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Toxicopathological Impacts of Chlorpyrifos on Sperm Qualities and Testicular Tissue Alterations and Their Modulation with Vitamin E and Zinc in Male Albino Rats

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ABSTRACT

This study was carried out on 30 mature male albino rats (2 months old, weighed about 130 g) in 3 groups of ten rats for each one in ARC. Group I, kept as control. Group II, daily intubated with CPF, at a dose level of 10.6 mg/kg BW in corn oil. Group III**,** daily intubated with CPF at a dose level of 10.6 mg/kg BW, zinc at a dose level of 227 mg/kg BW and vitamin E at a dose level of 75 mg/kg BW for 50 days equivalent to one spermatogenic cycle in rats. At the end of the experiment, the rats were sacrificed to take blood, semen and tissue specimens. Histopathologically, the testes and epididymis showed moderate degeneration and thickening of the tubular membrane with interstitial oedema in the CPF intoxicated group. These changes were ameliorated in the rats of group III. The semen analysis is aggressively badly affected in the CPF intoxicated group in comparison with the control group and improved in rats of group III. Testosterone hormone levels were assayed with ELISA technique as 3.871±0.31 ng/ml, 1.112±0.82 ng/ml, and 2.503±0.25 ng/ml in control, CPF, CPF plus vitamin E and Zn groups, respectively. Moreover, the DNA integrity of spermatozoa in the CPF group was severely affected in comparison with either the control group and group III.

Keywords: antioxidants, Chlorpyrifos, histopathology, semen, spermatogenic cycle.

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INTRODUCTION

The organophosphorus (OP) compounds represent a major class of insecticides used globally. As a quantity, they amounted to 44% of the total insecticides used in Egypt during the 90's. These insecticides are mainly acetylcholinesterase inhibitors affecting severely on the central or peripheral nervous system. Some of these OP pesticides are endocrine disruptors and affect male fertility **(Kovgac *et al.*,1995; Mansour and Mossa 2010).** Misuse of pesticides, especially in developing countries, contributes to the contamination of vegetables, fruits, water, soil and different environmental components by pesticide residues. Even when pesticides are used following good agricultural practices, their residues in plants may be unavoidable **(Maroni *et al.*,2000; Goel *et al.*,2007).** Chlorpyrifos, [O, O-diethyl-O-(3, 5, 6-trichloro2-pyridyl)-phosphorothioate; IUPACI is anticholine-sterase insecticides with contact, stomach, and respiratory action. They are used widely to combat insect pests in different crops as well as in buildings and public places **(Thomson, 1992).** Therefore, their residues may be found in the same food commodities such as vegetables and fruits produced from sprayed fields. Monitoring studies conducted in different countries revealed the presence of residues of both insecticides among other ones in different varieties of food commodities **(Akan *et al.*,2013; Al-Naggar *et al.*,2015).**

Recent studies identified reactive oxygen species (ROS) as a cause of toxic effects exerted by OP pesticides. These ROS are responsible for inducing oxidative stress in the tissues and chronic, permanent

damage **(Akhgari *et al.*,2003; Olorunshola *et al.*,2011).** This raised the interest of scientists to search for antioxidants, which might alleviate oxidative stress caused by pesticides. Several substances including OP pesticides-induced oxidative stress in experimental animals as an essential oil, wheat germ oil and grape seed oil, vitamin E (α-tocopherol) and zinc were used against CPF-induced oxidative stress in rats (**Mansour and Mossa 2009; Mansour and Mossa 2010; Khalifa *et al.*,2011; Mansour *et al.*,2011; Mansour and Gamet-Payrstre 2016).** The mammalian cells reduced the adverse effect of lipid peroxidation via the utilization of both enzymatic and non-enzymatic antioxidants, which scavenge for free radicals in the system. Oxidative stress results when the endogenous antioxidants have been overwhelmed by the rate and extent of free radical generation. Therefore, during oxidative stress, an increase in the exogenous supply of antioxidants improves the capacity of the tissue to cope with high antioxidant as zinc and vitamin E demands. (**Ambali *et al.*,2010).**

