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RESEARCH ARTICLE

Histopathological and genotoxic effects of chlorpyrifos in rats

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Abstract This study aims to investigate the effects of chlorpyrifos's sub-acute exposure on male rats. Two groups with six animals each were orally treated, respectively, with 3.1 mg/kg b w and 6.2 mg/kg b w of chlorpyrifos during 4 weeks. The genotoxic effect of chlopyrifos was investigated using the comet assay and the micronucleus test. Some hematological and liver's histopathological changes were also evaluated. Results revealed that chlorpyrifos induced histopathological alterations in liver parenchyma. The lymphoid infiltration observed in liver sections and the increase in white blood cells parameter are signs of inflammation. A significant increase in the platelet' count and in polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio was observed in chlorpyrifos-treated groups which could be due to the stimulatory effect of chlorpyrifos on cell formation in the bone marrow at lower doses. In addition, the increase of bone marrow micronucleus percentage and the comet tail length revealed a genotoxic potential of chlorpyrifos in vivo.

Keywords Chlorpyrifos · Genotoxicity · Hematological parameters · Liver · Organophosphate

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Introduction

The widespread use of insecticides is now a worldwide phenomenon. Environmental chemical pollution by insecticides has been increasing due to their extensive use in agriculture and pest control (Wang et al. 2011). So, the potential environmental contamination from pesticides is raising concerns for the public and regulatory bodies (Liu et al. 2010).

In 2003, they accounted for over 59 % (4645 tons) of pesticide sales in the EU, and chlorpyrifos (CPF) was the top-selling pesticide (15.6 %, 1226 tons) (Eurostat statistics 2015).

In Tunisia, after stopping the use of organochlorine insecticides, the organophosphorus (OP) insecticides have become important in mosquito control strategies. Many studies have reported that OP insect resistance in Tunisia is increasing (Ben Cheikh et al. 1998; Ben Cheikh et al. 2008; Ben Cheikh et al. 2009).

OP insecticides affect the nervous system by inhibiting acetylcholinesterase. This inhibition results in overstimulation of the parts of nervous system that contain acetylcholine-muscarinic receptor in post-ganglionic fibers of central and peripheral nervous systems (O'Malley 1997). This activity leads to an excessive accumulation of acetylcholine (Ach) and overstimulation of cholinergic neurons. If the concentration of the OPs is high, enough death results in a matter of minutes (Solberg and Belkin 1997).

CPF is an OP insecticide with a broad spectrum of insecticidal action, utilized extensively in agriculture and for residential pest control throughout the world (Mitra et al. 2008). Many reports demonstrated that CPF could generate a hepatic dysfunction, immunological abnormalities, embryotoxicity, genotoxicity, teratogenicity, neurochemical, and neurobehavioral changes (Dam et al. 1999; Gomes et al. 1999; Ki et al. 2013). In addition, an increased rate of chromosomal aberrations in the spleen cells of mice and in the bone marrow cells

of rats treated with CPF has been observed (Amer and Aly 1992; Amer et al. 1996). Moreover, CPF could induce DNA damage and cells apoptosis in vitro (Li et al. 2015).

To explore CPF-induced genotoxicity in rats, we investigated DNA-damaging potential of CPF using the comet assay and the micronucleus test. As well, some hematological indices and liver histological changes were evaluated.

Materials and methods

Chemicals

The commercial chlorpyrifos-ethyl (diethyl 3,5,6,-tricholoro-2-pyridyl phosphorothionate), (Supreme®480 g/l, SINOCHEM NINGBO LTD, China) is a liquid solution with amber color. The active ingredient of this product is chlorpyrifos (480 g/l) which is mixed with three inert ingredients (Isopropanol (110 g/l), clastor oil polyoxyethylene (80 g/l), and Xylene (fill to 1 l)). This product was purchased from a local market of agricultural products (Monastir, Tunisia).

