

Animal Model Study of Reproductive Toxicity of the Chronic Exposure of Dicofol

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Abstract

Dicofol is an organochlorine acaricide widely used in local market. The present study was conducted to evaluate the dicofol chronic toxicity of male fertility indices and reproductive toxicity resulted from acaricide through sexual and reproductive hormones as well as investigate the effect on testicular function and epididymal oxidative parameters in an animal model. In this investigation, two equal groups of male albino rats were orally administered dicofol, at 4.19 and 16.75 mg/kg body weight/day through drinking water for consecutive 90 weeks. Dosages represent $1/80$ and $1/20$ of LD_{50} of dicofol respectively. The third group was kept as control group. At the end of each experimental period (16, 28 and 90 weeks), blood samples were taken to examine sexual, reproductive and thyroid hormones. Also, animals were dissected and the reproductive organs (epididymus and testes) were taken to measure fertility indices, oxidative parameters and testicular biomarkers. The results indicated dicofol decreased testes and epididymus weights for both low and high doses. This effect was dose-related and should be associated with decline in epididymus sperm count, percent of sperm motility, viability and maturity and increased abnormal sperm morphology. Decline in serum testosterone, follicle stimulating hormone and luteinizing hormone levels concomitant with an elevation in estradiol and progesterone levels were observed. Dicofol-treated group demonstrated de-generation and atrophy of some seminiferous tubules associated with depression in luminal spermatozoal concentration. Meanwhile, dicofol increased oxidative stress by elevation of lipid peroxidation index associated with depletion in glutathione level. Concerning the testicular biomarkers, dicofol increased total protein level and decreased the activities of the enzymes responsible of spermatogenesis, *i.e.* lactate dehydrogenase, acid and alkaline phosphatase activities. [Life Science Journal. 2009; 6(3): 1 – 18] (ISSN: 1097 – 8135).

Keywords: Rats, pesticide, dicofol, chronic toxicity, fertility, testes, epididymus, lipid peroxidation, glutathione, testicular markers, hormones.

1 INTRODUCTION

Pesticides are agricultural chemicals used for controlling pests on the plant or animals. Problems associated with pesticide hazards to man and environment are not confined to the developing countries, but extended to developed nations and still facing some problems in certain locations^[1]. The severity of pesticide hazards is much pronounced in third world countries. A number of long persistent organochlorines (O'Ch), which have been banned or severely restricted are still marketed and used in many developing countries^[2]. The ideal pesticide is a pesticide which is effective only against the pests and be harmless to people, animals and environment. However, they have some side / non-target effects that may show undesired actions appears latter^[3]. A large number of chemicals occurring in our environment may have potential to interfere with the endocrine system of

animals^[4]. Many of these chemicals can disrupt development of the endocrine system and of the organs that respond to endocrine signals in organisms indirectly exposed during prenatal and/or early postnatal life; effects of exposure during development are permanent and irreversible^[5]. Several pesticides have been reported to produce gonadal toxicity, among these are persistent and bioaccumulative organochlorine pesticides (O'Ch). Increasing interest has been observed among environmental and health institutions regarding the potential reproductive effects due to exposure to occupational and environmental chemicals^[4]. Over the past decade, there has been an increasing focus on the effects of synthetic chemicals on human endocrine system—specially on effects related to androgen and estrogen homeostasis^[6]. An understanding of the developmental consequences of endocrine disruption in wildlife can lead

to new indicators of exposure to endocrine disrupting contaminants. Thus, wildlife serve as important sentinels of ecosystem health, including human public health^[7]. There is much concern that exposure to such environmental contaminants causes decreased sperm counts, impairment of sperm motility, reduced fertilization ability, producing abnormal sperm in men and wildlife^[8]. It has been reported that, pesticides with such properties have been shown to cause overproduction of reactive oxygen species (ROS) in both intra- and extracellular spaces, resulting in a decline of sperm count and infertility in wildlife and human^[9]. The antioxidant system plays an effective role in protecting testes and other biological tissues below a critical threshold of ROS thus preventing testicular dysfunction^[10]. ROS has been shown to damage macromolecules, including membrane bound polyunsaturated fatty acid (PUFA), causing impairment of cellular function^[11]. Spermatozoa are rich in PUFA, and, therefore, could be highly susceptible to oxidative stress. Dicofol, an organochlorine acaricide, is used widely on agriculture crops and ornamentals and in or around agricultural and domestic buildings for mite control^[12]. It tends to accumulate in steroid producing organs such as adrenal gland, testes and ovary^[13] and has antispermatogenic and antiandrogenic properties^[14]. Previous studies have suggested that O'Ch, pesticides impaired the testicular functions through altering the activities of relevant enzymes^[15,16]. Studies have been conducted on reproductive toxicity of dicofol in male rats following short-term exposure^[13,14]. However, few and olden literature in this respect following long-term exposure specifically in mammals are available^[17,18]. For the above mentioned, the objective of the current study was to characterize the endocrine-disrupting effects and reproductive toxicity of chronic daily exposure to dicofol in male albino rats via evaluation of male fertility indices and reproductive and sexual hormone levels. As well as oxidative parameters in cauda epididymus and testicular functions were studied

2. Materials and Methods

2.1 Experimental Animals

In the present study, a total of seventy two male Wistar albino rats, *Rattus norvegicus* were obtained from the Farm of General Organization of Serum and Vaccine, Egypt. Male rats initially weighing 150±10 g were used. Animals were acclimated to holding facilities for two weeks prior to the experiment. The rats were housed in groups and kept in room under controlled temperature (24°C), humidity (30-70 %) and light (12: 12 hr / light: dark). All animals were provided balanced diet throughout the experimental period, these diet were obtained from

Agricultural-Industrial Integration Company, Giza. Which formed of proteins, lipids, fibers, wheat, clover, maize, beans, crushed bones, molasses, choline, lysine, methionine, NaCl, Mn, Zn, Co, Mg, Cu, Fe, Se, I2 and many vitamins like A, E, D3, K, B1, B2, Biotin, B6, B12, Niacin and Folic acid. Animals were given food and water *ad libitum*.

2.2 Experimental Materials

Dicofol (an O'ch pesticide), formulation 18.5 % Emulsifiable Concentrate (EC), was received from El-Nassr Company, was used through this investigation. Commercial name (Kelthane). The oral median lethal dose (LD50) of dicofol (administered to rats *per OS*.) was 348.86 mg/kg b.w, according to Weil's method (Weil, 1952). In this investigation, the used dosages were chosen according to the maximum tolerated dose (MTD), which suppressed body weight gain slightly i.e.; 10 %, generally ¼ MTD and 1/16 MTD, are then selected for testing (Hayes, 1989). Accordingly, dicofol was administered to rats at 4.19 and 16.75 mg/kg body weight (representing 1/80 and 1/20 LD₅₀) in drinking water.

2.3 Experimental Design

Seventy-two adult albino rats were allocated into three groups- 24 each - and treated with dicofol through drinking water for 90 successive weeks as the following:

Group (1): Rats received tap water only as an emulsifier of the pesticide dicofol (Emulsifiable concentrate).

Group (2): Rats received 4.19 mg/kg b.w. /day (30 ppm dicofol, which represents the lower dose).

