

Chlorpyrifos induced testicular damage in rats: Ameliorative effect of glutathione antioxidant

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Abstract: This study investigated the induction of oxidative stress in the testis of adult rat exposed to Chlorpyrifos (CPF). CPF was administered orally, in a dose of 30 mg/kg body weight to male rats for 90 days/ twice/ weekly. Co-administration of water soluble non enzymatic antioxidant glutathione (GSH) was given in a dose of 100 mg/kg body weight, oral, for the same period. Another two groups of male rats were administered GSH and corn oil, respectively. The activities of superoxide dismutase and glutathione reductase were decreased while the levels of lipid peroxidation were increased in the testicular tissues of the exposed animals. Testosterone hormone level in the serum was significantly decreased. The decrease in the histochemical determination of testicular alkaline phosphatase was observed in CPF-treated rats. A significant decrease in all stages of spermatogenesis in the seminiferous tubules was recorded in the exposed animals. Co-administration of GSH restored these parameters.

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1. Introduction

Occupational exposure to pesticides is becoming a common and increasingly alarming worldwide phenomenon. Approximately 3 million acute poisonings and 220,000 deaths from pesticide exposure have been reported annually (Marrs, 1993; USDA, 1994; Yasmashita *et al.*, 1997). The health effects cause by this occupational exposure are enormous. The widespread use of organophosphorus compounds (OP) and the high rates of food contamination could lead to humans, animals, and birds being exposed to high levels of this pesticidal chemical (Suresh *et al.*, 2006). Chlorpyrifos (CPF), O,O-diethyl-O- (3,5,6-trichloro-2-pyridinyl) phosphorothioate, is classified as a moderately hazardous, Class II insecticide by the WHO (1997). It belongs to the phosphorothioate class of insecticides. Its acute toxicity varies according to the species and route of exposure; acute oral LD₅₀ for male rats is estimated to be 80 mg/kg bodyweight (Karanth *et al.*, 2004). The chief mechanism of action of OP pesticides occurs by the inhibition of neuronal cholinesterase activity, a key enzyme that is involved in neurotransmission (Richardson *et al.*, 1993). Pesticides are known to produce oxidative stress, and extensive data suggest that oxygen free radical formation can be a major contributor to the toxicity of pesticides (Lodovici *et al.*, 1994; Bagchi *et al.*, 2002). Many insecticides are hydrophobic molecules that bind extensively to biological membranes, especially phospholipid bilayers and they may damage the membranes by inducing lipid peroxidation (Lee *et al.*, 1991). Some OP pesticides have also been reported to affect the reproductive system (Frag *et al.*, 2000). CPF can induce adverse effects on reproductive

performance; showed fetotoxic and teratogenic effects at a maternal dose of 25 mg/kg-d, a dose that also produced maternal toxicity (Frag *et al.*, 2003). Exposure to CPF poses the greatest problem to pregnant women and small children, with evidence of neurophysiologic effects in humans. Indoor spraying of CPF may pose a considerable risk to human health especially to children (Gurunathan and Bukowske., 1998; Qiao *et al.*, 2003). Therefore, the relationship between maternal CPF exposure and birth defects has been controversial despite the importance to public health and concern over prenatal exposure (Tian *et al.*, 2005). Antioxidants have a number of biological activities, including immune stimulation and alteration of the metabolic activities of carcinogens. These vitamins can also prevent genetic changes by inhibiting the DNA damage induced by reactive oxygen metabolites (Verma *et al.*, 2007). Glutathione (GSH), the major water -soluble antioxidant, is known to protect cellular membranes and lipoproteins from peroxidation (Rana *et al.*, 2002). The aim of this study was to determine the effect of sub-chronic CPF exposure on the reproductive system of male rats and to assess whether these effects can be ameliorated by co-treatment with GSH.

2. Material and Methods

2.1. Chemicals

Chlorpyrifos of purity 96.5% (Technical Grade) and glutathione 100% (pure powdered form) were purchased from Sigma Chemicals Co. (St. Louis, USA). Superoxide dismutase, glutathione reductase and lipid peroxidation (thiobarbituric acid) were measured using commercial test kits supplied by Bio-

diagnostics (Bio-diagnostics, Cairo, Egypt). All other chemicals were of the highest grade available commercially.

