International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 5 (1): 51-57, 2009
ISSN 1811-7775

Evaluation of the Reproductive Toxicity of Chlorpyrifos Methyl, Diazinon and Profenofos Pesticides in Male Rats

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Abstract: The toxic effects of organophosphorus pesticides (i.e., chlorpyrifos methyl, diazinon and profenofos) on male reproductive system of rats were evaluated. Rats received pesticides mixed with powdered feed at concentrations of 5 and 50 ppm of each pesticide for 65 successive days. Sex organs weight, semen picture, concentrations of the hormones [i.e., testosterone, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)], activities of acetylcholinesterase (AChE) and histopathological changes in testes were the criteria used to evaluate the reproductive toxicity of the treated rats. Results showed that the effect of all tested pesticides on testes and seminal vesicles weights was dose-dependent since all tested pesticides at 50 ppm significantly decreased their weights. Serum AChE activity was inhibited with all tested pesticides. Both the concentrations of the tested pesticides decreased sperm count associated with increase in the number of morphologically abnormal spermatozoa of treated rats; however sperm motility was significantly decreased with the highest concentration of the tested pesticides. A decrease in the serum testosterone was observed in all treated groups; however LH and FSH levels were decreased with the highest concentration of the tested pesticides. Tissues of treated rat's testes showed slight alterations when histopathologically examined especially with the higher concentrations.

Key words: Organophosphorus pesticides, testosterone, sperm, gonadotropins testis

INTRODUCTION

Global concerns have been raised in recent years over the potential adverse effects that may result from exposure to chemicals that have the potential to interfere with the endocrine system which are called Endocrine Disrupting Chemicals (EDCs). The Natural Resources Defense Council (1998) defined an endocrine disrupter as synthetic chemicals that when absorbed into the body either mimic or block hormones and disrupts the body's normal functions through altering normal hormone levels, halting or stimulating the production of hormones, or changing the way hormones travel through the body. The hypothesis that environmental chemicals acting as EDCs could be causative agents of changes in population-based, reproductive health trends is relatively recent. Pesticides represent one of the better studied groups of EDCs (Johnson *et al*., 2000). Among common pesticides, organophosphorus (OP) compounds are widely used in agriculture and household applications. Currently the development and use of these compounds is greater than ever before and this trend will most likely continue, because new applications for these compounds have been discovered (Gupta, 2006). A profile reported from 66 poison control centers in the United States in 1997 indicated that organophosphorus pesticides were involved in top poisoning than any other classes of pesticides (Litovitz *et al*., 1998).

Chlorpyrifos-Methyl (CPM) has been suspected as endocrine disrupter by a few *in vitro* studies. CPM exhibited anti-androgenic activity in the Hershberger assay such as inhibition of the testosterone-stimulating increased weight of accessory sex organs (Kang *et al*., 2004). In adult men, 3, 5, 6-trichloro-2-pyridinol (TCPY), a metabolite of chlorpyrifos and chlorpyrifos-methyl, was associated with reduced testosterone levels (Kamijima *et al*., 2004). Abd el-Aziz *et al*. (1994) found that diazinon decreased the weights of most genital organs and motility associated with an increase in the percentage of dead and morphologically abnormal spermatozoa and decreased in the plasma testosterone level of treated rats for 65 consecutive days. Profenofos has a moderate order of acute toxicity following oral and dermal exposure. Profenofos considered as one of the male reproductive toxicants (Moustafa *et al*., 2007).

The aim of this study, therefore, was to assess the potential impacts of organophosphorus insecticides (i.e., chlorpyrifos-methyl, diazinon and profenofos, which are recommended universally and in Egypt to control various economic pests) on male reproductive system of

rats. Hence, the weights of sex organs, concentrations of certain sex steroidal hormones, spermatozoal morphology and testes histopathology were examined in treated rats.

MATERIALS AND METHODS

Pesticides used: Chlorpyrifos-methyl [O,O-dimethyl O-(3,5,6-trichloro-2-pyridyl) phosphoro-thioate], diazinon [O, O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate] and profenofos [O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate] were kindly provided from Central Agricultural Pesticide Laboratory (Dokki, Giza, Egypt) and all compounds were of 99% purity.

