Chlorpyrifos (from different sources): Effect on Testicular Biochemistry of Male Albino Rats.

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Abstract

Organophosphates are known primarily as neutrotoxins. However, reactive oxygen species (ROS) caused by organophosphates may be involved in the toxicity of various pesticides. Therefore, in this study we aimed to investigate the toxic effects of three trade names of chlorpyrifos (CPF) pesticide, from different local manufactures [chlorozan (K) pestpan (W) and pyriban (H)] on testicular weight , testicular oxidative stress and some testicular biochemical parameters in male albino rats. **Methods:** Three compounds (K, W and H) were administrated orally to rats at dose of 23.43, 21.40 and 17.43 mg/kg b.w., respectively (which represent the 1/4 LD_{50}) with 5 doses per week for 28 days. Twenty-four hours after the last treatment the rats were sacrificed using anesthetic ether. Testes were collected , cleaned and weighed. Right testes were fractionated and supernatant of testicular homogenate was obtained by centrifugation, lipid peroxidation (LPO), total glutathione, activities of alkaline and acid phosphatases, lactate dehydrogenase and total protein were measured. Moreover, the left tests were histologically examined. **Results:** The testes weights were significantly decreased in (W) group only. Chlorpyrifos treatments (K, W and H) alter markedly the testicular lipid peroxidation (LPO) levels, while, the decline in the total glutathione (GSH) was occurred only in (W and H) groups, in comparing with the control group. Also, there was significant decrease in the activities of alkaline and acid phosphatase (ALP and ACP) and lactate dehydrohenase (LDH) in all treated groups. Total protein (TP) level exhibited an elevation in testicular tissue in comparison with the control group. Treatment-dependent histopathological changes were seen in testes of CPF-W group only. **Conclusion:** Chlorpyrifos (CPF) alters testicular functions possibly by induction of testicular oxidative stress and inhibition of the activities of marker enzymes, thereby disrupting male reproduction. [Journal of American Science 2010;6(7):252-261]. (ISSN: 1545-1003).

Keywords: Chlorpyrifos; rats; lipid peroxidation; total glutathione; acid and alkaline phosphatase; lactate dehydrogenase; total protein ; tests.

1- Introduction

The widespread use of organophosphorus insecticides (OPIs) has long been shown to exert deleterious effects on living organisms. For instance, the exposure of laboratory animals to OPIs, in particular to chlorpyrifos [O, O-diethyl – O -(3, 5, 6-trichloro- 2- pyridyl) phosphorothionate], elicits a number of effects including hepatic dysfunction **(Gomes *et al.*, 1999 and El-Kashoury and El-Said, 2007)**, ciliotoxicity **(Swann *et al.*, 1996)**, immunological abnormalities **(Blakley *et al.* , 1999)**, genotoxicity **(Song *et al.* , 1998)** and testicular damage **(Joshi *et al.* , 2007)**.

Chlorpyrifos was introduced to the Egyptian market, since thirty years at least recently over ten trade names are available in the market but the most common used are the previous mentioned trade names (K, W & H), commonly used to control cotton pests.

Organophosphorus pesticides exert their biological effects mainly through electrophilic attack of cellular constituents with simulataneous generation of reactive oxygen species (ROS). ROS may be involved in the toxicity of various pesticides **(Dwivedi *et al.* , 1998)**.

Malondialdehyde (MDA) is a marker of membrane lipid peroxidation resulting from the interaction of ROS and the cellular membrane **(Aslan *et al.*, 1997)**. The final membrane damage can lead to a loss of cellular homeostasis by changing the membrane characteristics **(Swann *et al.*, 1991)**. ROS are produced by univalent reduction of dioxygen to superoxide anion (O^-_2), which in turn disproportionates to H_2O_2 and O_2 spontaneously or through a reaction catalyzed by superoxide dismutase (SOD). Endogenous H_2O_2 may be converted to H_2O either by catalase or glutathione peroxidase

(GSH-Px). Otherwise, it may generate a highly reactive free hydroxyl radical (-OH) via a Fenton reaction, which is responsible for oxidative damage. GSH-Px converts H_2O_2 or other lipid peroxides to water or hydroxy lipids, and during this process glutathione (GSH) is converted to oxidized glutathione **(Bachowshi *et al.*, 1997)**.