Group (3): Rats received 16.75 mg/kg b.w. /day (120 ppm dicofol, which represents the higher dose).

dicofol emulsified daily in drinking water in glass bottle, and the bottles were cleaned daily.

All animals were observed at least once daily for behavior; signs of intoxications, mortality, morbidity, and food and water consumption were monitored daily. Animals weighed weekly and the dose was adjusted accordingly.

2.4 Sampling

2.4.1 Blood samples

At the end of each experimental period, (16, 28 & 90 weeks), blood samples were collected, from fasted rats (control and treatanimals), from the orbital sinus vein using anesthetic ether by heparinized capillary tubes in plain tubes, according to Schalm^[19], and allowed to be clotted at room temperature to obtain serum for hormonal assay

2.4.2 Sacrifice and tissues preservation

Five animals/group were sacrificed by design 16, 28 and 90 weeks on study. Testes and epididymus from sacrificed rats were removed immediately, clean of adhering tissues and weighed. Then, epididymus prepared for fertility evaluation and determination of oxidative biomarkers. Testes samples were taken for histopathological examination through the light microscope^[20], and estimation of testicular functions.

2.5 Data Collection Techniques

2.5.1 Evaluation of Fertility

Spermatozoa were obtained by mincing the cauda epididymus in a known volume of physiological saline (w/v) at 37°C for evaluation of semen parameters under microscope (40X) as the following:

2.5.1.1 Sperm concentration (count)

The spermatozoa concentration was carried out by diluting the sperm suspension with water (1: 20), then mixed together, after that a drop of them delivered into the Neubauer haemocytometer in each side of the counting chamber. The haemocytometer is allowed to stand for 5 min. for sedimentation, then sperms were counted in the large five squares and expressed as sperm concentration in million, according to Feustan et al^[21].

2.5.1.2 Sperm motility

The motility of sperm was evaluated directly after mincing in drop of sperm suspension, microscopically. Non-motile sperm numbers were first determined, followed by counting of total sperm. Sperm motility was expressed as percent of motile sperm of the total sperm counted, according to Linder et al^[22].

2.5.1.3 Sperm viability by Eosin stain

This technique is used to differentiate between live and dead sperms. A drop of the Eosin stain added into sperm suspension on the slide and stand for 5 min. at 37°C, then examined under microscope. The head of dead spermatozoa stained with red color. While, the live spermatozoa unstained with Eosin stain. Sperm viability was expressed as percentage of live sperm of the total sperm counted, according to Krzanowska et al^[23].

2.5.1.4 Sperm maturation by aniline-blue

Nuclear maturation was evaluated by aniline-blue stain, according to Morel et al^[24]. Sperm nuclei that stained with blue color were considered to be immature. But nuclear mature sperm was not stained with aniline-blue. The percentage of immature sperm was calculated from the

observation of one hundred sperm preparation from each group.

2.5.1.5 Sperm morphology

A drop of Eosin stain was added to the sperm suspension and kept for 5 min. at 37°C. After that a drop of sperm suspension was placed on a clean slide and spread gently to make a thin film. The film was air dried and then observed under a microscope for changes in sperm morphology, according to the method of Feustan et al^[21]. The criteria chosen for head abnormality were; no hook, excessive hook, amorphous, pin and short head. For tail, the abnormalities recorded were; coiled flagellum, bent flagellum, bent flagellum tip. The result are the percentage overall abnormal form.

2.5.2 Hormonal assay

2.5.2.1 Determination of serum testosterone

Testosterone determination was performed according to the method adopted by Jaffe and Behrman^[25], by using the coat-A-count technique, (radioimmunoassay)

2.5.2.2 Determination of serum estradiol

Estradiol determination was performed according to the method of Xing et al^[26] by the coat-A-count technique, (radioimmunoassay)

2.5.2.3 Determination of serum progesterone

Progesterone determination was performed according to Yalow and Berson^[27], by the coat-A-count technique, (radioimmunoassay)

2.5.2.4 Determination of serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH)

Follicle stimulating hormone and Luteinizing hormone determination Were performed according to Santner et al^[28], by the coat-A-count technique, (immunoradiometric assay).

2.5.2.5 Determination of serum total thyroxine (T₄) and total tri-iodothyronine (T₃)

Thyroxine and tri-iodothyronine determination were performed in serum according to the method adopted by Britton et al^[29], by the coat-A-count technique, (radioimmunoassay)

2.5.3- Oxidative biomarkers in epididymus

After evaluation of fertility related parameters, the remaining sperm suspension was collected and centrifuged by using cooling centrifuge. Then, the supernatant separated and kept at (-40°C) until determination of oxidative biomarkers.

2.5.3.1 Lipid peroxidation assay

Lipid peroxidation (LPO) was measured in epididymus homogenates according to the method of Ohkawa et al^[30]. Based on the formation of thiobarbituric acid reactive substances (TBARs) and expressed as the extent of malondialdehyde (MDA) production.

2.5.3.2 Determination of total glutathione (GSH & GSSG) :Principle

Total glutathione (GSH) was measured according to the method of Bergmeyer and Graßl^[31] is based on the catalytic action of glutathione is a system, in which GSH undergoes periodical oxidation by DTNB and reduction by NADPH. The measure of the concentration of glutathione in samples is the velocity increase of absorbance (but not the end value).

2.5.4 Testicular functions

A 10 % homogenate (W/V) of testes was prepared in ice cold normal saline using a chilled glass-teflon porter-Elvehjem tissue grinder tube, then centrifuged at 10,000 xg for 20 min. at 4°C. The supernatant used for determination of protein contents^[32], alkaline phosphatase activity^[33], and acid phosphatase activity^[34]. Also, a 10 % homogenate of testes was prepared in ice-cold 0.1 M phosphate buffer, the homogenate was centrifuged at 12,000 xg for 30 min. at 4°C. The supernatant was used for determination of lactate dehydrogenase (LDH) activity^[35].

2.6 Statistical analysis

Data obtained were statistically analyzed using student's t-test at $p < 0.05$ or less was considered significant^[118].

3. RESULTS & DISCUSSION

3.1 Clinical observation and Mortality

In the present chronic toxicity study, dicofol was administered into male rats at 4.19 and 16.75 (ml/kg b.w), equal to 30 and 120 ppm respectively, in drinking water, for long-term exposure (90 weeks).

No visible signs of toxicity were noted during the experiment period, except emaciation (alopecia) and rough hair. The mortality were 30, 25, 13 % in the groups of rats dosed with higher and lower-dose and the control group, respectively.

3.2 Effect of dicofol as Endocrine Disruptor

3.2.1 Effect on male fertility indices

3.2.1.1 Testes and epididymus weight

The testes of humans and other animals are highly susceptible to damage produced by genetic disorders, environmental or occupational exposure to chemicals or other means. Specific causes of testicular damage have

been catalogued^[36-38].

Results demonstrated that dicofol at lower and higher-doses significantly decreased the weight of testes in all treated groups at week 16, 28 and 90 (Table 1). These changes were more marked at higher-dose and prolonged dicofol exposure. Meanwhile, decline in epididymus weight was observed only at the higher-dose level after 28 and 90 weeks (Table 1). In the present study, decline in testes weight was confirmed by the histopathological observations, since most of the seminiferous tubules were degenerated and atrophied, as shown in Figures 1, 2, 3. Table 1. Influence of Dicofol on testes, epididymus weight and semen parameters after chronic exposure in drinking water, for 90 weeks.