Test animals: Adult male Sprague-Dawley rats (240-270 g, 10 week old), *Rattus norvegicus albinus*, were purchased from the Biological Products and Vaccines Holding Company, Helwan Farm, Cairo. Rats were maintained under the laboratory conditions of 25+5°C and 65+5% R.H. for two weeks before starting the experiment for acclimatization. They were housed in metal cages (35×25×20 cm) with a 12:12 h light/dark cycle and maintained on *ad libitum* diet and water. All animals were in good health. Prior to administration of pesticides, rats were assigned to each group by randomization of body weights.

Experimental design: Rats were divided randomly to seven groups, each group had five rats. The (first and second), (third and fourth) and (fifth and sixth) groups received diazinon, chlorpyrifos-methyl and profenofos pesticides, respectively. All pesticides were blended with powdered feed at concentrations of 5 and 50 ppm for each pesticide. The seventh group received pesticide-free diet and considered as control. Feeding administration lasted for 65 successive days.

Diet preparation: The powdered feed containing the required concentrations of tested pesticides was prepared as described by Kimbrough *et al.* (1972) using the same solvent and mixing technique. Thus, the amount of each tested pesticide was dissolved in 50 mL of redistilled diethyl ether and mixed thoroughly with 50 g of corn starch. The solvent was evaporated and the amount of each tested pesticide impregnated in starch was then added to 950 g of the powdered feed to give the required concentrations. Control diet was prepared by the same technique without addition of any pesticide.

Parameters studied: In the experiment, clinical signs and weights of genital organs (i.e., testes, epididymis, seminal vesicles and prostate glands) were assessed as indices of reproductive toxicity. Clinical signs including any abnormal appearance of behavior were recorded twice a day in each animal. At the end of the experimental period (65 days of dietary pesticide treatment), rats were sacrificed by decapitation, blood samples were collected in non-heparinized tubes, left till clotting occurred and centrifuged at 4000 rpm for 10 min. The obtained sera were kept frozen till being used for activities of AChE assay and hormonal assay that include the determination of levels of testosterone, LH and FSH. Epididymal spermatozoa were also examined. Testes were prepared and kept for histopathological examination. The various parameters were determined by the following methods.

Cholinesterase (ChE) activity: Serum cholinesterase (ChE) activity was determined according to the kinetic method of Den Blawen *et al.* (1983).

Sperm analysis: Epididymis was excised and weighed from each animal. The right cauda epididymis was used for sperm count and left one for sperm motility and sperm morphological analysis according to the method described by Jeong *et al.* (2005).

Hormonal assay: Levels of testosterone, LH and FSH were determined in serum of male rats by Elecsys Analyzer, D-Vi-S, using kits of Roche Diagnostics GmbH, D-68298, Mannheim, Germany.

Histopathological examination: The testes were fixed in Bouin's solution and processed by dehydration in different concentrations of alcohol, cleared with xylol and embedded in paraffin blocks, then sectioned at 4 μ thicknesses. The paraffin sections were stained by haematoxylin and eosin (Harris, 1898) and then histopathological examination was carried out microscopically.

Statistical analysis: Data are expressed as Mean±S.D. Statistical significance of differences was determined using the program SPSS 12 (SPSS, USA) by performing one-way ANOVA with post hoc comparisons between the control group and each of the treated group followed by Duncan's multiple comparison tests. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Clinical symptoms and mortality during the test period: Adults male albino rats fed on contaminated rations with two concentrations (5 or 50 ppm) of each tested pesticide

Table 1: Genital organs weights of male rats treated with dietary daily concentrations of pesticides for 65 days

		Testes		Epididymis		Seminal vesicles		Prostate gland	
Treatments	Concentration	Mean	Control (%)	Mean	Control (%)	Mean	Control (%)	Mean	Control (%)
Diazinon	5	4.3±0.57^{b}	95.5	0.61±0.04^{a}	96.8	0.76±0.13^{b}	96.2	0.63±0.1^{a}	88.7
	50	2.8±0.22^{a}	62.2	0.61±0.03^{a}	96.8	0.51±0.06^{a}	64.5	0.64±0.06^{a}	90.1
Chlorpyrifos- methyl	5	4.1±0.74^{b}	91.1	0.68±0.08^{a}	107.9	0.74±0.05^{b}	93.6	0.64±0.09^{a}	90.1
	50	2.9±0.06^{a}	64.4	0.59±0.02^{a}	93.6	0.56±0.09^{a}	70.8	0.63±0.09^{a}	88.7
Profenofos	5	4.0±0.49^{b}	88.8	0.60±0.04^{a}	95.2	0.77±0.03^{b}	97.4	0.67±0.02^{a}	94.3
	50	2.9±0.04^{a}	64.4	0.63±0.05^{a}	100.0	0.53±0.06^{a}	67.1	0.68±0.09^{a}	95.7
Control	-	4.5±0.3^{b}	100.0	0.63±0.06^{a}	100.0	0.79±0.11^{b}	100.0	0.71±0.03^{a}	100.0