Oral administration of CPF to male rats at the doses of 3, 6 and 9 mg kg-1d-1 for 90 days showed minor histopathological changes in testis, epididymis, brain, liver, and adrenal with no change in sperm motility and sperm morphology except a significant decrease in sperm counts in comparison with control. It is suggested that CPF caused toxicological changes along with mild testicular and spermatotoxic effects in male rat**s (Akhtar *et al.*,2009).** CPF administration caused severe damage to the reproductive system of the male rat. However, the mechanism is not clear (**Viswanath *et al.*,2010).** CPF exposure on clinical, hematological and biochemical parameters in mice and the possible ameliorative effect of co-administration of vitamins C and E was estimated in male mice. Vitamins pretreatment ameliorated toxic cholinergic signs induced by CPF on testicular functions, sperm concentration and motility, and serum testosterone concentration **(Ambali *et al.*,2011).**

The reproductive toxicity of male rats with CPF was in the form of a marked reduction in testicular sperm counts, motility and significant growth of sperm malformation rate in exposed males. Histopathological examination of testes showed mild to severe degenerative changes in seminiferous tubules. The levels of testosterone showed a decreasing tendency in association with CPF administration **(El-Sharkawy *et al.*,2014; Sai *et al.*,2014).**

The use of pesticides expected to cause indirect effects on human health to evaluate the implications of toxicological effects of subchronic CPF exposure on reproductive function in male rats. Aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and creatinine concentrations were significantly increased on CPF exposure with a significant reduction in blood plasma acetylcholine esterase (AchE) enzyme. Also, it resulted in reduced sperm counts, motility and an increase in sperm abnormalities with a significant reduction in serum testosterone. The results demonstrated that prolonged exposure of CPF induces spermatogenesis damage, possibly through interference with sex hormones and AchE enzyme resulting in the decrease of fertility **(Peiris and Dhanushka 2017)**.

In comparison with calves, steers, and cows, bulls are highly susceptible to a single dose of CPF as one of the organo-phosphorus compounds. The maximum tolerated dose of CPF in sheep is 750 mg/kg. Sheep given 850 mg/kg died five days after dosing, those given 900 mg/kg died on the third day, and a dose of 1,000 mg/kg was lethal within 30 hours. The onset of poisoning signs is usually delayed compared with that of many other commonly used organophosphates **(Gupta, 2018).**

Consequently, the present investigation was carried out to assess some toxicological effects in male albino rats following exposure to CPF and to evaluate the ameliorative effect of Co-administration of vitamin E and Zn sulphate.

MATERIALS AND METHODS

Animals

Animal maintenance and care conformed with recommended International Guiding Principles for Biochemical Research Involving Animals (National Research Council ,US, Institute for Laboratory Animal Research, 2004.

The present study is carried out on 30 mature male albino rats (2 months old, weighed about 130 g) purchased and housed in the laboratory animal's farm of Agriculture Research Center at Giza, Egypt. They were given food and water adlibitum. All rats were housed in metal cages and received a balanced ration diet.

Chemicals

Chlorpyrifos liquid was obtained from the Kafr El-Zayate Company for Agrochemicals, while zinc sulphate powder and liquid vitamin E (tocopherol) were purchased from El-Gomhoria Company for Chemical Products.

Experimental design

Animals were classified into three groups. Group I: Ten rats were daily intubated with corn oil (2 ml) and kept as control. Group II: Ten rats were daily

intubated with Chlorpyrifos at a dose level of 10.6 mg/kg body weight (minimal toxic dose) in corn oil (2 ml). Group III, daily intubated with CPF at a dose level of 10.6 mg/kg body weight in corn oil (1 ml), zinc sulphate (Zn) at a dose level of 227 mg/kg body weight in distilled water (0.5 ml) and vitamin E at a dose level of 75 mg/kg body weight in corn oil (0.5 ml). In the morning before the presentation of feed and water, the total daily dose per animal was emulsified in corn oil "CPF and vitamin E" and Zn in water (shaken well before use) and given orally. Regarding our schedule, semen (by squeezing the epididymal and testicular tissue) and tissue specimens from animals of each group were sacrificed after anesthesia with ether at 50 days (one spermatogenic cycle) to get blood, semen and tissue samples.