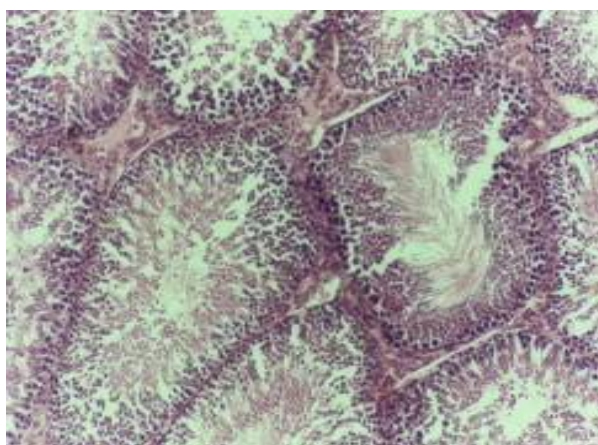


Figure 1. Light photomicrograph of a section of the Testes of rats in control group, showing the normal mature seminiferous tubules with complete series of spermatogenesis and high spermatozoal concentration in the lumen. (H & E X40)

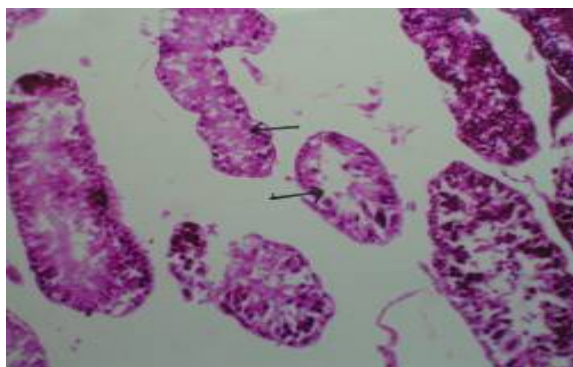


Figure 2. Light photomicrograph of a section of the Testes of rats administrated 30 ppm of dicofol (lower dose) for 28 weeks, showing degeneration and atrophy of some seminiferous tubules (arrow). (H & E X40)

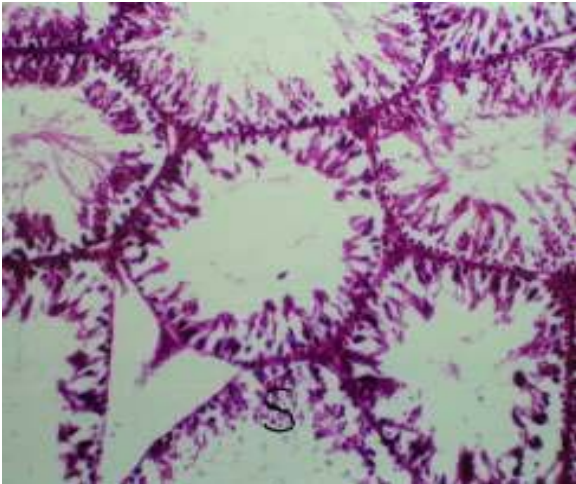


Figure 3. Light photomicrograph of a section of the Testes of rats administrated 120 ppm of dicofol (higher dose) for 16 weeks, showing degeneration of the seminiferous tubules (S) with depression in luminal spermatozoal concentration. (H & E X40)

Similar results were recorded with O'Ch pesticides at different experimental period: i.e. DDT^[39], lindane^[40,41] and endosulfan^[42]. Much data have been reported on the reproductive toxicity of chlorinated hydrocarbons and confirm our results. Brown and Casida^[43] and Jadaramkunti and Kaliwal^[14] showed that reduction of testes and epididymus weights in rats treated with the highest dose of dicofol for long-term are the result of reduction diameter of somniferous tubules, spermatogenic, Leydig and Sertoli cells. On discussing the results with previous reports, it is proposed that, dicofol probably impeded the activity of testes and epididymus by inhibition of androgen production, its antiandrogenic nature or its direct action on these organs^[44]. Moreover, the deleterious effects of dicofol on reproductive organ weights might be due to a decrease in testosterone T level after 16, 28 and 90 weeks from the onset of the treatment^[45].

Several studies have shown that the epididymis and accessory sex organs require a continuous androgenic stimulation for preservation of their normal structural and functional integrity^[46]. Thus, the slight reduction in the weight of the epididymis and accessory sex organs in the treated rats may be due to lower bioavailability of androgens^[47].

Also, Sujatha et al^[41] reported that, the decrease in testicular weight of lindane-treated rats (O'Ch) may be due to reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells. Furthermore, there is much concern that exposure to estrogen –or estrogen-like chemicals induce major pathological effects in epididymus in men and experimental animals^[40]. It could be concluded that dicofol may be acting on testes and accessory reproductive organs by blocking androgen biosynthesis and/or by antagonizing the action of

androgens^[48]. Also, the same authors mentioned that dicofol may be acting directly on the normal function of the hypothalamo-pituitary-gonadal axis.

3.2.1.2 Epididymal sperm count

Sperm count is one of the most sensitive tests for spermatogenesis and it is highly correlated with fertility. Our results showed that, treatment of rats with dicofol at the lower and higher-dose levels for three durations; 16, 28 and 90 weeks, significantly, reduced the total sperm count in all treated groups (Table 1), the effect was dose and time dependent. Histological structure of the testes confirmed this; where it revealed degeneration and atrophy in some of somniferous tubules associated with low luminal spermatozoal concentration. These findings go hand in hand with those of Jadaramkunti and Kaliwal^[14] who found that the number of spermatogenic, spermatocytes, spermatides and Leydig cells were significantly decreased with higher-dose of dicofol and thus reduced sperm count. Also, the authors reported that quantity and quality of sperm production has been adversely affected following exposure of certain drugs and chemicals, particularly mutagens and teratogens. Furthermore, there is a clear correlation between the degree and duration of exposure to pesticides and the extent of spermatogenic arrest and hormonal imbalance.

3.2.1.3 Motility and viability of sperm

The assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperm are immotile, to distinguish between immotile dead sperm and immotile live sperm^[49].

Our results revealed that, at week 16, after the administration of dicofol, a dose-dependent reduction in percentage of motile and live were observed. Also, at week 28 and 90, significant decrease was observed, the changes noted did not follow the expected dose-relationship. As regards the effect of time, dicofol has time dependent effect, however, not in uniform fashion (Table 1) and Figures (4, 5). Choudhary and Joshi^[42], also stated that, oral administration of rats with endosulfan (O'Ch) at doses of 5, 10 and 15 mg/kg b.w./day for 30 days, significantly, decreased the spermatozoal motility and density in cauda epididymus and testes in dose-dependent manner. Decline in sperm motility and density after oral administration of endosulfan is may be due to androgen insufficiency^[16,50], which caused impairment in testicular functions by altering the activities of the enzymes responsible for spermatogenesis, this clearly suggests an antiandrogenic effect of endosulfan^[15,51].