Animals

Male Wistar rats weighing 120–160 g, obtained from SIPHAT (Pharmaceutical Industrial Society of Tunisia, Ben Arous, Tunisia), were used in this study. Animals were randomly divided into three groups of six rats each, one control group and two CPF treated groups. Diets and water were given ad libitum to rats. Animals were housed at 24±3 °C and maintained during the experimental period in accordance with the guidelines for animal care of the Faculty of Medicine of Monastir, Tunisia.

Study design

1. Treated groups:
   CPF was dissolved in corn oil, and we have proceeded to give by gavage 5 ml/kg of body weight of insecticide for 4 weeks, 5 days per week.

Group1 (CPF 1)	six rats were treated with 3.1 mg/kg of CPF (1/50 DL50).
Group 2 (CPF 2)	six rats were treated with 6.2 mg/kg of CPF (1/25 DL50).

2. Control group: six rats received an equivalent volume of corn oil as previously described for treated groups.
   Twenty-four hours after the treatment with the last dose, animals were sacrificed after ether anesthesia, and a blood sample was taken from each rat by heart tapping.

Follow-up of body and liver weight

Animal body was weighed every week, whereas liver weight was immediately taken after sacrifice for establishing:

Body weight gain (%)	(final body weights−initial body weights* 100)/initial body weight.
Relative liver weight (%)	organ weight×100/final body weight.

Histological study

After blood sampling, rats were decapitated, and the livers were removed, post-fixed in alcoholic bouin and embedded in paraffin. Five micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E) for optic microscopy analysis. Histopathologic evaluations (six slides for each group, two sections were included on each slide per animal) were conducted without prior knowledge of treatment group. Hepatic changes were graded based on severity from normal to severe. Normal (0) represented the least detectable lesion, mild (+) represented an easily discernible lesion, moderate (++) represented a change affecting a large area of the tissue represented on the slide, and severe (+++) represented the maximal lesion observed.

Hematological study

Freshly collected blood samples were analyzed for hematological parameters analysis using an automatic hematological assay analyzer (Beckman Coulter, USA). The following parameters were tested: red blood corpuscles (RBC), white blood corpuscles (WBC), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet counts (PLT), hemoglobin (Hb) and hematocrite (HCT) levels, and mean platelet volume (MPV).

Micronucleus test

Bone marrow micronucleus slides were prepared by using the modified method of Schmid (1975). We used bovine serum albumin (BSA) prepared in phosphate buffered saline (PBS) as suspending medium instead of fetal calf serum (Seetharama Rao et al. 1983).

The bone marrow of both femur and tibia was flushed out using 2 ml of 5 % BSA centrifuged at 1000 rpm for 10 min. The pellet was resuspended in a required volume of BSA (5 %), mixed thoroughly, and a drop of the suspension was smeared on clean slide. The slides were fixed in methanol and stained with Giemsa (5 %).

The slides were scored for the presence of micronucleus in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). About 1500 PCE were scanned from each animal to evaluate the percentage of MN-PCE and 500 PCE and NCE to estimate PCE/NCE ratio.

Comet assay or alkaline single-cell gel electrophoresis

The alkaline version of the comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly, a mixture of whole blood and low melting point agarose (LMA) (prepared in 0.6 % PBS (pH 7.4)) was dropped into a frosted slide precoated with a layer of 1 % normal melting point agarose.

Slides were placed in ice for 10 min and allowed to solidify. Cover slips were then removed and slides were immersed in freshly prepared lysing solution (2.5 M NaCl, 100 Mm EDTA, 0.25 M NaOH, Triton X100 (1 %), DMSO (10 %), pH 10) for 3 h at 4 °C, in the dark. After lysis, slides were placed in the electrophoresis tank. The tank was filled with freshly made alkaline electrophoresis solution (20 mM EDTA, 10 N NaOH, pH 10) to cover the slides, and then they were left for 20 min in the dark to allow DNA unwinding and alkali-labile site expression.

