sperm motility and morphology were analyzed from the left one. Epididymis was excised and minced in 1 ml of phosphate buffered saline (pH 7.2) to obtain sperm suspension. The suspension was filtered through a nylon mesh [46]. The other testes and epididymes were frozen in −20 °C until usage.

2.8.2. Sperm count

The cauda epididymal sperm count was performed according to Vega et al. [47] and Narayana et al. [46] using a Neubauer hemocytometric chamber. Layered slides with semen were viewed by bright-field microscope with magnification of 400×. The total sperm count in squares of 1 mm^2 each was determined to express the number of sperm/epididymis. Epididymal sperm counts were expressed as number of sperms per epididymis. To minimize the error, the count was repeated three times on each sample [48].

2.8.3. Sperm motility

Approximately 10 μl of sperm suspension with a micropipette was layered onto a warmed microscope slide. Sperm motility was assessed by counting all progressive motile (effective), the non-progressive motile (non-effective or sluggish) and the immotile (dormant) spermatozoa in the same microscopic field (400×). In each semen sample, at least 10 microscopic fields were examined with at least 100 sperm/field was counted. The number of motile sperm cells in each field was divided by the total number, and the average of the fields was assayed. The percentage of motile spermatozoa was determined [49].

2.8.4. Sperm viability

Sperm viability was assessed using the eosin-negrosin staining [50]. The staining was performed with one drop of freshly collected semen (10 μL) placed on a slide and stained with two drops of freshly prepared staining solution (20 μl) of eosin–nigrosin (1 g eosin + 5 g nigrosin/100 ml deionized water). The live unstained (intact) and dead (purple to red-stained head with damaged membranes) spermatozoa were analyzed under the microscope at 400×. The dye exclusion was evaluated in 100 spermatozoa. Sperm viability was defined as the percentage of dead sperm cells. Viability was evaluated according to WHO guidelines [51].

2.8.5. Sperm morphology

To assess the spermatozoa morphological abnormalities, a drop of sperm suspension was smeared on a slide and air-dried and made permanent. The smeared slide was stained with 1% eosin Y and 5% nigrosin. Morphological sperm defects were evaluated and examined on optical microscope using 400× magnification [52]. At least 100 spermatozoa from different fields in each slide were examined and classified for criteria of morphological abnormalities (head, tail and tail-head) according to Filler [53]. Abnormal sperm cells were counted and the percentage was calculated.

2.8.6. Assessment of sperm production

From the previously frozen testes and epididymides, sperm content per gram was determined using slight modifications on the method described previously [46,47]. Briefly, after thawing at temperature (25–27 °C), the whole epididymal and the testicular tissues were homogenized for 5 min in 5 ml of physiological saline (0.9% NaCl) containing 0.05% (v/v) Triton X-100 using a manual homogenizer. The homogenates were diluted with 1.5 ml of the saline solution; spermatozoa and spermatid were counted at 400× magnifications by light microscopy in a Neubauer hemocytometer. Three counts per sample were averaged [54]. These count values were used to obtain total number of spermatids per testis and sperm per epididymis, which was then divided by the testis or epididymis weight to determine the number per gram of testis or epididymis.

2.9. Pregnancy outcomes

On the 20th day of gestation, the pregnant females were anesthetized with diethyl ether and killed by decapitation. After collection of the uterus, the number of implantation sites, resorptions (a conceptus is defined as a late resorption if it is grossly evident that organogenesis has occurred; if this is not the case, the conceptus is identified as an early resorption), dead, and live fetuses (a live fetus is defined as one that responds to stimuli; a dead fetus is defined as a term fetus that does not respond to stimuli and that is not markedly autolyzed; dead fetuses demonstrating marked to extreme autolysis are considered to be late resorptions) were recorded [55]. Uteri that appeared non-gravid were stained with ammonium sulfide [56] to confirm pregnancy status and examined for evidence of implantation sites.

2.10. Histopathological examination

Histological preparations were performed in all previously Bouin's fixed testes and epididymides, dehydrated and embedded in paraffin wax. Five-micron thick paraffin sections were performed in a microtome and stained with haematoxylin and eosin [57]. Stained sections were examined under light microscope and general histopathologic appearance was assessed.

2.11. Statistical analysis

The data were collected and entered to the personal computer. The results obtained are expressed as mean values ± SD. Statistical significance was assessed using one-way analysis of variance (ANOVA) and unpaired Student's *t*-test. Values of $P < 0.05$ were considered significant. Statistical analysis was done using statistical package for Social Science (SPSS/Version 17) software, USA (1989–2002) LEAD Tech. Inc. To determine the diazinon–response relationship for each dose treatment on toxicological variables, a Pearson correlation was performed. $P < 0.05$ was the level for accepting the significance.

3. Results

The findings depict the indices of androgenicity in male mice after administration of three different dosage levels of anticholinesterase insecticide, diazinon for 28 days.