Each value represents mean of five replicates ±SD. ^{a,b}Values across each column having the same superscript letter(s) were not significantly different ($p>0.05$)

for 65 consecutive days were daily examined physically and clinically. The observations included changes in skin, eyes, orifices and clinical signs of respiratory behavior changes. Results showed that no clinical symptoms of toxicity or mortalities were observed in treated rats throughout the experimental period.

Evaluation of the reproductive organs weight: Genital organs weights were among of the criteria used to evaluate the reproductive toxicity of the tested pesticides to rats. Data pertaining to the impact of the tested pesticides on weights of male genital organs are shown in Table 1.

Perusal of these results clearly exhibited that the effect of all tested pesticides on testes and seminal vesicles weights was dose-dependent since all tested pesticides at 50 ppm significantly decreased their weights and maximum percent of decrease was occurred with diazinon i.e., 62.2 and 64.5% of control for testes and seminal vesicles weights, respectively. Otherwise, the effect was not significant with the lowest concentration of all tested pesticides. On the other hand, there were no significant differences in weight of epididymis and prostate glands at the end of the experimental period among the treated groups as compared with control.

Useful information on male reproductive capacity of laboratory animals can be obtained by measuring weights and the volume of testis, prostate, seminal vesicles, epididymis and coagulating glands (Doul *et al*., 1980). The weights of testes and accessory sex organs are known to be reliable indices of testicular androgen production (Price and Williams-Ashman, 1961; Rind *et al*., 1963). Sub-lethal chronic administration (7-14 mg kg^{-1} a day for 15 days) of quinalphos resulted in decreased testicular mass **(**Sarkar, 2000). Significant decline in testicular weight may be due to decrease in the number of spermatogenic elements and spermatozoa (Sherins and Hawards, 1978; Takihara *et al*., 1987). Abd El-Aziz *et al*. (1994) found that diazinon decreased the weights of most genital organs when administered at two different doses 1.5 and 3 mg kg^{-1} body weight in male rats for 65 consecutive days. Furthermore, Ray (1991) indicated that relative weights of the testes and accessory sex organs exhibited a significant reduction in all rats treated with quinalphos. On the other hand, Kang *et al*. (2004) revealed that the weight of relative and absolute androgen-dependent accessory sex organs, seminal vesicle ventral and prostate gland were unchanged by the treatment of chlorpyrifos-methyl at 2, 10, 50 and 250 mg kg^{-1}. Generally, maintenance of weights of accessory reproductive glands depends on testosterone level (Jana *et al*., 2003). Several pesticides have reduced the organ weights by affecting either hypothalamus or pituitary or both (Okazaki *et al*., 2001; Latchoumycandane *et al*., 2002).

Table 2: Serum cholinesterase activity of male albino rats fed on contaminated rations for 65 successive days

		Cholinesterase activity (U L^{-1})	
Treatments	Concentration	Activity	Control (%)
Diazinon	5	185.3±4.10^{bc}	54.2
	50	111.8±25.3^{d}	32.7
Chlorpyrifos- methyl	5	174.4±17.6^{bc}	51.0
	50	130.6±12.1^{cd}	38.2
Profenofos	5	218.9±13.5^{b}	64.1
	50	169.7±25.3^{bc}	49.7
Control	-	341.7±29.4^{a}	100.0

Each value represents mean of five replicates ±SD. $^{a-d}$ Values across each column having the same superscript letter(s) were not significantly different ($p>0.05$)

Effect on serum cholinesterase (ChE) activity: Data shown in Table 2 revealed that the impact of prolonged administration of tested pesticides for 65 successive days in rats resulted in significant inhibition of serum ChE activity with all tested pesticides. Maximum percent of inhibition occurred with the high concentration of diazinon followed by chlorpyrifos-methyl and profenofos, i.e., 32.7, 38.2 and 49.7% of control, respectively. There are many environmental toxicants inducing alteration of reproductive functions concurrently with impaction on the central nervous system and behavior, which are so called neuroendocrine disrupters operating through hypothalamo-pituitary-gonadal axis (Sarkar *et al*., 2000; Gore, 2001).

