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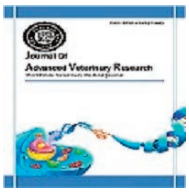

Cytogenetic and Genotoxic Effects of Penconazole and Chlorpyrifos Pesticides in Bone Marrow of Rats

Doha Yahia[1], Marwa F. Ali[2*]

[1]*Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Assiut University, Egypt*
[2]*Department of Veterinary Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt*

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ABSTRACT

The current study was undertaken to explain the cytogenetic and genotoxic effects of penconazole (PEN) fungicide and chlorpyrifos (CPF) insecticide in male rats. Rats (n=10/group) were treated with 500 mg/kg body weight PEN orally for 24 h, 48 h and 100 mg/kg body weight for 30 days and the control animals were administered distilled water only. Chlorpyrifos exposed groups received 39 mg/kg body weight for 24 h and 48 h and 8 mg/kg body weight for 30 days by oral gavage and the control group was received corn oil. Blood samples were collected for complete blood count. Bone marrow was flushed from the femur bones for chromosomal aberration (CA) and comet assay. Spleen samples were preserved in 10% formalin for histopathological examination. The level of DNA damage was measured using DNA damage index. The results showed that PEN and CPF caused significant hematological changes, significant increase in DNA damage index and increased the number of nuclei with I, II, III and IV degrees of damage. Different types of CA were recorded in PEN and CPF exposed groups including chromosomal break, deletion, attenuation, chromosome ring, gap and fragments. In addition, numerical aberration as polyploidy appeared in CPF exposed groups only. PEN and CPF caused histopathological changes in spleen in the form of apoptosis, congestion, thrombosis and hemosiderosis. In conclusion, PEN and CPF induced genotoxic and cytogenetic effects in bone marrow. DNA damage index and the percentage of CA were higher in CPF than PEN groups.

Introduction

The wide spread use of pesticides in agriculture and forestry is a significant source of environmental pollution. The target of using pesticides is the eradication of pests but other adverse effects were announced in none target organisms (Joy *et al*., 2005). Commercial formulations of pesticides may have severe toxic effects on the living organisms rather than the pure agents; as they contain solvents and some active compounds (Sanchez *et al*., 2006; Chan *et al*., 2007). The reported toxic effects were ranged from acute to chronic toxicity, e.g. endocrine disruptions or cancer in humans (Berrada *et al*., 2010).

Penconazole (PEN) (1-(2-(2,4-dichlorophenyl)-pentyl)-1H-1,2,4-triazole, CAS registry No. 66246-88-6) is a triazole fungicide used in agriculture for control of foliar pathogens (Kenyon *et al*., 1997). PEN belongs to conazoles, a class of azole-based fungicides, which is widely used in human and veterinary medicine for the treatment of local and systemic fungal infections (Chaâbane *et al*., 2017). The mechanism of action of these fungicides is the inhibition of lanosterol 14α-demethylase (CYP51) activity, a key enzyme for ergosterol biosynthesis in fungi, causing membrane dysfunction and disability to ensure substrate intake (Buchenauer, 1987; Zarn *et al*., 2003).

Penconazole is sprayed directly onto plants, absorbed and distributed rapidly inside the leaves (Kim *et al*., 2002). It is susceptible to accumulate in soils and can resist degradation (Singh, 2005). Moreover, residual amounts of PEN above the maximum residue limits were detected in some crops (Güdücü *et al*., 2011). Some reports demonstrated oxidative stress, hepatic, renal, genotoxic and testicular damages induced by PEN in rats (El sharkawy and Elnisr, 2013; Chaâbane *et al*., 2017; Ferri *et al*., 2018).

Chlorpyrifos-ethyl (CPF) (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS registry No. 2921-88-2) is a broad spectrum widely used organophosphate (OP) insecticide for control a wide range of insects. It acts through inhibition of acetylcholinesterase, and consequently accumulation of acetylcholine in cholinergic synapses and in neuromuscular junctions (Kwonq, 2002; Mitra *et al*., 2008). OP insecticide have demonstrated genotoxic, clastogenic and alkylating proper-

*Corresponding author: Marwa F. Ali
E-mail address: marwafa28@gmail.com

ties; they are potentially mutagenic and clastogenic (Mehta *et al*., 2008; Sarabia *et al*., 2009; Ezzi *et al*., 2016).

Several studies demonstrated that CPF could generate genotoxicity, teratogenicity, hepatic dysfunction, immunological abnormalities, embryotoxicity, neurobehavioral, neurochemical changes and testicular damage (Dam *et al*., 1999; Gomes *et al*., 1999; Ki *et al*., 2013; Elsharkawy *et al*., 2014). In addition to an increased rate of CAs in bone marrow cells of rats and spleen cells of mice (Amer *et al*., 1996), and caused DNA damage in brain and liver of rats (Mehta *et al*., 2008; Yahia and Ali, 2018).

Bone marrow is suitable for both comet assay (Sasaki *et al*., 1997) and CA assay, because large numbers of dividing cells are easily prepared for analysis (Kirkland *et al*., 1990). Chromosome aberrations are the best validated and most widely used biomarker for early detection of biological effects of genotoxic agents (Bonassi and Au, 2002).

Several studies used comet assay and/ or micronucleus assay to study the genotoxic effect of pesticides (Mehta *et al*., 2008; Ojha *et al*., 2013; Muranli *et al*., 2015; Ezzi *et al*., 2016). A recent study by Ferri *et al*. (2018) had assessed the oxidative DNA damage induced by CPF and PEN in human lymphocytes by using the comet assay.

According to the authors knowledge, studies that use the comet assay together with CA assay for evaluation of the genotoxic effect of PEN and CPF in bone marrow of rats are limited, so the current study aimed to investigate the genotoxic effect of acute and subacute exposure to commercial formulations of PEN and CPF in bone marrow of rats, in addition to studying the hematological effects and histopathological changes in the spleen.

