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The Pathophysiology and Neurobehavioral Effects of Chlorfenapyr Insecticide in Lactating Female Sprague Dawley Rats and in HepG2 Cell Line

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Research Article

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Abstract

Chlorfenapyr (CFP) is good candidate insecticide for control of vectors blood borne diseases like malaria. As little information about CFP toxicity and possibility of its residue's presence in food stuffs, milk and environment, we explore the postnatal toxic effects of CFP in female Sprague dawley rats and its pups. CFP was given orally at doses of 0,54 and 108 mg/kg to female albino rats immediately at first day after delivery till 21 days of lactation. All dams and its pups were weighted, euthanized and blood was separated for serum separation and tissues were preserved either at 4c as tissue homogenate for measurements of oxidant/antioxidants levels or in buffered formalin for histopathological examination. The highest dose of CFP induced hepatorenal toxicity in dams and its pups with evidence of increase liver enzymes and creatinine level when compared to control groups. Also, CFP displayed histopathological changes in liver, kidney, brain and spleen tissues of dams as well as rats' pups after 21 days of treatment. The toxic effects resulted from secretion of CFP in milk and increased the free radicals' production and oxidants like MDA in tissues of rats' pups. Also, CFP had a cytotoxic effect on HepG2 cells indicated by induction of oxidative stress and lethality to cell line. Taken collectively, chlorfenapyr is a good candidate insecticide in vector control but had a cytotoxic effect in female albino rats, its pups, and in HepG2 cells.

Keywords: CFP; Pro-Insecticide; Cytotoxic Effects; Oxidative Stress; Albino Rats; HepG2 Cells

Abbreviations: CFP: Chlorfenapyr; WP: Wettable Powder; PBS: Phosphate Puffer; RBC: Red Blood Cell Count; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCHC: Hemoglobin Concentration; MCH: Mean Corpuscular Hemoglobin; GHS: Glutathione; SOD: Superoxide Dismutase.

Introduction

Chlorfenapyr is a pro-insecticide used since 1995 for control of agriculture pest. Human toxicity from CFP was little as only few cases were reported with nausea, vomiting, fever,

rhabdomyolysis and nervous system toxic manifestationafter ingestion of current insecticide [1-5].

CFP is a member of a new class of insecticide, of pyrroles group (chemical name: 4-bromo-2-(4-chlorophenyl)-1-(ethoxymethyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile; trade name: Pylon miticide-insecticide) [6]. The uses of the CFP were removal of mites, caterpillar pests, thrips, and fungus gnats by foliar spray on ornamental crops in greenhouses and its mode of action was through inhibition of oxidative phosphorylation in the mitochondria, resulting in reduction of ATP production, cellular death, and ultimately, death of the organism [7].

The CFP is a light tan or light-yellow solid powder. While CFP toxicity has not yet been studied in humans and animals resulted that classification of CFP toxicity as category III chemical. Recently, In few studies recorded that CFP induced developmental and maternal toxicity in female albino rats [7,8].

CFP sources or residues were recorded in environment, in food products, water, animal derived foods and in tissue of treated rats, analyzed by different methods of chromatography like GLC and HPLC. The rationale of this study to explore the Pathophysiology effects of chlorfenapyr in female albino rats and its pups [9-15].

Materials and Methods

Animals

Sprague Dawley rats obtained from Experimental Unit in the Faculty of Pharmacy, Mansoura University; Animals weighed about 250 ±10 gm and were obviously healthy then grouped and housed in plastic cages with soft wood shavings as a bedding material then adapted for about 2 weeks and maintained on a balanced ration before the experiment.

Tested Chemicals

CFP is a light green wettable powder (WP) with slight chlorine odor and kindly obtained from Central Agricultural Pesticide Laboratory, Ad Doki, Giza, Giza Governorate after HPLC analysis to confirm the percentage. HepG2 (85011430, sigma, USA) was gifted to us was provided to us from faculty of medicine, Mansoura university, Egypt

Experimental Design

Eighteen (18) pregnant female *Sprague Dawley* rats were separated into three groups with six females for each. CFP was given orally and daily at doses of 0,54 and 108 mg/kg (equivalent to 1/20 and 1/10 of LD50) to female albino rats immediately at first day after delivery till 21 days of lactation. The neonates litter size recorded and both dams and neonates weighed daily and kept under observation until weaning.