Pathological examination

Postmortem examination of sacrificed animals was carried out and gross findings for each organ were recorded. Tissue specimens from testes and epididymis were fixed in formol saline solution (10% formalin in 0.9% NaCl) then routinely processed in an automated tissue processor (dehydrated in grades of ethyl alcohol and cleared in xylene), embedded in paraffin, sectioned at 3-5 μm, and stained with haematoxylin and eosin stain (**Bancroft and Gamble 2018)**, then examined by light microscopy.

Semen evaluation

The following parameters were evaluated immediately after semen collection:

1- Sperm individual motility in percentage using a light microscope at X100 magnification after dilution of the semen with 2.9 % sodium citrate dihydrate solution according to **Woston and Martin (1972)**.

2- Assessment of abnormal sperms and acrosomal abnormalities in percentage using fast green stain according to **Wells and Awa (1970).**

3- Live dead ratio of spermatozoa using the eosin-nigrosin staining technique according to **BjoÈrndahl *et al.* (2003).**

4- Sperm concentration in number with millions using the improved Neubauer hemocytometer slide after staining with eosin according to **Smith and Mayer (1955)**.

Assessment of sperm DNA integrity (Comet/Single cell gel electrophoresis assay)

DNA integrity and the incidence of DNA strand breaks or fragmentation was detected using the alkaline single cell gel electrophoresis (comet) assay according to **Boe-Hansen *et al.* (2006).** Briefly, frozen-thawed spermatozoa were diluted in PBS, embedded in agarose, followed by cell lysis. DNA decondensation, electrophoresis, and DNA staining with ethidium bromide. The cells visualized by fluorescent microscopy. Intact nuclei appeared to have compact and brightly fluorescent heads; in contrast, strand breaks in damaged cells allow DNA migration during electrophoresis, and a tail of DNA could be seen behind the head, giving the appearance of a comet **(Hughes *et al.*,1996).** After subjecting the spermatozoa to the comet assay, sperm nuclei were analyzed by a computer software program (Comet-Score Program).

Testosterone hormone analysis

Blood samples were taken from the eye veins of rats and kept at room temperature for complete clotting and centrifuged at 3000 rpm for the separation of serum and serum was stored at - 20ºC. Testosterone hormone levels were assayed with ELISA technique using a commercial kit (Byk, Dietzenbach, Germany) in a private lab (**Olayemi, 2007**).

Statistical analysis

The obtained data of semen pictures and testosterone hormone was statistically analyzed using **Costat Computer Program (1986)** Cottort Software.

RESULTS

1. Histopathology findings

Testes

Grossly the testes have mild to moderately congested blood vessels in the CPF intoxicated group. Microscopically, normal histological picture of seminiferous tubules and interstitial tissue in the control group was seen (group I), while the rats treated with CPF (group II) showed moderate to severe lesions in the testis. The testicular changes were in the form of congestion associated with mild to moderate oedema of the interstitial tissue. Moreover, moderate to severe degenerative changes characterized by thickening and hyalinization of basement membranes of seminiferous tubules, irregularity and shrinkage of seminiferous tubules, vacuolar degeneration of many spermatogenic cells and hypospermatogenesis (reduction in germ cells). Also, the increased number of the degenerated spermatids and residual bodies in the lumen of the seminiferous tubules appeared as fused spermatids were noticed (Fig. 1&2). These histopathological changes were reduced in the rats treated with CPF plus Zn or CPF plus vitamin E (groups III). In addition, the spermatogenic cells and the interstitial congestion of blood capillaries were ameliorated (Fig. 3).

Epididymis

Histologically, the epididymal tubules lined with one layer of ciliated cuboidal epithelial cells in the control group, while in group II showed congestion, interstitial oedema and moderate to severe vacuolar degeneration of some ductal cells (Fig. 4). Hyperplasia of the lining epithelial cells of tubules with more than one cuboidal cell layer (Fig. 5) was seen in some epididymal tubules with dark focal areas of concentrated degenerated spermatids were noticed in tubules. Rats in group III exhibited noticeable improvements in epididymal tissues (Fig. 6).