Virtually, no available literatures concerning the comparison of the toxic effects of the three trade products of chlorpyrifos (K, W and H) on the testicular toxicity, therefore, the present study is an effort aimed:

1- To investigate the adverse effects of three trade names of chlorpyrifos on oxidative status.
2- To verify the effect of subacute exposure to chlorpyrifos on the activity of specific enzymes that responsible of spermatogenesis (ALP, ACP and LDH), TP level in testicular tissue of male rats and the histopathology of the testes.

2-1- Materials

Three trade names of chlorpyrifos active ingredient, 48 % EC., (an organophosphorus group), locally formulated in Egypt and were used in this study:
First: Trade name chlorzan (K), obtained from Kafr El-Zyat Co.
Second: Trade name pestban (W), obtained from El-Watania Co.
Third: Trade name pyripan (H), obtained from El-Helb Co.

2-2- Preparation of the dose:

Each emulsifiable concentrate (EC) was emulsified in water immediately before use and orally administrated to animals by esophageal intubation (per os).

The calculated median lethal dose (LD_{50}) of the three trade names of chlorpyrifos (K, W and H) were 93.75, 85.58 and 71.13 mg/kg b.w., respectively, according to Weil's method **(Weil, 1952)**.

Sub-acute dose, represents 1/4 LD_{50} for each emulsifiable concentrate was diluted in water and used for dosing through experimental priod.

2-3- Animals:

Male albino rats weighting 150 $\pm$ 10 g obtained from the farm of General Organization of Serum and Vaccine (Helwan Farm), Egypt, were used for the study. The animals were housed in plastic cages and allowed to adjust to the new environment for a week before starting the experiment. Rats were fed standard food pellets and tap water ad *libitum*. The rats were housed at 23 $\pm$ 2ºC and in daily dark/light cycle.

2-4- Experimental design:

Animals were randomly divided into four groups of twenty animals each as the following: Group-C: animals served as control group and given tap water instead of pesticide in parallel to the treated group. Group-K: animals were given CPF-K at 23.43 mg/kg b.w. Group-W: animals were given CPF-W at 21.40 mg/kg b.w. Group-H: animals were given CDF-H at 17.83 mg/kg b.w.

Each rat was given orally repeated dose of chlorpyrifos over period of 28 days (5 doses / week). Clinical signs were monitored daily and animals were weighed twice weekly throughout the experiment and the dose was adjusted accordingly.

2-5- Sampling

After completion of treatment period (28 days), five animals from each group were anaesthetized with ether and sacrified. The testes were removed immediately, cleaned of adhering tissues and weighed. The right testes were kept in a deep freezer (-40ºC) for biochemical estimations. Left testes were removed and fixed in 10 % formaline for routine histopathology.

2-6- Biochemical estimations:

Frozen testes were washed with saline solution, then minced and homogenized (10 % w/v) in ice-cold saline, using a chilled glass-teflon porter-Elvehjem tissue grinder tube. Te homogenate was centrifuged at 10,000 Xg for 20 min. at 4ºC and the resultant supernatant used for determination of lipid peroxidation (LPO) which is represented by malondialdehyde (MDA), total glutathione (GSH), alkaline phosphatase (ALP), acid phosphatase (ACP) and total protein (TP). 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 4ºC, the supernatant used for determination of lactate dehydrogenase (LDH). The different assays were carried out using diagnostic kits (Table 1).

Table (1): Procedures adopted for biochemical parameters in the testicular tissues.

Parameters	Reference
Lipid peroxidation (LPO)	**Ohkawa *et al.* (1979)**
Total glutathione (GSH)	**Akerboom and Sies (1981)**
Alkaline phosphatase (ALP)	**Babson (1965)**
Acid phosphatase (ACP)	**Babson and Read (1959)**
Lactate dehydrogenase (LDH)	**Moss and Handerson (1994)**
Total protein (TP)	**Bradford (1976)**

2-7- Histopathological studies

For histopathological observations at light microscopic level, fresh testes were immersed in 10 % formalin saline.

Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5 µm thick sections were double stained with hematoxylin and eosin and observed under microscope **(Banchraft *et al.* , 1996)**.

2-8- Statistical analysis

Data analysis and evaluation of statistical significance among different values was done using Student's-test **(Snedecor and Cochran, 1980)**.