3.1. Mortality and macroscopic symptoms of toxicity

In the present study, all animals survived until the end of the experimental period in 2.0, 4.1 and 8.2 mg/kg bw/day treated groups. Additionally, no abnormal behavior was observed. Diazinon exhibited cholinergic signs of adverse toxicological effects at

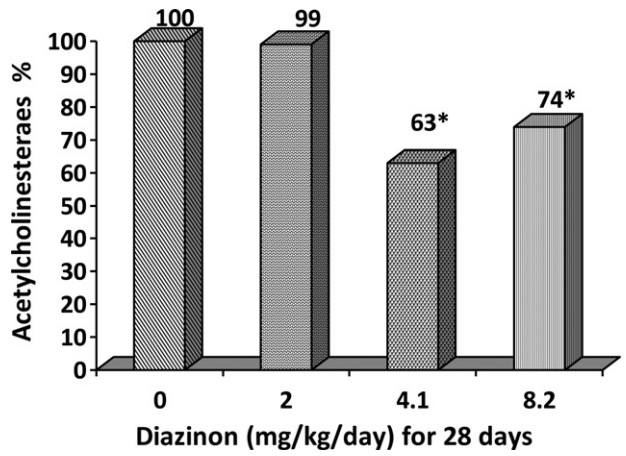

Fig. 1. Effects of different doses of diazinon on plasma AChE activities in male CD-1 mice. Data are expressed as percent of mean control values ± SD for each dose. *Statistical analysis indicates significant differences at $P < 0.05$.

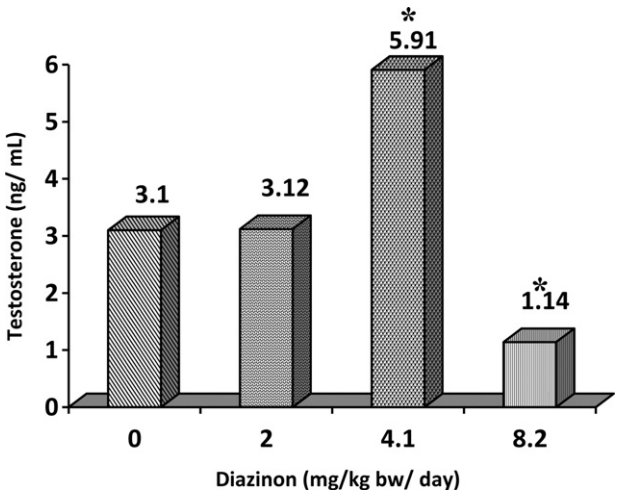

Fig. 2. Mean ± SD of the plasma concentration of testosterone in adult male CD-1 mice after oral administration of saline or different doses of diazinon once a day for 28 days. *Statistical analysis indicates significant differences at $P < 0.05$.

4.1 and 8.2 mg/kg bw/day doses, such as sluggishness, muscular tremors, irregular movement, and abdominal tremble. The progression of these signs proceeds to the last week of experiment of such treated males. At necropsy, no macroscopic alterations that could be attributed to treatment with three dosage levels of diazinon were found in the testes and/or reproductive accessories.

3.2. Body and reproductive organ weights

There was a significant correlation between symptoms of toxicity of both body and reproductive organ weights at doses of 0, 2.0, 4.1 and 8.2 mg/kg bw/day of diazinon exposure ($P < 0.05$) (Table 1); however, relative testes and seminal weights showed an insignificant Pearson correlation ($r = 0.106$ and 0.107, respectively) (Table 1). Furthermore, diazinon 4.1 mg/kg bw and 8.2 mg/kg bw (28 days) caused a significant decrease in body weight of 26% and 30%, respectively in respective to control ($P < 0.05$) (Table 1). A significant decrease in the absolute and relative weight of the epididymis (50%, 27% and 50%, 27%) and prostate (50%, 30% and 50%, 30%) was observed after treatment with diazinon 4.1 mg/kg bw/day and 8.2 mg/kg bw/day, respectively, comparable to control. On the other hand, exposure to diazinon 4.1 mg/kg bw/day and 8.2 mg/kg bw/day for 28 days caused a reduction of 31% and 35% in the absolute testis weight, respectively ($P < 0.05$) (Table 1). In animals treated with diazinon at 4.1 mg/kg bw/day and 8.2 mg/kg bw/day there was a significant decrease in the absolute weight of seminal vesicle of 32% and 37%, respectively ($P < 0.05$) (Table 1). Treatment with 2.0 mg/kg bw/day diazinon did not alter the body, testis or accessory reproductive organ weights as compared to control.

3.3. Evaluations of biochemical findings

3.3.1. Plasma acetylcholinesterase percent (AChE)

Plasma acetylcholinesterase (AChE) activity is depicted in Fig. 1. There is no significant difference in level of plasma AChE activity in the animals treated with 2.0 mg diazinon/kg bw/day as compared to control. After 28 days of the treatment with diazinon, significant inhibition in the activity of plasma AChE by 37% and 26% ($P < 0.05$) was observed in mice treated with 4.1 and 8.2 mg/kg bw/day, respectively comparable to control.