2.2 Animals

Sexually mature male Spurge Dawely rats (weighing approximately 200–250 g), obtained from the Laboratory Animals House, Assiut University, were used. The animals were housed in plastic cages, fed a standard laboratory diet and water *ad libitum*, exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 24 ± 2 °C. The animals were quarantined for 10 days before beginning the experiments. All rats were handled in accordance with the standard guide for the care and use of laboratory animals.

2.3. Animal treatment schedule

Animals were randomly divided into four groups of fifteen animals each. Animals in CPF-treated group: were given twice/ weekly a dose of CPF alone (30 mg/kg body weight, oral) for 90 days. Animals in CPF plus GSH-treated group: were simultaneously given GSH as an aqueous solution in a dose of (100 mg/kg body weight, oral); twice/ weekly, during the 90 days and exposed to the CPF at the previous dose. Animals in GSH-treated group: were given aqueous solution of GSH alone; twice/ weekly for 90 days. Control group: were given only standard pellet diet and corn oil.

2.4. Oxidative status in testicular tissues

2.4.1. Superoxide dismutase (SOD)

Superoxide dismutase (EC.1.15.1.1) was assayed by the method of Marklund and Marklund, (1974). Briefly, the assay mixture contained 2.4 mL of 50 mM Tris HCl buffer containing 1 mM EDTA (pH 7.6), 300 mL of 0.2 mM pyrogallol and 300 mL enzyme source. The increase in absorbance was measured immediately at 420 nm against a blank containing all the components except the enzyme and pyrogallol at 10 s intervals for 3 min on a Systronics Spectrophotometer.

2.4.2. Glutathione reductase (GSR)

Glutathione reductase (EC. 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1975). Disappearance of NADPH was measured immediately at 340 nm against a blank containing all the components except the enzyme at 10 s intervals for 3 min on a Systronics Spectrophotometer.

2.4.3. Lipid peroxidation

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS), was measured by the method of Buege and Aust (1978).

2.5. Determination of protein

Protein concentrations were measured by the method of Bradford (1976), using bovine serum albumin as a standard. Protein concentration used in the concentration of SOD, GSR and TBARS can be expressed as activity per mg of protein by dividing the units /ml of protein concentration.

2.6. Testosterone hormone assay

Serum testosterone levels were measured by RIA following extraction with diethyl ether as described previously Muroño *et al.* (1989).

2.6. Histochemical study

Histochemical determination of alkaline phosphatase activity in frozen sections of testicular tissues was demonstrated according to Rustin and Peters (1979).

2.7. Quantitative analysis of seminiferous epithelial cycle and leydig cells

The identification of the different stages of seminiferous epithelial cycle (50 seminiferous tubules for each animal) was based on the morphological changes of the germ cell nuclei and the local arrangement of the spermatids (Franca *et al.*, 1995). The number of Leydig cells/mm² (unit area) was counted using the square grid (area/mm²) at 1000X magnification and 35 unit areas were counted. The nuclear volume of the Leydig cells was performed using ocular micrometer at 1000X magnification. The total numbers of 300 nuclei were measured /animal. The measurement was carried out by concerning rounded nuclei of Leydig cells, where the formula of the sphere was applied as ($V=4/3 \pi R^3$), where R=nuclear diameter/2.

2.8. Histopathology

For histopathological examination, the testicular tissues were dissected and the tissue samples were fixed in Zenker solution for 24 hrs, processed by using a graded ethanol series, and embedded in paraffin. The paraffin sections were cut into 5 ml thick slices and stained with hematoxylin and eosin for light microscopic examination.

2.9. Statistical analyses

Data were analyzed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test. All data were expressed as mean \pm SD for all experimental and control animals/dose. $P > 0.05$ was considered significant compared to control.

3. Results

3.1. Oxidative status in testicular tissues

The activities of superoxide dismutase and glutathione reductase of the testicular tissues showed a significant ($p<0.05$) decrease while the levels of lipid peroxidation increased in both CPF-treated and GSH plus CPF-treated rats in comparison with the control. There is a significant ($p<0.05$) difference between the CPF-treated group and GSH plus CPF-treated rats (Table 1).

3.2. Measurement of testosterone

The control and GSH-treated rats had equivalent of testosterone levels at the end of the 90 day, but the CPF-treated and GSH plus CPF-treated rats had significantly ($p<0.05$) lower serum testosterone levels than the control rats (Table2). However, the GSH plus CPF- treated rats don't differ significantly ($p<0.05$)

from the CPF- treated rats in terms of serum testosterone levels (Table 2).