2. Semen evaluation

The following parameters of the semen analysis were statistically analyzed to compare the data of each parameter of the CPF intoxicated group to the control and CPF plus Zn and vitamin E groups through one spermatogenic cycle.

Data regarding the effect of treatment on the semen evaluation 50 days' post intoxication presented in table (1) and showed significant ($P<0.05$) reduction in the progressive motility in the CPF treated group (39.00±2.08%) compared with the control, & group III (73.00±1.70%, and 55.00±1.50%, respectively). Similarly, there was a significant reduction in the sperm cell concentration ($P<0.05$) among the CPF intoxicated, control & group III ($63.40±4.04×10^6$), ($118.70±4.04×10^6$) & ($116.30±6.65×10^6$), respectively. There was no significant difference between the control and CPF plus Zn and vitamin E group. Regarding the changes in the life / dead percentage ($P<0.05$) between the CPF treated, control and group III. There was a significant increase in comparison with the other two groups as (31.20±0.44%); (11.80±0.42%) & (25.00±1.22%), respectively. Moreover, there was a significant enhancement of the dead life ratio in a comparison between the CPF group and CPF plus Zn and vitamin E group. Data also indicated that CPF plus Zn and vitamin E group reduced significantly ($P<0.05$) sperm abnormalities (25±1.22 and 11.8± 0.42%, respectively) compared with control and CPF treated group (11.8± 0.42% and 31.20± 0.44%, respectively). Data regarding the effect of treatment on the acrosomal abnormalities revealed that CPF plus Zn and vitamin E group reduced significantly ($P<0.05$) acrosomal abnormalities (16.50±1.30% and 36.50±1.91%, respectively) compared with control and CPF treated group (16.5±1.30% and 53± 1.53%, respectively).

Table 1: Semen picture in the spermatogenic cycle (50 days) post intoxication:

Treatments	Motility %	Life / Dead %	Sperm Abnormalities %	Acrosomal Abnormalities %	Concentration per millions
Control	$73.00±1.7^a$	$83.1±1.04^a$	$11.8.±0.42^a$	$16.5±1.30^a$	$118.7×10^6±4.04^a$
CPF Treated	$39.00±2.08^b$	$55±1.29^b$	$31.20±0.44^b$	$53±1.53^b$	$63.40×10^6±4.04^b$
CPF Plus vitamin E & Zn	$55.00±1.5^c$	$63.5±1.07^c$	$25.00±1.22^c$	$36.00±1.91^c$	$116.30×10^6±6.65^a$

Values with different superscripts in the same column are significantly different at ($P<0.05$).

3. Assessment of sperm DNA integrity

Upon the comparison of the intoxicated group to the other two groups on the DNA integrity in the table (2). There was a significant drastic increase ($P<0.05$) in Comet % in the treated group with either control and group III as (19.89±0.35%), (8.55±0.14%) & (11.75±019), respectively. Also, data indicated that CPF group showed either a significant increase ($P<0.05$) in DNA in tail and tail moment with either control and group III as (6.38±0.56), (4.73±0.12) & (4.85±0.97) and (0.82±0.037), (0.43±0.004) & (0.41±0.10), respectively.

Table 2: Assessment of sperm DNA integrity

Treatments	Comet%	Tail Length (Pixel)	DNA% in the tail	Tail moment	Olive moment
Control	8.55±0.14[a]	7.3±0.35[a]	4.73±0.12[a]	0.43±0.004[a]	0.84±0.647[a]
CPF Treated	19.89±0.35[b]	8.35±0.31[a]	6.38±0.56[b]	0.82±0.037[b]	1.01±0.125[a]
CPF Plus vitamin E & Zn	11.75±0.19[c]	8.09±0.38[a]	4.85±0.97[ab]	0.41±0.10[c]	0.86±0.14[a]

Values with different superscripts in the same column are significantly different at (P<0.05).