3.3.2. Steroid hormones

RIA assays revealed that plasma testosterone levels were drastically increased (90%) in the animals treated with 4.1 mg/kg bw/day diazinon ($P < 0.05$) (Fig. 2). Conversely, plasma level of testosterone was reduced (63%) after treatment with 8.2 mg/kg bw/day diazinon ($P < 0.05$) (Fig. 2). Similarly, plasma levels of estradiol showed a significant increase of 84% after treatment with 4.1 mg/kg bw/day diazinon ($P < 0.05$) (Fig. 3C). At other dosage (2.0 mg/kg bw/day) of diazinon exposure, the plasma testosterone and estradiol concentrations were similar to control ($P < 0.05$) (Figs. 2 and 3C).

3.3.3. Pituitary hormones

Compared to the control, plasma levels of both LH (45% and 49%) and FSH (56% and 65%) decreased in animals exposed to 4.1 mg/kg bw/day and 8.2 mg/kg bw/day of diazinon, respectively ($P < 0.05$) (Fig. 3A and B). In addition, plasma PRL content significantly increased in 4.1 mg/kg bw/day group (96%), but decreased (11%) in 8.2 mg/kg bw/day group ($P < 0.05$) (Fig. 3D).

3.4. Evaluations of reproductive performance quality

3.4.1. Mating and fertility indices

In Table 2 fertility indices of the male mice given diazinon at doses of 4.1 and 8.2 mg/kg bw/day for 28 consecutive days were 86% and 60%, respectively, as compared to 100% for control normal group ($P < 0.05$). In male mice for diazinon given at the lowest tested dose (2.0 mg/kg bw/day), the fertility indexes were 96% with respect to control.

3.4.2. Semen analysis

Administration of male mice with 4.1 and 8.2 mg/kg bw/day diazinon resulted in paucity of epididymal spermatozoa counts and testicular spermatid enumeration (Table 2) and reduction of sperm motility (Table 2, Fig. 4), while the number of dead sperms increased. As shown in Table 2, a significant decrease in the percentage of the total sperm viability is dose dependent ($P < 0.05$). The lowest dose did not reach the level of statistical significance of sperm quantity. Morphological abnormalities of spermatozoa were categorized by head or tail (Figs. 5 and 6) as shown in mostly monitored in smears of mice administrated 4.1 or 8.2 mg/kg bw/day diazinon were mainly significantly increased (Table 2, Figs. 5 and 6). To determine the diazinon effects on different semen analysis patterns for each dosage concentration (0, 2.0, 4.1 and 8.2 mg/kg bw/day) a Pearson correlation was obtained ($P < 0.05$) (Table 2). Diazinon displayed significant varying correlation effects on semen analysis.

Table 1
General reproductive toxicology of different doses of diazinon on male CD-1 mice.

	Diazinon (mg/kg bw/day)				
	0	2.0	4.1	8.2	*r*
Initial body weight (g)	34.4 ± 2.65	33.7 ± 2.69	34.5 ± 3.21	33.8 ± 2.98	
Final body weight (g)	39.9 ± 3.01	38.8 ± 3.12	29.6 ± 3.41*	28.2 ± 2.77*	−0.42**
Absolute organs weights (g)					
Testes	0.23 ± 0.013	0.22 ± 0.013	0.16 ± 0.021*	0.15 ± 0.001*	−0.58**
Epididymides	0.06 ± 0.011	0.06 ± 0.0032	0.03 ± 0.001*	0.03 ± 0.001*	−0.475**
Prostate	0.08 ± 0.0032	0.07 ± 0.001	0.04 ± 0.003*	0.04 ± 0.003*	−0.485**
Seminal vesicles	0.22 ± 0.021	0.21 ± 0.017	0.15 ± 0.001*	0.14 ± 0.012*	−0.521**
Relative organ weights					
Testes	0.58 ± 0.036	0.57 ± 0.021	0.54 ± 0.002	0.53 ± 0.001	0.106
Epididymides	0.15 ± 0.018	0.15 ± 0.01	0.10 ± 0.001*	0.10 ± 0.002*	−0.42**
Prostate	0.20 ± 0.032	0.18 ± 0.0098	0.14 ± 0.002*	0.14 ± 0.012*	−0.48**
Seminal vesicles	0.55 ± 0.042	0.54 ± 0.013	0.51 ± 0.001	0.50 ± 0.03	0.107

Data are presented as mean ± SD (n = 25 male mice), there is no mortality during the experiment. g: weights per grams
* Significantly different from control value at $P < 0.05$.
** (r) Pearson correlation of toxicology with different doses of diazinon ($P < 0.05$).