Materials and methods

Chemicals

Penconazole:1-(2-(2,4-dichlorophenyl)-pentyl)-1H-1,2,4-triazole, CAS No. 66246-88-6. Fungicide, commercial grade with trade name Topas® 10% (Syngenta Co, Switzerland). Chlorpyrifos: O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS No. 2921-88-2 an organophosphate insecticide, commercial form with a trade name Clorzane 48% EC (Kafre Elzayat KZ CO, Egypt).

Animals

Adult male Sprague-Dawley rats (aged 9-10 weeks) with 90-110 g average body weight were used in this study. Animals were delivered from the Experimental Animal Unit, Faculty of Medicine, Assiut University, Egypt. The animals were randomly housed in plastic cages and allowed to adapt for a week before the experiment. Animals were maintained in a suitable condition, with a 12 h dark and 12 h light cycle and temperature (22-25°C). Rats were supplied with standard dry food pellets and tap water ad libitum. The animals were handled in accordance with the institutional guidelines for care and use of experimental animals in Assiut University.

Experimental design

Penconazole exposure

Groups of ten rats were used for each treatment. The first and the second groups received ¼ LD_{50} (500 mg/kg b.w.) of PEN by oral gavage. The first group exposed to one dose for 24 h, while the second group exposed to 2 doses (once/day) for 48 hours. The third group exposed to 1/20 LD_{50} (100 mg/kg b.w.) twice a week for 30 days. Control group was administered distilled water only for 24 h, 48 h and 30 days. Penconazole oral LD_{50} in rat is 2000 mg/kg b.w. (FAO/WHO, 1991).

Chlorpyrifos exposure

Groups of ten rats were used. The first and the second groups received ¼ LD_{50} (39 mg/kg b.w.) of CPF by oral gavage. The first group was exposed once for 24 h, while the second group was exposed to two doses, one dose per day for 48 hours. The third group was exposed to 1/20 LD_{50} (8 mg/kg b.w.) twice per week for 30 days. Control group was administered corn oil only at 24 h, 48 h and 30 days. Oral LD_{50} of CPF is 155 mg/kg in adult male rats (Worthing and Walker, 1987).

Necropsy

Five animals per group were euthanized by diethyl ether inhalation 24 h after the last dose. Blood samples were collected from the descending aorta in Vacutainer tubes coated with EDTA anticoagulant for hematological analysis. Bone marrow was flushed out from one femur using 1 ml of homogenizing buffer (0.024 M Na_2EDTA, 0.075 M NaCl), centrifuged at 1500 rpm for 15 min. at 0°C, and the supernatant was used for comet assay. Spleen tissue was collected and kept in 10% neutral buffered formalin for histopathological examination.

Hematological analysis

Blood samples were analyzed using Vet. Hematology analyzer (Medonic CA 620, Sweden). Hematological variables measured were red blood cells count (RBCs), hemoglobin concentration (HGB), red blood cells distribution width (RDW), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells count (WBCs), lymphocytes count, granulocytes and monocytes.

Comet assay (Single cell gel electrophoresis)

Comet assay was performed according to Tsuda *et al*. (1998); fully frosted slides were layered with 100 µL normal agarose 1% GP-42 (Nacalai Tesqe, Inc. Kyoto, Japan). Equal volume of nuclear suspension was mixed with 2% low melting agarose (Nacalai Tesqe, Inc. Kyoto, Japan) at 45°C, layered on the slide and covered with a cover slide. Finally, agarose GP-42 was layered on the surface and allowed to gel for 10 min.

The slides were immersed into a chilled lysing solution (10 mM Tris base, 2.5 M NaCl, 0.1% Sodium lauryl sulfate (SDS), 100 mM Na_4EDTA, Triton X-100, and 10% dimethyl sulfoxide) and kept in the dark at 4°C for 3 hours. Then the slides were immersed in chilled alkaline solution (1mM Na_2 EDTA and 300 mM NaOH, pH 13) in a horizontal gel electrophoresis platform (Cleaver Scientific Ltd, U.K.) in the dark for 20 min, electrophoresis was conducted at 25V and approximately 300 mA for 20 min. after that the slides were neutralized with 400 mM Tris buffer (Wako Pure Chemical Industries, Ltd. Japan) pH 7.5 for 7 min. dehydrated in ethanol 95% for 5 min. and allowed to dry at room temperature.

Slides were stained with 50 µL (20 µg/mL) ethidium bromide (Wako Pure Chemical Industries, Ltd.) immediately before microscopical examination then examined by fluorescence microscope (Olympus BX-43, Japan) using the green filter at 200 fold magnifications, 50 cells in one slide were examined by Comet Assay Software Project (CASP, University of Wroclaw, Institute of Theoretical Physics). Tail length, head radius, comet length (tail length + 2xhead radius), %DNA in tail and tail moment were used as indicators of DNA dam-

age. DNA damage index was calculated according to DNA % in the tail. Zero % indicated no damage, < 5% I, 5-20 % II, 20 - 40 % III; more than 40% is IV degree damage. DNA damage index = # 0 + # I + # II+ # III + # IV/ # of scored cells (Ojha *et al*., 2013).

Chromosomal aberration assay

Chromosomal aberration assay was performed according to Adhikari and Grover (1988) with some modifications. Five animals per group were injected with colchicine (4 mg/kg b.w.) intraperitoneally 2 h before sacrifice to arrest mitosis; the animals were euthanized after anesthesia with diethyl ether. Bone marrow was collected from the femur in 3 mL normal physiologic saline. The cell suspension was centrifuged at l000 rpm for 10 min, and the bone marrow pellet was resuspended in 0.56% potassium chloride, incubated for 30 min. then centrifuged at 1000 rpm for 10 min. The cells were fixed in Carnoy's solution (1: 3, acetic acid: methano1) for 30 min. then centrifuged and the cells again were centrifuged and resuspended in the same fixative for 24 h. The fixed cells were dropped on dry slides, stained with 10% Giemsa for 30 min. Slides were examined at 100 X magnifications (oil immersion lens), 100 well spread metaphase for each group were scored to detect CAs.

