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Original Article

Genotoxicity evaluation of chlorpyrifos: a gender related approach in regular toxicity testing

Mansur A. Sandhu[1], Abdullah A. Saeed[1], Muhammad S. Khilji[1], Anwaar Ahmed[2], Malik Shah Z. Latif[3] and Nauman Khalid[4]

[1]Department of Veterinary Biomedical Sciences, Faculty of Veterinary and Animal Sciences, PMAS, Arid Agriculture University, Rawalpindi, 46300, Pakistan
[2]Department of Food Technology, Faculty of Crop and Food Sciences, PMAS, Arid Agriculture University, Rawalpindi, 46300, Pakistan
[3]Department of Biochemistry, Khawaja Muhammad Safdar Medical College, Sialkot, Pakistan
[4]Department of Global Agricultural Sciences, Graduate School of Agricultural and Life Sciences, 1-1-1 Yayoi, Bunkyo-ku, 113-8657, Tokyo, Japan

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ABSTRACT — The oral intubation of chlorpyrifos, an extensively used organophosphate insecticide, was tested for its capability to induce *in vivo* genotoxic upshot in blood lymphocytes of 24 male and female Wistar rats using biomarker of genotoxicity. Rats were orally administered with daily doses 3 and 12 mg/kg body weight (BW) of chlorpyrifos (CPF). The blood lymphocytes were harvested after 7 and 14 days of treatment and subjected to bi-nucleus (BN), multi-nucleus (MN) and single cell gel electrophoresis (comet assay) to evaluate the extent of DNA damage. Other than BN and MN assay, damage to DNA was assessed through comet length, height, area, head diameter, head DNA percentage and tail DNA percentage along with tail movement. A significant boost was noticed in the frequency of BN cells formation after 12 mg/kg BW CPF treatment. However, the propensity to produce MN cells was significantly more ($P \leq 0.05$) in males than that of females. Likewise, the frequency of comet formation, mean comet length, height and area were more ($P \leq 0.05$) in males than females even with 12 mg/kgBW. Comet head DNA % and tail length remained non-significant. Olive movement also revealed a significant increase ($P \leq 0.05$) in males than females. The study inferred that the CPF can induce DNA damage in both male and female subjects but more pronounced in the male individuals.

Key words: Chlorpyrifos, DNA damage, Gender, Comet, Multinucleus, Binucleus

INTRODUCTION

Pesticides are one of the known sources of environmental pollutants and have the ability to affect human and animal health other than insects through inhibition of acetylcholinesterases. Increased use of insecticide to sustain high yielding crops may lead to irreversible ecosystem damage (Reinecke and Reinecke, 2007) though; these chemicals have increased worldwide production of food and fiber. Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)-phosphorothioate] is a broad-spectrum organophosphorus and is effectively used against fire ants, turf, cockroaches, mosquitoes, termites, lice and fleas (Eaton *et al*., 2008). Chlorpyrifos (CPF) induces hepatic and immunological modulation, genotoxicity, embryotoxicity (Yin *et al*., 2009), a cholinesterase inhibitor and results in increase of acetylcholine in the neuronal junction (Jeyaratnam and Maroni, 1994). In rats, CPF is moderately toxic and its LD_{50} ranges from 95-270 mg/kg BW (Kamrin, 1997) and is a known anti-androgenic compound (Kang *et al*., 2004). Cometa *et al*. (2007) reported that in mice CPF dose at 12.5 mg/kg BW can induce inhibition of blood and brain acetylcholine. Among customary techniques to detect potential genetic and genotoxic effects, comet assay is one of the most promising and wide spreading tests. However, the formation of comet may be due to oxidative damage to various cells (Wielgomas and Krechniak, 2007) and there is a potential association

