Original Research Article

Reproductive parameters and oxidative stress marker impacts associated with oral and inhalation exposure to Chlorview

ABSTRACT

Aim: Factory workers, pesticide applicators and farmers are the most susceptible group to reproductive problems due to continuous exposure to pesticides. Farmers now increasingly deploy pesticides in their agronomic practices. This research aimed to assess the impact on reproductive markers and stress markers of acute exposure to chlorview, an organophosphate pesticide through oral and inhalation routes using animal model.

Study design: A total of 64 male wistar rats were used for the experiment. The animals were divided into two groups for oral (36 rats) and inhalation (28 rats) routes of exposure. Acute toxicity studies and the median lethal dose were carried out using a modified method.

Methodology: Standard methods were used to determine oxidative stress markers of lipid peroxides, catalase activity and glutathione peroxidase activity. Immunoassay test kit was used to determine the reproductive toxicity studies**.**

Results: The results from the 24 hour acute toxicity studies revealed that oral exposure to pesticide gave a median lethal dose (LD_{50}) of 155 mg/kg b.w. while inhalation exposure gave a median lethal concentration (LC_{50}) of 1414 mg/kg b.w. for 60 minutes. Oxidative stress markers (GSH, MDA, CAT and GPx) show that the pesticide induced appreciable oxidative imbalance in the system. In oral exposure, there was a significant ($p<0.05$) increase in the activity when compared to control. The reproductive marker toxicity studies revealed that oral exposure to pesticide led to a significant ($p<0.05$) increase in the Cholesterol (which has a correlation with estradiol) and a significantly ($p<0.05$) decreased in testosterone and sperm count when compared to control. This research concludes that exposure to pesticides can pose a reasonable risk to reproductive and stress markers through oral and inhalation routes of exposure which affect many biochemical processes.

Keywords: Reproductive toxicity, Chlorpyrifos, Enzyme activity, Reactive oxygen species (ROS), Agrochemicals

1. INTRODUCTION

Pesticides are chemicals utilized agricultural practice and in the household for the prevention and control of pest [1] They may also be used as insect repellants that are directly applied to the skin or clothing [2]. The dramatic increase in use of pesticides for agricultural, industrial, and household purposes has created an increasing concern about this class of chemicals. Pesticide residues are now among the most common synthetic chemicals in our environment, detectable in the tissues of humans, animals, aquatic life, and wildlife worldwide [3]. Chemicals assault or enter the body at almost every hour of the day. They may come through air, food, products use on the body, and in drinking water. Toxic buildup of these chemicals has been shown to cause several damages in the body and minimize health. Many modern pesticides (synthetic) persist in soil for years and compound the store of toxins such as heavy metals and other metabolites in the soil, air and water [4, 1]

The major mechanism of organophosphate (OP) toxicity is the irreversible inhibition of acetylcholinesterase activity that results in accumulation of acetylcholine and acute muscarinic and nicotinic effects. In subchronic or chronic organophosphate exposure, oxidative stress induction has been recorded as the main mechanism of organophosphate toxicity [5]. Reactive oxygen species (ROS) production and antioxidant barrier attenuation are both the likely to induce oxidative stress. Oxidative stress can be therefore be defined as a state of imbalance between the body antioxidant defense system–enzymatic and non-enzymatic and the production of free radicals [6].

Activities of cytochrome P450s (monooxygenases) may result in the production of reactive oxygen species (ROS) by addition of one atom of molecular oxygen into a substrate (organophosphate) through the pathway electron transport [6]. This reaction generates reactive oxygen species and alters normal antioxidant homeostasis resulting in antioxidant depletion, if the requirement of continuous antioxidants is not maintained [7]. Milatovic *et al*. [3] described other way of ROS generation in OP toxicity which involves inhibition of oxidative phosphorylation and induction of glycogenolysis to increased release of glucose in the liver to meet the energy requirement (ATP) of the body [8]. Consumption of high energy reduces the capacity of energy level maintenance by the cells. Hence, ROS may be generated in large amounts in different organs [3]. Thus, another mechanism implicated in ROS generation in chlorpyrifos (CPF) an organophosphate exposure is disturbance in the cell redox system [6], leading to MDA increase on cell membrane [9].

Human and animal organisms utilize different mechanisms to counteract ROS damage. There are two main categories of antioxidant compounds; First, the enzymatic system which include enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) [10].