4. Blood serum testosterone hormone profile

There was a significant (P<0.05) reduction in testosterone concentration in the CPF treated group (1.112±0.82 ng/ml) compared with the CPF plus Zn and vitamin E (2.503±0.25 ng/ml) and highly reduction (P<0.01) compared with the control group (3.871±0.31 ng/ml).

Table 3: Sera testosterone concentration of the treated groups 50 days' post intoxication:

Treatments	Control	CPF Treated	CPF Plus vitamin E & Zn
Testosterone (ng/ml)	3.871±0.31[a]	1.112±0.82[b]	2.503±0.25[c]

Values with different superscripts in the same row are significantly different at (P<0.05).

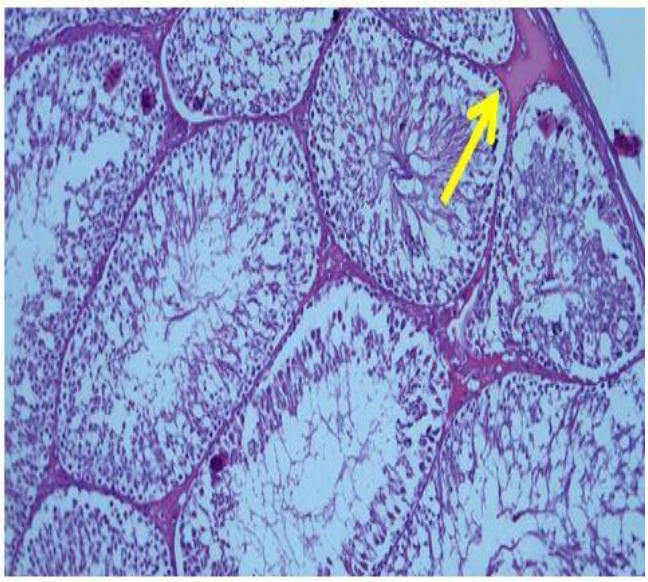

Fig. 1: Albino rat testis of CPF treated group showing moderate degenerated seminiferous tubules with interstitial oedema (arrow) and hypospermatogenesis (H&E, X100).

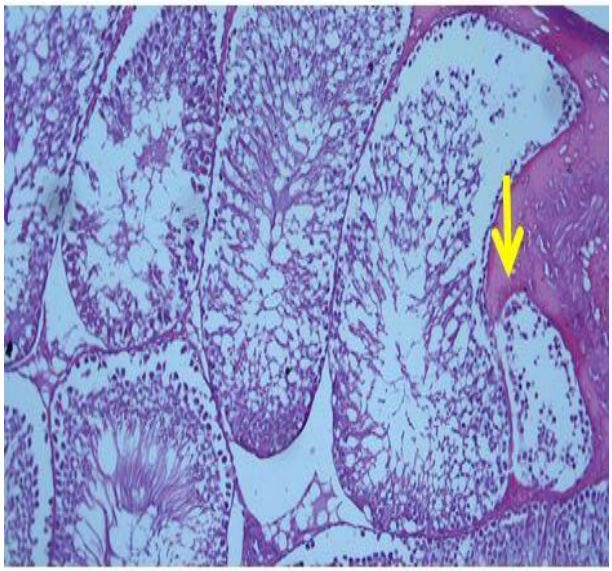

Fig. 2: Albino rat testis of CPF treated group showing severe degenerated seminiferous tubules with interstitial oedema and necrosed tubule (arrow) (H&E, X100).

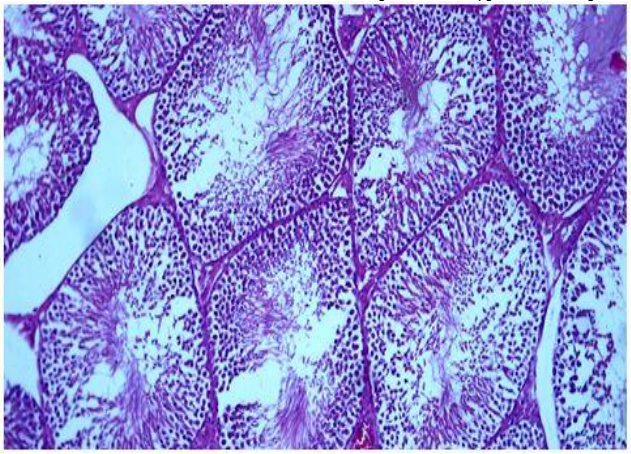

Fig. 3: Albino rat testis of CPF plus Zn & vitamin E treated group showing ameliorated seminiferous tubules with spermatids in the tubules and normal interstitial tissues (H&E, X100).