Histopathological examination

Fresh specimens from spleen of rats of all experimental groups were collected and fixed in 10% neutral buffered formalin. The tissues were dehydrated in a graded alcohol series, cleared with methyl benzoate, embedded in paraffin wax, sectioned at 4 µm thickness and stained with hematoxylin and eosin (Bancroft *et al*., 1996) for histopathological examination by light microscopy (Olympus CX31, Japan) and photographed using digital camera (Olympus, Camedia C-5060, Japan).

Statistical analysis

The normality of data was checked using Shapiro- wilk test. All the normally distributed data were checked for homogeneity of variance by using Levene's test. Then statistical analyses were done using SPSS software package version 16.0. Data were analyzed by using one-way analysis of variance (ANOVA) followed by post-hoc least significant difference (LSD) multiple range test for comparison among control and exposed groups. All data were expressed as Mean±SE for all experimental and control animals ($P < 0.05$).

Results

Hematological findings

Effect of penconazole on hematological parameters

Exposure of rats to penconazole for 24 h significantly *($P < 0.01$)* reduced RBCs count, HGB, HCT, MCHC, RDW%, WBCs, lymphocytes and monocytes. After 48 h exposure showed significant increase of MCV ($P < 0.05$), reduced RDW% *($P < 0.01$)* and lymphocytes ($P < 0.05$) while 30 days exposure caused significant changes in MCV (P < 0.05), MCHC, RDW%, WBCs, granulocytes *($P < 0.01$)* and lymphocytes ($P < 0.05$). PEN hematological parameters are shown in Tables 1 and 2.

Effect of chlorpyrifos on hematological parameters

Oral exposure of rats to CPF for 24 and 48 h showed significant *($P < 0.01$)* increase in MCV and a significant *($P < 0.01$)* decrease in MCHC if compared with control group. WBCs, lymphocytes and monocytes significantly *($P < 0.01$)* decreased at 24 h, while at 48 h lymphocytes decreased ($P < 0.05$) but granulocytes and monocytes significantly *($P < 0.01$)* increased. At 30 days exposure, animals showed significant *($P < 0.01$)* decrease in RBCs count, HGB, HCT, MCHC ($P < 0.05$) and RDW%

Table 1. Effect of penconazole exposure on red blood cells profile of rats

	RBCs ($\times 10^6/mm^3$)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)
Control	7.37±0.23a	12.90±0.63a	35.65±1.70a	48.25±0.72a	17.45±0.25a	37.00±0.46a	27.35±0.89a
24 hours	5.47±0.73b	10.05±0.66b	28.55±1.93b	50.17±1.27ab	17.85±0.43a	35.30±0.15b	23.87±0.58b
48 hours	7.17±0.23a	13.55±0.20a	37.30±0.57a	52.00±0.81b	16.00±0.69a	36.35±0.02a	21.85±0.25c
30 days	6.78±0.05a	12.80±0.34a	36.20±0.95a	52.60±1.15b	18.90±0.40a	35.52±0.44b	19.90±0.46d

Data are displayed as Mean±SE. In each column, different letters a,b,c and d, indicate significant ($P < 0.05$) differences among exposed groups. n=5 animals/group, rats administered ¼ LD_{50} of PEN for 24 and 48 h and 1/20 LD_{50} for 30 days.

Table 2. Effect of penconazole on leucocytes count

	WBCs ($10^3/mm^3$)	Lymphocytes ($10^3/mm^3$)	Granulocytes ($10^3/mm^3$)	Monocytes ($10^3/mm^3$)
Control	10.40±0.63a	9.25±0.66a	0.60±0.05a	0.55±0.03a
24 hours	4.45±0.77b	3.60±0.63b	0.62±0.11a	0.17±0.02b
48 hours	7.70±2.25ab	5.70±1.73b	1.65±0.37a	0.35±0.14ab
30 days	6.95±0.54b	5.45±0.14bc	0.85±0.60b	0.65±0.08a

Data are displayed as mean±SE. In each column, different letters a,b,c and d, indicate significant ($P < 0.05$) differences among exposed groups. n=5 animals/group, rats administered ¼ LD_{50} of PEN for 24 and 48 h and 1/20 LD_{50} for 30 days.

accompanied with significant *(P < 0.01)* decrease in WBCs, lymphocytes and monocytes. CPF hematological parameters are displayed in Tables 3 and 4.

Findings of DNA damage in the exposed groups

DNA damage in penconazole exposed groups

Exposure of rats to PEN for 24 h, 48 h and 30 days showed significant marked DNA damage in bone marrow, represented as significant increase in number of nuclei with I, II, III and IV degrees of damage, while control group showed only type 0 (typical condensed, round nuclei with intact DNA) Fig.1b and type I damage. DNA damage index increased by 2.81, 3.55 and 2.75 folds at 24 h, 48 h and 30 days respectively compared with control group. Results also showed significant *(P < 0.01)* increase in all parameters of DNA damage including tail length, comet length, % DNA in tail and tail moment. The 48 h group showed highest degree of DNA damage (Table 5 and Fig. 1).

Table 3. Effect of exposure to chlorpyrifos on red blood cells profile

	RBCs ($\times 10^6/mm^3$)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)
Control	7.07±0.38a	13.10±0.66a	35.70±1.70a	50.63±2.49a	18.60±1.17a	36.73±0.53a	25.76±1.81a
24 hours	6.75±0.25a	13.0± 0.23a	38.45± 1.29a	57.00±0.23b	19.3 ±0.40a	33.85±0.54b	29.35±0.89ab
48 hours	6.97±0.02a	12.5±0.05a	38.85± 0.14a	57.70± 1.15b	17.95±0.02a	31.95 ±0.20c	23.45±1.12ac
30 days	5.13 ± 0.40b	9.60± 0.94b	26.86±2.68b	52.53±1.12a	18.23±0.39a	35.06±0.08d	20.06±0.54c

Data are displayed as Mean±SE. In each column, different letters a,b,c and d, indicate significant (*P < 0.05*) differences among exposed groups. n=5 rats/group, rats administered ¼ LD50 of CPF for 24 and 48 h and 1/20 LD50 for 30 days.