3.3. Quantitative analysis of seminiferous epithelial cycle and Leydig cells

The quantitative results of the seminiferous tubules cycles are presented in Table (3). There was a significant decrease in the mean number of different germ cell types in all stages of the seminiferous tubules in both exposed groups versus those of controls. The mean number and nuclear volume of Leydig cells were significantly decreased as shown in Table (4).

3.4. Histopathology

The seminiferous tubules of the GSH-treated group and control rats were structurally normal, and they were found appeared to be uniform in size and shape. They were lined by regularly arranged rows of spermatogenic cells at different stages of maturation (Fig. 1A). In contrast, after 90 days of CPF-exposure, there were fewer spermatogenic cells in some of the seminiferous tubules which dissociated from each other (Fig.1B), necrosis in some seminiferous tubules

accompanied with edema in the interstitial tissue (Fig. 1C) and the testicular blood vessels were engorged with blood and had very thick walls (Fig. 1D) associated with connective tissue proliferation (Fig. 1E). Moreover, in the GSH plus CPF- treated group, the seminiferous tubules had more or less normal structure with mild changes in spermatogenic cells accompanied with mild edema in the interstitial tissues (Fig. 1F).

3.5. Histochemical study of alkaline phosphatase in testicular tissues

Figure 2 shows that alkaline phosphatase activities in rat testis were strongest in the peritubular compartment and in occasional interstitial cells. There was also strong reactivity in the vascular endothelium in control group (Fig.2.a). After 90 days of CPF exposure, the intensity of staining indicates that a decrease of testicular alkaline phosphatase activity in CPF-treated rats and GSH plus CPF-treated rats (Fig.2. b,c) in comparison of control.

Table (1): The effect of the CPF on the oxidative status in the control and exposed groups of rats.

	Total lipid peroxide (nmol/mg protein)	Superoxide Dismutase (nmol/mg protein)	Glutathione reductase (nmol/mg protein)
CPF	0.29 ± 0.07*	0.36 ± 0.02*	0.59 ± 0.14*
CPF+GSH	0.10 ± 0.03*a	0.43 ± 0.02*a	1.07 ± 0.04*a
GSH	0.14 ± 0.01ab	1.22 ± 0.11ab	1.14 ± 0.03ab
Control	0.17 ± 0.02	0.98 ± 0.14	1.72 ± 0.07

Values are expressed as means ± SE (n=15). * Significance at $P < 0.05$ in comparison of control. *a* Significance at $P < 0.05$.in comparison of CPF – treated group. *b* Significance at $P < 0.05$ in comparison of CPF+GSH- treated group.

Table (2): The effect of the CPF on the testosterone hormone level in exposed and control groups of rats.

	CPF	CPF+GSH	GSH	Control
Testosterone (µg/dl)	2.86 ± 0.04*	2.94 ± 0.05*	3.86 ± 0.07ab	3.92 ± 0.09

Values are expressed as means ± SE (n=15). * Significance at $P < 0.05$ in comparison of control. *a* Significance at $P < 0.05$.in comparison of CPF – treated group. *b* Significance at $P < 0.05$ in comparison of CPF+GSH- treated group.

Table (3): The effect of the CPF on the different stages of the seminiferous epithelial cycle in control and exposed groups of rats.

	Sertoli cells	Spermatogonia		Primary spermatocyt	Secondary spermatocyt	Spermatids		Spermatozoa
		Type A	Type B			Round	Elongated	
CPF	1.52 ± 0.16*	17.5 ± 1.5*	3.7 ± 2.4*	33.12 ± 1.7*	4.5 ± 0.22*	35.6 ± 1.87*	8.5 ± 0.45*	10.4 ± 0.77*
CPF+GSH	1.0 ± 0.11*a	13.8 ± 1.0*a	3.0 ± 5.8*	28.6 ± 1.4*	3.6 ± 0.24*	28.6 ± 1.47*a	8.5 ± 0.60*	8.78 ± 0.66*a
GSH	3.74 ± 0.10	22.5 ± 1.1	8.7 ± 2.8	43.73 ± 1.3	7.6 ± 0.21	44.82 ± 2.78	9.0 ± 0.35	11.99 ± 0.54
Control	3.54 ± 0.13	23.6 ± 1.2	8.8 ± 2.6	46.76 ± 1.6	7.2 ± 0.23	46.88 ± 2.48	9.4 ± 0.55	12.56 ± 0.62

Values are expressed as means ± SE (n=15). * Significance at $P < 0.05$ in comparison of control. *a* Significance at $P < 0.05$.in comparison of CPF – treated group. *b* Significance at $P < 0.05$ in comparison of CPF+GSH- treated group.