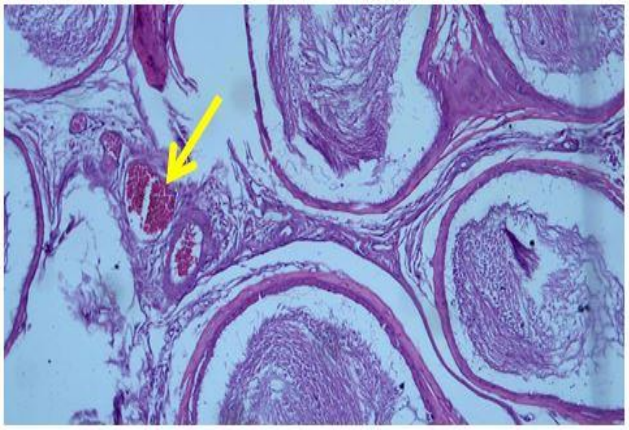

Fig. 4: Albino rat epididymis of CPF intoxicated group showing moderate degenerated tubules, congested blood vessels (arrow), and oedema in the interstitial tissues (H&E, X100).

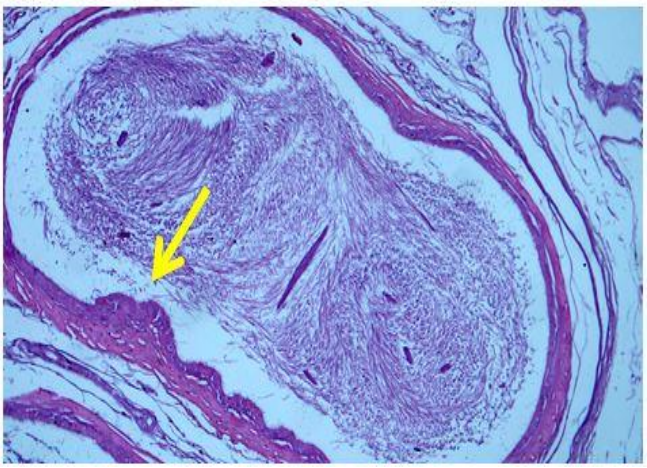

Fig. 5: Albino rat epididymis of CPF intoxicated group showing hyperplasia of the lining epithelial cells of tubules with more than one cuboidal cell layer (arrow) with dark focal areas of concentrated degenerated spermatids (H&E, X100).

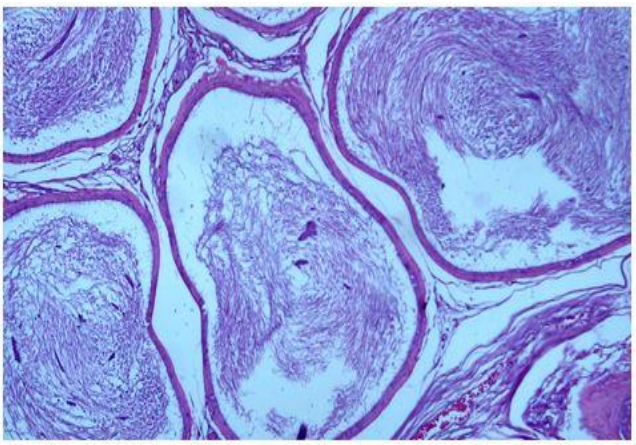

Fig. 6: Albino rat epididymis of CPF plus Zn & vitamin E group showing ameliorated epididymal tubules with spermatids in the lumen (H&E, X100).