Correspondence: Mansur A. Sandhu (E-mail: mansoorsandhu@uaar.edu.pk)
Nauman Khalid (E-mail: nauman_khalid120@yahoo.com)

of long term use of CPF and carcinogenicity. Chlorpyrifos is reported to be one of heavily used insecticides in the USA, followed by its restricted use by the Environmental Protection Agency in June 2001 due to its exposure risk to children (Eaton *et al*., 2008). However, CPF is still used extensively for agricultural and house pest control. Therefore, an emergent concern over the occurrence of genotoxins (pesticides) in the food is important to proceed for its detection and mutagenicity in multiple ways for the protection of human and animals. Although the studies of acute toxic effects of CPF have been done earlier (Rahman *et al*., 2002; Jamil *et al*., 2004), but the results of these studies appear to be controversial (Bhaskar Gollapudi *et al*., 1995; Akcha *et al*., 2008). Since, there are only handfuls studies on multiple low doses and gender related manipulate of CPF. The current study used a time-honored genotoxicity evaluation animal model to compare gender differences in multiple low doses of CPF and DNA damage in rat's blood lymphocytes.

MATERIALS AND METHODS

Chemicals

For present study, chlorpyrifos (99.2%) was afforded by Four-Brother Pharmaceuticals Industry Ltd., Lahore, Pakistan. All other chemicals i.e. Dimethylsulfoxide (DMSO) was from Mallinckrodt (Mexico City, Mexico); Triton X-100 (Scharlau, Gato Perez, Spain); normal (NMA) and low melting point (LMP) agarose, ethylenediaminetetraacetic acid (Na_2EDTA) and Tris-HCl from Invitrogen (Carlsbad, CA, USA); sodium chloride (NaCl), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), methanol, May Grünwald-Giemsa and trypan blue from Merck (Darmstadt, Germany); ethidium bromide (EtBr) from MP Biomedicals, Inc (Fountain Pkwy, Solon, OH, USA),. Lymphocytes were isolated using Histopaque 1077 was obtained from Sigma, St. Louis, MO, USA. LMA and NMA were prepared in phosphate-buffered salt solution.

Animal husbandry and treatments

A number of 36, Wistar male and female rats of same age group (120 ± 10 days) and weight (250 ± 15 g) were procured from National Institute of Health, Islamabad, Pakistan. The rats were maintained at 25 ± 2°C, relative humidity (40-50%) with 12 hr dark/day cycle in separate stainless steel wire mesh rat cages and fed/ watered *ad libitum*. After 01 week of becoming accustomed, rats were divided into six groups of six rats each. In first four groups (male and female) rats were treated by oral intubation of CPF at a dose rate of 03 and 12 mg/kg BW. That is sub lethal dose and is equivalent to 1/67 and 1/17 respectively, according to the LD_{50} of CPF, since the oral LD_{50} of CPF in rats was evaluated as 200 mg/kg BW (Savithri *et al*., 2010). Group IV and V (male and female) served as control, in these two groups the rats received only vehicle (corn oil) by oral gavages. Prior to oral gavage, CPF was assorted with 0.5 ml corn oil. The rats were dosed on daily basis and sampling was carried out after 07 days of treatment and then after 14 days of treatment. All animal procedures were conducted in compliance with protocols approved by the PMAS Arid Agriculture University Institutional Animal Care and Use Committee.

Blood collection and lymphocyte isolation

A total volume of 2 ml blood was collected from anesthetized (inhalant anesthetic ether) animal by cardiocentesis at 07 days and 14 days post CPF exposure. The blood was straight away transferred to sodium-heparinized vacutainers. The blood lymphocytes were isolated by whole blood density centrifugation on Histopaque. The lymphocytes were auxiliary washed with PBS (pH-7.4) and sustained as 2.6 x 10^3/ml of MEM with 5% fetal bovine serum and 50 mg/ml of gentamicin for further use in comet. No mortality was observed throughout the experiment.