Electrophoresis was carried out for 20 min at 37 V and 300 mA. The slides were then washed for 10 min with 1 ml of neutralizing solution (0.4 M TrisBase, pH 7.5). After neutralization, gels were stained with 100 μl of ethidium bromide solution (20 μg/ml) and covered with cover slips for 20 min. The slides were scored without knowledge of the dose group. The extent of DNA migration was determined for each sample by capturing the image of 100 cells (50 cells on each of two slides) magnified at ×400 under a fluorescence microscope. Image analysis was performed with Tri Tek Comet Score software. The comet tail length (TL) was evaluated. TL indicates the extent of migration of genetic material in the direction of the anode (Singh et al. 1988).

Statistical analysis

Results are presented as mean±SD. Statistical analysis has been carried out using one way ANOVA (SPSS 21). Comparison between groups was done using Tukey's post test. Differences between groups were considered significant when $p<0.05$.

Results

Body weight gain (%) and relative liver weight (%)

In the current study, after 4 weeks of treatment, we observed an increase of body weight of treated rats. This increase was significant in rats treated with the higher dose in comparison with the control group (Fig. 1a). However, we noted a decrease in the relative liver weight of treated rats in comparison with the controls (Fig. 1b). That decrease was significant ($p\leq0.01$) only in group treated with the higher dose.

Histological studies

Light microscopic observation of the liver tissue of the control rats exhibited a normal histological architecture with adjacent sinusoids radiating from the central veins (CV) toward the periphery of the liver lobules and normal portal triad. The hepatocytes were of normal shape with dispersed chromatin (Fig. 2a, b).

Dilated sinusoids, dilated central vein (Fig. 2c, e), and a dilated portal triad (Fig. 2d, f) were observed in both treated groups. These alterations increased with the increase of the CPF dose.

The administration of the lower dose of CPF induced moderate alterations in hepatic parenchyma. A lymphoid infiltration and cytoplasm vacuolization (Fig. 3a, c) were observed.

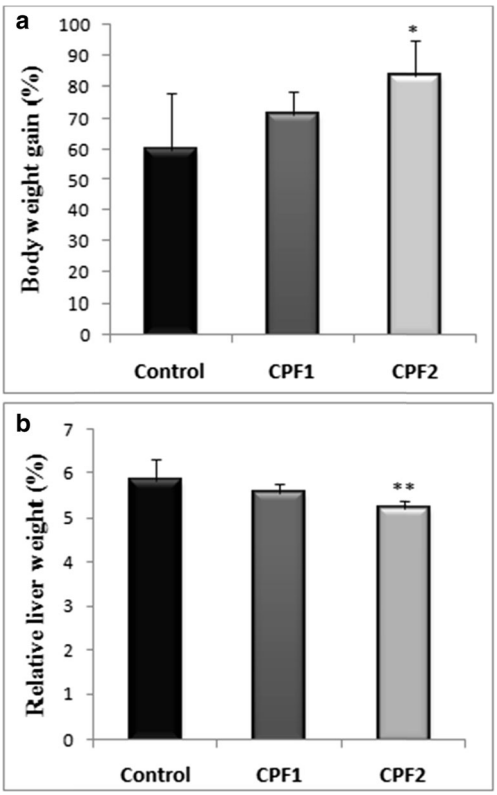

Fig. 1 Effect of CPF on body weights (**a**) and on liver weight (**b**) in the treated and control groups. *Significantly different from control ($p\leq0.05$); **Significantly different from control ($p\leq0.01$)