3- Results

3-1- Testes weights

The variations in the testes weights of rats subjected to chlorpyrifos (CPF) for 28 days are shown in Table (2).

There was significant decrease ($P < 0.05$) in the testes weights of the group W only as compared to control group.

Table (2): Effect of oral administration of chlorpyrifose (K, W and H) on testicular weights of rats after sub-acute exposure (28 days).

Parameter	Control group	Chlorpyrifos		
		K-group	W-group	H-group
Testes weight (g)	**2.713 ± 0.074**	**2.845 ± 0.073**	**2.479 ± 0.036***	**2.605 ± 0.105**

The data expressed as mean ± SE , n = 5., * $P < 0.05$ (Student's test).

3-2- Testicular oxidative stress:

The influence of sub-acute toxicity of three trade names of chlorpyrifos (K, W and H) administration on testicular lipid peroxidation (LPO) and total glutathione (GSH) levels are shown in Table (3). The results showed significant increase ($P < 0.001$, $P < 0.01$ and $P < 0.01$) in LPO as measured by the amount of MDA formed after treatment with chlorpyrifos (K, W and H, respectively) in comparing with the control group.

In addition, chlorpyrifos treatment (W and H) caused a significant decrease ($P < 0.01$) in the levels of testicular total glutathione (GSH), compared with the control group.

Table (3): Effect of oral administration of chlorpyrifos (K, W and H) on testicular oxidative status in rats after sub-acute exposure (28 days)

Treatment / Parameters	Control group	Chlorpyrifos		
		K-group	W-group	H-group
Malondialdehyde (MDA) n mol/g wet w	78.86 ± 2.185	126.44 ± 4.963***	103.57 ± 4.304**	103.57 ± 6.164**
Total glutathione (GSH) µ mol/g wet w	31.44 ± 2.786	28.57 ± 0.698	17.98 ± 1.399**	18.48 ± 1.416**

The data expressed as mean ± SE , n = 5. ** P < 0.01, *** P < 0.001 (Student's test).

3-3- Biochemical assays

Testicular biochemistry have been recorded in Table (4). Decline in alkaline phosphatase activity (P < 0.01) in the three chlorpyrifos-treated groups was recorded as compared to control group. Also, acid phosphatase activity was decreased (P < 0.05, P < 0.01 and P < 0.05) in the chlorpyrifos-treated groups (K, W and H, respectively) when compared with the control group. Moreover, the results showed significant decrease (P < 0.01, P < 0.05 and P < 0.01) in the levels of lactate dehydrogenase (LDH) after sub-acute exposure to chlorpyrifos (K, W and H, respectively).

In addition, total protein levels were found to be significantly raised (P < 0.001, P < 0.05 and P < 0.01) in chlorpyrifos-treated animals (K, W and H groups, respectively) in comparing with the control group.

Table (4): Effect of oral administration of chlorpyrifos (K, W and H) on some testicular biochemical parameters in rats after sub-acute exposure (28 days)

Treatment / Parameters	Control group	Chlorpyrifos		
		K-group	W-group	H-group
Alkaline phosphatase (U/mg protein)	0.096 ± 0.006	0.052 ± 0.005**	0.066 ± 0.003**	0.072 ± 0.001**
Acid phosphatase (U/mg protein)	0.110 ± 0.011	0.065 ± 0.006*	0.062 ± 0.004**	0.071 ± 0.007*
Lactate dehydrogenase (U/mg protein)	1.650 ± 0.080	1.090 ± 0.059**	1.280 ± 0.061*	1.120 ± 0.051**
Total protein (mg/g tissue)	16.870 ± 0.876	23.080 ± 0.252***	20.110 ± 0.668*	23.380 ± 0.961**

The data expressed as mean ± SE, n = 5. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student's test).

3-4- Testicular histopathology:

In addition to the findings listed above, we have observed many microscopic changes in the testes of male albino rats after sub-acute exposure to chlorpyrifos.

Histological findings of testes from control and treated groups (K, W and W) are presented in Figures 1, 2, 3 & 4 respectively.

Control and CPF-treated groups (K and H) testes revealed normal mature semineiferous tubules with complete series of spermatogenesis and high spermatozoal concentration in the lumen (Fig. 1, 2 and 4). Meanwhile, the testes of chlorpyrifos-treated group (W) showed degeneration and atrophy of non functioning seminifrous tubules with few numbers of sperm cells in the lumen of the seminiferous tubules (Fig. 3).