Analysis of nuclear abnormalities

For each CPF dose, earlier than seclusion of lymphocytes, whole blood smear slides were primed on new and cleaned glass slides. After fixation in methanol (10 min) the slides were air dried at room temperature and stained with 6% May Grünwald-Giemsa in Sorenson buffer (pH-6.9). Five slides were arranged for each animal; all the slides were coded and scored blind under 1000X magnifications. The frequency of bi-nucleated (BN) and multi-nucleated (MN) lymphocytes was scored until 100 lymphocytes / slide was observed and calculated as follows:

$$\text{BN/MN\%} = \frac{\text{Number of damaged cells}}{\text{Total number of cells}} \times 100$$

Single cell gel electrophoresis (Comet Assay)

The procedure followed the guidelines and general principles reported by Singh *et al*. (1988). However, the procedure was slightly modified according the recommendation of Smith *et al*. (2008). The 100 µl of HMP agarose 0.9% was placed on fully frosted slides. To prepare second layer, 25 µl of lymphocyte suspension was mixed with 0.8% LMP agarose and poured to form thin layer on previously formed layer of HMP agarose. During the polymerization of each gel layer, slides were kept on ice.

After gel solidification, the slides were fully immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4°C. After 1.5 hr, slides were washed with deionized water (3-4 times) and placed in chilled alkaline buffer (300 mM NaOH, 1 mM Na_2EDTA; pH-13) for 30 min at room temperature for DNA unwinding. Electrophoresis was accomplished on a horizontal electrophoresis platform in fresh and ice-cold alkaline electrophoresis buffer for 25 min at 320 mA. After electrophoreses the slides were engrossed in neutralization buffer (Tris–HCl; pH-7.5) and the neutralizing procedure was repeated three times for five minutes. All the steps for comet assay were performed under faint light and the electrophoresis tank was covered with black paper to shun additional DNA damage due to stray light. For production of positive control comets, a small number of lymphocytes from control group were treated with 150 µM of H_2O_2 for 1 hr at 4°C.

Staining procedure

Each slide was stained with 10% EtBr for 10 min. Subsequently, the slides were curved in chilled distilled water to take out excess EtBr, and the cover slips were sited over it. For scoring the slides were placed in a dark humidified chamber to prevent gel drying. The slides were scored within 5 hr of staining.

Scoring of slides

The slides were analyzed by using a Ceti Magnum-T epifluorescence microscope outfitted with an excitation filter of 460-550 nm. Microphotographs were taken with 40X objective. The parameters preferred for the quantification of DNA damage were the frequency of DNA damage (% cells), comet length and height (µm), comet area (μm^2), head diameter (µm), head DNA (%), comet tail DNA (%), tail length (µm), and olive tail moment (OTM) determining tail length and the fraction of total DNA in tail. The data was based on 500 erratically selected cells per group sample, i.e. 100 cells were from each animal.

Statistical analyses

Data is articulated as mean values ± S.D. (n = 5). For comet assay, five animals were considered as an experimental unit. For BN and MN assay of 500 cells per animal were analyzed. Statistical analysis was performed using two and three-way analysis of variance (ANOVA) to assess significant differences among treatment groups using SAS for Windows 2003 package program. If a divergence was found between the means the data was subjected to the Duncan Multiple Range Test (DMRt) to find out the significance of the means in consideration. For all the