Table 4. Effects of chlorpyrifos exposure on total and differential leucocytes counts

	WBCs ($10^3/mm^3$)	Lymphocytes ($10^3/mm^3$)	Granulocytes ($10^3/mm^3$)	Monocytes ($10^3/mm^3$)
Control	9.60±1.02a	8.33±1.13a	0.66±0.08a	0.60±0.05a
24 hours	5.42±0.36b	4.77±0.33b	0.45±0.02a	0.20±0.06b
48 hours	9.80±0.17a	6.10±0.17c	2.30± 0.05b	1.40±0.05c
30 days	6.00±0.43b	4.70±0.26b	1.03±0.18c	0.27±0.03b

Data are displayed as Mean±SE. In each column, different letters a,b,c and d, indicate significant (*P < 0.05*) differences among exposed groups. n=5 rats/group, rats administered ¼ LD_{50} of CPF for 24 and 48 h and 1/20 LD_{50} for 30 days.

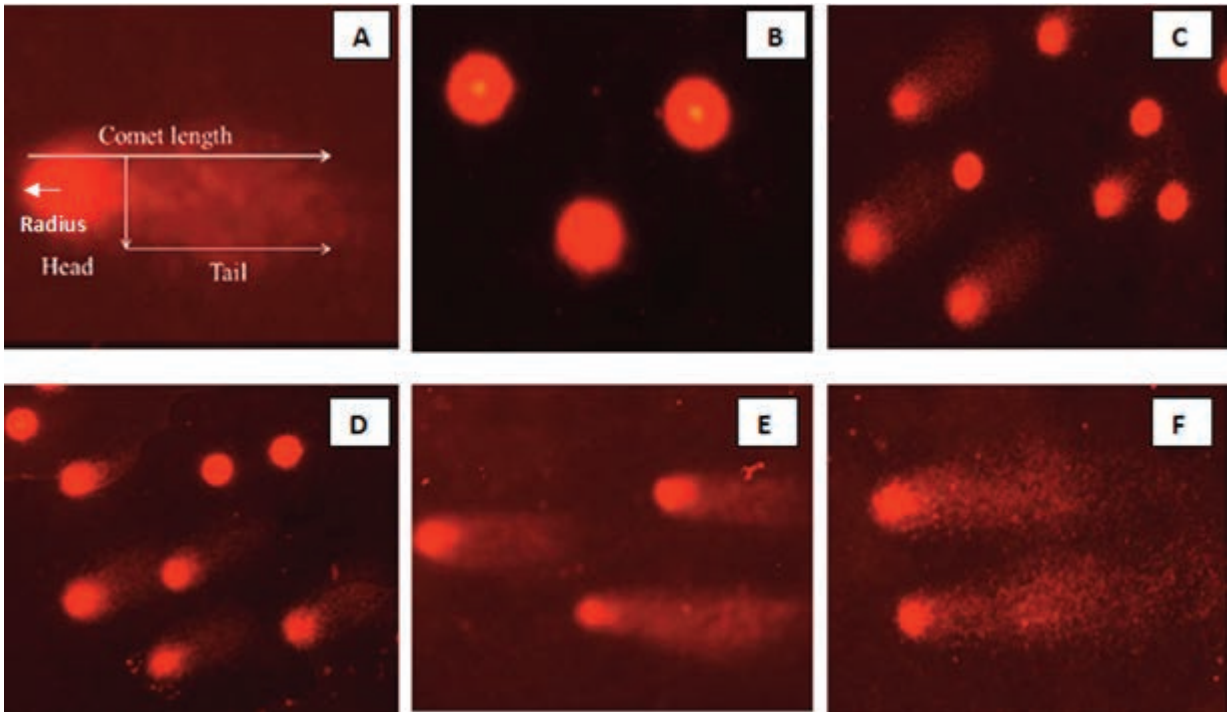

Fig. 1. DNA damage in bone marrow of rats exposed to ¼ LD_{50} penconazole for 24 h and 48 h, and 1/20 LD_{50} for 30 days. A) Comet length = 2x head radius + Tail length. B) Control, round nuclei indicated intact DNA. C and D) different degrees of DNA damage. E and F) Extensive DNA damage indicated by long tail.

DNA damage in chlorpyrifos exposed groups

Rats exposed to CPF for 24 h, 48 h and 30 days showed significant time related DNA damage regardless of dose fraction from LD_{50}. DNA damage index increased by 2.87, 3.49 and 4.11 folds at 24 h, 48 h and 30 days respectively as compared with control group. The number of nuclei with type I, II, III and IV was increased in all exposed groups. On the other hand, tail length, tail moment and % of DNA in the tail significantly *(P < 0.01)* increased in a time related manner (Table 6 and Fig. 2).

Chromosomal aberrations in the exposed groups

Chromosomal aberrations in penconazole exposed groups Percentage of CAs was significantly increased in all exposed groups. Different types of CAs were detected in PEN exposed rats; chromosomal breaks were the most obvious type than other types of CA such as chromosome ring, attenuation, fragments, gap and deletion were detected (Table 7 and Fig. 3).

Table 5. DNA damage index and comet parameters in bone marrow of rats exposed to penconazole fungicide

	Control	24 hours	48 hours	30 days
DNA damage index	0.49±0.10a	1.38±0.06b	1.74±0.19c	1.35 ±0.20 b
Tail length (µm)	6.30±0.57a	28.06±1.42b	43.22±1.99c	30.43 ±1.55b
Head radius (r)	50.54±0.42a	51.10±0.35a	157.31±0.97b	156.35±0.83b
Comet length	107.72±1.09a	130.60±1.73b	445.31±6.71c	405.01±5.29b
%DNA in tail	1.13±0.14a	6.45±0.44b	10.73±0.65c	8.32±0.55d
Tail moment (µm)	0.14±0.02a	3.61±0.38b	8.21±0.73c	6.10±0.50d

Data are displayed as Mean±SE. n= 100 nucleus/animal (5 rats/group). In each row, different letters a,b,c and d, indicate significant (*P < 0.05*) differences among exposed groups. Rats administered ¼ LD_{50} of PEN for 24 and 48 h and 1/20 LD_{50} for 30 days. DNA damage index = # I+ 2#II +3#III+ 4# IV/# scored cells. Comet length = tail length + 2r.