The increased acetylcholine (ACh) in pituitary gland and hypothalamus by organophosphate induced-inhibition of acetylcholine esterase (AChE) can variably affect anterior pituitary functions and the release of

secondary neurotransmitters, especially dopamine or gonadotrophins (Sarkar *et al*., 2000). Organophosphates may act as neuroendocrine disruptors via inhibition of AChE activity and increase of acetylcholine level in brain (Herken and Neubert, 1953). These results are in accordance with those findings obtained by (Karanth and Pope, 2003; McDaniel and Moser, 2004; Farag *et al*., 2007) who concluded that ChE activity was significantly decreased after exposure of rats to organophosphorus pesticides.

Effect on spermatozoal morphology and viability: The percentages of sperm motility decreased significantly in treated rats with each pesticide at the highest concentration and the least incidence was noticed with diazinon (i.e., 20.9%). Both concentrations of all tested pesticides decreased significantly sperm count of treated rats where, the highest effect was noticed for diazinon at 50 ppm (37%). Total sperm abnormalities were significantly increased for all tested pesticides at both concentrations (total sperm abnormalities ranged from 294.7-663.1% versus control). Generally, the most pronounced malformations which were observed in sperms are bent tail, coiled tail and protoplasmic droplets (Table 3). The abnormalities appeared as bent tail, constitute the highest percentages of the total deformities. Sperm morphology is considered as a better discriminator between fertile and infertile males than sperm concentration (Guzick *et al*., 2001). Sperm morphology and motility could also be useful markers of toxic damage even in the absence of any effect on fertility. Spermatogenesis is controlled by two main regulatory processes, i.e., endocrine regulation via the gonadotropin hormones and local regulation via inter-cellular communications (Holdcraft and Braun, 2004). The obtained results are in accordance with those found by Abd El-Aziz *et al*. (1994), who revealed that diazinon given orally to male rats for 65 consecutive days decreased sperm motility associated with an increase in the percentage of dead and morphologically abnormal spermatozoa. Methyl Parathion has been shown to induce reproductive abnormalities in both wild life and humans with reduction in sperm counts (Mathew *et al*., 1992). Furthermore, Sarkar (2000) found that Sub-lethal chronic administration (7-14 mg kg^{-1} a day for 15 days) of quinalphos resulted in severe disruption of spermatogenesis with increasing doses of pesticide. Remarkable reduction in the sperm count was observed in Wistar rats following treatment with quinalphos (250 μg kg^{-1}, i.p.) for approximately one (13 days) and two cycles (26 days) of the seminiferous epithelium (Ray *et al*., 1992). Prior epidemiologic work on Chinese pesticide factory workers showed that OP exposure was associated with decreased sperm concentration and motility (Padungtod *et al*., 2000). Sperm production and percentage of motile sperm were decreased in the 15 and 28 mg/kg/day treated male mice groups with dimethoate compared to the control (Farag *et al*., 2007).

Hormonal status: The results quite indicate that, all tested pesticides caused significant decrease in testosterone levels of male rats. The highest reduction was observed in case of diazinon-treated rats at 50 ppm (48.3%) followed by chlorpyrifos methyl (55%) and profenofos (61.6 %). The effects were less pronounced with the lowest concentrations (Table 4). In addition, serum LH and FSH levels in all pesticides-treated rats were significantly decreased with the high concentration only.