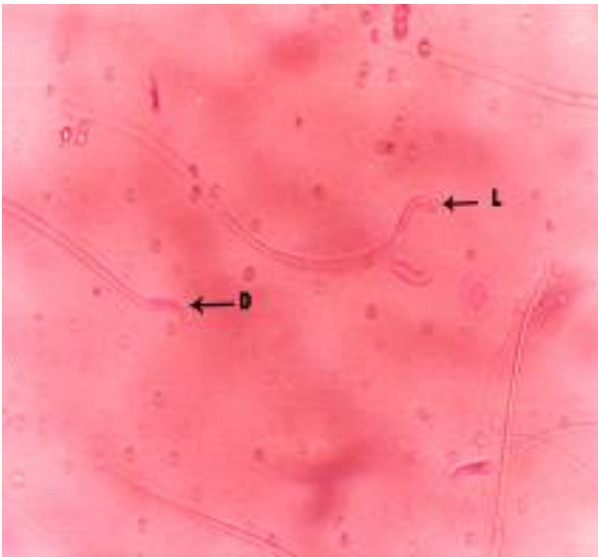


Figure 4. Showed live unstained sperm (L) and dead sperm (D) stained with eosin stain in rats treated with dicofol in drinking water. (40X)

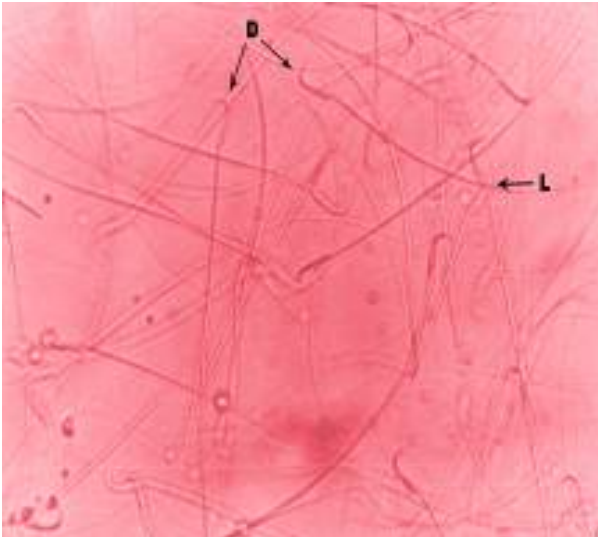


Figure 5. Showed live unstained sperm (L) and dead sperm (D) stained with eosin stain in rats treated with dicofol in drinking water. (40X)

3.2.1.4 Maturation of sperm

From the aforementioned presentation, there were meaningful changes in the number of mature sperms in epididymus. Where, at week 16 and 28 results revealed marked decline in number of mature sperms, the differences seen were dose dependent. Furthermore, a time-related decreased in number of mature sperms was observed in dicofol-treated rats at the lower-dose; whereas, a trend (not significant) for this effect in dicofol-treated rats at the higher dose (Table 1) and Figures (6,7). Morel et al^[24], found a positive correlation between chromosomal abnormalities at the time of meiosis that cause disturbance during the transition of nucleoprotein and percentage of

sperm nuclei that stained with aniline blue. The acidic aniline blue stains lysine-rich nucleoprotein of immature sperm. During spermatogenesis lysine rich histones are replaced by intermediate nucleoproteins which then are replaced by arginine and cysteine-rich protamines. Then, abnormal chromosome segregation at the time of meiosis allows the persistence of lysine-rich nucleoproteins in spermatozoa. It has been concluded that immature sperms were usually increased in infertile men^[52].

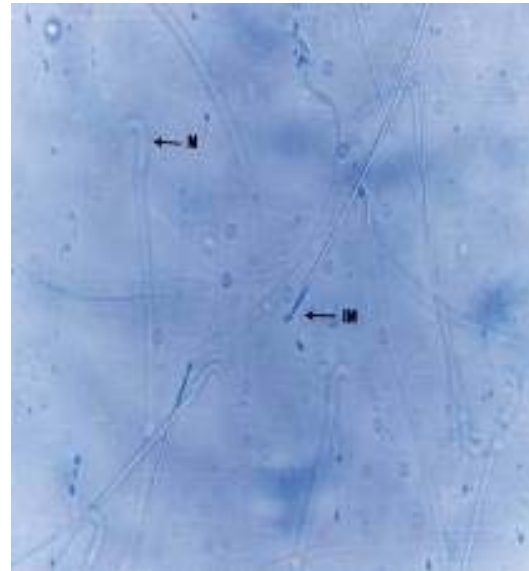


Figure 6. Showed nuclear mature sperm (M) was not stained and immature sperm (IM) with abnormal chromosomes stained with aniline blue in rats treated with dicofol in drinking water. (40X)



Figure (7) Showed immature sperm (IM) stained with aniline blue color in rats treated with dicofol in drinking water. (40X)

3.2.1.5 Sperm morphology

Abnormal form percent was significantly increased in dose and time-dependent manner in all treated groups at the three durations (Table 1) and Figures (8, 9, 10 and 11). This occurred as a result of toxic injury of dicofol to somniferous tubules as postulated from the histological examinations of testes in the treated animals.

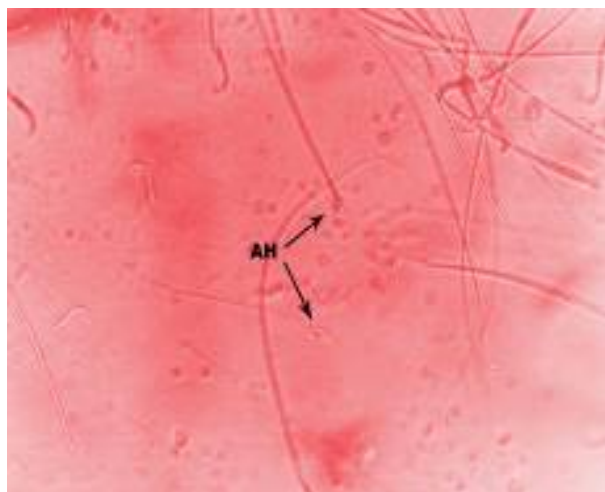


Figure 8. Showed abnormal head sperm miss-shape (AH) stained with eosin stain in rats treated with dicofol in drinking water. (40X)

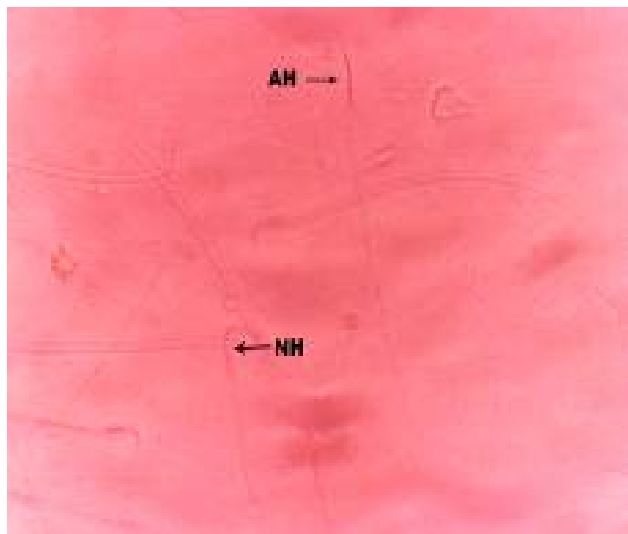


Figure 9. Showed normal sperm with normal head hook shape (NH) and abnormal head sperm no hook (AH) in rats treated with dicofol in drinking water. (40X)