Clinical Signs and Behavioral Test

The treated dams and neonates observed daily throughout the experimental period for any abnormal behavior, findings or alteration. All rats were trained for behavioral assessment of gait movement to its cage with marking of its toe with ink and then all rats were tested after end of treatment and before sacrifice.

Maternal And Neonatal Body Weight Gain

The initial body weight determined from the day of parturition for both dams and neonates and then throughout the experiment the body weight calculated before each administration. The body weight gain % determined according to the following formula [16].

Sample Collection

At day 21 postpartum, dams and weaned rats euthanized with thiopental Na (40 mg/Kg i.p). For hematological examinations fresh blood sample collected from the heart with a sterile syringe and then collected in centrifuge tubes contain K3EDTA as anticoagulant.

The fresh blood collected in gel tube (not contain anticoagulant) for serum separation in centrifuge at 3000 rpm for 15 minutes then 20. Also, the liver tissue sample removed from dams and weaned rats and washed with saline solution then one gram of tissues homogenized in falcon tube with 9 ml ice cold phosphate puffer (PBS) PH7.4 through homogenizer then centrifuged at 3000 rpm for about 15 minutes at 4, the supernatant separated, collected and 20 in Eppendorf tubes [17].

The liver, kidney, spleen and brain specimen from both dams and weaned rats collected and kept in 10% neutral buffered formalin for histopathlogical processing, and analysis.

Hematological Examination

Blood sample analysis carried out by Mindray BC-1800 hematological analyzer whereas hemoglobin (Hb), red blood cell count (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) evaluated besides total and differential white blood cells were also measured [18].

Biochemical Analysis

Gamma glutamyl transferase activity, Alanine aminotransferase activity, Glucose, serum total protein, albumin, creatinine, urea and cholesterol level were measured in serum of treated and control group.

Antioxidant and Oxidative Stress Biochemical Analysis

Liver homogenate of all treated groups and pups were analyzed for GSH, GST, SOD, CAT and MDA levels.

Histopathologic Examination

Specimens from liver, kidney, spleen and brain were fixed in 10% formalin and 5µ thickness sections of specimens prepared then stained with hematoxylin and eosin (H&E) and examined microscopically.

Cytotoxicity of CFP On HepG2 Cells

The stock solution of CFP (100 mmol/L) was prepared in ethanol and stored at 4°C. Working solutions were prepared by dissolving the stock solution in the culture medium. We exposed HepG2 cells to different concentrations (0, 10,20, 40 ng/mL) of CFP for 24 hours to determine its toxic effects. The HepG2 cells were cultured according methods described earlier [19].

Cell Viability and Oxidative Stress Tests

The viability of cells was detected by quantification of formed formazan salt. In this regard, HepG2 cells (2 × 10^5 cells) were seeded in 96-well plates. Later 24 h, the medium solution was changed with other medium containing different concentrations (0, 10, 20,40 ng/mL) of chlrfenapyr and solvent (ethanol) were added for 24. MTT (50 µg/mL) was added to each well. After 4 h incubation at 37°C, the later solution was discarded and formed formazan crystals was dissolved in DMSO (100 µL). The color developed was measured at 570 nm using a multiplate reader (Synergy HT, Bio-Tek, Winooski, Vermont).

Additionally, the cell extract was centrifuged (10000 g, 10 min, 4ºC) and supernatant was used for oxidative stress assays such as glutathione (GSH), superoxide dismutase (SOD), and MDA.

Statistical Analysis

Statistical analyses were carried out using SPSS software program (13, USA). Homogeneity of the groups was tested by Kruskal Walis test. One-way ANOVA was used to define significance between groups at $p< 0.05$ (20).