Table (4): The effect of the CPF on Mean of Leydig cells number and nuclear volume in exposed and control group of rats

	Mean number of Leydig cells	Mean nuclear volume of Leydig cells (μm^3)
CPF	$1.47 \pm 0.1^*$	$1.61 \pm 0.2^*$
CPF +GSH	$4.54 \pm 0.5^* a$	$8.68 \pm 0.3^* a$
GSH	$7.17 \pm 0.3 ab$	$12.01 \pm 0.4ab$
Control	7.28 ± 0.5	11.90 ± 0.5

Values are expressed as means \pm SE (n=15). * Significance at $P < 0.05$ in comparison of control. *a* Significance at $P < 0.05$ in comparison of CPF – treated group. *b* Significance at $P < 0.05$ in comparison of CPF+GSH- treated group.

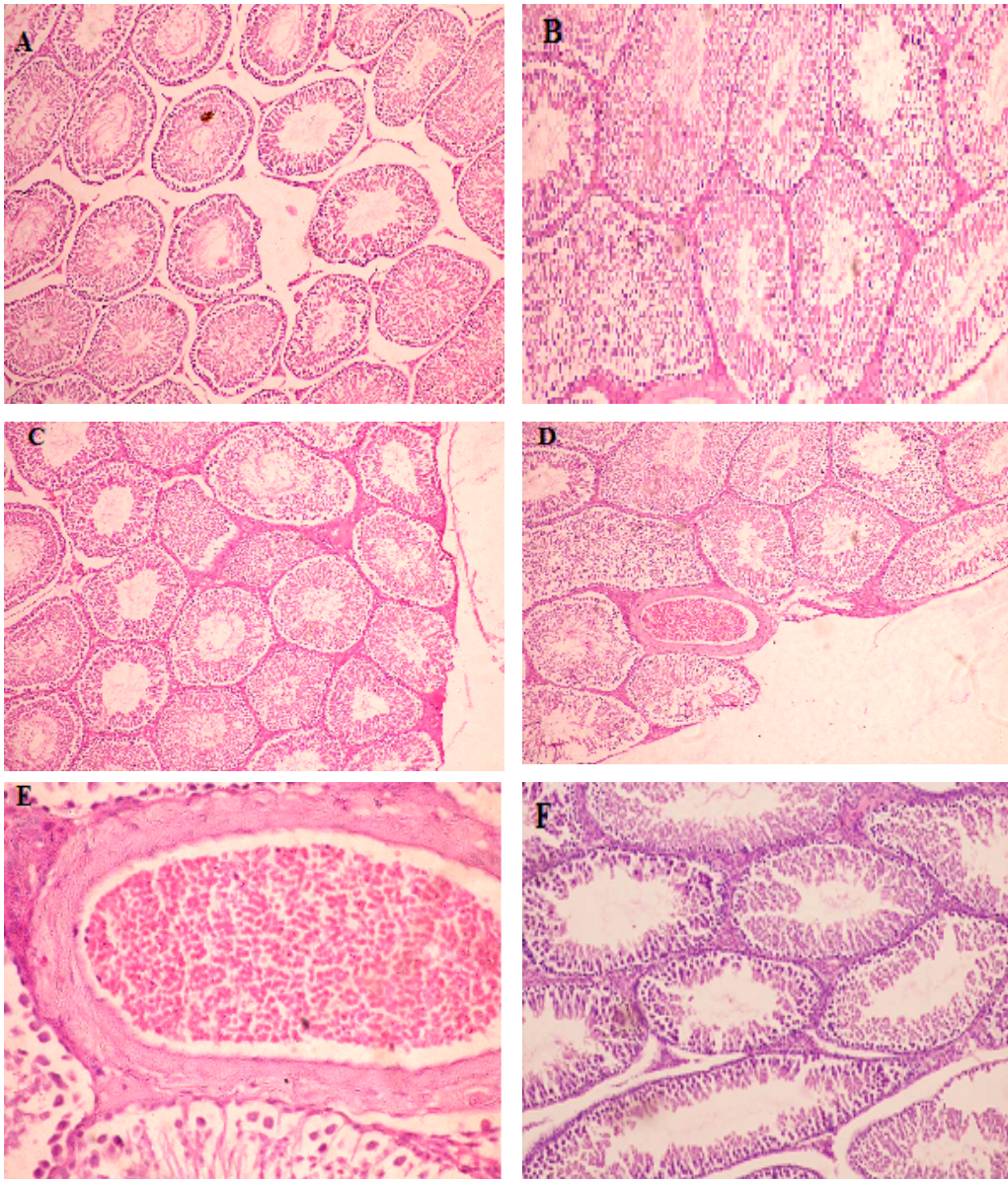


Fig.1 (A. Testes of CPF- treated groups showed seminiferous tubule dissociated from each other with decreasing in spermatogenic cells H&E X10. (B. Interstitial edema is present among the seminiferous tubule from the same group H&E X25. (C. Sever congestion of the testicular blood vessel which has thick wall H&E X10. (D Thick wall and congested blood vessel associated with proliferating connective tissue H&E X40.(F. Testes of GSH plus CPF- treated group showed seminiferous tubules had mild changes in spermatogenic cells accompanied with mild edema in the interstitial tissues H&E X10.