Table 6. DNA damage index and comet parameters in rats exposed to chlorpyrifos insecticide

	Control	24 hours	48 hours	30 days
DNA damage index	0.45±0.13a	1.29±0.07b	1.57±0.10c	1.85±0.23d
Tail length (µm)	7.85±0.77a	18.94±1.29b	25.40±1.58c	39.05±1.69d
Head radius (r)	50.51±0.44a	44.66±0.27b	48.93±0.51c	52.40±0.37d
Comet length	109.21±1.43a	108.61±1.49a	123.60±2.11b	144.19±2.10c
%DNA in tail	1.24±0.14a	5.24±0.53b	8.64±0.83c	9.41±0.58c
Tail moment (µm)	0.19±0.03a	1.56±0.22a	3.43±0.46b	7.78±0.49c

Data are displayed as Mean ± SE. n= 100 nucleus/ rat (5 rats/group). In each row, different letters a,b,c and d, indicate significant (*P < 0.05*) differences among exposed groups. Rats administered ¼ LD_{50} of CPF for 24 and 48 h and 1/20 LD_{50} for 30 days. DNA damage index = # I+ 2#II +3#III+ 4# IV/# scored cells. Comet length = tail length + 2r.

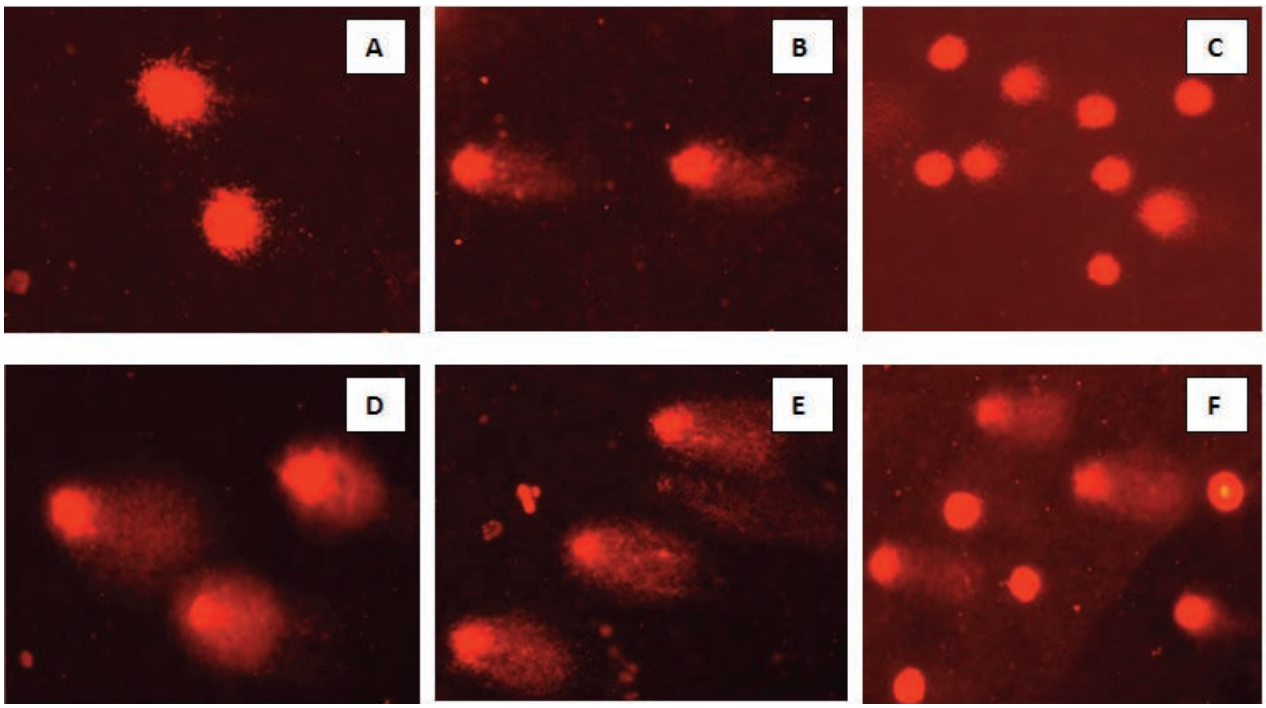

Fig. 2. DNA damage in bone marrow of rats exposed to CPF. A and B) 24 h exposed group showing mild DNA damage. C and D) 48 h exposed group showing different degrees of damage. E and F) animals exposed to CPF for 30 days showing severe DNA damage.

Chromosomal aberrations in chlorpyrifos exposed groups

Chlorpyrifos induced several types of CAs including break, deletion, attenuation, chromosome ring, gap and fragments. The numerical aberration was observed in the form of polyploidy in CPF groups at 24 h and 30 days.

There was significant *(P < 0.01)* increase in the percent of chromosomal aberrations in all exposed groups. Data are shown in Table 8. Different types of chromosomal aberrations are shown in Fig. 3.