The second antioxidant system is the Non-enzymatic which include vitamins like vitamin C, beta-carotene and vitamin E and peptides like reduced form of glutathione (GSH). Their synergistic work aids to enhance the antioxidant capacity of the organism. To play the antioxidant role GPx utilizes reduced glutathione (GSH) and converts it to oxidized glutathione form (GSSG). Glutathione reductase (GR) is used to recycle GSSG back to GSH. GR in turn requires NADPH (from the pentose phosphate pathway) for reduction GSSG to GSH. Nicotinamide adenine dinucleotide phosphate (NADP) is converted to NADPH by NADPH Glucose 6-phosphate dehydrogenase with glucose as substrate [8].

Pesticides persistent residues in the environment eventually enter the aquatic ecosystem and bioaccumulate in fatty tissues of aquatic organisms and terrestrial vertebrates through the food chain. These pesticides residues present beyond the permissible limit are suspected to be causing malfunctioning in the gonads at receptor levels affecting the reproductive function of the species and decrease in fertility of future fauna in vertebrate [11]. Decreased plasma levels of sex steroid hormones have been reported in several fish species with the tendency to bioaccumulate DDT and HCH in gonads [11].

Faraga *et al*. (2010) reported that delayed neurotoxicity was not observed on exposure to chlorpyrifos for doses below double of the oral $LD_{50.}$ Experiment with mice recorded that chlorpyrifos showed no evidence of selective developmental neurotoxicity up to 5 mg/kg/d and not carcinogenic for both sexes up to 10 mg/kg/d. Also, no reproductive effect was reported in teratology and reproduction study for oral administration of up to 3mg/kg/d in both male and female rats and no teratogenic effect on the progeny [12].

In contrast, Qiao *et al*. [13] reported evidence of neurophysiologic effects in humans with pregnant women and small children being at the greatest risk of exposure to chlorpyrifos through Indoor spraying of

chlorpyrifos. However, the link between chlorpyrifos exposure to pregnant women and birth defects has been unresolved notwithstanding its relevance to public health [14]. On the other hand, significant increase in polychromatic erythrocytes was reportedly induced by oral treatment with chlorpyrifos when compared to the control [12]. In addition, basic in vivo and in vitro studies recognized cytogenetic toxicity and significant increase in the formation rate of micronucleus after exposure to chlorpyrifos [14]. Furthermore, Chlorpyrifos showed fetal toxicity, teratogenic, adverse effects on reproductive performance and maternal toxicity at dose of 25 mg/kg/d [15]. However, no work has shown how different routes of exposure can affect these biochemical parameters. Therefore, this work was done in other to assess the impact of chlorpyrifos on reproductive markers and oxidative stress markers exposed via oral cavity or inhalation.

2. Materials and Methods

The pesticide Chlorview® (Chlorpyrifos 40% E.C.) was purchased from commercial agro-chemical vendor at Ogige market in Nsukka, Enugu State, Nigeria. Wistar rats used were purchased from the animal house of the University of Nigeria Nsukka and fed with top finisher feed throughout the period of the experiment. The experiment was carried out in the Department of Biochemistry and other laboratories in the University of Nigeria Nsukka, as well as the Central Research Laboratory and Diagnostic Laboratory, Ilorin, Kwara state, Nigeria.

2.1 Experimental Design

A total of 64 male wistar rats were used for the experiment. The animals were divided into two groups for oral (36 rats) and inhalation (36 rats) routes of exposure. Acute toxicity studies were carried out using 16 animals for oral exposure and 16 animals for inhalation exposure. The four experimental groups consist of 3 treatment groups and a control group. The treatment doses were obtained by dividing the value of the median lethal dose (LD50) established from the acute studies by a factor of 40, 25 and 10 to get 3 treatment doses representing groups 1, 2 and 3 respectively and group 4 (control group) for both routes of exposure. The treatment doses were as follows:

Oral
Group 1 - 3.8mg/kg b.w
Group 2 - 6.2mg/kg b.w
Group 3 - 15.5mg/kg b.w
Group 4 - Control

Inhalation
Group 1 - 35.36 mg/kg b.w
Group 2 - 56.57 mg/kg b.w
Group 3 - 141.421 mg/kg b.w
Group 4 - Control