Results

Postnatal Maternal and Pups' Body Weight Gain% upon Exposure to CFP

A relative significant decrease in the body weight gain in all treated group, lactating dams throughout the study in respect to the control group especially groups of 1/10 LD_{50} of CFP results illustrated in Table 1. Additionally, there was a significant decrease in the body weight gain of pups in all treated groups in respect to the control group (Tables 1 & 2). Additionally, it was noticed that CFP had toxic effect on neurons indicated by abnormal movement especially at highest dose when compared to control group (data not shown).

Group	Mean Initial body weight	Mean Final body weight	Body weight gain %
Group 1 (Control)	158.67±2.33	206.67±0.88a	30.25
Group 2	156.67±2.03	180.23±1.53e	15.04
Group 3	158.21±0.58	189.67±1.76c	19.88

Table 1: The initial and final body weight mean and body weight gain % in lactating female rats administered orally different doses of CFP postnatally from 0^{th} to 21^{th} days postpartum daily in comparison to the control group (mean ± SE).

Group	Mean Initial body weight	Mean Final body weight	Body weight gain %
Group 1 (Control)	5.47±0.12	34.83±0.73a	536.75
Group 2	5.37±0.29	24.13±0.58c	349.35
Group 3	5.87±0.12	30.67±0.88b	422.49

Table 2: The initial and final body weight mean and body weight gain % in pups of maternally treated dams orally with different doses of CFP postnatally from 0th to 21th days postpartum daily in comparison to the control group (mean ± SE).

Postnatal Maternal and Pups' Biochemical Analysis

Metabolic, Liver and Kidney Functions' Biomarkers in Lactating Dams and Pups

a) **Metabolic, Liver and Kidney Functions' Biomarkers in Lactating Dams**: In lactating dams, the results showed that a significant decrease in glucose, cholesterol and total protein in all treated groups in respect to the control group especially group of 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. Also, the group of 1/10 LD_{50}, 1/20 LD_{50} of CFP equivalent to 108 mg/kg Bw. and 54 mg/kg Bw respectively showed a significant decrease of albumin and globulin levels in comparison to control group (Table 3).

In table 3, there was a significant increase of all biomarkers (ALT, AST and GGT) in most of treated groups in comparison to the control group especially higher doses groups (1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw.). In addition, there was a significant increase in blood urea nitrogen in most of treated groups in comparison to the control group especially groups 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. On the other hand, only in groups 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. showed a significant increase creatinine level.

	ALT (U/I)	AST (U/I)	GGT (U/I)	Glucose (mg/dl)	Cholesterol (mg/dl)	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Control	24.06±1.97c	34.67±1.7d	16.63±2.49c	152.47±2.66a	93.9±1.84a	8.53±0.52a	5.77±0.45a	2.77±0.28a	39.33±0.85c	0.56±0.02b
Group 1	44.14±2.04a	64.4±2.46b	37.01±2.86a	92.77±2.83c	72.8±3.59c	4.93±0.09c	3.32±0.63cd	1.61±0.31b	67.97±4.36a	1.33±0.23a
Group 2	34.5±2.47b	40.1±1.14cd	25.23±3.19b	143.03±0.9b	74.13±2.58c	7.07±0.21b	4.32±0.78bc	2.73±0.23a	54.29±3.32b	0.72±0.04b

Table 3: The postnatal maternal biochemical metabolic, liver and kidney biomarkers changes after administration of different doses of CFP orally from 0th - 21th days postpartum daily in comparison to the control group.

b) **Liver and Kidney Functions' Biomarkers in Pups**: Liver and kidney functions estimated in pups of maternally treated dams for the same groups as illustrated in table 4.

In table four, there was a significant increase in ALT and GGT in all maternally treated groups in comparison to the control group especially maternally treated groups of 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. Also, there was a significant increase in blood urea nitrogen and creatinine in all treated groups in comparison to the control group especially group of 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw.

	ALT (U/I)	GGT (U/I)	Urea (mg/dl)	Creatinine (mg/dl)
Control	35.27±1.22c	24.30±0.96c	47.33±1.65c	0.69±0.02c
Group 1	60.74±3.73a	53.97±2.92a	77.62±2.12a	1.82±0.08a
Group 2	44.43±2.47b	33.34±1.44b	59.02±1.56b	0.86±0.04b

Table 4: The biochemical liver and kidney biomarkers changes in maternally treated pups with different doses of CFP orally from 0th - 21th days postpartum daily in comparison to the control group.
A, b, c, d: Different letters are significantly different between groups at P≤0.05.