Results of the histopathological examinations

Spleen of penconazole exposed groups

Spleen of rats exposed to PEN for 24 h showed apoptosis in the white pulp accompanied with vascular changes in the form of congestion of some blood vessels and thrombosis in others. Mixed thrombus consists of fibrin, RBCs and WBCs (Fig. 4C). Marked apoptosis of lymphocytes was seen in most of white pulps of spleen in rats exposed to PEN 48 h (Fig. 4D). At

Table 7. Chromosomal aberrations in bone marrow of penconazole exposed rats

	Control	24 hours	48 hours	30 days
% of aberrations	3.99± 0.41a	32.58±0.48b	31.82±0.97b	37.22±0.71b
Break	1.18±0.65a	11.94±0.78b	11.98±1.24b	14.16±1.02b
Ring	0.87±0.61a	8.74±0.31b	6.03±0.51c	5.57±0.34c
Attenuation	0.66±0.54a	5.43±1.31	5.87±1.25b	4.58±1.18b
Fragment	0.57±0.40a	6.08±0.65b	7.94±1.04b	3.26±0.81c
Gap	0.46±0.30a	0.39±0.39a	0.00±0.00a	0.72±0.39a
Deletion	0.25±0.21a	0.00±0.00	0.00±0.00	8.93±0.07b

All aberrations are expressed as Mean ± SE. n= 5 animals in each group, 100 metaphases were examined per group. In each row, different letters a,b and c, indicate significant ($P < 0.05$) differences among exposed groups.

Table 8. Chromosomal aberrations in bone marrow of chlorpyrifos exposed rats

	Control	24 hours	48 hours	30 days
% of aberrations	3.67±0.20a	33.47±1.21b	39.89±2.40 c	40.21±0.42c
Break	1.26±0.23a	10.27±2.64b	7.93±0.79b	8.37±0.47b
Ring	0.80±0.27a	8.13±0.80b	10.75±0.92bc	6.70±0.47b
Attenuation	1.03±0.39a	5.83±1.01b	6.66±0.47b	17.38±1.18c
Fragment	0.00±0.00a	2.10±0.58b	6.98±2.32b	6.04±1.16b
Gap	0.58±0.42a	0.78±0.39a	1.07±0.54a	0.84±0.41a
Deletion	0.00±0.00a	6.00±1.85b	6.50±1.95b	0.43±0.43a
polyploidy	0.00±0.00a	0.36±0.36b	0.00±0.00a	0.45±0.45b

All aberrations are expressed as Mean ± SE. n= 5 animals in each group, 100 metaphases were examined per group. In each row, different letters a,b and c, indicate significant ($P < 0.05$) differences among exposed groups.

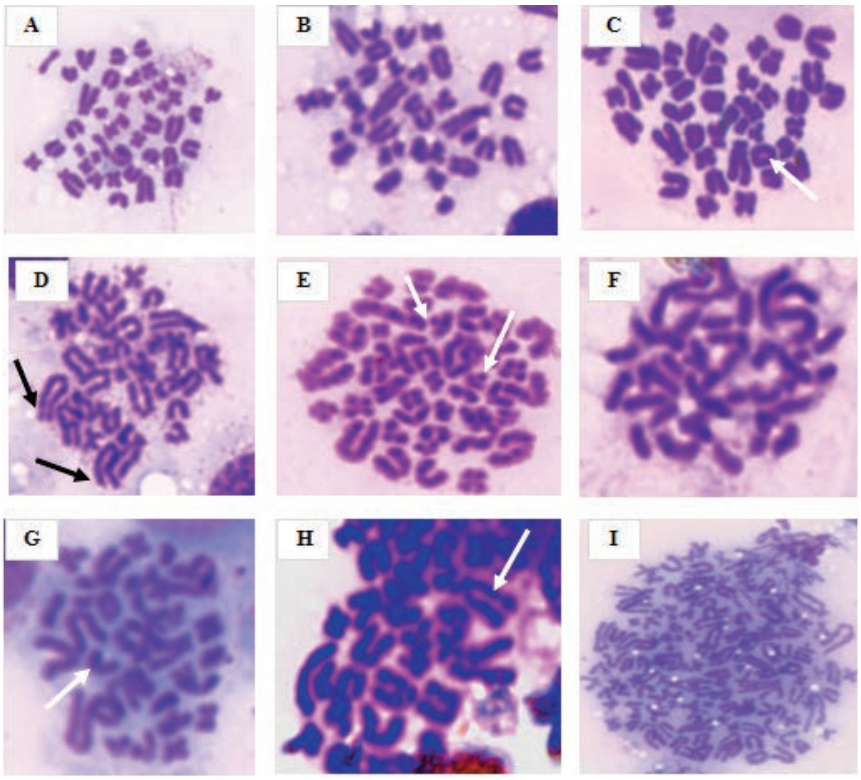

Fig. 3. Different types of chromosomal aberrations. A and B) Normal metaphase. C) chromosomal ring (white arrow). D) chromosome break (black arrows). E) Chromosomal Fragments. F) Attenuation. G) Chromatid deletion. H) Chromatid gap (white arrow). I) Polyploidy.

30 days, extra medullary hematopoiesis was very characteristic showing megakaryocytes within a background of maturing granulocytic elements (Fig 4E).

Spleen of chlorpyrifos exposed groups

Histopathological examination of spleen after 24 h from CPF exposure showed vascular lesions demonstrated with congestion, thrombosis and vasculitis. Vasculitis appeared by infiltration of the wall of blood vessels in spleen with mononuclear inflammatory cells and variable fibrinoid degeneration of tunica media (Fig. 4F). Hemosiderosis was observed at 48 h as intracellular brown granules in white and red pulp associated with apoptosis of lymphocytes in some white pulps which characterized by shrinkage of individual lymphocytes, condensation of nucleus and fragmentation of apoptotic cells (Fig. 4G). At 30 days, angiopathic changes were the most obvious changes such as vacuolation of tunica media in some blood vessels (Fig 4H).