2.2 Acute toxicity Studies

The investigated acute toxicity were using a modified Lorke's [41] method. Oral acute toxicity study was done with 16 wistar rats of weighing 140g to 200g. They were divided into 4 groups of 4 animals each. Adjusted doses of 50, 120, 200 and 270 mg/kg b.w. of the pesticide were administered to each group representing groups 1, 2, 3 and 4 respectively. Inhalation acute toxicity was carried out using 12 wistar rats of weight range 133g to 223g divided into 4 groups of 3 animals each, adjusted doses of 1000, 2000, 3000 and 6000 mg/kg b.w. of the pesticide was administered to each group representing groups 1, 2, 3 and 4 respectively. This reflects the range of LD_{50} values already reported in various literatures. Experimental animals were observed for signs of sub-acute and acute toxicity for and the median lethal dose (LD_{50}) determined after 24 hours.

2.3 Determination of median lethal dose (LD_{50})

The median lethal dose was determined using the following formula [9]

$LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = Highest dose that gave no mortality

D_{100} = lowest dose that gave mortality

Converting Emulsifiable Concentration (E.C.) to mg/ml

$$(g/L) \div 10 = E.C.$$

$$(g/L) = E.C. x\, 10$$

For 40% E.C.

$$(g/L) = 40\, x\, 10 = 400 g/L$$

- To convert g/L to mg/m

1g = 1000mg

1L = 1000ml

$$400\text{g/L} = (400 \text{ x } 1000) / 1000 = 400\text{mg/ml}$$

∴ there is 400mg of chlorpyrifos in 1ml of the pesticide.

2.4 Determination of Reduced Glutathione Concentration

Concentration of reduced glutathione was estimated using the method proposed by Moron *et al*. [16]. 0.5g of sample was homogenized in 2.5 ml of 5% TCA to obtain a 20% serum. 125 µl of 25% TCA was added to 0.5 ml of serum to precipitate the protein and the precipitated protein centrifuged at 1000rpm for 10 mins. The serum was cooled on ice and 0.1 ml of the supernatant was taken for the estimation. The supernatant was made up to 1 ml with 0.2M sodium phosphate buffer (pH 8.0). 2.0 ml of freshly prepared DTNB solution was added to the tubes and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 mins. A standard curve of GSH was prepared using concentrations ranging from 2-10 nmoles of GSH in an electronic calculator set to the linear regression mode and the values of the samples were read off it. The values are expressed as nmoles of GSH /g serum.

2.5 Determination of Malondialdehyde Concentration

The level of Lipid peroxides was estimated by Thiobarbituric acid reaction method described by Ohkawa *et al*., [17]. To 0.2 ml of test sample, 0.2 ml of SDS, 1.5ml of acetic acid and 15 ml of TBA were added. The mixture was made up to 4 ml with water and then heated in a water bath 95^{o} C for 60 minutes. After cooling, 1ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was taken and its absorbance was read at 532 nm. The level of lipid peroxides was expressed an nmoles of MDA released/g wet tissue.

2.6 Assay for Catalase Activity

Catalase activity was measured by the method of Aebi. [18]. 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate butter (pH 7.0). Reaction was started by the addition of l.0ml of freshly prepared 50 mM H_20_2. The rate of decomposition of H_2O_2 Was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein. A unit is defined as the velocity constant per second. The reaction occurs immediately after the addition of H_20_2. Solutions are mixed well and the first absorbance (A1) is read after 15 seconds (t1) and the second absorbance (A2) after 30 seconds
(t2). The absorbance is read at wave length 240 nm.

2.7 Assay for Glutathione Peroxidase activity

About 3-ml cuvette containing 2.0 ml of phosphate buffer(75mmol/L, PH 7.0) , 50ul of (60mmol/L) glutathione reductase solution, 50ul of (0.12mol/L) NaN_3, 0.I ml of (0.15mmol/L) Na^{2+} EDTA , 100uL of (3.0 mmol/L) NADPH, and 100ul of tissue supernatant was added. Water was added to make a total volume of 2.9ml. The reaction was started by the addition of 100uL of (7.5 mmol/L) H_20_2, and the

Conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-minutes interval for 5 min. Enzyme activity of GPx was expressed in terms of mg of proteins [19].