Oxidative Stress Biomarkers in Lactating Dams and Pups

a) Oxidative Stress Biomarkers in Lactating Dams: The lactating dams shown a significant decrease in GSH, GST and CAT in most of treated groups when compared with the control group especially higher doses groups (1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. The treated groups (1/10 LD_{50} CFP equivalent to 108 mg/kg Bw. showed significant decrease in level of SOD. All treated groups showed a significant increase MDA oxidant in respect to the control group especially groups of 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw (Tables 5 & 6).

Groups	GSH mg/g. tissue	GST U/g. tissue	SOD U/g. tissue	CAT U/g. tissue	MDA nmol/g. tissue
Group 1 (Control)	28.69±0.95a	10.78±0.81a	27.42±1.04a	17.96±0.94a	30.97±0.93c
Group 2	15.91±1.42b	6.05±0.47c	16.08±1.53b	10.94±0.71c	73.96±1.90a
Group 3	19.67±1.29b	7.69±0.44b	24.82±1.39a	13.54±0.76b	61.14±2.72b

Table 5: The biochemical oxidative stress biomarkers changes in lactating dams after administration of different doses of CFP group orally from 0th - 21th days postpartum daily in comparison to control group.

b) Oxidative Stress Biomarkers in Pups: The pups of maternally treated dams displayed a significant decrease in GST, GSH, SOD and CAT in most of treated groups when compared with the control group especially higher doses groups (1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. On the other hand, all maternally treated groups in respect to the control group especially groups of 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw. showed a significant increase in MDA level when compared to control group.

	GSH mg/g. tissue	GST U/g. tissue	SOD U/g. tissue	CAT U/g. tissue	MDA nmol/g. tissue
Group 1 (Control)	18.94±1.10a	8.04±0.44a	14.63±1.17a	9.37±0.51a	49.45±0.96c
Group 2	10.11±0.39c	4.37±0.45b	8.74±1.13b	5.49±0.29b	87.38±1.03a
Group 3	14.33±1.06b	5.53±0.29b	13.73±0.64a	8.84±0.71a	69.29±0.65b

Table 6: The biochemical oxidative stress biomarkers changes in pups of maternally treated dams with different doses of CFP orally from 0th - 21th days postpartum daily in comparison to control group.
A, b, c, d: Different letters are significantly different between groups at P≤0.05.

Hematological Finding in Lactating Dams

There was a significant decrease in the Hb content in the groups received 1/10 LD_{50} of CFP equivalent to 108 mg/kg Bw in comparison to the control group. Also, total leukocytic count showed a significant increase in the groups treated with 1/10 LD50 of CFP equivalent to 108 mg/kg Bw. in comparison to the control group. On the other hand, there was no significance change in all treated groups in PCV, MCV, MCH and MCHC levels in respect to the control values (Table 7).

	RBCs (million cells/uL)	Hb (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	TOTAL WBCs (1000 cells/uL)
Group 1 (Control)	8.42±0.02a	14.76±0.05a	45.75±0.05a	54.34±0.20	17.53±0.10	32.26±0.10	7.67±0.10b
Group 2	8.22±0.15a	14.22±0.12b	44.94±0.03a	54.68±0.97	17.32±0.22	31.65±0.25	8.82±0.07a
Group 3	8.42±0.14a	14.56±0.10 ab	45.76±0.10a	54.36±0.98	17.30±0.16	31.83±0.28	7.63±0.17b

Table 7: The hematological finding in lactating dams after administration of different doses of CFP orally from 0th - 21th days postpartum daily in comparison to control group.

Histopathological Findings

The histopathological changes were observed in lactating dams and pups of maternally treated dams with different doses of CFP (108 mg/kg Bw. and 54 mg/kg Bw.) in comparison to the control group and the results showed that there were severe pathological changes especially at the higher doses groups.