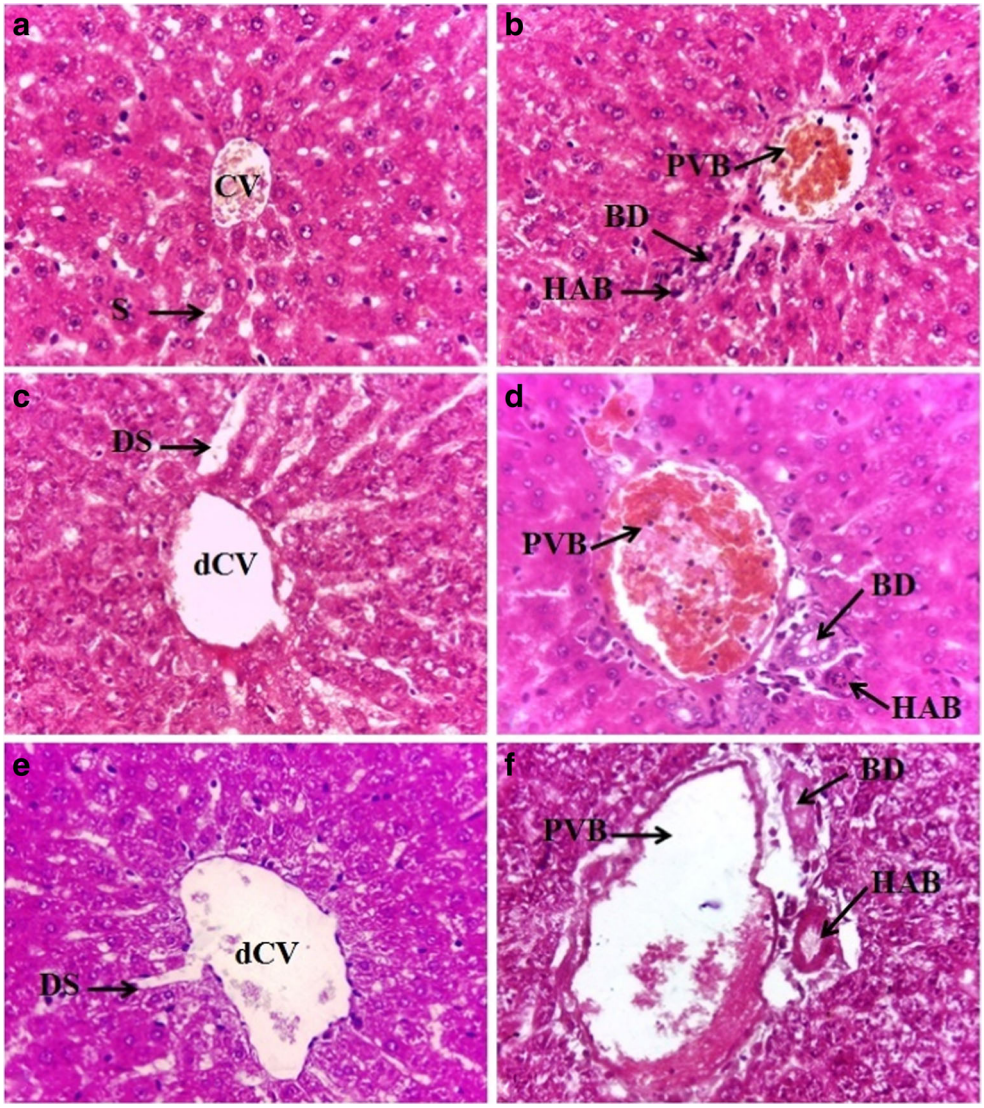

Fig. 2 Light micrographs of rat liver tissue stained by hematoxylin-eosin (H&E) in control and treated groups. The control reveals a normal liver parenchyma and a normal portal triad (**a**, **b**) (×400). Rats treated with lower dose (CPF1) show changes in the liver parenchyma (**c**) (×400) and hyperthrophy of portal triad (**d**) (×400). Severe alterations in the liver parenchyma (**e**) (×400) and an important hypertrophy of portal triad (**f**) (×400) are present in rats treated with higher dose (CPF2). *CV* central vein, *S* sinusoids, *DS* dilated sinusoid, *dCV* dilated central vein, portal triad (*PVB* portal vein branch, *HAB* hepatic artery branch, *BD* bile duct)

The higher dose of CPF induced the formation of oedema and the dislocation of the wall of the central vein (Fig. 3e). Also, we have observed a hepatocellular necrosis randomly distributed throughout the parenchyma (Fig. 3e, f). In addition, a hepatocyte's proliferation and hyperchromatic nuclei were detected only in liver sections of treated rats with higher dose (Fig. 3f), as well as an increase in inflammatory cell infiltration and cytoplasmic vacuolation were observed (Fig. 3b, d). Our findings showed that histological changes of the liver were proportional to the dose (Table 1).