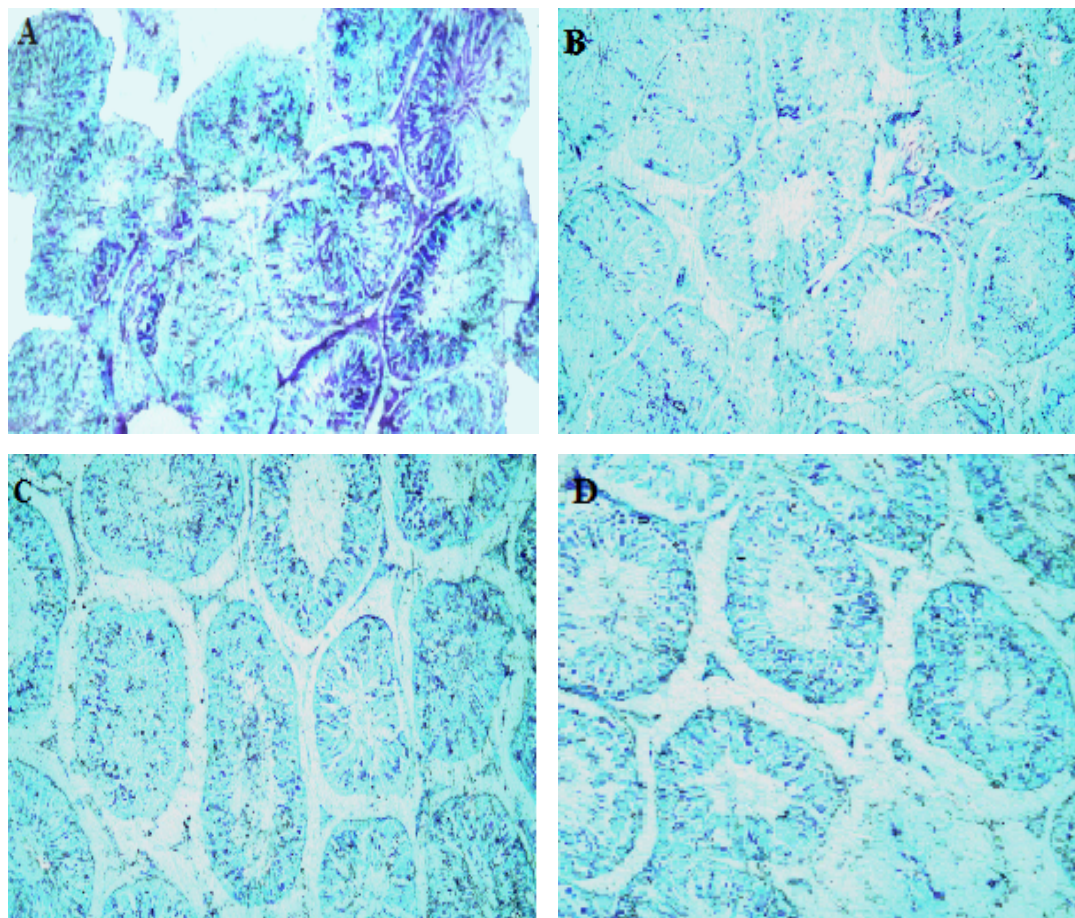


Fig. 2 Section of the testis stained for alkaline phosphates showed (A) The dark deposits indicating enzyme activity are limited to selected interstitial cells and peritubular elements in the control group (X 40). (B) Seminiferous tubules with faint staining inside the tubular epithelium and interstitial space indicating weak enzyme reaction in CPF -treated group (X 40). (C) Seminiferous tubules with moderate staining inside the tubular epithelium and interstitial space indicating to improve in enzyme reaction in CPF plus GSH - treated group (X 40).

4. Discussion

A role of ROS in infertility due to defective sperm function has been reported (Aitken and Clarkson, 1987; Aitken *et al.*, 1989; Peltola *et al.*, 1994). The excessive generation of ROS has been shown to cause per-oxidative damage to the plasma membrane, which leads to impaired sperm function (Kobayashi *et al.*, 1991). Superoxide dismutase depletion is thought to be associated with sperm immobility (Kobayashi *et al.*, 1991). Testicular production of free radicals and activation of the antioxidant defense system have been reported after exposure to toxic chemicals (Aitken *et al.*, 1989 and Peltola *et al.*, 1994) and the epididymal production of free radicals and function of the antioxidant defense system require further studies. Previous studies have shown that lindane induces oxidative stress in the testis as well as in the epididymis and epididymal sperm of adult rats (Chitra *et al.*, 2001 and Sujatha *et al.*, 2001). In the present study, we have sought to determine the

effect of CPF on the testicular antioxidant system of adult rats by measuring the antioxidant enzymes and lipid peroxidation (LPO) in various regions of the testicular tissues. Our results revealed that a pronounced increase in the products of LPO while a significant decrease was obtained in the levels of SOD and GSR. CPF is known to generate oxidative stress, induce LPO, and cause depletion of the GSR, increase in oxidized glutathione (GSSG), and decrease in the ratio of GSR/GSSG in rat erythrocytes and tissues (Gultekin *et al.*, 2001; Verma and Srivastava, 2001, 2003). It has been known to generate oxidative stress by inhibiting enzymatic antioxidant defense (Verma and Srivastava, 2003). Jett and Navoa (2000) reported that an increase in H₂O₂ independent NADPH consuming activity in brain of pups of CPF exposed dams. The exposure to CPF in rats causes oxidative stress in all parts of brain (Verma *et al.*, 2009). Our result strengthens the hypothesis and suggests that induction of oxidative stress is perhaps the central