Liver: The lactating dams treated with different doses of CFP displayed degenerative changes and intralobular histiocytic infiltration with intralobular fibroblastic proliferation in the hepatic tissue. While the pups of treated dams with different doses of CFP shown a focal histiocytic and lymphocytic infiltration besides congestion of portal vein and margination of leukocytes in a dose dependent manner in respect to the control group, results illustrated (Figure 1).

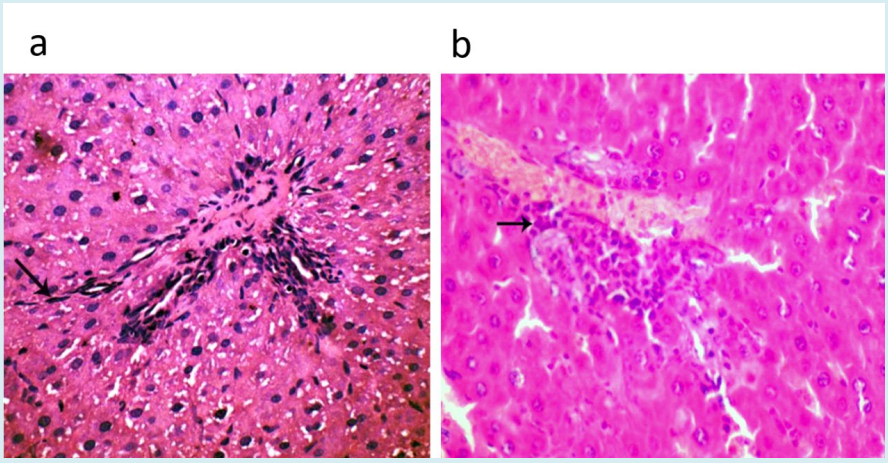

Figure 1: The depicted figure shown **(a)** Liver of lactating dams treated with 1/10 LD_{50} of CFP orally from 0th - 21th days postpartum showing intralobular fibroblastic and histiocytic infiltration in the hepatic tissue (arrow) in (HE, 400x). (HE, 400x) **(b)** Liver of suckling pups of treated dams with 1/10 LD_{50} of CFP orally from 0th - 21th days postpartum showing mild lymphocytic infiltration in hepatic tissue (arrow) (HE, 400x).

Brain: The lactating dams treated with different doses of CFP there was neuronal necrosis, neuronophagia and astrocytosis in the brain tissue with degenerative changes of purkinje cells in cerebellum and the pathological changes were more sever in respect to the control group, results illustrated in Figures 2a & 2b.

The pups of treated dams with different doses of CFP there was focal edema in the neutrophils with focal hemorrhagic areas in the brain tissue in a dose dependent manner in respect to the control group, results illustrated in Figure 2c.

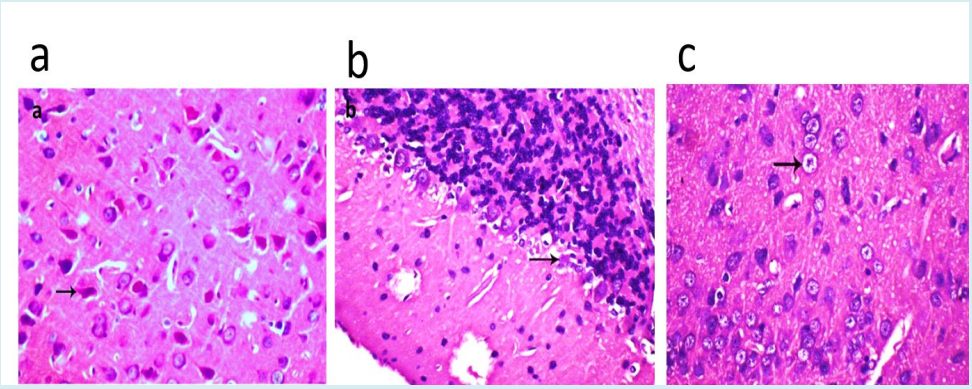

Figure 2: The depicted figure shown **(a)** Brain of lactating dams treated with 1/10 LD_{50} of CFP orally from 0th - 21th days postpartum showing central chromatolysis and neuronal necrosis in the brain tissue (arrow) (HE, 400x) **(b)** Brain of lactating dams treated with 1/20 LD_{50} of CFP orally from 0th - 21th days postpartum showing degenerative changes of purkinje in cerebellum (arrow) (HE, 400x) **(c)** Brain of suckling pups of treated dams with 1/10 LD_{50} of CFP orally from 0th - 21th days postpartum showing cytotoxic edema of neurons (arrow) in (HE, 400x).