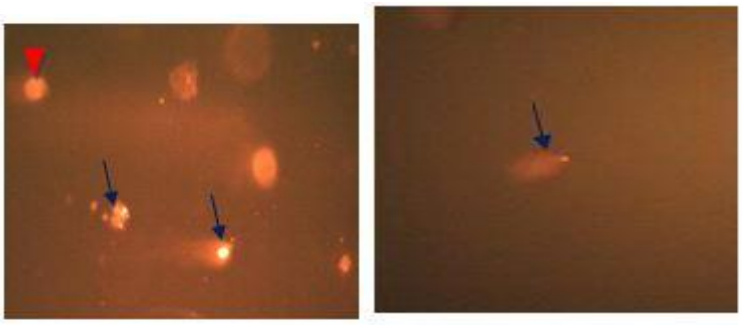

Fig. 7&8: Comet picture of Albino rat spermatozoa in CPF intoxicated group showing a high tendency of DNA fragmentation (arrow) than without DNA fragmentation (redhead arrow).

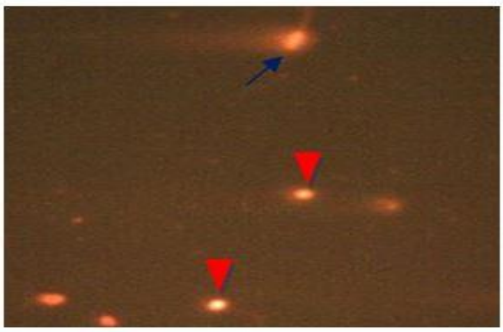

Fig. 9: Comet picture of Albino rat spermatozoa in CPF plus Zn & vitamin E group showing a low tendency of DNA fragmentation (arrow) than without DNA fragmentation (redhead arrow).

DISCUSSION

Different OP insecticides are in extensive use worldwide and 5 % of the world's populations are directly exposed to these insecticides. According to recent reports, this population is calculated to be 2.60 million persons. The OP pesticides are fat-soluble macromolecular substances that can be absorbed through the lungs, skin and gastrointestinal tract and bind to red blood cell AChE. The OPs inactivate AChE by phosphorylating the serine hydroxyl group located at the active site of AChE. The current study has shown mild to moderate histopathological changes in the testicular tissue in the form of congestion associated with moderate to severe oedema in the interstitial tissue. In contrast, the epididymal tissue showed congestion, interstitial oedema and moderate to severe degeneration of some ductal cells. Moreover, these findings were consistent with **Debnath and Mandal (2000); Akhtar *et al.*(2009); Farag *et al.*(2010); El-Sharkawy *et al.*(2014); Sai *et al.*(2014).**

They reported that CPF caused testicular lesions characterized by markedly decreased testes weight with moderate to severe widening of interstitial spaces and partial arrest of spermatogenesis. The obtained decrease in the testicular spermatogonia and spermatids indicated that spermatogenesis was deteriorated in the CPF-exposed groups. These results are consistent with the published data that reported that CPF caused adverse reproductive effects in male rats, including severe testicular damage, and resulted in a reduction in sperm count and thus affected the fertility.

Generally, **Li *et al..*,(2009)** explained the pathogenesis of the toxicity by multiple factors, but it appeared to be initiated through the production of Reactive Oxygen Species(R.O.S.) by the activated CPF iron oxygen complex and **Goel *et al..*,(2015)** added that oxidative damage of the tissues appeared to be an essential mechanism in the pathogenesis of tissue injury. On the other hand, it is well known that Zn is a **necessary** component of the oxidant defense system with participation at multiple cellular levels **(Bray and Bettger 1990).** Several studies have shown that zinc possesses antioxidant properties and thus can protect the cell from oxidative damage induced by certain xenobiotics that similar to the protective role of zinc by **Mansour *et al..*,(2017)**. Apart from its direct antioxidant effect by occupying iron and copper-binding sites on lipids, proteins and DNA, zinc also plays a structural role in maintaining the integrity of Cu-Zn-SOD as a cofactor, and in glutathione regulation which is vital to cellular defense (**Powell, 2000)**.