mechanism by which OP pesticides exert their cellular action. Oxidative damage primarily occurs through production of reactive oxygen species, including hydroxyl radicals and hydrogen peroxide that subsequently react with biological molecules as well as causing damage to membranes and other tissues (Banerjee *et al.*, 1999). A significant decrease in the GSR level and a concomitant increase in LPO level following administration of the pesticide dose were observed in the present study. The decrease in GSR level leads to a net suppression in the total antioxidant capacity since it plays a key role as a substrate for the enzyme glutathione S-transferase (GST) and as a cofactor for a variety of enzymes including glutathione peroxidase (GPx). Also, GSR depletion has been shown to intensify LPO and predispose cells to further oxidant damage (Maellaro *et al.*, 1990).

In the present study, there is a decrease in the serum testosterone level and reduction in spermatogenesis in CPF- exposed groups. These results are in consistent with the published data reported that CPF at 17.5 mg/kg-d caused adverse reproductive effects in male mice included severe testicular damage and resulted in reduction in sperm count and thus affect the fertility (Joshi *et al.*, 2007). On the other hand, the reduction in spermatogenesis which observed in the exposed groups may simply represent the effects of CPF exposure on sperm parameters, and testis histological changes. Spermatogenesis and fertility are critically dependent upon the maintenance of adequate levels of testosterone (Kidd and James, 1991). Therefore, the effects of CPF on the spermatogenesis in this study can be attributed to its ability to reduce serum testosterone levels and activity of some testicular enzymes. Concerning of the reproductive toxicities of OP pesticide, the methyl parathion caused increasing in the levels of abnormal sperm and testosterone, whereas the luteinizing hormone level and total number of seminiferous tubules decreased in the testis besides a few tubules showed exfoliation of epithelium and vacuoles in the testis of the treated newborn Wistar rat (Narayana *et al.*, 2006). The pesticide vinclozolin, which is known as an endocrine disrupting chemical regarding spermatogenesis and gene expression revealed a significant increase in the expression level of mRNAs of the testicular steroidogenic enzyme genes; cytochrome P450 (CYP) side chain cleavage (P450scc or CYP11A) and CYP17A (Kubota *et al.*, 2003).