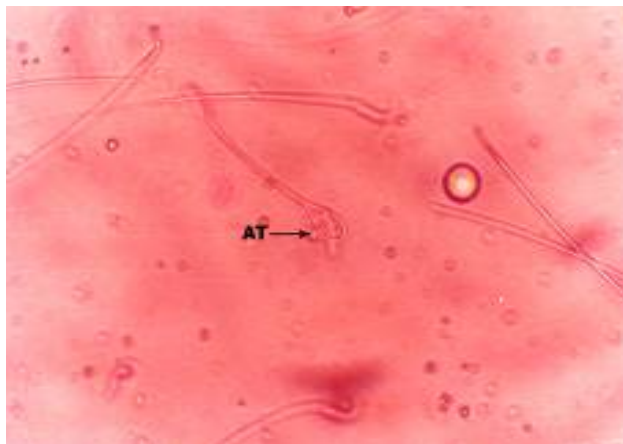


Figure 10. Showed bent tail tip sperm (AT) stained with eosin stain in rats treated with dicofol in drinking water. (40X)

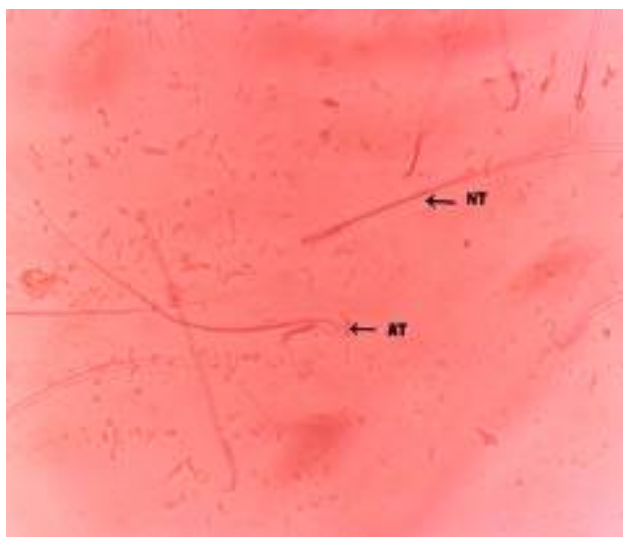


Figure 11. Showed normal tail sperm (NT) and bent tail sperm (AT) in rats treated with dicofol in drinking water. (40X)

Similar results were reported with certain organochlorine pesticides (O₂C) at different experimental periods such as (i.e.), DDT^[39], lindane^[40,41], dicofol^[14], and endosulfan^[42].

Our results are in accordance with those of Tag El-Din et al^[53], who reported that treatment with dicofol in higher dose (16.75 mg/ kg b.w./day, 5 days/week), for 6 months dosing period, significantly, reduced testes and epididymus weight, the total sperm count and percentage of motile and live sperms than lower dose (4.19 mg/kg b.w.). As well as, a significant increase in the percentage of abnormal forms was seen in higher-dose group, and this effect was dose-dependent. The authors noticed a marked depletion in number of mature sperm in the higher-dose group.

From the obtained results, it is interesting to notice that, dicofol seems to be more hazardous at higher dose and prolonged exposure period than lower dose and short exposure period, as revealed from its powerful effects on the weight of testes and epididymus or other semen parameters measured including; sperm motility, total sperm count, cauda epididymus sperm count, percentage of mature and live sperm, as well as abnormal forms.

Generally, the differences in fertility index data including statistical significant differences believed to dicofol effect. Notably, dicofol as well as, the administration periods played an important role in this respect, *i.e.* the effect was treatment and time dependent. The present study reveals that an exposure to dicofol may affect the histology of testes and sperm morphology. Accordingly, this testicular and spermatotoxic changes may be responsible for observed male mediated developmental toxic effects.

3.2.2 Effect on sex hormones

Persistence of chlorinated insecticides and their congeners in the tissues of man and animals and in the environment post health problems of toxicological importance. One of these problems is the endocrinal dysfunction^[54,55]. Recent evidence had suggested that organochlorine pesticides, even at low concentrations, may disrupt the endocrine system, which was responsible for proper hormone balance^[56,57].

3.2.2.1 Testosterone

Testosterone T is the main steroid sex-hormone in male albino rats, it secreted by leydig cells of the testes under the control of complex neuroendocrine interactions^[58,59].

In the present study, T level, significantly, decreased in all treated groups at lower and higher-doses at 16, 28 and 90 weeks of administration, except, in lower-dose group at 16 weeks, where T level unchanged after dicofol treatment (Table 2). Notably, there were no dose-related changes in T level, while a time-dependent reduction in higher-dose group was observed.

The significantly decrease of testosterone level, may be as a result of direct damage of dicofol on leydig cells, which are the main site of testicular androgen biosynthesis.

Results of the present work agree with those found by Krause^[60], Desaulniers^[61], Lafuente^[62], Ben Rhouma^[39], and Choudhary and Joshi^[42], who noted that T level was significantly decreased in male rats treated with organochlorine pesticides at different doses, *i.e.* DDT, PCB-126 and 153, methoxychlor, DDT, endosulfan, respectively.

Contrary to the results of the present investigation are those reported by Foster^[63] and Desaulniers^[64], who mentioned that, rats administered with tris (4-chlorophenyl) (TCPM), that is structurally related to DDT and dicofol,

and PCB-28 (2, 4, 4'-trichlorobiphenyl) or PCB-77 (3, 3', 4, 4'-tetrachlorobiphenyl) did not exhibit meaningful changes in T level.

3.2.2.2 Estradiol and progesterone:

Organochlorine pesticides have some estrogenic properties, and may modify the feed-back mechanism of steroids on the hypothalamus and pituitary^[62]. Exogenous estrogens (natural or synthetic) elicit all the pharmacologic responses usually produced by endogenous estrogens.

Estradiol (E₂) determinations have proved of value in a variety of contexts, including the investigation of precocious puberty in girls and gynecomastia in men^[65]. The present study also revealed that, significant, increase in E₂ level was observed in all dicofol-treated groups at the three durations (16, 28 and 90 weeks) of administration. In addition, dicofol significantly increased progesterone (P₄) level in treated groups; the effect was restricted only in duration of 16 and 28 weeks, and also there were no positive trend in this respect. (Table 2)

The present investigation in comparable to other (O'Ch) pesticides on account of exhibiting estrogenic activity of dicofol when used in higher-dose for long-term^[66-69]. The significant elevation of steroids for example, P₄ and E₂ in male rats which received lower and higher-doses of dicofol for long-term exposure could be attributed to increase the incidence of hypertrophy and/or vacuolation (empty cavities) of the adrenal cortex that enhanced the steroidogenic activity^[70,71]. Moreover, Jadaramkunti and Kaliwal^[4] and Tag El-Din^[53] suggested that dicofol mimic estrogenic activity when compared to other chlorinated pesticides (O'Ch) which may have a direct effect on the testes or indirectly through the hypothalamo-hypophyseal testicular axis or by desensitizing the testes to gonadotropins. Furthermore, it has been reported that, the estrogenic like effects may be produced as a result that dicofol binds to estrogen receptors and exhibits estrogenic activity^[72] or by direct effect on sertoli cells resulting decreased FSH receptor binding and decreased 3-hydroxy-steroid-dehydrogenase activity that change estradiol to androgen, thus raising estradiol levels^[5, 73].