Kidney: The lactating dams treated with different doses of CFP displayed fibrous tissue proliferation of renal glomeruli with degenerative changes in renal tubular epithelium results illustrated in Figure 3a. while the pups of treated dams with different doses of CFP shown a degeneration in the renal tubular epithelium and interstitial lymphocytic infiltration with congestion of the renal glomeruli in a dose dependent manner in respect to the control group, results illustrated in Figure 3b.

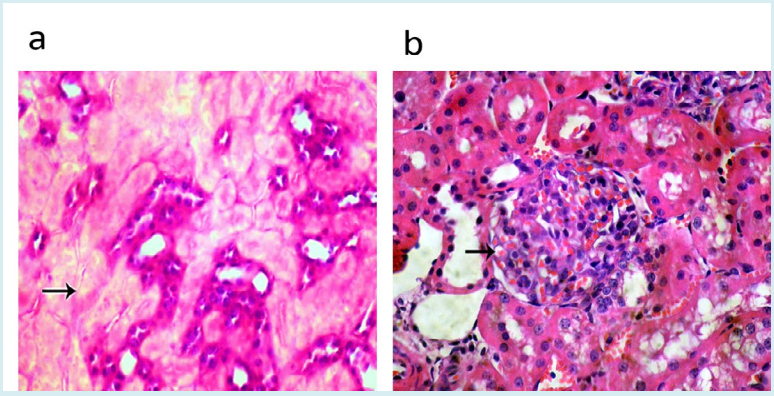

Figure 3: The depicted figure shown a) kidney of lactating dams treated with 1/10 LD_{50} of CFP orally from 0^{th} - 21^{th} days postpartum showing degenerative changes and necrosis of renal tubular epithelium (HE, 400x). (b) Kidney of suckling pups of treated dams with 1/10 LD_{50} of CFP orally from 0^{th} - 21^{st} days postpartum showing congestion of renal glomeruli (arrow) (HE, 400x).

Spleen: For lactating dams treated with different doses of CFP there was marked lymphoid depletion in the splenic tissue and the pathological changes were more sever in the dose of highest dose of CFP in respect to the control group, results illustrated in Figure 4a.

The pups of treated dams with different doses CFP displayed a severe lymphoid depletion with congestion of the splenic sinusoids in a dose dependent manner in respect to the control group, results illustrated in Figure 4b.

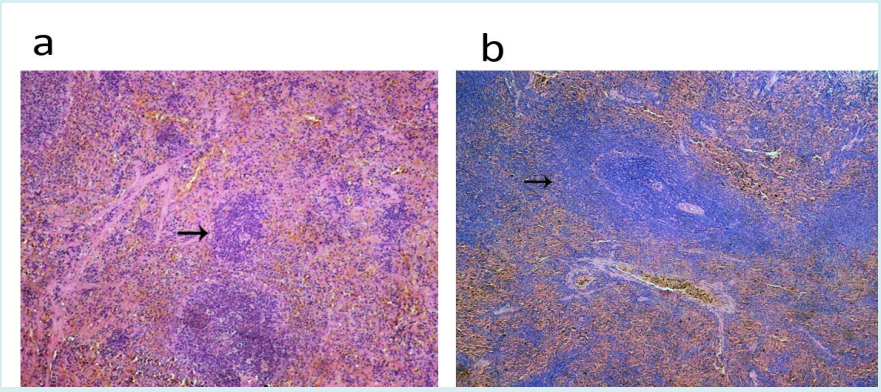

Figure 4: The depicted figure shown **(a)** Spleen of lactating dams treated with days postpartum showing severe depletion of the splenic lymphoid tissue (arrow) (HE, 100x). **(b)** Spleen of suckling pups of treated dams 1/10 LD_{50} of CFP orally from 0th - 21th days postpartum showing lymphoid depletion (arrow) (HE, 400x).