2.8 Determination of Cholesterol Concentration

Cholesterol level was determined using the method of Allian *et al*., [20]. Reagents were prepared according to instructions on the vial label, Test tubes were labeled: blank, standard, control, sample. 1.0ml of the reagent was added to all tubes and pre warmed at 37°C for at least two minutes. 0.01 ml of sample was added to respective tubes, mix and returned to 37°C. All tubes were incubated at 37°C for ten minutes. The reagent blank was used to zero the spectrophotometer at 520 nm and then absorbance read and recorded for all tubes.

2.9 Determination of Testosterone Concentration

Testosterone level concentration was determined using the method of Ekins, [21]. The desired number of coated wells was secured in a holder, 25µl of the standard, specimen and control was dispensed into appropriate well. 50µl of rabbit anti-testosterone reagent was dispensed to each well and mixed thoroughly for 30 minutes. A 100µl of testosterone-HRP conjugate reagent was dispensed into each well and incubated at 37°C for 60 minutes. The microwells were rinsed and flicked 5 times with washing buffer (1x). 100µl of TMB substrate was dispensed to each well and mixed gently for 10 seconds and then incubated at room temperature (18-22°C) for 20 minutes. The reaction was stopped by adding 100µl of stop solution to each well and gently mixed for 30 seconds for complete color change. The absorbance was read at 450 nm with a microtiter well reader within 15 minutes.

2.10 Sperm Analysis

Sperm analysis carried out using the method of Ochei and Kolhatkar [22]. The Neubauer chamber having a grid containing 1-5 large squares was used. The central square is subdivided into 25 smaller squares of which the 4 corner squares are designated 5a, 5b, 5c, 5d and the central small square as 5e. The depth of the chamber in 0.1 mm, so the volume of fluid held between cover slip and chamber is 0.1cu.mm, and the volume in 5a, 5b, 5c, 5d and 5e is 0.02 cu.mm

To calculate the number of spermatozoa per ml counted in the chamber, a multiplication factor is used. The multiplication factor for square 5 is 10,000, for all large squares 1-5, the factor is 2000: for the smaller squares 5a, 5b, 5c, 5d and 5e, the multiplication factor is 50,000.

2.11 Calculation

No of sperm cells counted in 5a, 5b, 5c, 5d and 5e = n

Multiplication factor = 50,000

Dilution factor = 20

Sperm count per ml = n x 50,000 x 20

= n x 10^6

Total sperm count = n x 10^6 x volume of semen

2.12 Statistical Analysis

IBM SPSS software version 23 was used to carry out the statistical analysis. A one way analysis of variance (ANOVA) was carried out at $\alpha = 0.05$, and Duncan's multiple range test was used to show the source of the observed differences.

3.0 RESULTS AND DISCUSION

3.1 Oxidative stress

3.1.1 Oral oxidative stress

Table 1 shows the effects of oral exposure to Chorview on the oxidative stress markers and antioxidant enzymes of wistar rats. The results suggest that the pesticide induced appreciable oxidative imbalance in the system. In oral exposure, Catalase activity of group 3 (6.82±1.10 U/mg) significantly ($p<0.05$) increased when compared to group 4 (4.68±0.71 U/mg), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Glutathione peroxidase activity was non-significantly ($p>0.05$) decreased across the treatment groups when compared to group 4 (control). There was a significant ($p<0.05$) increase in the concentration of Malondialdehyde of group 3 (1.59±0.43 mg/dL) when compared to group 4 (1.12±0.10 mg/dL), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Also the concentration of Glutathione showed a non-significant ($p>0.05$) decrease across the treatment groups when compared to group 4 (control).

3.1.2 Inhalation oxidative stress

Table 2 shows the effects of Inhalation exposure to Chorview on the oxidative stress markers and antioxidant enzymes of wistar rats. The results that the inhalation exposure showed similar trend to oral in the oxidative stress markers in wistar rats exposed to Chlorview. There was a significant ($p<0.05$) increase in the activity of Catalase of group 3 (195.73±47.09 U/mg) when compared to group 4 (112.04±9.57 U/mg), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Glutathione peroxidase activity of group 2 (151.56±1.25 U/mg) and group 3 (130.61±2.39 U/mg) significantly ($p<0.05$) decreased when compared to group 4 (334.52±16.14 U/mg), however, there is a non-significant ($p>0.05$) increase in group 1 when compared to group 4. There was a significant ($p<0.05$) increase in the concentration of Malondialdehyde of group 3 (1.33±0.110 mg/dL) when compared to group 4 (0.61±0.83 mg/dL), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Also, the concentration of Glutathione showed a significant ($p<0.05$) decrease across the group 1 (146.39±0.50 mg/dL), group 2 (142.14±0.50 mg/dL) and group 3(140.02±1.50 mg/dL) when compared to group 4 (149.94±1.50 mg/dL).