These findings are in close agreement with those reported by Tag El-Din^[53], who stated that dicofol at two doses 4.19 and 16.75 mg/kg b.w./day, in drinking water, for 6 months increased E₂ and P₄ levels in male rats in a dose-dependent manner.

Many pesticides are able to block or activate the steroid hormone receptors and/or to affect the levels of sex hormones, thereby potentially affecting the development or expression of the male and female reproductive system or both. This emphasizes the relevance of screening pesticides for a wide range of hormone-mimicking effects^[74].

3.2.3 Effect on reproductive hormones:

3.2.3.1 Luteinizing hormone and follicle-stimulating hormone

Luteinizing hormone (LH) is glycoprotein released from the anterior pituitary; it stimulates T production by leydig cells of the testes in males. Hypothalamic control of LH appears to be by a common releasing hormone termed gonadoliberein (GnRH, LHRH), with negative feedback control at the hypothalamic level by E₂ in the female and T in the male^[58].

Our results revealed that, short-term dicofol exposure (16 weeks) did not exert appreciable changes in LH and FSH levels in both lower and higher-dose groups. On the contrary, prolonged dicofol-exposure (28 and 90 weeks) significantly decreased LH and FSH levels in dose and time-dependent manner (Table 2). These results go hand in hand with those of Tag El-Din^[53] who reported that dicofol at 4.19 and 16.75 mg/kg b.w./day, for 6 months, induced significant decrease in LH level in male rats.

According to the suggestion reported by Singh and Pandey^[67], the changes in the pattern of the steroidogenic enzymes 3 β -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase lead to inhibition of testicular androgen biosynthesis in adult rats, which is required for spermatogenesis in seminiferous tubules and sperm maturation in the epididymus. A complementary proposed mechanism, could explain dicofol induced toxicity, is blocking gonadotropin production and/or release by the pituitary, thereby testosterone production by leydig cells is not stimulated, causing spermatogenesis arrest^[75]. This mechanism is supported by the data previously reported by Mably^[76] who recorded that, the alteration of LH had led to destruction of seminiferous epithelium and loss of germinal elements results in the reduction of the number of spermatids, sperm production in the testes as well as increase abnormality of sperms.

Marked decline in LH and FSH levels in the present study confirm the findings of Desaulniers^[61] and Lafuente^[62], who investigated the toxicological influences of PCB (126 and 153) and methoxychlor, O'Ch, at different concentrations on male rats. On the other hand, the results of the present study disagree with the findings of Tag El-Din^[53], who mentioned that FSH level increased significantly after treatment with dicofol at lower and higher-doses (4.19 and 16.75 mg/kg b.w./day), in drinking water, for 6 months. It was proved that certain O'Ch pesticides did not alter LH and FSH levels when administered into rats at different doses for short-term intervals, such as : TCPM^[63], DDT^[60] and PCB^[28] and 77^[64].

An elevation in circulating levels of inhibin, a glycoprotein of primarily sertoli cell origin which inhibits FSH synthesis and secretion by the pituitary^[77], could

account for the observed decrease in serum FSH level in the current study which was confirmed histopathologically by degeneration and atrophy of seminiferous including leydig and sertoli cells. FSH stimulates the sertoli cells of the seminiferous tubules to produce androgen binding protein, probably moves via the sertoli cells to other germ cells and to the epididymus where the testosterone is released to exert its physiological effects in sperm maturation^[76].

3.2.4 Effect on thyroid hormones

3.2.4.1 Tri-iodothyronine and thyroxine

The disruption of thyroid hormone homeostasis by a variety of xenobiotics has been associated with thyroid follicular cell hypertrophy, hyperplasia, and the development of thyroid tumors in rats^[78,79]. Thyroid toxicants affect circulating levels of thyroid hormone by either direct action on the thyroid gland or by increasing peripheral elimination of thyroid hormone^[79].

Concerning the thyroid hormones; dicofol, at lower and higher-doses, induced significant decrease in thyroxine (T₄) and tri-iodothyronine (T₃) levels throughout the experimental periods (16, 28 and 90 weeks), except at 16 weeks T₄ level did not alter significantly in lower-dose group (Table 2). These changes were more marked at higher dose of dicofol as well as, prolonged dicofol exposure. It is worth to say, results revealed evident affection of thyroid gland, such affection was dose and time-dependent.

Hypothyroidism significantly reduced seminiferous tubule and lumen diameter, where in hypothyroid rats, the proliferation and differentiation of germ cells were arrested and their number was decreased^[80], the present study clearly indicates that hypothyroidism adversely affects spermatogenesis; it also indicates that thyroid hormones are essential for normal spermatogenesis.

In accordance with the findings of the present study, El-Kashoury^[81] described similar changes in T₄ and T₃ levels after dicofol exposure at lower and higher-doses. They also mentioned that the decrease in T₄ levels may be a result of iodine deficiency, the gland fails to synthesize T₄ and hypothyroidism occurs. Another suggestion reported by Hotz^[82] who reported that, pesticide increased deiodination and biliary excretion of thyroid hormone T₄ which led to increased rate of T₄ elimination from the blood.

A complementary proposed mechanism, could explain organochlorine (O'Ch) induced toxicity, is attributed to their ability to deplete stores of vitamin A and thyroid hormones from the body by 30-50 %, through interaction with a common plasma protein carrier called transthyretin (TTRs) and alteration of their metabolism in the liver and other organs. T₄ and Vit. A are known to be important regulators of normal epithelial differentiation and

proliferation^[83]. Another support to the interaction of O`Ch with TTRs was established by Van den Bery^[84] who mentioned that hydroxylated PCBs and number of halogenated industrial chemicals, mainly pesticides (O`Ch) may decrease thyroid hormone levels in rats through interference with hormone transport carriers (TTRs).

An alteration in thyroid hormone T₄ and/or T₃ level in the present study confirm the findings of Desaulniers^[64]. Desaulniers^[61] and Fisher^[85], who investigated the toxicological influences of certain organochlorine pesticides "O`Ch" at different concentrations, *i.e.*, PCB-28 (2, 4, 4'-trichlorobiphenyl) and PCB-126 (3, 3', 4, 4', 5-pentachlorobiphenyl).

Another mechanism was postulated by Villa^[86] who stated that "O`Ch" might alter the expression of a membrane of genes by a direct receptor mechanism. This receptor is made of a basic protein and known as aryl hydrocarbon receptor (AHR) which is maintained in a ligand binding state in association with cytosolic protein^[87]. Exposure to "O`Ch" and related compounds leads to dissociation of AHR from the binding protein^[88], which is transferred to the nucleus and then it binds to specific DNA leading to severe harmful effects such as induction of cytochrome P₄₅₀ (CYP₄₅₀) 1A1 gene^[89]. Lastly, bioactivation of these compounds can make them more toxic which may modulate the expression of the related genes in the tissues^[90].