Table 3: Effect of pesticides on epididymal sperm characters in male rats

		Sperm motility (%)		Sperm count ($\times 10^6$ mL^{-1})		Sperm abnormality (%)	
Treatments	Concentration	Mean	Control (%)	Mean	Control (%)	Mean	Control (%)
Diazinon	5	70.0±10.0^{d}	87.5	43.3±7.1^{bc}	58.7	6.6±1.6^{b}	347.3
	50	16.7±11.5^{a}	20.9	27.3±2.5^{a}	37.0	12.6±1.25^{c}	663.1
Chlorpyrifos- methyl	5	73.3±15.2^{d}	91.6	48.7±5.0^{c}	66.1	5.9±1.1^{b}	310.5
	50	43.3±15.2^{bc}	54.1	36.0±3.5^{ab}	48.8	10.3±0.64^{c}	542.1
Profenofos	5	63.3±5.7^{cd}	79.1	35.3±4.7^{ab}	47.9	5.6±1.2^{b}	294.7
	50	23.3±15.2^{ab}	29.1	31.3±3.2^{a}	42.4	10.9±1.3^{c}	573.7
Control	-	80.0±5.0^{d}	100.0	73.7±8.4^{d}	100.0	1.9±0.23^{a}	100.0

Each value represents mean of five replicates ±SD. $^{a-d}$Values across each column having the same superscript letter(s) were not significantly different ($p>0.05$)

Table 4: Concentration of certain serum hormones in male rats exposed to the pesticides for 65 days

		Testosterone (ng mL^{-1})		LH (mIU mL^{-1})		FSH (mIU mL^{-1})	
Treatments	Concentration	Mean	Control (%)	Mean	Control (%)	Mean	Control (%)
Diazinon	5	5.3±0.45^{c}	88.3	3.3±0.11^{c}	100.0	1.20±0.15^{b}	100.0
	50	2.9±0.14^{a}	48.3	1.9±0.14^{a}	57.5	0.92±0.03^{a}	76.6
Chlorpyrifos methyl	5	5.4±0.45^{c}	90.0	3.2±0.06^{c}	96.9	1.20±0.2^{b}	100.0
	50	3.3±0.15^{ab}	55.0	2.1±0.05^{b}	63.6	0.93±0.03^{a}	77.5
Profenofos	5	5.2±0.51^{c}	86.6	3.1±0.08^{c}	93.9	1.10±0.05^{ab}	91.6
	50	3.7±0.22^{b}	61.6	2.3±0.04^{b}	69.7	0.97±0.008^{a}	80.8
Control	-	6.0±0.27^{d}	100.0	3.3±0.32^{c}	100.0	1.20±0.19^{b}	100.0

Each value represents mean of five replicates ±SD. $^{a-d}$Values across each column having the same superscript letter(s) were not significantly different ($p>0.05$)

Testosterone (17 β-hydroxy-4 androsten-3-one) is a C19 hormone with a molecular weight of 288.4 daltons. It is the principal male hormone produced by the interstitial Leydig cells of the male testes and in smaller amount by the adrenals and the female ovaries. Thus, the testes are responsible for the synthesis of the male sex hormones, or androgens and for the production of spermatozoa. The most important androgen, both in terms of potency and the amount secreted in testes is the steroidal compound, testosterone, a powerful anabolic hormone. It is a vital to the development of secondary sexual characteristics in males and is essential for spermatogenesis (Guyton, 1991; Mycek *et al*., 1997). Testosterone is secreted as mentioned earlier by the leydig cells of the testis under the influence of Luteinizing Hormone (LH). Krause (1977) reported that the decreased testosterone levels might be due to a direct damage of leydig cells or to a lowered stimulation of these cells by LH. Disorders of male genital function (hypogonadism) are manifested by a decrease in plasma testosterone level. Hypogonadism may occur with defective seminiferous tubules function or defective leyding cell function and this leads to infertility through decreased production of spermatozoa, but masculinization is usually normal. Defective leydig cell function also results in failure of testosterone dependent functions including spermatogenesis. Sertoli cells, the major epithelial component of the seminiferous epithelium, are essential for the control of spermatogenesis, by supplying nutrients which ensure germ cell proliferation and differentiation and by responding to endocrine stimuli (Saunders, 2003). Many sertoli cell functions are regulated by Follicle-Stimulating Hormone (FSH) (Simoni *et al*., 1999). There are several reports representing that organophosphates decrease testosterone levels in rats treated at different doses, the effects were always accompanied by defects in gonads and suppress LH and FSH (Ray *et al*., 1991, 1992; Abd El-Aziz *et al*., 1994; Kang *et al*., 2004). Other studies also showed that organophosphate pesticides such as chlorpyrifos, fenthion, fenitrithion and dimethoate act like androgen receptor antagonists or suppressor of gene expression related to gonadotropin synthesis (LH and FSH) or steroidogenesis (Walsh *et al*., 2000; Gore, 2001; Kitamura *et al*., 2003). Kang *et al*. (2004) revealed that chlorpyrifos-methyl showed anti-androgenic activity without estrogenic activity in rats. Chlorpyrifos-methyl suppressed androgenic activity in Hershberger assay using castrated rats (Jeong *et al*., 2006). Furthermore, chlorpyrifos was reported to affect profoundly hypothalamic GnRH gene expression and reduce LH and FSH (Padungtod *et al*., 1998; Guven *et al*., 1999; Gore, 2001). Suppression of gonadotrophins might have caused decrease in sperm density in testes (Sinha *et al*., 1995). With regard to pesticides that act on the brain, both organophosphate and carbamate pesticides can reduce acetylcholinesterase activity and hence block nerve impulses. This effect may be linked to the suppression of the brain's release of hormones that stimulate the gonadotrophic hormones (LH and FSH) (Lyons, 1999).