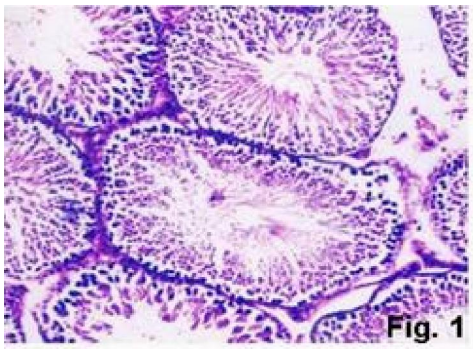

Fig..(1):Testis of rat in control group showing the normal histological structure of mature seminiferous tubules with complete spermatogenic series. (H&E X40)

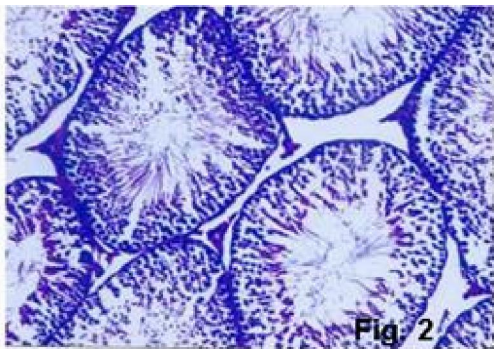

Fig.(2):Testis of rat administrated chlorpyrifos(K) showing mature testicular tissue with full spermatogenesis in the seminiferous tubules . (H&E X40).

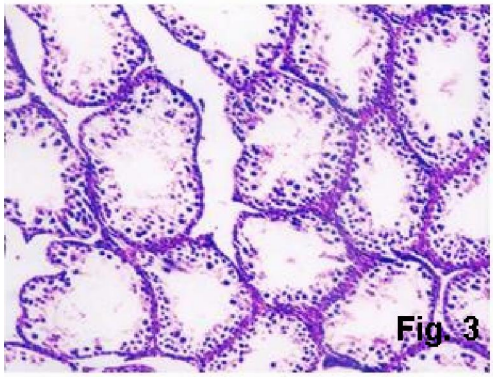

Fig.(3): Testis of rat administrated chlorpyrifos (W) showing degeneration and atrophy of non-functioning seminiferous tubules.(H&E X40).

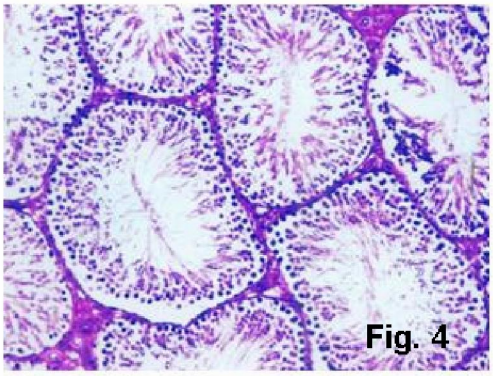

Fig.(4):Testis of rat administrated chlorpyrifos (H) showing normal functioning mature seminiferous tubules .(H&E X40).

4- Discussion

Organophosphates (OPIs) are among the most widely used synthetic pesticides. The wide spread use of OPIs has stimulated research into the possible extence of effects related with their reproductive toxic activity **(Joshi *et al.* , 2007)**.

Our results showed that the weights of testes were significantly decreased in chlorpyrifos-treated group (W) only, as compared to the control group. The decrease in testicular weight in the treated rats may be due to reduced tubular size as confirmed by the histopathological findings of the testes which show degeneration and atrophy of non functioning seminiferous tubules (Fig. 3). This was in accordance with **Joshi *et al.* (2007)** who found mild to sever degenerative changes in seminiferous tubules at various dose levels (7.5, 12.5 & 17.5 mg/kg b.w./day) for 30 days and decreased testes weight.

Also, spermatogenic arrest and inhibition of steroid biosynthesis of leydig cells , a site of steroid biosynthesis may contribute to the decline of testes weight **(Sujatha *et al.* , 2001)**. Another, explanation was reported by **Chitra *et al.* (1999)** which indicate that the decrease in testicular weight may be a result of impairment at testicular, pituitary, or hypothalamic level.