3.3 Effect on Oxidative Parameters

Pesticides and environmental chemicals may induce oxidative stress leading to generation of free radicals and alteration in antioxidants or oxygen free radical (OFR) scavenging enzyme system^[91].

3.3.1 Lipid peroxidation

Generation of oxidative stress and consequent lipid peroxidation (LPO) by pesticides is reported in many species. It has been reported that increase in ROS can cause the destruction of all cellular structures including membrane lipid^[92]. Hence in the present study, lipid peroxidation is used as an index of oxidative stress. Several drugs, xenobiotics and environmental pollutants are known to cause imbalance between formation and removal of free radicals^[93].

ROS such as superoxide anions (O₂⁻), hydroxyl radical (OH) and H₂O₂ enhance oxidative process and produce lipid peroxidative damage to cell membranes. The (OH) radical has been proposed as an initiator of LPO through an iron-catalysed Fenton reaction^[94]. LPO is the process of oxidative degeneration of polyunsaturated fatty acid (PUFA) and its occurrence in biological membranes causes impaired membrane function, structural integrity^[7], decrease in membrane fluidity and inactivation of a several

membrane bound enzymes.

Results in (table 3) showed that, an administration of rats with dicofol, at lower and higher doses for two durations 28 and 90 weeks, increased oxidative stress in cauda epididymus of rats, as evidenced by enhanced levels of malondialdehyde (MDA) level.

An elevation of LPO in cauda epididymus, as evidenced by increased production of MDA in the present study, suggests participation of free radical-induced oxidative cell injury in mediating the toxicity of dicofol. These intentionally introduced environmental xenobiotics are known to have a strong affinity for interaction with membrane phospholipids^[95]. An elevation in LPO caused by other O`Ch in different experiments has also been reported; methoxychlor^[96], methoxychlor^[97], endosulfan^[98] and 2, 3, 7, 8-tetrachlorobenzo-P-dioxin (TCDD)^[99].

3.3.2 Glutathione level

Glutathione (GSH) one of the most abundant antioxidant in cells has been found to decrease during apoptosis. GSH has been hypothesized to play a role in the rescue of cells from apoptosis, by buffering an endogenously induced oxidative stress^[100]. In our study, a decrease in GSH levels in dicofol-intoxicated animals may be responsible for enhanced LPO^[101, 102]. Our results confirm the findings of Tithof^[103], Selzak^[104], Luna Samanta^[105], Latchoumycandane^[96], and Saradha and Mathur^[106], who reported that repeated administration of several organochlorine at different concentrations (dicofol, 2, 3, 7, 8-tetrachlorodibenzo-P-dioxin (TCDD), hexachlorocyclohexane (HCH), methoxychlor and lindane, respectively) induced disturbances in the activities of the enzymes regulating GSH metabolism.

As regards GSH level and lipid peroxidation in normal rats (control group), it is worth to mention that, there were significant differences between the control groups (28 and 90 weeks). A noticeable decrease in GSH level accompanied by concomitant increase in LPO in 90 weeks compared with their levels in 28 weeks was observed. It is well documented that advancing age an organism is under greater oxidative stress as the result of impairment of the function of mitochondrial respiratory chain^[107]. This leads to an accumulation of DNA, RNA and protein free radical damage^[108] and causes alterations in antioxidant enzyme levels^[109].

3.4 Effect on Testicular Functions

Organochlorine pesticides (O`Ch), having estrogenic property, impaired the testicular functions through altering the testicular biochemistry^[15, 16, 42].

3.4.1 Total protein

The testicular fluid contains both stimulatory factors as well as inhibitory factors that selectivity alters the protein secretions^[110]. Thus, the changes in protein suggested that there is a reduction in the synthetic activity in testes.

From Table 4, it is clear that, an administration of dicofol into rats leads to elevation of protein concentration in testes at two doses (lower and higher). Except, at higher-dose for 28 weeks, no appreciable changes in protein content were observed.

Similar elevation in protein content caused by other "O'Ch" has also been reported^[111,112].

The accumulation of protein occurred in testes and epididymus due to androgen deprivation to target organs. This deprivation effect also led to a reduction in testicular and cauda epididymal sperm population, loss of motility in the latter and an increase in number of abnormal spermatozoa, thereby manifesting 100 % failure in fertility in treated animals^[113].

Concerning the protein content, our findings, are in accordance with those reported by El-Kashoury and Mansour⁸¹ who studied the effect of dicofol at two doses, for long term, on testicular biochemistry.

3.4.2 Acid and alkaline phosphatase (ACP & ALP)

Acid phosphatases are enzymes capable of hydrolyzing orthophosphoric acid esters in an acid medium. The testicular acid phosphatase gene is up-regulated by androgens and is down-regulated by estrogens^[114]. Activities of free lysosomal enzymes have been shown to rise when testicular steroidogenesis is increased^[47].

Based on the data obtained in this study, dicofol when administered into rats, at lower and higher doses for different duration (28 and 90 weeks) induced significant decrease in ALP activity (Table 4). It is clear that, the changes noted did not follow the expected dose and/or time relationship. A decrease in ALP activity indicated that dicofol treatment produced a state of decreased steroidogenesis where the inter- and intracellular transport was reduced as the metabolic reactions to channelize the necessary inputs for steroidogenesis slowed down^[115].

As regards ACP enzyme, results of the present study showed significant decrease in its activity during two durations (28 and 90 weeks). While, there were no treatment-related changes, but a time-dependent effect in

higher-dose groups was detected.

As regards ALP and ACP activities, results of the present investigation were similar to those reported by Chitra^[116]. A decrease in the ACP in Free State would thus reflect decreased testicular steroidogenesis in rats and this may be correlated with the reduced secretion of gonadotrophins^[115].

3.4.3 Lactate dehydrogenase (LDH)

Testicular LDH is an essential component of the metabolic machinery of spermatozoa and involved in the energy generation processes. An administration of rats with dicofol at both doses (lower and higher) decreased significantly LDH activity in testicular tissues. All changes in LDH activity mean that no dose relationship. On the other hand, the higher-dose exhibited time-dependent effect (Table 4). The decreased in LDH activity in dicofol-treated rats points toward the interference of dicofol with the energy metabolism in testicular tissues^[116].

The correlation between LDH and motility and living sperm could be a sign that extracellular LDH ensures metabolism of spermatozoa, perhaps even in anaerobic conditions. This hypothesis is underlined by the significant negative correlation between LDH and pathomorphology of sperm^[117].

In the present study, male fertility indices were measured confirm the above-mentioned suggestion where, marked declined in count, motility and viability of sperm were observed as well as an elevation in abnormalities of sperm. It means that LDH enzyme has an important role in the normal energy supply in spermatogenesis.

Notably, decline in serum T level was observed with a reduced reproductive organ weights, which means that male reproductive toxicity induced by dicofol would be augmented by decreased serum T level as well as a decreased function of sertoli and leydig cells, in addition to the direct cytotoxic effect on germ cells^[45].