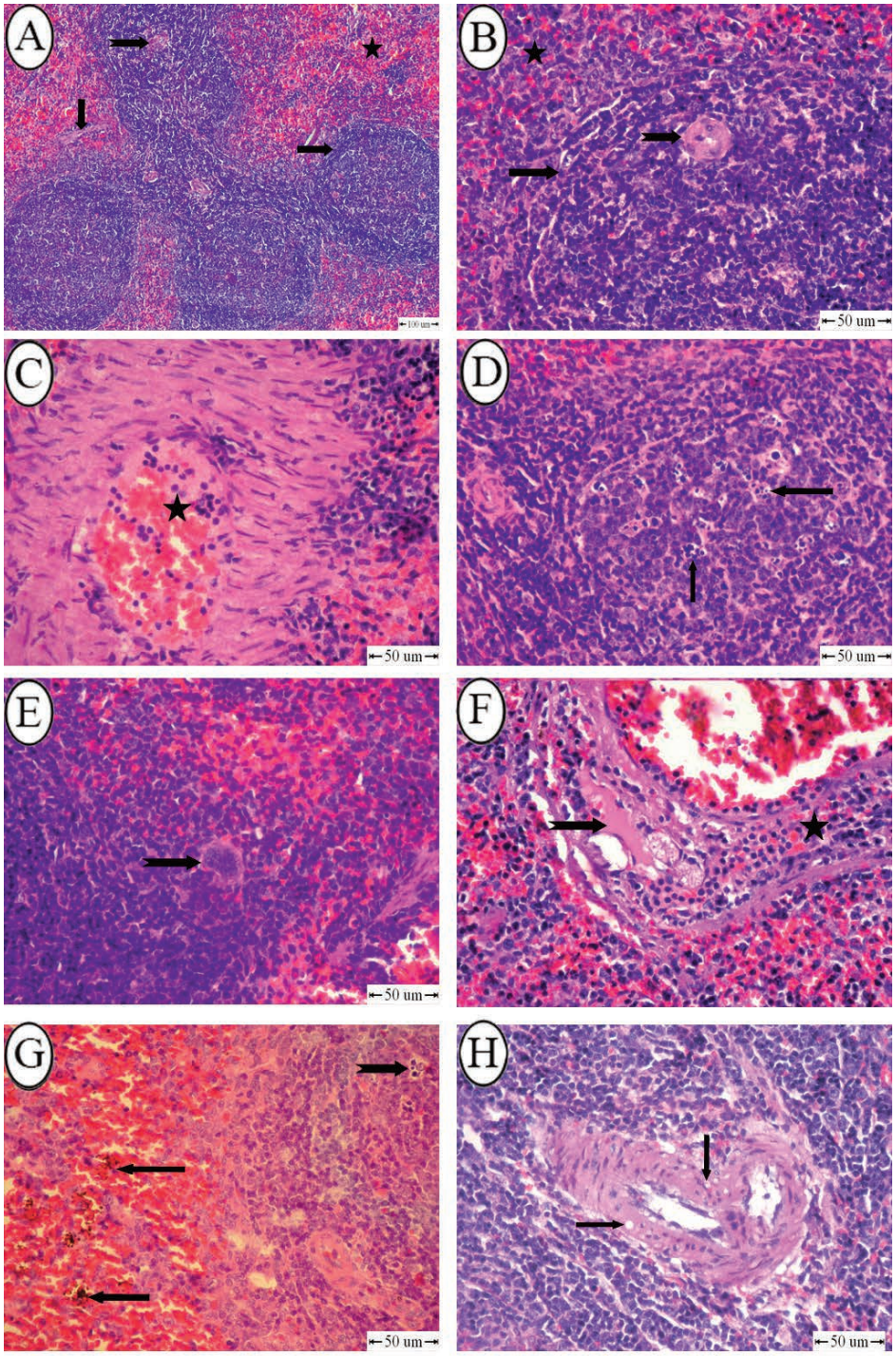

Fig 4. Histopathology of spleen: A) Control group showing red pulp (star), white pulp (right arrow), central arteriole (notched arrow) and trabecula (down arrow) bar = 100 µm. B) Control group in higher power bar = 50 µm. C) PEN exposed rats for 24 h showing thrombosis (star). D) PEN exposed rats 48 h showing marked apoptosis of lymphocytes in most of the white pulps of spleen (arrow). E) PEN exposed rats 30 days showing extra medullary haematopoiesis, formation of megakaryocytes (notched arrow). F) CPF exposed rats at 24 h showing vasculitis characterized by infiltration of the wall of blood vessels with inflammatory cells (star) and fibrinoid degeneration of tunica media (arrow). G) CPF exposed rats for 48 h showing hemosiderosis (arrow) and apoptosis of lymphocytes in some white pulps (notched arrow). H) CPF exposed rats 30 days showing vacuolation of tunica media in some blood vessels. H&E. bar = 50 µm

Discussion

The current study investigated the cytogenetic and genotoxic effect of commercial formulations of triazole fungicide (Topas-penconazole) and organophosphate insecticide (Clorzane-chlorpyrifos) on the bone marrow of rats by using CA assay and comet assay. The comet assay is used for the detection of DNA damage and genotoxicity of chemical agents (Sasaki *et al*., 1997; Tsuda *et al*., 1998; Undeger and Basaran, 2005).

DNA damage index was used for evaluation of the degree of DNA damage; it depends mainly on the percentage of DNA in tail, so it is considered as an accurate measure for the degree of DNA damage as reported by Dogan *et al*. (2011). In penconazole groups, the DNA damage index increased by 2.81, 3.55 and 2.75 folds at 24 h, 48 h and 30 days respectively compared with the control group in addition to other comet parameters, which are all indicators of DNA damage and expressed the genotoxic potential of PEN. The mechanism of DNA damage by PEN may be related to its ability to induce oxidative stress as reported by Chaâbane *et al*. (2015, 2017), who found that exposure of rats to PEN induced oxidative stress indicated by increased level of reactive oxygen species (ROS) and reduced levels of antioxidants; excessive production of ROS could damage critical molecules such as proteins, lipids and nucleic acid bases, and resulting in cell cycle inhibition and apoptosis (Perry *et al*., 2001; Barnham *et al*., 2004; Ting *et al*., 2010). On the other hand, the current results disagree with European Food Safety Authority (EFSA) Scientific Report (2008), which concluded that PEN has no genotoxic potential either in vitro or in vivo (bone marrow micronucleus test) studies but they did not use comet assay for this evaluation.