The increase in catalase activity observed in this study can be as a result of the increase in the superoxide radicals generated by the pesticide or the as a result of the direct binding of the heavy metal present in the pesticide. These heavy metals can also disrupt the enzyme activity by binding to the sulphydryl group (SH) inhibiting its activity [25]. The reactive oxygen species (ROS) may be produced as the result of the metabolism of organophosphates by cytochrome P450s. The P450s are monooxygenases and catalyze oxidation by addition of one atom of molecular oxygen into a substrate (organophosphate) by an electron transport pathway. Organophosohates change normal antioxidant homeostasis resulting in antioxidant depletion, if the requirement of continuous antioxidants is not maintained [6]. These findings observed with regards to oxidative stress in both inhalation and oral exposure showed substantial oxidative imbalance. This unearthing were seen in the decrease observed in the stress marker GSH and GPx and also supported by the report of Uzun and Kalender [9] who reported a decreased GPx activity in rats treated with chlorpyrifos. Reduced glutathione GSH is utilized by GPx and it's converted to oxidized glutathione form (GSSG). Decreased GSH regeneration and conjugation reactions also reduce the level of GSH [6]. The GSH which plays a pivotal role in biotransformation and detoxification of chemicals and drugs in the system is an important antioxidant. The antioxidant strength is seen in its sulfhudryl group (SH) which can be easily used up by the cells during stress and become oxidized. The little non-significant decrease observed in GSH can be attributed to the fact that the increase in the generated reactive oxygen species may have not totally over-whelmed the antioxidant defense system at the time. The GSH counters this effect by donating its hydrogen in the SH group to the radicals and gets converted to its oxidized form GSSG. This is facilitated by the antioxidant enzyme glutathione peroxidase [23].

Many xenobiotics cause oxidative damage through the process of lipid peroxidation [24]. MDA increases observed during this investigation represents an indicator of oxidative damage or cellular toxicity due to increases in generation of hydroxyl and hydroperoxyl radicals that mainly attack lipids (poly unsaturated lipids) which are also major constituents of the cell membrane. The damages leads to the increased production of MDA. [6, 9].

3.2 Reproductive Marker Toxicity

3.2.1 Oral exposure

Table 3 shows the effects of oral exposure to Chlorview on the reproductive toxicity markers of wistar rats. The results from the reproductive toxicity studies revealed that that oral exposure to Chlorview led to a significant ($p<0.05$) increase in the Cholesterol concentration of group 3 (224.65±6.57 mg/dl) when compared to group 4 (151.96±32.01 mg/dl), however, there was a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4(control).The testosterone concentration of group 3 (1.30±0.12 ng/ml) significantly ($p<0.05$) decreased when compared to group 4 (3.76±1.09 ng/mg). Although there was a non-significant ($p>0.05$) decrease in Testosterone concentration of group 1 (3.8mg/kg b.w.) and group 2 (6.2mg/kg b.w.) when compared to group 4 (Control). There was a significant ($p<0.05$) decrease in the Sperm count of group 1 (70.00±2.00 $X10^6$/mL), group 2 (66.00±1.00 $X10^6$/mL) and group 3 (60.67±5.77 $X10^6$/mL) when compared to group 4 (82.00±6.00 $X10^6$/mL).

3.2.2 Inhalation reproductive toxicity

Table 4 shows the effects of inhalation exposure to Chlorview on the reproductive toxicity markers of wistar rats. The results show that inhalation exposure to Chlorview led to disruption of vital markers of reproductive toxicity. There was a significant ($p<0.05$) increase in the cholesterol concentration of group 1 (196.10±21.80 mg/dl), group 2 (237.07±22.90 mg/dl) and group 3 (300.29±0.28 mg/dl) when compared to group 4 (86.28±5.68 mg/dl). Testosterone concentration showed a significant ($p<0.05$) decrease in group 1 (10.18±0.23 ng/mL), group 2 (9.73±0.36 ng/mL) and group 3 (9.20±0.17 ng/mL) when compared to group 4 (10.96±0.06 ng/mL). The Sperm count of group 1 (71.50±2.12 $X10^6$/mL), group 2 (63.50±0.71 $X10^6$/mL) and group 3 (53.00±1.41 $X10^6$/mL) was significantly ($p<0.05$) decreased when compared to group 4 (86.50±0.71 $X10^6$/mL).