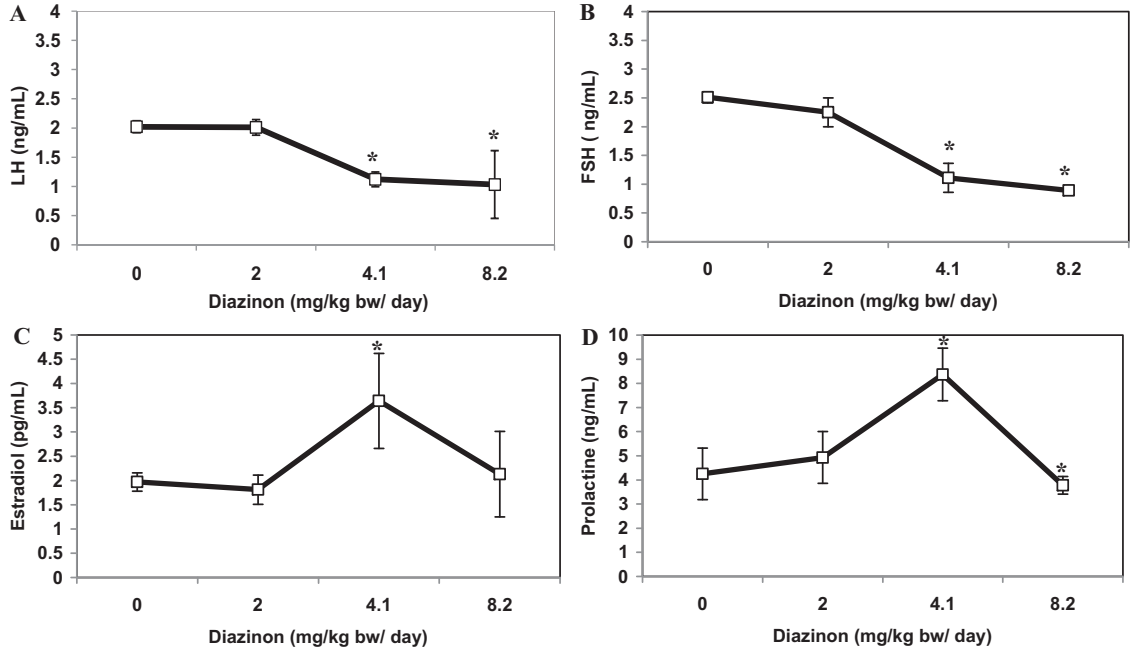

Fig. 3. (A–D). Effects of DZN on plasma LH, FSH, estradiol and PRL profiles. Note the attenuation of hormones dose dependently by DZN.

Table 2
Functional seminal parameters of male CD-1 mice exposed to different doses of diazinon.

	Diazinon (mg/kg bw/day)				
	0	2.0	4.1	8.2	*r*
Mating index (%)[a]	25/25 (100)	25/25 (100)	22/25 (88)*	15/25 (60)*	
Fertility index (%)[b]	25/25 (100)	24/25 (96)	19/22 (86)*	9/15 (60)*	
Sperm count/epididymis (mean $\times 10^6$)	6.3 ± 0.985	5.9 ± 0.68	3.9 ± 0.69*	2.1 ± 0.65*	−0.51**
Sperm count/(g) epididymis (mean $\times 10^6$)	215 ± 19.8	213.0 ± 22.65	45.0 ± 3.98*	22.0 ± 1.99*	0.210
Spermatid count/testis (mean $\times 10^6$)	57 ± 3.65	56.0 ± 5.98	35.0 ± 3.85*	18.0 ± 1.65*	−0.712**
Spermatid count/(g) testis (mean $\times 10^6$)	365 ± 39.5	360.0 ± 31.2	183.0 ± 8.65*	96 ± 6.98*	−0.698**
Motile sperms (%)	77.1 ± 4.85	74.7 ± 8.98	46.7 ± 4.65*	23.3 ± 2.85*	−0.588**
Abnormal sperms (%)	14.7 ± 1.065	22.5 ± 1.99*	47.6 ± 4.68*	52.6 ± 4.65*	0.521**
Viability (%)	78.5 ± 8.01	76.04 ± 9.01	38.27 ± 3.98*	17.73 ± 1.85*	−0.658**

Data are presented as mean ± SD.
Number of males which used for mating (n = 25).
[a] Number of males inseminated females/total number of males cohabited with females × 100.
[b] Number of pregnant females/total number of inseminated females with evidence of vaginal plug × 100.
* Significantly different from control at $P < 0.05$.
** (r) Pearson correlation of semen analysis with different doses of diazinon ($P < 0.05$).

Table 3
Reproductive outcome parameters of untreated female mice after cohabitation with diazinon treated male mice.