Similar results were recorded by **Choudhary and Joshi (2003)** who reported significant reduction in the testes weight after exposure of rats to endosulfan (organo-chlorine pesticide) at the dose levels of 5, 10

and 15 mg/kg b.w./day for 15 and 30 days. In addition, **El-Kashoury (2009)** showed that the weight of testes was significantly lowered in male rats exposed to profenofos (OPIs) at the dose of 23.14 mg/kg b. w. for 60 days.

The present results confirm the previous reports of **El-Kashoury and El-Far (2004)** who mentioned that administration of rats with profenofos at 23.14 and 46.30 mg/kg b.w. for 28 and 60 days, respectively, induced significant decrease in thyroid hormone levels. There is ample evidence that thyroid hormone is essential to the normal development of testes **(Cook *et al.* , 1994 and Hardy *et al.* , 1996)**.

Links between oxidative stress and adverse health effects have been suggested for several diseases such as cardiovascular, respiratory and neurological as well as for the general aging process. Several drugs, xenobiotics and environmental pollutants are known to cause this inbalance between formation and removal of free radicles. Testes is the main organ of male reproduction. So, the principle objective of the present study was to assess the oxidative damage sustained by testes following sub-acute exposure to three trade names of chlorpyrifos. The present study revealed an elevation in malondialdehyde levels (an indicator of LPO) in three of CPF-treated groups (Table, 3).

Lipid peroxidation, is an oxidative deteriorative process of unsaturated fatty acids, due to excess generation of free radical. Our results suggest but don't prove that free-radical mediated lipid peroxidation, may be involved in toxic manifestation of chlorpyrifos. Similar results were reported by **Gultekin *et al.* (2000) and Gultekin *et al.* (2001)** who indicated that increasing chlorpyrifos concentration caused asignificant reduction in the activities of superoxide dismutase (SOD) and catalase (CAT) and asignificant increase in the level of malondialdehyde and glutathione peroxidase. SOD catalyses the conversion of superoxide radical (O^{-}_{2}) to hydrogen peroxide (H_2O_2), while CAT converts H_2O_2 to H_2O. So, these enzymatic antioxidants may counteract oxidative stress and can alleviate the toxic effects of reactive oxygen species, ROS **(Bagchi *et al.* , 1995)**. Also, **Joshi *et al.* (2007)** reported that sub-acute exposure to chlorpyrifos induce oxidative stress in testes of rats. Furthermore, OPIs such as phosphomidon, trichlorfon and chlorvos have been reported to induce oxidative stress as shown by enhanced MDA production **(Naqvi and Hasan, 1992)**.

The most important non-protein thiol in the cells, the tripeptide glutathione (GSH), which is not only partner in the recycling of oxidized α-tocopherol and ascorbic acid but it is an important water-phase antioxidant and essential cofactor of antioxidant enzymes.

Oxidants such as hydrogen peroxide (H_2O_2) activate specific gene expression through the antioxidant responsive elements. Also, elevation of glutathione level in the testes, may help to preserve physiological integrity of the testes **(Rushmore *et al.*, 1991)**.

In the present study, a significant decrease in the total GSH level was observed following treatment with CPF (W and H) compared with the control group (Table, 3). This result go hand-to-hand with **Gultekin *et al.* (2000)** who found that GSH-Px was indirectly activated by chlorpyrifos inducing ROS. Therefore, the decreased level of total GSH in the testicular tissue after sub-acute exposure to chlorpyrifos may be due to activation of GSH-Px. This enzyme converts H_2O_2 or other lipid peroxides to water or hydroxy lipids, and during this process glutathione (GSH) is converted to oxidized glutathione **(Bachowshi *et al.*, 1997)**.

Meanwhile, it has been reported that OPIs, such as phosphomidone, trichlorfom and dichlorvos caused a decrease in glutathione peroxidase (GSH-Px) activity **(Naqvi and Hasan, 1992)**. Also, administration of mixture of pesticide including chlorpyrifos reduced the activities of GSH-Px in rat testes **(Mattson *et al.*, 1996)**.

In addition, **Latchoumycandane *et al.* (2002)** indicated that methoxychlor (O'ch), a widely used pesticide, has been shown to induce reproductive abnormalities in male rats causing reduced fertility by inducing oxidative stress in the epididymis and epididymal sperm due to decreased antioxidants enzymes (superoxide dismutase, catalase and glutathione reductase) and increased levels of hydrogen peroxide and lipid peroxidation after 4 or 7 days of treatment.