In the present study, the histochemical expression of the alkaline phosphatase enzyme in the testes of albino rats is decreased due to the toxic effect of CPF exposure. Almost similar observations have also been reported by Seven *et al.* (2004) that the activity of alkaline phosphatase in the liver of rats due to adverse toxic effects of CPF exposure; while Linn (1998) due to inhibition of testicular steroideogenesis by the

induction of cytochrome P-450, which is the result from decrease steroid hydroxylases and decline lysosomal enzyme alkaline phosphatase or the activation of CPF by microsomal monooxygenase in the testes, in the presence of NADPH and oxygen, which might result from decline the enzymes in the target tissue (Kubota *et al.*, 2003). The mechanism of action of some OP on the testes involves induction of cytochrome P-450 and significant increase in the expression level of mRNAs of the testicular steroidogenic enzyme genes; cytochrome P450 (CYP) side chain cleavage (P450scc or CYP11A) and CYP17A resulting in decreases in the activities of steroid hydroxylases and enzymes. The metabolites of CPF are appeared to be important in the biological activity of the pesticide and are responsible for the toxic side effects of the pesticide on the testicular cytochrome P-450 and enzymes level (Mutch and Williams, 2007).

The testicular histomorphological studies of CPF- exposed group revealed that a significant decrease in spermatogenic cells of the seminiferous tubules which dissociated from each other and also there is necrosis in some seminiferous tubules accompanied with edema in the interstitial tissue. In addition, there was a significant decrease in the mean number of different germ cell types in all stages of the seminiferous tubules. Moreover, the mean number and nuclear volume of Leydig cells were significantly decreased as compared with control. These findings were in consistent with Debnath and Mandal (2000) they detected that the OP insecticide pollutant quinalphos induced shrinkage of the tubular diameter and testicular atrophy leading to degenerative changes in the germinal epithelium in albino rats. The reproductive toxicity of CPF in male mice was studied by Farag *et al.* (2010). 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 at the high level dose of CPF at 25 mg/kg-d. The obtained decrease in the testicular spermatogonia and spermatid indicated that spermatogenesis was partially arrested at the CPF exposed group. These results are in consistent with the published data reported that CPF at 17.5 mg/kg-d caused adverse reproductive effects in male mice included severe testicular damage and resulted in reduction in sperm count and thus affect the fertility (Joshi *et al.*, 2007). Indeed, the decrease in Sertoli, Leydig and germ cell numbers, which observed in this study, is related indicator for hypothyroidism and lowered testosterone level at 17.5 mg/kg-d. CPF was reported to be a potential endocrine disrupter by depression of sperm T4 level (Rawlings *et al.*, 1998). Recent study has suggested that one critical molecular target of thyroid hormone in the Leydig cell is the steroidogenic acute regulatory (StAR) protein, which is

responsible for cholesterol transport across the outer mitochondrial membrane for testosterone production (Walsh *et al.*, 2000 and Manna *et al.*, 2001 a, b).

Glutathione, the major water-soluble antioxidant, is known to protect cellular membranes and lipoproteins from peroxidation (Rana *et al.*, 2002). It may minimize LPO in biological systems (Selvam, 2002) and acts to protect both cytosol membranes against free radical attack (Hogg *et al.*, 1998). Its high electron donating capacity combined with its high intracellular concentration endows GSH with great reducing power, which is used to regulate a complex thiol exchange system.

Our results suggest that exposure to CPF induces depletion in antioxidant defense systems in the testes indicating CPF-induced oxidative stress in the testes. This effect may lead to disruption in the functional integrity of cell organelles. The present study also suggests that co-administration of GSH with CPF prevents the adverse effects of CPF on antioxidant status as well as improving testosterone hormone level, spermatogenesis, testicular histopathological alterations in adult rats. In conclusion, the adverse effect of CPF on male reproduction of rats may be due to the induction of oxidative stress. These effects could be alleviated by non-enzymatic water-soluble antioxidant glutathione.

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