Hematological study

The hematological parameter data are presented in Table 2. A non-significant difference between treated groups and control group was observed in RBC, MCV, HCT, MPV, HGB, MCH, and MCHC levels.

However, we observe an increase in PLT and WBC count in treated groups compared to control group. These findings showed that PLT and WBC count changes were proportional to the dose. These parameters increased significantly ($p \leq 0.01$) after the administration of both doses of CPF.

Micronucleus test

The results of micronucleus assay carried out on rat bone marrow cells to assess the potential of CPF to induce cytogenetic damage are listed in Table 3. The cytotoxic effects of CPF were evaluated by the determination of PCEs in total erythrocytes (PCEs and NCEs). A significant difference ($p \leq 0.01$) between the

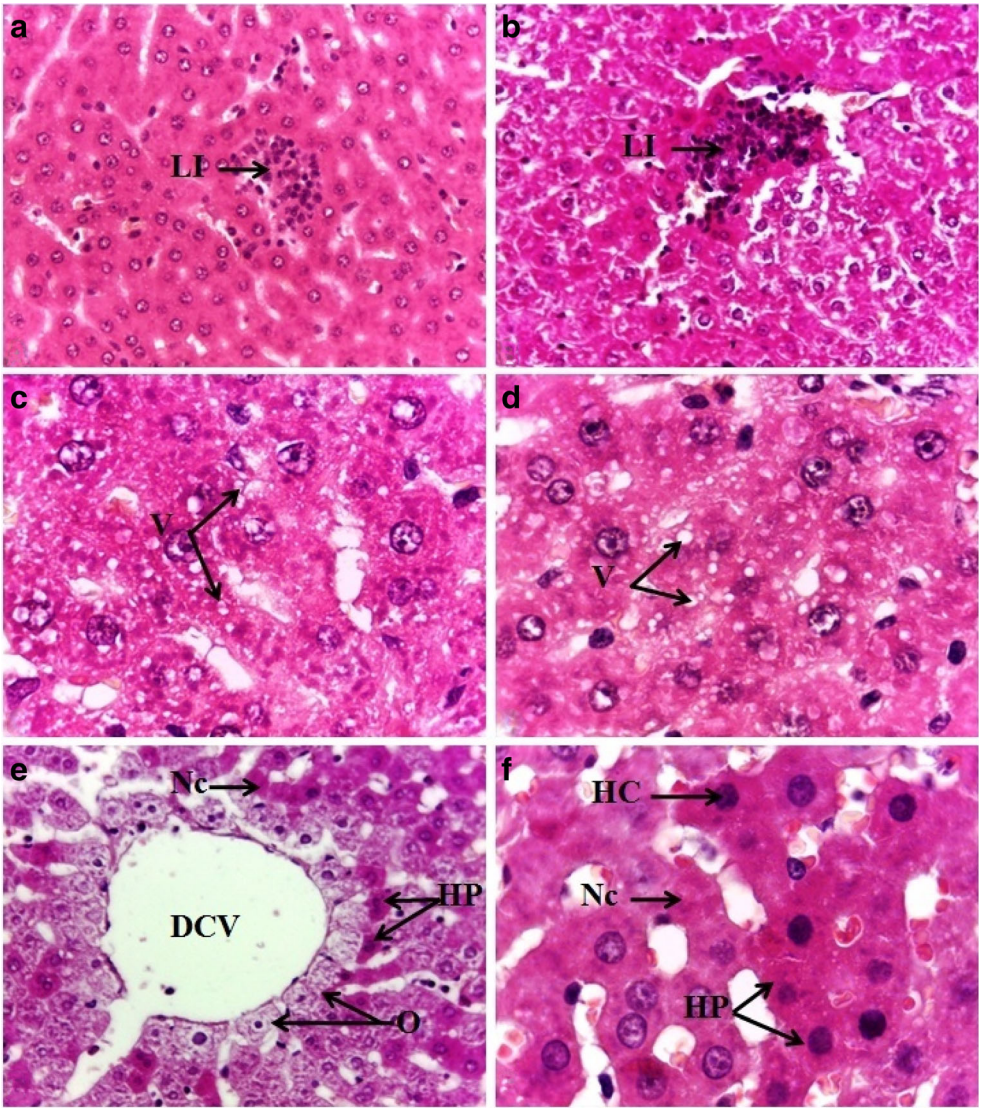

Fig. 3 Light micrographs of rat liver tissue stained by hematoxylin-eosin (H&E) in treated groups. Rats treated with the lower dose (CPF1) show a moderate changes in the liver parenchyma (**a**, **c**) (×400, ×1000, respectively). Rats treated with the higher dose (CPF2) reveal a severe alteration in the liver parenchyma (**b**, **d**, **e,** and **f**) (×400, ×1000, ×400, and ×1000, respectively). *DCV* dislocated central vein, *LI* lymphoid infiltrate, *V* vacuoles, *Nc* necrotic cell, *O* oedema, *HP* hepatocyte's proliferation, and *HC* hyperchromatic nuclei

control group and the treated groups was observed in P/N ratio.

Table 1 Grading of the histological changes in the liver sections

	Control group	CPF1	CPF2
Dilated sinusoid	0	+	++
Dilated central vein	0	++	+++
Hepatocyte's proliferation	0	0	++
Necrotic cells	0	0	++