Histological evaluation: The testes of control rat exhibited normal testicular structures on histological examination (Fig. 1A). However, at the high concentration of all tested pesticides only, the tissues revealed normal testicular

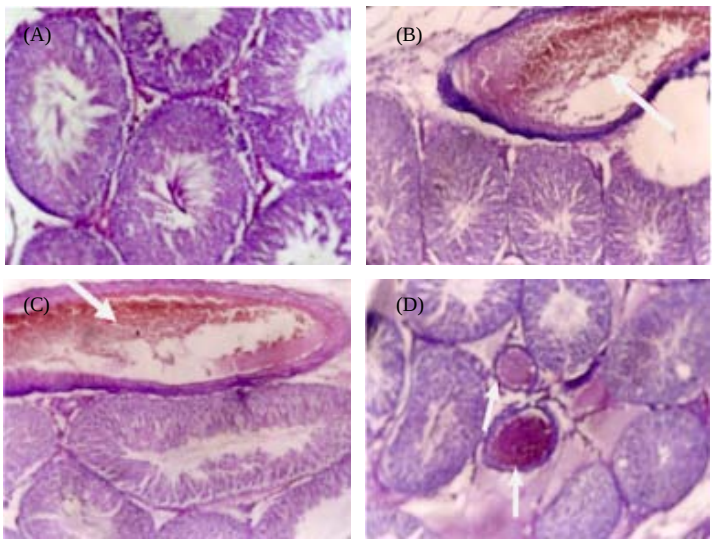

Fig. 1: Testes from (A) control rat showing normal structure, with all the successive stages of spermatogenesis (H and E10), (B) chlorpyrifos methyl treated rats, (C) diazinon treated rats and (D) profenofos treated rats, showing congestion in seminiferous tubules (H and E10)

structures with congestion in seminiferous tubules (Fig. 1B-D). Okamura (2005) found that histopathologically the testes in all DDVP treated groups (0, 1, 2 or 4 mg kg^{-1}) 6 days a week for 9 weeks were not significantly different from those of the male rats control group. No histological changes appeared in mice testis in the 7 mg/kg/day treated group with dimethoate compared to the control group (Farag *et al*., 2007). Moustafa *et al*. (2007) showed that profenofos caused congestion in testes blood vessels with edema among seminiferous tubules in male rats which orally administered at dose of 17.8 mg kg^{-1} twice weekly for 65 days.

CONCLUSION

These results suggest that all tested pesticides decreased testosterone levels in treated rats at both concentrations, the effects were accompanied by decreasing in the testes and seminal vesicles weights and suppression of LH and FSH with the highest concentrations. Significant inhibition of serum ChE activity with both concentrations of tested pesticides was observed as well. These pesticides may act as neuroendocrine disruptors via inhibition of AChE activity and increase of acetylcholine level in brain and this effect may be linked to the suppression of the brain's release of hormones that stimulate the gonadotrophic hormones (LH and FSH). Also the results quite indicate that, the percentages of sperm motility decreased significantly in treated rats with each pesticide at the highest concentration. Sperm count decreased significantly and total sperm abnormalities increased significantly in treated rats with all tested pesticides at both concentrations. All the above mention effects were more pronounced and significantly with the higher concentration of diazinon. Thus, we have to be aware that diazinon has detrimental effects on the male reproductive system of rats.

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