Notably, the chlorfeapyr treatment to HepG2 cells at highest concentration of clorfenapyr at 20 and 40 is cytotoxic as increased numbers of dead cells versus viable HepG2 cells figure 6. Moreover, the cell lysis shown a significant reduction of glutathione superoxide dismutase, and increased MDA level especially at high concentration of chlorfenpayr at doses of 20 and 40 ng/ml Table 8 & Figure 5.

Lo0	GSH mg/g. tissue	SOD U/g. tissue	MDA nmol/g. tissue
0	20.69±1.095a	8.42±0.054a	14.97±0.93c
10 ng/ml	18.69±1.09a	7.42±0.034a	13.97±0.93c
20	10.69±1.03b	3.42±0.054b	33.97±0.93b
40	8.91±1.042b	2.08±0.53b	75.96±1.90a

Table 8: Effect of CFP on oxidative stress in (HepG2) cells.

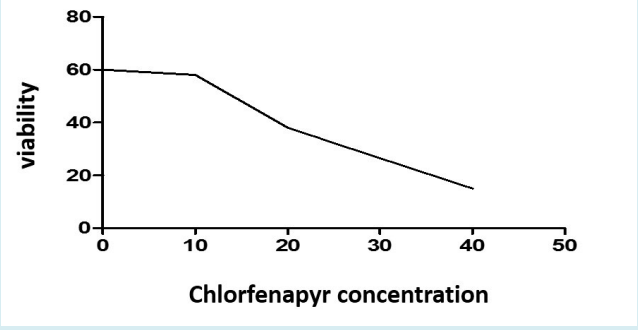

Figure 5: Showed that chlorfenpayr at high concentration induce lethal action to hepG2 cells

Discussion

CFP has a household and agriculture uses but also have a hazardous effects on animals and human health (1-4) especially when contaminated the environment [9,10], or remained as residues in food products (11-13) , water, animal derived foods [14,15].

CFP, a pyrrole insecticide has displayed a potential role to control of parathyroid resistant *insects* [21] and it was considering as a candidate insecticide for targeting malaria vectors that were resistant to pyrethroids [22]. Even chlorfenaapyr considered a good candidate's insecticides, it had developmental and maternal toxicity reported earlier [7,8].

Here, we are the first report of postnatal effects of chlofenapyr in female albino rats and its pups through hepatorenal toxicity, increased the oxidant, reduction of antioxidant levels in tissue of dams and pups and pathological changes in both tissue of dams and its pups. In this regard, CFP could pass through placental barrier as it evidence its presence in milk as residues [15]. In consistent to current study, maternal toxicity of oral exposure CFP at doses of 1/10, 1/20 of LD50 in female albino rats recorded earlier with evidence of induction of pathological features in liver, kidney, placenta, increased activity of liver enzymes, urea and creatinine levels, MDA as oxidant and reduced the levels of antioxidants [8]. Notably, CFP induced a significant inhibition in the activity of GST in the antioxidant in CHO_{K1} cells which retain GST levels after treatment with vitamin C or vitamin E [23]. Additionally, CFP had a cytotoxic effect in the different antioxidant assays. CFP is a pyrrole pro-insecticide; it is bio transformed by phase 2 oxidative elimination of *N*-ethoxymethyl group, which induced ablation of the mitochondrial ATP production through uncoupling of the mitochondrial oxidative phosphorylation that might enhanced the reactive oxygen radicals' production [23,24]. Notably, the cytotoxic effect of CFP recorded on CHO-K1 cell line as well as indicated cases of human intoxicated with CFP [25]. Finally, the pathological effect of CFP on brain was translated on neurobehavioral changes. In conclusions, chlorfeapyr is a good candidate insecticide in vector control but had a cytotoxic effect in female albino rats, its pups, and in HepG2 cells

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Conflict of Interest: All authors have no conflict of interest

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