The present results suggest that chlorpyrifos treatment caused a marked decrease in the activities of alkaline, acid phosphatase and lactate dehydrogenase in all treated groups (Table, 4), which reflect suppression in testicular function. Activities of these marker enzymes are considered to

be functional indicators of spermatogenesis **(Johnson *et al.*, 1970)**.

The results in this study go hand-to-hand with the finding of **Salem *et al.* (1989)** who investigated the influence of methamidophos (OPIs) on rats at a dose of 100 ppm in drinking water for 9 and 45 days. They found that acid, alkaline phosphatase and lactate dehydrogenase were reduced significantly in the testicular tissue.

Also, **Moustafa *et al.* (2007)** reported that profenofos is considered as one of the male reproductive toxicants. Moreover, **El-Kashoury (2009)** found significant decrease in the activities of ALP, ACP and LDH in testicular tissues of rats exposed to profenofos for 60 days at a dose of 23.14 mg/kg b.w. per day.

ALP is primary of testicular and epididymal origin and, therefore suitable for differentiation of oligo-and azoospermia **(Turner and McDonell, 2003)**. Decline in ALP activity indicated that chlorpyrifos treatment produced a state of decreased steroidogenesis where the intercellular transport was reduced as the metabolic reactions to channelize the necessary inputs for steroidogenesis slowed down **(Latchoumycandane *et al.* 1997)**.

Acid phosphatase is an enzyme capable of hydrolyzing orthophosphoric acid esters in an acid medium. The testicular acid phosphatase gene is up-regulated by androgens and down-regulated by estrogens **(Yousef *et al.* , 2001)**.

LDH is associated with the maturation of germinal epithelial layer of seminiferous tubules and associated with post meiotic spermatogenic cells **(Sinha *et al.* , 1997)**. An inhibition in the activity of LDH in testes of chlorpyrifos-treated rats points to the interference of chlorpyrifos with the energy metabolism in testicular tissues **(Mollenhauer *et al.* , 1990)**.

Concerning the testicular protein level, results of the present study exhibit an increase in its level in all chlorpyrifos-treated groups (K, W and H). The testicular fluid contains both stimulatory factors as well as inhibitory factors that selectivity alter the protein secretions **(Brooks, 1983)**. Thus the changes in protein suggested that there is a reduction in the synthetic activity in testes.

The elevation in testicular protein level in the present study confirms the previous results by **Choudhary and Joshi (2003), Joshi *et al.* (2007) and El-Kashoury (2009)** who mentioned that the protein content of the testes was raised at significant levels in endosulfan (O'ch), chlorpyrifos and profenofos-treated rats, respectively.

Gupta *et al.* (1981) ; Singh and Pandey (1989) found that an elevation in testicular protein may be due to the hepatic detoxification activities caused by endosulfan which results in inhibitory effect on the activities of enzyme involved in the androgen biotransformation.

In accordance with the findings of the present study, **Rao and Chinoy (1983)** suggested that the accumulation of protein occurred in testes and epididymus due to androgen deprivation to target organs. This deprivation effect led to a reduction in testicular and cauda epididymus sperm population, loss of motility in the latter and an increase in number of abnormal spermatozoa, thereby manifesting 100 % failure in treated animals.

From the afore-mentioned results, it could be concluded that chlorpyrifos (W) decreased the testes weight in comparing with chlorpyrifos (K and H). Meanwhile, chlorpyrifos (K, W and H) had induced a distinct oxidative stress in testicular tissues except (K) for total glutathione. Moreover, chlorpyrifos (K, W and H) induced adverse effects on testicular function by altering biomarker enzymes. In general, CPF-W had a pronounced effect on testicular function more than the other two emulsifiable concentrate (CPF-K and CPF-H). Thus care should be taken and more studies should be done to increase the validity of those information.

Abbreviation used

OPIs, organophosphorous insecticides; O'ch, organochlorine, LPO, lipid peroxidation; GSH, glutathione; CPF, chlorpyrifos; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase,; GSH-Px, glutathione peroxidase; K, chlorzan; W, pestban; H, pyripan; ALP, alkaline phosphatase; ACP, acid phosphatase; LDH, lactate dehydrogenase, TP, total protein; EC, emulsifiable concentrate.

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2010/4/4