	Diazinon (mg/kg bw/day)				
	0	2.0	4.1	8.2	*r*
Number of mated females	25	25	25	25	
Number of pregnant females	25	24	19	9	
No of litters	25	24	19	9	
No of implantations/litter	10.66 ± 1.62	10.67 ± 1.06	10.16 ± 0.98	10.39 ± 1.01	
Live fetuses/litter (%)	10.00 ± 0.98 (94)	10.02 ± 0.132 (94)	9.44 ± 0.952 (91)	7.05 ± 1.29* (68)	−0.398**
Dead fetuses	0	0	0	1.05 ± 0.012 (10.2)	
Early resorptions/litter (%)	0.14 ± 0.106 (1.3)	0.15 ± 0.013 (1.4)	0.13 ± 0.013 (1.3)	0.39 ± 0.022* (3.9)	0.254
Late resorptions/litter (%)	0.52 ± 0.065 (4.9)	0.50 ± 0.002 (4.7)	0.59 ± 0.425 (5.8)	1.90 ± 0.684* (18.3)	0.71**
Post-implantation loss (%)[a]	6.19 ± 1.85	6.09 ± 1.05	8.15 ± 0.98	21.77 ± 1.065*	0.62**
Fetal body weight (g)/litter	1.01 ± 0.021	0.99 ± 0.013	0.87 ± 0.013*	0.75 ± 0.011*	−0.65**
Sex ratio (male/female)/litter	1.06 ± 0.011	1.27 ± 0.014	1.08 ± 0.003	1.07 ± 0.003	0.106

Data are presented as mean ± SD.
[a] Post-implantation loss (%) = ((no. of implantation sites − no. of live fetuses)/no. of implantations) × 100.
* Significantly different from control at $P < 0.05$.
** (r) Pearson correlation of reproductive outcomes with different doses of diazinon ($P < 0.05$).

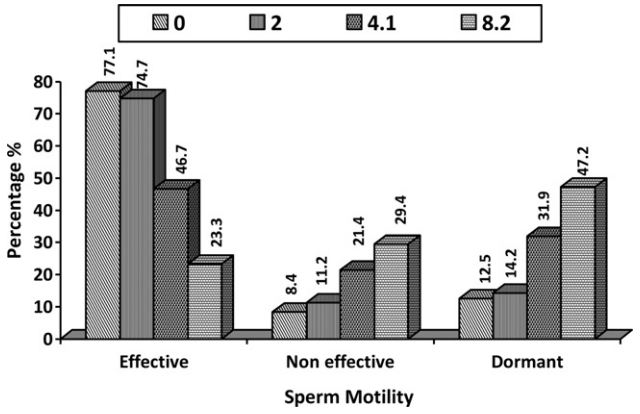

Fig. 4. Mean percentage ± SD of the sperm motility in semen of adult male CD-1 mice after oral administration of saline or different doses of diazinon once a day for 28 days. *Statistical analysis indicates significant differences at $P < 0.05$.

3.4.3. Female pregnancy outcomes

Significant reduction was observed in number of live fetuses, statistically significant ($P < 0.05$) increases in the number of resorbed fetuses in untreated females that mated with males exposed to the dose of 8.2 mg/kg bw/day, as compared to the control (Table 3). Diazinon exposure caused no obvious change in the reproductive performance in the treatment groups of 2.0 and 4.1 mg/kg bw/day. Correlation analyses of reproductive outcome parameters showed significant positive correlation between late resorptions and post-implantation loss ($r = 0.71$ and 0.62, respectively $P < 0.05$) (Table 3) but negative significant correlation between live fetuses and fetal body weight ($r = -0.389$ and $r = -0.65$, respectively $P < 0.05$) (Table 3).

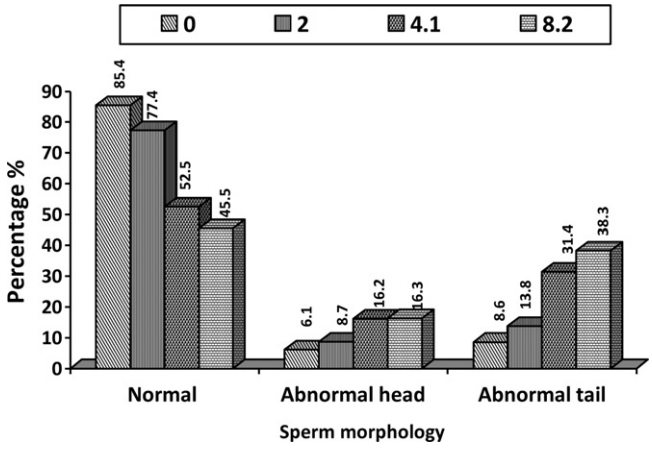

Fig. 5. Mean percentage ± SD of the sperm morphology in semen of adult male CD-1 mice after oral administration of saline or different doses of diazinon once a day for 28 days.*Statistical analysis indicates significant differences at $P < 0.05$.