Hyperchromtic nuclei	0	0	++
Dislocation of the wall of the central vein	0	0	++
Vacuoles	0	++	+++
Lymphoid infiltrate	0	+	++
Dilated portal triad	0	++	+++
Oedema	0	0	++

0 normal, + mild, ++ moderate, +++ severe

The micronucleus induction in the bone marrow of rats exposed to CPF was also observed. There was a significant

Table 2 Effect of CPF on hematological constituents of male rats

Items (unit)	Control	CPF 1 (3,1 mg/kg)	CPF 2 (6,2 mg/kg)
RBC (10^6/mm)	7.79±0.58	7.58±0.44	7.64±0.07
MCV (μm)	53.02±0.87	52.90±1.41	54.16±0.44
HCT (%)	41.98±1.10	41.08±1.40	41.50±0.44
PLT (10^3/mm)	315±59.45	545.50±77.16**	617.33±139.19**
MPV (μm)	8.20±0.99	7.40±0.19	7.82±0.52
WBC (10^3/mm)	11. 27±0.81	14.62±1.54**	16.35±0.60**
HGB (g/dl)	13.37±0.54	13.92±0.73	14.07±0.20
MCH (pg)	18.35±0.84	17.08±1.63	18.30±0.30
MCHC (g/dl)	34.65±2.08	33.48±1.64	33.87±0.62

**Significantly different from control ($p \leq 0.01$)

Table 3 Results of micronucleus test in rat bone marrow cells

Groups	% de MN-PCEs	PCE/NCE ratio
Control	1,82±0,6	2,40±0,1
CPF 1	41,86±5,2**	3,37±0,2**
CPF 2	55,58±6,9**	4,17±0,4**

**Significantly different from control ($p \leq 0, 01$)

($p \leq 0.01$) increase of MN-PCE percentage in the treated groups compared with control group. Thus, all dose levels can be considered mutagenic.

Results of comet assay

All doses of CPF could induce DNA damage at different levels. Figure 4 displays the results of DNA damages in rat blood cells according to comet analysis. A statistically significant ($p \leq 0.01$) increase of comet tail length was observed in the group treated with the higher dose of 6.2 mg/kg (b/w) compared with control group. Figure 4 also demonstrates that DNA damage increased with the increase of the CPF dose.