On the other hand, CPF increased DNA damage index by 2.87, 3.49 and 4.11 folds at 24 h, 48 h and 30 days respectively as compared with the control group. This result is in agreement with Goldoni *et al*. (2017), who found that exposure to fenthion organophosphate insecticide increased DNA damage index in peripheral blood cells of rats. Also, these results are in harmony with Ezzi *et al*. (2016), who found that exposure of rats to CPF in a dose of 3.1 mg/kg and 6.2 mg/kg for 4 weeks induced DNA damage in rat blood cells indicated by increased tail length. In addition, Rahman *et al*. (2002) recorded DNA damage in the lymphocytes of mice exposed to CPF. Yahia and Ali (2018) found that CPF induced extensive DNA damage in brain and liver of rats. CPF is bioactivated by a cytochrome P450- mediated desulfuration reaction to a highly potent cholinesterase inhibitor known as chlorpyrifos oxon, which might be responsible for DNA damage in exposed animals (Linn, 1998). Also, Braun *et al*. (1982) explained that OP compounds act as alkylating agents that cause alkylation of DNA bases and induce DNA damage. It was observed that longer time exposure to low doses of CPF produced extensive DNA damage than acute exposure this results is similar to that obtained by Mehta *et al*. (2008), who found that DNA damage index was increased in rats' brain and liver after exposure to CPF in a time related manner.

Chromosomal aberrations were detected in all exposed groups and the percentage of aberrant cells was significantly increased *($P < 0.01$)* at PEN and CPF exposed groups in comparison with the control groups. The most common types of aberrations in PEN groups represented as chromosome break, ring, attenuation, fragments and gap but chromatid deletion was observed at 30 days only. On the other hand, CPF exposed groups showed several types of structural CAs such as break, ring, attenuation, fragments, gap and deletion besides numerical aberration represented as polyploidy was observed at 24 h and 30 days.

Pfeiffer *et al*. (2000) stated that the majority of CAs are lethal to the cell and others may lead to oncogenic transformation by several mechanisms, as inactivation of a tumor suppressor gene or by generation of novel fusion proteins capable of initiating carcinogenesis. A direct relation was observed in this study between CAs and DNA damage in bone marrow cells of rats exposed to PEN or CPF. These results were similar to that obtained by Costa *et al*. (2015) they found a relation between CAs and % of DNA in the tail.

The current results may be related to the LD_{50} of each compound; PEN has higher LD_{50} (2000 mg/kg) compared with CPF (155 mg/kg) so the degree of DNA damage was higher in CPF group than PEN group at 30 days while the number of aberrant cells and types of aberrations were higher in CPF groups at 24 h, 48 h and 30 days.

Hematological changes were observed as transient decreases in RBCs indices after exposure to PEN for 24 h may be attributed to the acute toxic effect of PEN on the spleen, as shown by the histopathological examinations; which showed apoptosis in the white pulp accompanied with vascular changes as congestion of some blood vessels and thrombosis. The significant decrease in total WBCs count was attributable to the significant decrease in lymphocytes count, which may associate the apoptosis of lymphocytes in the spleen.

On the other hand, exposure to CPF produced significant decreases in RBCs count and hemoglobin concentration. These results agree with that obtained by Patil and Govindwar (2003) and Elsharkawy *et al*. (2013). Furthermore, WBCs count lymphocytes were decreased this result disagree with several researchers who stated that exposure to OP insecticides increased the WBCs count (Celik and Suzek, 2008; Celik *et al*., 2009; Elsharkawy *et al*., 2013; Ezzi *et al*., 2016), they found that exposure of rats to CPF increased the WBCs and lymphocyte count and they attributed this increase to the response of the immune system. The toxic effect of CPF on the spleen and lymphocytes stands behind the decreases in the measured hematological parameters.

Histopathological examination of spleen of rats exposed to PEN for 24 h showed apoptosis in white pulp accompanied with vascular changes. Marked apoptosis of lymphocytes was seen in most of white pulps of spleen in rats exposed to PEN for 48h. Apoptosis is a physiological process by which the cellular and tissue homeostasis are controlled; it removes the damaged or unwanted cells (Orrenius *et al*., 2011). It is a programmed cell death that is controlled at the biochemical level. Apoptosis is characterized by condensation of nuclear chromatin, DNA fragmentation, cell shrinkage and the formation of membrane-enclosed apoptotic bodies. Tanel and Averill-Bates (2005) stated that exposure to low concentrations of the toxicants can induce apoptotic cell death while exposure to higher concentrations will cause necrosis. Apoptosis arises mainly through two major signaling pathways that involve death receptors and mitochondria (Orrenius *et al*., 2015). Séïde *et al*. (2016) reported that thiabendazole fungicide activated both the mitochondrial and death receptor pathways of apoptosis in rat hepatocytes after 60 min.

In CPF groups, histopathological examination of spleen after 24 h from exposure showed angiopathic changes demonstrated with congestion, thrombosis and vasculitis, which became more obvious after 30 days. Apparently, it is potential that CPF caused severe toxic injury to capillary endothelium resulting to the development of widespread vascular lesions in various organs (Yadav *et al*., 2018). In the present study, hemosiderosis was observed at 48 h in white and red pulp associated with apoptosis of lymphocytes in some white pulps. These changes may be due to the suppressive effect of CPF on the immune system. These results are similar to another study of CPF toxicity in broilers showed disorganization of follicular patterns and congestion, cytoplasmic

vacuolation, degenerative changes as well as increased hyperplasia among reticular cells (Shahzad *et al*., 2015). Histopathological damage to spleen might be due to the inflection of nervous system results into altered production of lymphocytes, phosphorylation and oxidative damage (Galloway and Handy, 2003). Slight depletion of lymphoid cells in spleen clearly suggests that chlorpyrifos has a cytotoxic effect, so that immunosuppression may be a possible outcome of sub-acute chlorpyrifos intoxication. Also, these changes may be due to the effect of CPF exposure on oxidative stress.

Conclusion

The current study concluded that PEN and CPF induce severe DNA damage and chromosomal aberrations in bone marrow of rats. DNA damage index and the percentage of chromosomal aberrations are higher in CPF than PEN groups.

Conflict of Interest

The authors declare that they have no conflict of interest.

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