3.5. Histological findings

Testicular histopathology showed a dose-dependent effect of diazinon on spermatogenesis (Fig. 7). The control mice showed a normal process of spermatogenesis, a regular arrangement of spermatogenic epithelium existed in seminiferous tubules (Fig. 7A). The mice treated with diazinon at 2.0 mg/kg bw/day did not show a significant testicular damage (Fig. 7B). The mice exposed to 4.1 and 8.2 mg/kg bw/day provoked severe alterations in the seminiferous tubules, namely the loss, derangement and sloughing of the germ cells, the vacuolization of germ cell cytoplasm and the disruption of spermatogenic cells (Fig. 7C and E) more evident in 8.2 mg/kg bw/day group (Fig. 7E). Most of the tubules showed hypoplasia and dispersion of the germ cells. In addition to focal areas of irregular seminiferous tubules were noted (Fig. 7C and E) and mild to severe vacuolation (Fig. 7D and F). Testicular damage in these diazinon treated mice was evident as interstitial edema and increased interstitial space (Fig. 7C and D). Leydig cells with degenerated nuclei were evident (Fig. 7D and F).

4. Discussion

Data from the foregoing results indicate that administration of diazinon to male mice by oral gavage resulted in significant adverse effects including cholinergic signs, decreased acetylcholinesterase activities and LH and FSH levels, and histopathologic alterations of testes in the 4.1 and 8.2 mg/kg bw/day groups. Testicular effects were characterized by markedly decreased testis weight with reduction in mating and fertility indices may simply represent the effects of diazinon exposure on sperm parameters and testis histological changes with the 4.1 and 8.2 mg/kg bw/day treatments. An effect on body weights was also observed in these treated groups. Dose 2.0 mg/kg bw/day, however, did not cause observed adverse effects in these parameters. Although 4.1 and 8.2 mg/kg bw/day diazinon groups produced marked testicular toxicity and reduction of sperm parameters, adverse effects on pregnancy outcomes were observed only in the 8.2 mg/kg bw/day group. Therefore, the male-mediated effects of diazinon on pregnancy outcomes cannot be directly attributed to the testicular toxicity but to damage of sperm DNA. Therefore, the present results are consistent with the

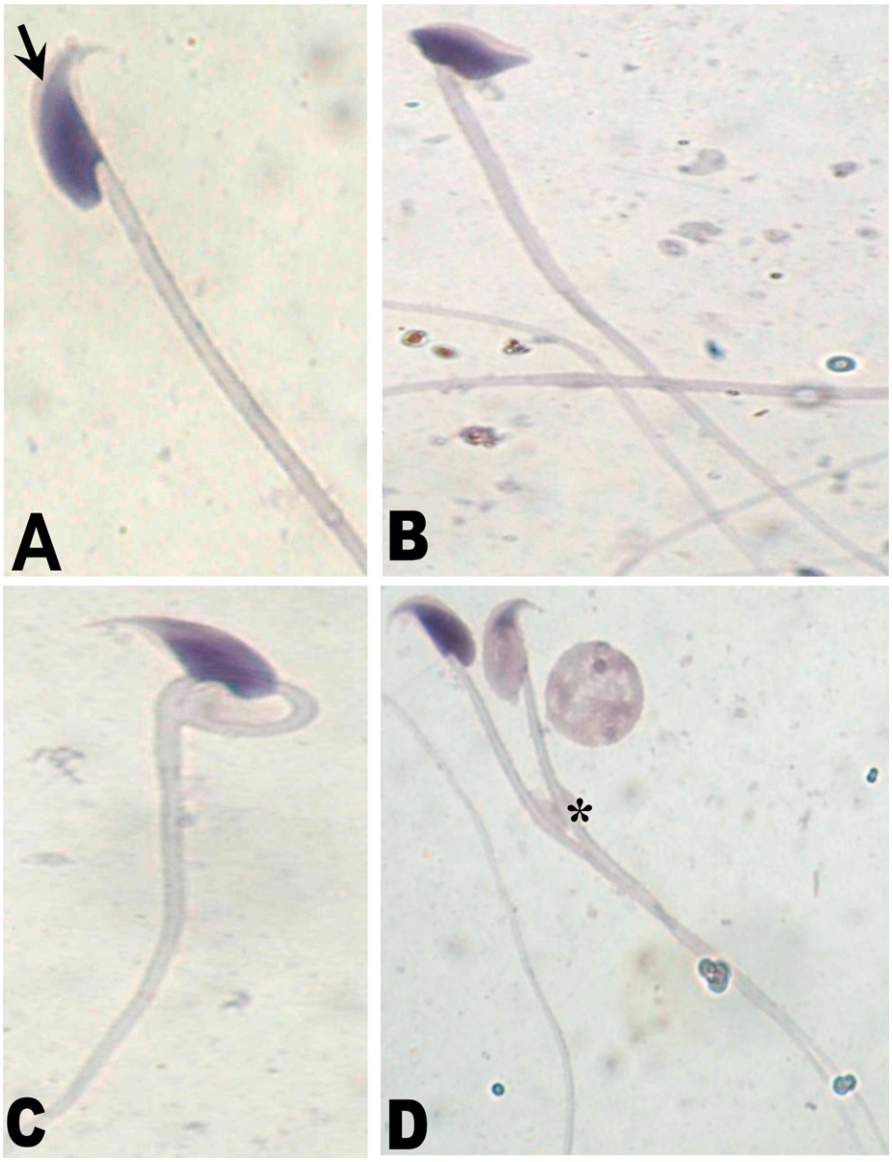

Fig. 6. Patterns of sperm abnormalities. (A) normal sperm with acrosome (arrow), (B) clumped head with normal tail, (C) normal head with looped tail, (D) unstained megacephaly head and normal tail with cytoplasm droplet (*). Haematoxylin stain.