The sperm analysis, testosterone and cholesterol levels were the reproductive markers or parameters investigated for the impact of chlorview via oral and inhalation exposure. The findings observed during this investigation showed an observable trend of increasing toxicity with increasing dose of the pesticide. There was a decrease in testosterone concentration and sperm levels observed in both forms of exposure to chlorview. There was also an increased level of cholesterol which impairs testicular function. The decrease observed is consistent with the published report that administration of chlorpyrifos to male mice by oral gavage resulted in significant adverse effects that included cholinergic signs, decreased acetylcholinesterase and testosterone levels, and histological changes in testis and epididymis in the 15 and 25 mg/kg-d treated groups. Testicular spermatid and epididymal sperm counts indicated that spermatogenesis was partially arrested at the middle and high dose groups (15 and 25 mg/kg-d) [43]. Li et al. [26] reported to have significantly decreased total sperm count, serum testosterone and gonadotropin levels and the activity of enzymes involved in spermatogenesis, as well as lead to oxidative damage in the testis [26]. When chronically exposed to a low dose of CPF, there was a disturbance in the secretion of endocrine hormones [27] that led to an obese [28] or diabetic [29] phenotype. This was also observed in this study, with excess fat deposition on the treatment groups, observed in the present study. This increased fat deposition may have also contributed to the alterations observed in the testosterone concentration as there is a negative correlation between plasm cholesterol levels and plasma testosterone levels [42]. The mechanism of CPF-induced reproductive toxicity was mainly attributed to the decreased content of testosterone [30, 31, 32 and 33], or oxidative damage caused by CPF to Leydig cells [30, 34] where testosterone is synthesized. The metabolites of CPF, such as the active sulfur atom, inhibit the activity of cytochrome P450 3A4 (CYP3A4) [35] an enzyme involved in testosterone metabolism [32], which leads to the decrease in testosterone. The values of the sperm count in the present study, there was a significant decrease, the maybe due to short duration of the present study. Sai et al. [43] also reported a decrease in the sperm count. The decrease in the sperm count can be attributed to increase in generation of reactive oxygen species by chlorview. The generation of these radicals occurs during metabolism by the cytochromes in the mitochondria. The spermatozoa is also made up of poly unsaturated fatty acid which is also the main target of these generated radicals. The center piece of the spermatozoa contains a lot of mitochondria which also generates peroxides, these peroxides are the most potent radicals which can combine with nitric oxide to form peroxynitrites and a continuous reactions that leads to the activation of apoptosis or necrosis of sperm cells. The peak effect of the exposure may be seen more in the next cycle of

spermatogenesis. A study that investigated chronic exposure to chlorpyrifos resulted in a significant decrease in sperm count and motility and an increase in the ratio of immotile and morphologically abnormal sperm in male rats [37, 38, 39, and 40].

Conclusion

This research suggests that exposure to Chlorview® (Chlorpyrifos 40% EC) poses a reasonable reproductive risk both through oral and inhalation routes of exposure affecting many biochemical processes. Exposure to chlorpyrifos induced an appreciable oxidative stress on the test animals. Hence, Induction of oxidative stress represents one of the major mechanisms of chlorpyrifos toxicity.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table 1: The effect of Oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on antioxidant biomarkers

GROUPS	GSH (mg/dL)	MDA (mg/dL)	CAT (U/mg)	GPx (U/mg)
Group 1	2.96±0.79^{y}	1.22±0.14^{y}	5.29±0.50^{y}	29.97±2.05^{y}
Group 2	2.94±0.41^{y}	1.26±0.04^{y}	5.50±0.80^{y}	28.93±0.57^{y}
Group 3	2.71±0.57^{y}	1.59±0.43^{z}	6.82±1.10^{z}	28.58±1.16^{y}
Group 4	3.09±0.19^{y}	1.12±0.10^{y}	4.68±0.71^{y}	30.48±3.61^{y}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 3