In view of this data, it can be concluded that dicofol induced disorders of reproductive system result from a disturbance of the androgen-estrogen balance, as well as oxidative stress and impairment in testicular functions. Although, it is not possible link all these events together, it is assumed that their collective impact to ultimately leads to a perceptible change in sex hormone balance and arrest of spermatogenesis. Further studies are need for better understanding of the cause of reproductive toxicity induction of dicofol, and possibly of o'ch as a whole.

Table 1. Influence of Dicofol on testes, epididymus weight and semen parameters after chronic exposure in drinking water, for 90 weeks

Periods Parameters	16 Weeks			28 Weeks			90 Weeks		
	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)
Testes weight (g)	1.55±0.035	1.35±0.016****	1.30±0.071**	1.58±0.034	1.50±0.041	1.38±0.051* ^a	1.95±0.011	1.48±0.137***	1.19±0.092****
Epididymus weight (g)	0.20±0.007	0.19±0.011	0.18±0.010	0.35±0.020	0.30±0.019	0.25±0.014** ^a	0.47±0.005	0.39±0.037** ^b	0.24±0.025****
Sperm count	100.0±3.536	90.0±2.236** ^a *** ^b	60.0±3.162****	90.0±2.915	35.0±3.536****	30.0±2.550****	110.0±3.742	20.0±3.536****	15.0±3.536****
Motility (%)	90.0±1.581	65.0±3.536** ^a ** ^b	40.0±3.536****	85.0±3.317	20.0±2.915****	15.0±2.550****	85.0±2.449	35.0±8.367****	25.0±8.660****
Viability (%)	90±2.236	70±2.550**** ** ^b	50±3.536****	90±2.550	40±3.536****	30±3.317****	90±1.225	40±3.742****	35±6.708****
Mature sperm (%)	90±2.550	80±3.53 ^a ** ^b	60±3.536****	80±3.536	65±2.550** * ^b	50±5.100** ^a	90±1.225	55±5.099****	50±6.403****
Abnormal forms (%)	15±2.236	25±3.536** ** ^b	40±1.871****	20±1.871	35±3.536** *** ^b	65±3.536****	20±1.225	65±2.236**** ** ^b	50±3.742****

Values represent means ± SE, n = 5; * P <0.05; ** P <0.01; *** P <0.001 (Student's t-test).
a: treated group versus control group; b: lower dose group versus higher dose group

Table 2. Influence of dicofol on sex steroid, reproductive and thyroid hormones after chronic exposure in drinking water for 90 weeks

Periods Parameters	16 Weeks			28 Weeks			90 Weeks		
	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)
Testosterone (ng/ml)	1.30±0.230	1.10±0.311	0.60±0.141* ^a	3.60±0.311	1.40±0.270*** ^a	1.30±0.230*** ^a	2.10±0.241	1.30±0.230* ^a	1.20±0.270* ^a
Estradiol (Pg/ml)	9.40±0.540	23.0±0.707*** ^a ** ^b	17.00±1.414** ^a	14.80±0.354	21.70±0.212*** ^a ** ^b	23.80±0.707*** ^a	8.70±0.283	13.20±0.270*** ^a *** ^b	9.90±0.396* ^a
Progesterone (ng/ml)	11.63±0.396	18.42±0.544*** ^a	18.89±0.652*** ^a	7.23±0.326	9.00±0.707* ^b	11.44±0.439*** ^a	4.84±0.334	4.97±0.369	6.00±0.500
FSH (ng/ml)	2.00±0.354	1.40±0.070	1.20±0.141	2.50±0.354	1.90±0.192 *** ^b	0.30±0.071*** ^a	3.20±0.184	0.80±0.071*** ^a *** ^b	0.25±0.050*** ^a
LH (ng/ml)	3.53±0.212	3.10±0.283	2.90±0.241	3.20±0.184	1.34±0.114*** ^a	1.18±0.141*** ^a	2.90±0.200	0.97±0.130*** ^a	1.40±0.184*** ^a
Thyroxine (ng/ml)	26.88±3.942	18.8±2.628	14.45±0.987* ^a	29.37±1.170	21.00±1.043*** ^a *** ^b	14.41±0.544*** ^a	43.83±0.472	37.82±2.381* ^a *** ^b	20.02±2.038*** ^a
Tri-iodothyronine (ng/ml)	0.46±0.046	0.32±0.029* ^a	0.27±0.012** ^a	0.41±0.012	0.36±0.016* ^a * ^b	0.29±0.019*** ^a	0.33±0.014	0.28±0.016* ^a *** ^b	0.16±0.003*** ^a

Values represent means ± SE, n = 5; * P <0.05; ** P <0.01; *** P <0.001 (Student's t-test).
a: treated group versus control group; b: lower dose group versus higher dose group.

Table 3. Influence of Dicofol on oxidative parameters in cauda epididymus and testicular functions

Parameters	28 Weeks			90 Weeks		
	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)	Control (0 ppm)	Lower-dose (30 ppm)	Higher-dose (120 ppm)
Malondialdehyde ($\mu\text{mol/g}$ tissue)	93.07 \pm 1.516	146.84 \pm 11.048** a	166.76 \pm 14.210** a	181.44 \pm 9.610	219.75 \pm 8.740* a	199.69 \pm 7.814
Total glutathione ($\mu\text{mol/g}$ tissue)	374.26 \pm 1.692	280.36 \pm 3.382*** a *** b	321.91 \pm 4.368*** a	291.41 \pm 5.074	154.69 \pm 4.227 *** a *** b	220.98 \pm 2.537*** a
Total protein (mg/g tissue)	17.90 \pm 0.511	22.09 \pm 0.371** a ** b	18.19 \pm 0.686	16.57 \pm 0.399	20.41 \pm 1.220* a	20.14 \pm 0.621** a
Alkaline phosphates (U/mg protein)	0.085 \pm 0.007	0.054 \pm 0.001** a	0.057 \pm 0.003* a	0.094 \pm 0.003	0.070 \pm 0.004** a	0.074 \pm 0.005* a
Acid phosphates (U/mg protein)	0.112 \pm 0.002	0.084 \pm 0.003*** a * b	0.099 \pm 0.004* a	0.125 \pm 0.003	0.089 \pm 0.004*** a	0.084 \pm 0.004*** a
Lactate dehydrogenase (U/mg protein)	1.55 \pm 0.043	1.11 \pm 0.046*** a *** b	1.53 \pm 0.036	1.60 \pm 0.034	1.28 \pm 0.090* a	1.33 \pm 0.045** a

Values represent means \pm SE, n = 5; * P <0.05; ** P <0.01; *** P <0.001 (Student's t-test)
a: treated group versus control group; b: lower dose group versus higher dose group.

ABBREVIATIONS USED

O`Ch, Organochlorine insecticides; ROS, Reactive oxygen species; PUFA, Polyunsaturated fatty acid; EC, Emulsifiable concentrate; T₄, Thyroxine; T₃, Triiodothyronine; T, Testosterone; P₄, Progesterone; E₂, Estradiol; FSH, Follicle-Stimulating hormone; LH, Luteinizing hormone; ALP, Alkaline Phosphatase; ACP, Acid Phosphatase; LDH, Lactate Dehydrogenase; LPO, Lipid Peroxidation; GSH, Glutathione.

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