mutagenic activity of diazinon [58] and with the recent conclusion that diazinon is a potential mutagen and carcinogen [59,60].

Inhibition of plasma AChE activities in the middle and high dose groups is in consistent with the principal mode of action of organophosphorus compounds [61] leading to accumulation of acetylcholine and subsequent activation of cholinergic, muscarinic, and nicotinic receptors and producing neurological deficits [62].

Considering the increase in serum estradiol level could be attributed to increased activity of the aromatase enzyme. This enzyme is responsible for estrogen production by converting testosterone and androstenedione to estrogens [63]. The crucial androgen/estrogen balance is necessary for normal development, even in the male [64], in many species. Therefore, it is likely that the resultant local balance between testosterone and estradiol may be responsible for some of the reproductive effects induced by diazinon. Investigation of alteration in the estrogen level is warranted to clarify whether or not the long-term effects on the testis are primary to diazinon exposition. However, the possibility remains that some of the adverse effects on the testis are resultant of changes in Leydig cells and testosterone withdrawal. Spermatogenesis and fertility are critically dependent upon the maintenance of adequate levels of testosterone [65].

Diazinon has been proven to alter steroidogenic hormones [5] which result in impairment to the reproductive physiological mechanisms [12]. Similar to the effects of other organophosphates (OP) [66], it is probable that the production of gonadotropins has been affected by diazinon in male mice by disruption of the hypothalamic–pituitary–gonadal axis and could be due to sensitivity of neuroendocrine neurons in the anterior hypothalamus [67].

The decrease of LH and FSH secretion observed in this study agrees with estrogenicity of diazinon [68], with positive feedback of increased estrogen, as normal levels of estrogens reduce LH release [69] or with impairment in their production and secretion [70]. One of the main reasons for the decrease in LH and FSH secretion could be correlated with increase in serotonin content in anterior hypothalamus level in relation to a possible decrease of testosterone level [67]. Another reason for decreasing a GnRH release could be explained by increase in dopamine concentration in the anterior hypothalamus [71]. In addition, the stimulatory effects of the pesticide on prolactin release [72] along with