Table 2: The effect of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on antioxidant biomarkers

GROUPS	GSH (mg/dL)	MDA (mg/dL)	CAT (U/mg Protein)	GPx (U/mg Protein)
Group 1	146.39 ± 0.50^{y}	0.64 ± 0.001^{x}	138.81 ± 19.15^{x}	315.39 ± 6.99^{x}
Group 2	142.14 ± 0.50^{z}	0.67 ± 0.010^{x}	155.13 ± 3.39^{x}	151.56 ± 1.25^{y}
Group 3	140.02 ± 1.50^{z}	1.33 ± 0.110^{y}	195.73 ± 47.09^{y}	130.61 ± 2.39^{y}
Group 4	149.94 ± 1.50^{x}	0.61 ± 0.83^{x}	112.04 ± 9.57^{x}	334.52 ± 16.14^{x}

Means with the same superscript across the same groups are non-significantly (p>0.05) different. n = 2

Table 3: The effect of Oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on reproductive toxicity markers

GROUPS	CHOL (mg/dl)	TESTOS (ng/ml)	SPERM ($X10^6$/mL)
Group 1	165.87 ± 17.78^{p}	2.87 ± 0.29^{p}	70.00 ± 2.00^{q}
Group 2	185.59 ± 13.92^{p}	2.78 ± 0.27^{p}	$66.00\pm1.00^{p,q}$
Group 3	224.65 ± 6.57^{q}	1.30 ± 0.12^{q}	60.67 ± 5.77^{p}
Group 4	151.96 ± 32.01^{p}	3.76 ± 1.09^{p}	82.00 ± 6.00^{r}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 3

Table 4: The effect of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on reproductive toxicity markers

GROUPS	CHOL (mg/dl)	TESTOS (ng/mL)	SPERM ($X10^6$/mL)
Group 1	196.10 ± 21.80^{b}	10.18 ± 0.23^{b}	71.50 ± 2.12^{c}
Group 2	237.07 ± 22.90^{b}	$9.73\pm0.36^{a,b}$	63.50 ± 0.71^{b}
Group 3	300.29 ± 0.28^{c}	9.20 ± 0.17^{a}	53.00 ± 1.41^{a}
Group 4	86.28 ± 5.68^{a}	10.96 ± 0.06^{c}	86.50 ± 0.71^{d}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 2

EQUATIONS:

The median lethal dose was determined using the following formula

Equation (1). $LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = Highest dose that gave no mortality

D_{100} = lowest dose that gave mortality

Converting Emulsifiable Concentration (E.C.) to mg/ml
Equation (2). $(g/L) \div 10 = E.C.$
$(g/L) = E.C.x\ 10$

For 40% E.C.
$(g/L) = 40\,x\ 10 = 400g/L$

- To convert g/L to mg/m
  1g = 1000mg
  1L = 1000ml
  $400\text{g/L} = (400\text{ x }1000)\ /1000 = 400\text{mg/ml}$

∴ there is 400mg of chlorpyrifos in 1ml of the pesticide.

Calculation of MDA
Equation (3)

$$\text{The concentration of MDA} = \frac{\text{Absorbance at 532 nm x D}}{\text{L x }\varepsilon}$$

Where,
L= light path (1cm).
ε = extinction coefficient 1.56 x 105 M-1.Cm-1.
D: dilution factor = Total Vol (10ml)/ Vol of the sample (0.2ml)

Calculation of catalase activity
Equation (4)

$$\text{K} = \frac{\text{Vt}}{\text{Vs}}\text{x}\frac{2.3}{\text{t}}\text{ x Log}\frac{\text{A1}}{\text{A2}}\text{ x 60}$$

Where,
K= Rate constant of the reaction.
t= (t2- t1) = 15 seconds.
Al= absorbance after 15 seconds.
A2 = absorbance after 30 seconds.
Vt = total volume (3 ml).
Vs=volume of the sample (0.1ml).

Calculation of glutathione peroxidase

Equation (5)

$$\text{Enzyme activity } (\text{M/min/ml}) = \text{A340/min x } \frac{\text{Vt}}{\text{e}} \text{ x d x Vs}$$

Where,
e= 6.22 x 10^6 $M^{-1}cm^{-1}$
d = 1cm
Vt = Total volume (3.0)
Vs = Sample volume (0.1 ml)