Discussion

In the present study, an increase in body weight gain was observed. That increase was significant only in groups treated with the higher dose. Our results are in accordance with other studies (Ambali et al. 2011; Meggs and Brewer 2007; Slotkin 2011) which suggested that CPF induces an increase of body weight gain in rats. However, other works have found that OP insecticides cause reduction of body and organ weights in experimental animals (Jayusman et al. 2014; Ogutcu et al. 2006). The weight increase represents an increase in adipose tissue. OP insecticides may induce accelerated differentiation of immature adipocytes into mature fat cells. This is one potential mechanism of weight gain (Meggs and Brewer 2007).

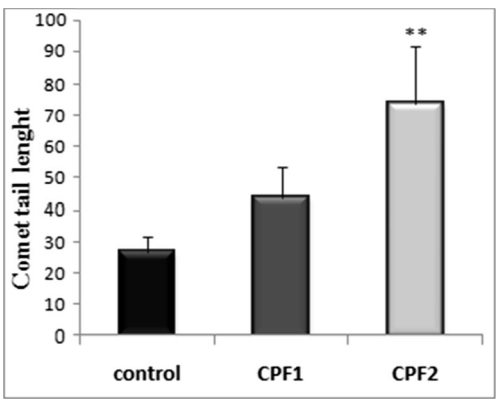

Fig. 4 Comet tail length in treated and control groups. **Significantly different from control ($p \leq 0.01$)

In treated groups, the relative liver weight decrease is an indicator of hepatic toxicity. That decrease was significant only in rats treated with the highest dose. It may be due to enhanced catabolic processes such as glycogenolysis, lipolysis, or proteolysis (Belaid-Nouira et al. 2013).

Our histological results demonstrated that CPF caused structural changes in the liver tissue of Wistar rats. CPF treatment generated an intense cytoplasmic vacuolation and appearance of moderate necrotic cell's foci. Necrosis is produced as a result of cell degeneration accompanied by organelles swelling and amorphous cytoplasm followed by shrinking and dissolution of nuclei (Campos-Pereira et al. 2012). Cytoplasmic vacuolation is known as an excess of lipids accumulation in cytoplasmic vesicles, but it was also suggested that vacuolar formation is a cellular defense mechanism against toxic substances. The segregation of these substances in vacuoles could be a way to prevent them from interfering with cellular metabolism (Mollendorf 1973).

Also, all liver sections of the treated rats have shown an abundance of dilated sinusoids, dilated central veins, and hypertrophy of portal triad. However, variable intensities of these changes were noticed depending upon the dosage of the treatment. These results are consistent with those of Tripathi and Srivastav (2010) which have shown that oral administration of CPF causes hepatocytic vacuolation, sinusoidal dilation, and focal necrosis.

In addition, the lymphoid infiltration observed in liver sections of CPF treated groups is a sign of inflammation and hypersensitivity to the used toxicant. The presence of lymphoid infiltration is in perfect agreement with WBC count results which proved the presence of inflammation. These observations are in agreement with those reported by Tripathi and Srivastav (2010); Heikal et al. (2012) and Elzoghby et al. (2014) which have shown that treatment with OP insecticides induces lymphocyte infiltration.

Moreover, oedema was only observed in the group treated with the higher dose. A study carried out by Mansour and Mossa (2010) on male and female rats revealed that oral administration of CPF caused the appearance of oedema in the portal area. Another study of Elhalwagy et al. (2008) has pointed out that the administration of fenitrothion induced the formation of oedema and some hemorrhagic areas in liver cells.

Furthermore, the dislocation of the wall in the central vein, the hepatocyte's proliferation, and the hyperchromatic nuclei was observed in liver sections of treated rats with a higher dose. Similar investigations have also been reported by Tripathi and Srivastav (2010) which have noticed increased chromatin content in hepatocytes of rat orally treated with 10 mg/kg of chlorpyrifos in 2 weeks.

As we have shown in the results section, a significant increase in the platelet' count was observed in treated rats with CPF. This result is evidenced by the result of the micronucleus test which found an increased PCE/NCE ratio in bone marrow cells. This increase could be due to the stimulatory effect of CPF on cell formation in the bone marrow at lower doses (Suzuki et al. 1989; Suzuki et al. 1993).