Vitamin E is an essential intracellular antioxidant in the cytomembranes responsible for the maintenance of cellular integrity (**Baker, 1996**)**.** Reactive oxygen species (ROS) is a term used to designate oxygen-derived free radicals (e.g., superoxide, hydroxyl radical, nitric oxide), and non-radical oxygen derivatives of high reactivity (e.g., singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite). The human body possesses molecules known as antioxidants that can counteract the harmful effects of these free radicals. If the generation of free radicals exceeds the protective capacity of antioxidants, this can cause a case of oxidative stress leading to chronic damage in the tissues and age-dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders, and other chronic conditions **Bhattacharya, (2015)**. Xenobiotics, such as pesticides, enhance the formation of ROS, which has been implicated in inducing oxidative stress in the tissues and chronic damage. Such damage occurs. In cases of excessive accumulation of ROS or insufficient protective antioxidant as zinc.

Therefore, the membrane stabilization by vitamin E may have played a significant role in the improvement of the cellular integrity of the neutrophils, preventing the release of the cell-damaging free radicals. **Liu *et al.*(1998)** have demonstrated that Zn has a protective effect on histological damage by maintaining membrane integrity due to its direct action on free radicals. Concerning with the semen picture alterations, there was a significant reduction in sperm motility and concentration and a significant increase in life/dead ratio, sperm abnormalities and acrosomal abnormalities the similar results were obtained by some other organophosphorus pesticides **Bardin *et al.*(1988); Sinha *et al.*(1995); Sai *et al.*(2014).**

The testosterone level in our study was significantly reduced; the same result was obtained by **Blankvoort *et al.*(2001),** who concluded CPF was reported to show anti-androgen activity by Hershberger assay in rats. The reduction of T content may be due to the hypothalamus-pituitary-testicular axis, which was damaged by CPF. In males, FSH is produced by the anterior pituitary gland. It acts on the Sertoli cell of the testes, stimulating them to synthesize and secrete the male sex hormone. Meanwhile, FSH was regulated by the negative feedback of T (**Rocha *et al..*,2007).**

Regarding to the DNA integrity, our study showed a significant drastic increase in Comet % in CPF intoxicated group with either control and CPF plus Zn and vitamin E group. Also, indicated that CPF group showed either a significant increase in

DNA% in tail and tail moment with either control and CPF plus Zn and vitamin E, these results were similar to the results obtained with **Anugya *et al.*,.(2008)** who stated that rats were administered 50 mg and 100 mg CPF/kg body weight daily for 1, 2, and 3 days, as well as 1.12 mg and 2.24 mg CPF/kg bodyweight for 90 days, showed DNA damage was estimated by scoring 100 cells per animal, dividing into five types: types 0, I, II, III, and IV.

The results indicate that exposure to CPF, acutely, or chronically, caused a dose-dependent increase in DNA damage in the liver and brain of rats. It can be concluded that CPF exhibits genotoxic potential in vivo. Also, our results come in contact with **Li *et al.*,. (2015),** who found that organophosphate insecticide chlorpyrifos (CPF) is known to induce neurological effects, malformation, and micronucleus formation, persistent developmental disorders, and maternal toxicity in rats and mice. The binding of CPF with DNA to produce DNA adducts using the alkaline comet assay leads to an increasing social concern about the genotoxic risk of CPF in humans, but CPF-induced cytotoxicity through DNA damage and cell apoptosis is not well understood.

CONCLUSION

It can be concluded that**,** CPF has a deleterious effect on the genital organs of mature albino male rats at the sublethal dose (10.6 mg/Kg body weight). This means that the male animals may be subclinically intoxicated with CPF (do not exhibit clinical signs of toxicity) and, at the same time, able to perform normal mating with low fertility. The use of antioxidants like vitamin E and zinc ameliorate the harmful effect of CPF-induced reproductive toxicity and internal organs in male rats. Recommendation, zinc, and vitamin E must be added to the feeding program of animals in the farm used organo-phosphorus compounds, especially chlorpyrifos as insecticides or pesticides.

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