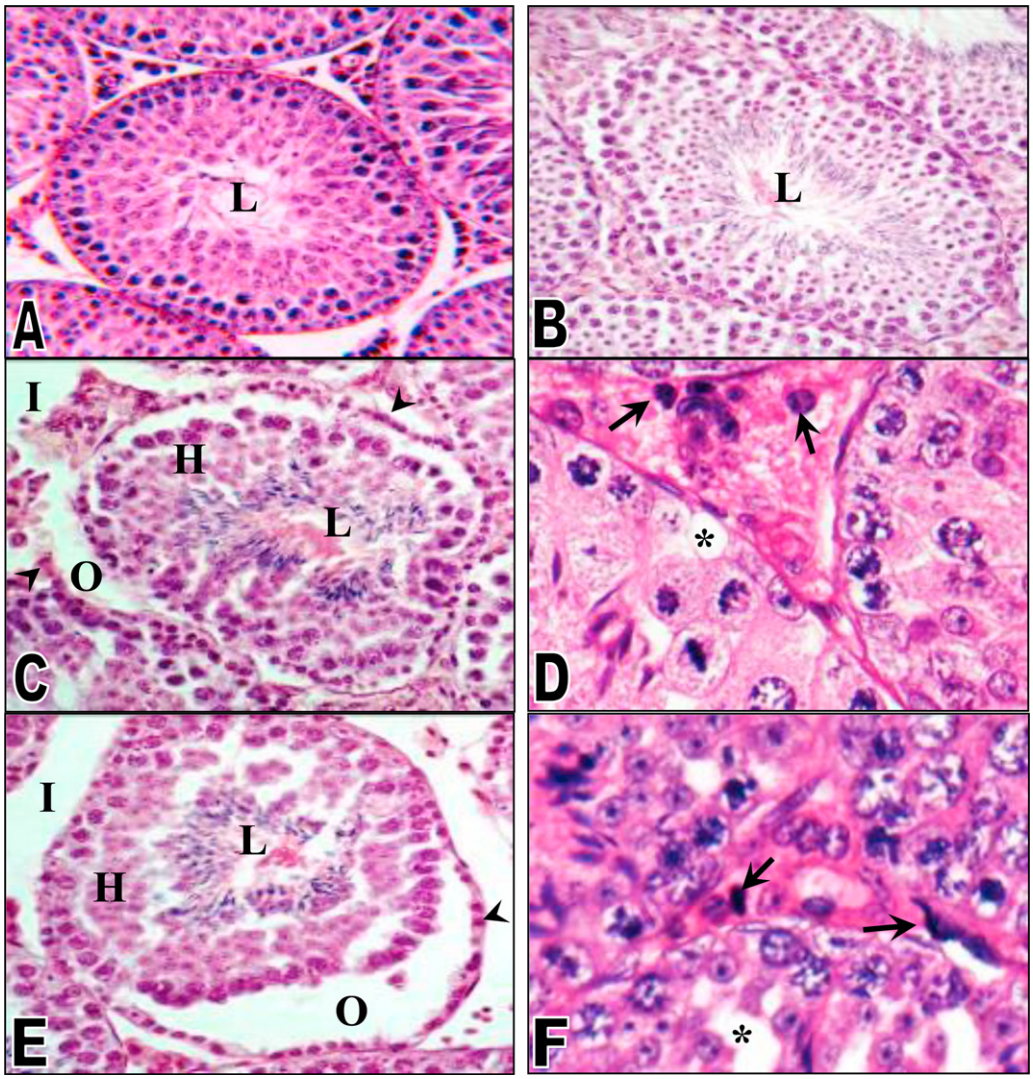

Fig. 7. Histopathology of testis after treated with diazinon at different doses. Control (A), 2.0 mg/kg (B), 4.1 mg/kg (C, D), and 8.2 mg/kg (E, F). in A&B groups, testicular tissue with normal architecture. In C-F groups, testicular tissue shows hypoplasia (H), vacuolization (*) and detachment of germ cells (arrowhead), degenerated Leydig cells in the testis interstitium with picnosis of their nuclei (arrow) odema (O), narrow lumen (L), dilated interstitium interlobular space (I). H&E staining, 400×.

hyperprolactinemic states they were associated with low LH levels [73]. The fluctuations of ACh in the present results are consistent with the reported findings indicated that acute doses of OP in laboratory animals caused alterations in concentrations of other neurotransmitters implying ACh [74].

The inhibition of testosterone secretion could be due to the inability of neuroendocrine cells of hypothalamic–pituitary axis to respond to the feedback when testosterone level decreased [75]. This implies that ACh do not play a crucial effect on the interaction between the nervous and endocrine system. Furthermore, the reduction in testosterone secretion may simply represent the participation of noradrenergic and serotoninergic transmission, the direct neural pathway between the anterior hypothalamus and the testes [17]. The increase in testosterone levels in dose group 4.1 mg/kg bw/day may be due to the direct stimulatory action of OP on Leydig cells through its effect on AChE [12]. The histologic changes in testes were attributable directly to AChE inhibition; mice giving 4.1 and 8.2 mg/kg bw/day diazinon demonstrate decreased plasma AChE activity, otherwise histologic alterations of testes appeared in these treated groups in a dose-related pattern. Chronic exposure to diazinon has been shown to alter the activity of the cholinergic system without altering noncholinergic activity [76]. The impaired Leydig cell function is displayed by a decrease in testosterone production as a consequence of reduced expression of several important steroidogenic factors, including StAR, CYP17A1, CYP11A1, and 3β-HSD. All these factors being important components of the steroidogenic machinery may serve as susceptible targets of endocrine disrupter actions [77].

5. Conclusion

The present data indicate that diazinon exposure at the doses of 4.1 and 8.2 mg/kg/day for 28 days: (1) could exert potential effects in neurotransmitters concentrations other than ACh in hypothalamus and pituitary; (2) could inhibit or increase testosterone or increase estrogen secretion with changing cytotoxic dose administration; (3) and finally, potential pathways might be involved in diazinon effects on reproductive toxicity (changing testosterone metabolic pattern; modifying neurotransmitters implying ACh; altering the activity of the direct hypothalamus–pituitary–gonadal neural pathway; and/or by a direct effect of DZN in testes which affect spermatogenesis in turn sperm patterns and reproductive performance. The current findings declare that 2 mg/kg bw/day diazinon did not induce adverse effects, but on the basis of a

JMPR/WHO codex alimentarius, the human acute daily intake (ADI) and acute reference dose (RfD) for diazinon are 0.002 and 0.03 mg/kg, respectively. Together, these data demonstrate that 2 mg/kg bw/day is about 1000 times the ADI and 0.02 times the recommended safety factor of 100 [78,79]. This raises awareness that such human exposures might occur occupationally or under normal circumstances, particularly for applicators and farmers. Especially, when appropriate industrial and agricultural hygiene regulations are not followed.

Conflict of interest statement

The authors have disclosed no potential conflicts of interest regarding this manuscript.

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