In addition, this study revealed a significant increase in the WBC count in all treated rats. This increase may indicate an activation of the animal's defense mechanism (Kalender et al. 2006). However, no significant difference was observed in RBC, MCV, HCT, MPV, HGB, MCH, and MCHC levels.

Insecticides may induce oxidative stress leading to the generation of free radicals which could produce DNA damage. In fact, the micronucleus test has been performed to evaluate the genotoxic effects of pollutants. In the present study, the micronucleus test has proved that CPF induced the formation of bone marrow micronuclei in a dose-dependent manner. That could be via clastogenic and aneugenic mode of action (Hayashi et al. 1989). The clastogenic mechanism of action of OP insecticides was also observed in vivo studies of mice (Cicchetti et al. 1999). These results are in concordance with those reported by Sarabia et al. (2009) and Hariri et al. (2011) which proved that organophosphate insecticides induce the formation of bone marrow micronuclei.

Furthermore, the PCE/NCE ratio is regarded as an indicator for toxicity affecting cell formation in the bone marrow. A stimulatory effect at lower doses was observed in the present study, as reflected by an increased PCE/NCE ratio. This could be due to the induction of erythropoietin, a growth factor for erythropoiesis, by low doses of CPF. Suzuki et al. (1989, 1993) and Gandhi et al. (1995) have reported similar observations with other mutagens in the micronucleus test.

Several studies have shown the involvement of reactive oxygen species (ROS) and oxidative stress in pesticide toxicity, and it is generally accepted that ROS eventually cause DNA damage (Bertram and Hass 2008; Heikal et al. 2012). Although, micronucleus test can detect clastogenic and aneugenic potential of chemicals, its inability to detect single strand breaks pose practical limitations to its use (Vikram et al. 2008).

Comet assay is commonly used as a biomarker for DNA damage; it has been widely used in the field of genetic toxicology and environmental biomonitoring. The DNA damage detected by the comet assay could be due to DNA single strand breaks, double strand breaks, adduct formations, DNA–DNA and DNA–protein cross-links (Mitchelmore and Chipman 1998). However, the genetic damage induced by aneugens is not readily detected by the comet assay (Mughal et al. 2010).

In our case, CPF induced erythrocyte DNA damage in a dose-dependent manner. The increase of comet tail length was significant only in treated rats with the higher dose compared with controls. Our results are consistent with those reported by Ojha et al. (2013) and Muller et al. (2014) which showed that treatment with OP insecticides caused severe DNA damage. Also, Sandhu et al. (2013) revealed that oral administration of 3 and 12 mg/kg b/w of CPF for 7 days and then 14 days induced a non-significant increase of DNA damage in blood cells via comet test and micronucleus test. However, in our study, the administration of 6.2 mg/kg b/w of CPF by the same route, for 4 weeks, induced a significant increase of DNA damage compared to the control group. This DNA damage was detected in blood cells by comet test and by micronucleus test in bone marrow cells. So, the greatest difference between our study and the previous one (Sandhu et al. 2013) concerns CPF time exposure.

Here, we demonstrate that both assays can successfully detect the DNA damage. Comet assay can determine the short lived-DNA damage (Giannotti et al. 2002) while the micronucleus test detects the structural and numerical chromosomal damage (Krishna and Hayashi 2000). This explains the necessity of tests, micronucleus test and comet assay, in the detection of DNA damage.

In conclusion, this study has exposed the consequences of CPF exposure on body weight gain and in relative liver weight. Also, we have found that the administration of low doses of CPF induced DNA damage in blood cells detected by comet test. In addition, the micronucleus test detected DNA damage in bone marrow cells. These results prove that CPF is genotoxic. Moreover, the WBC count is correlated with the important lymphoid infiltration observed in the liver of treated rats which is a sign of inflammation. Besides, an increased blood platelet count and PCE/NCE ratio in bone marrow cells might be due to the stimulatory effect of CPF at low doses. Further explorations at the molecular levels are justified to evaluate the full magnitude of the impact of CPF exposure on human health.

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Conflict of interest The authors declare that they have no competing interests.

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