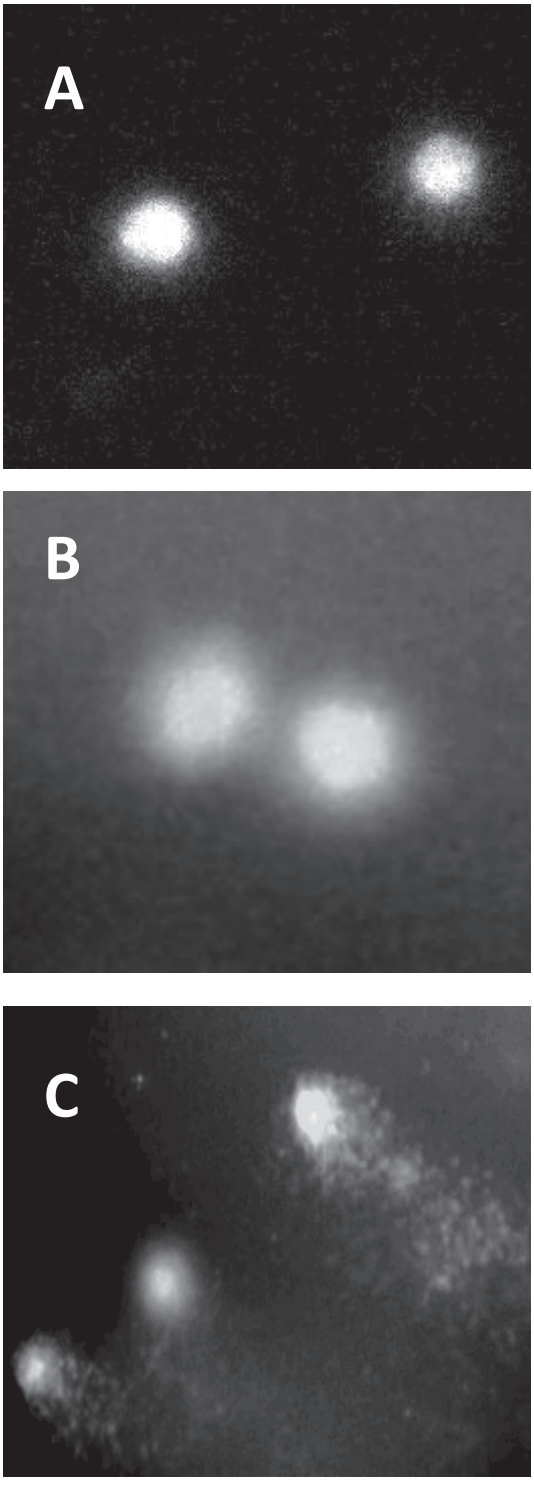

Fig. 1. Digitized images of lymphocytes with various degrees of DNA damage after *in vivo* chlorpyrifos treatment, Panel A: undamaged cells. Panel B: slightly damaged cells with diffused DNA. Panel C: damaged cells with comet.

experiments, the significance level was set at P≤0.05.

RESULTS

Bi-nucleus and multi-nucleus assay after CPF exposure

The outcome of BN assay was significant (P ≤ 0.05) with elevated dose rate of CPF. There was a greater generation of BN lymphocytes in both male and female rats due to exposure to 12 mg/kg BW treatment of CPF for 14 days than lower dose and control group (Table 1). The formation of BN cells was more in males with lower dose of CPF, however, non-significant values were observed in comparison to control group. Further, a significant (P ≤ 0.05) effect of duration was observed on induction of BN and MN lymphocyte development as given in Table 1. An interesting aspect was noticed that concentration and duration of pesticide use have almost similar effects on induction of MN lymphocytes in the blood circulation. The high-flying effect of CPF on the formation of MN was observed in male rats rather than females.

CPF exposure and blood lymphocyte Comet assay

The blood lymphocyte viability was above 90% after taking apart from blood and was accessed by trypan blue (data not given). The rats exposed to CPF showed a significant smash up to blood lymphocytes irrespective of the dose (Fig. 1), while, the damage was significantly higher (P ≤ 0.05) in males rather than females. However, the frequency of lymphocyte damage was more in CPF treated male as compared to female rats (Fig. 2A). The overall comet length was significantly high (P ≤ 0.05) in both male and female rats as compared to control showing a dose dependent relationship after 07 days of CPF exposure as shown in Fig. 2B. Males lymphocyte DNA was more prone to CPF induced shatter in terms of comet length with long-drawn-out dosage of CPF (Fig. 2B). Comet height was significantly higher (P ≤ 0.05) in male rats after 7 days of CPF exposure (12 mg/kg BW) and was least in low dose CPF treated rats irrespective of their gender (Fig. 2C) along with control group. Similarly, to comet length, the comet height was lesser (P ≤ 0.05) in females as compared to males even with amplified dose treatment (Fig. 2C). The comet area and head diameter were higher (P ≤ 0.05) in male rats treated with CPF for 14 days at the dose level of 12 mg/kg BW as given in (Figs. 2D and E) respectively and remained non-significantly lesser subsequent to 7 days treatment of CPF at the dose rate of 3 mg/kg BW.

Comet head DNA % and comet tail length remained non-significant at both CPF dose levels irrespective of gender and treatment duration while, control male and female rats lymphocytes showed a significant decrease in comet tail length (Figs. 3F and G). The tail DNA% was

Table 1. Frequencies of bi-nucleus and multi-nucleus lymphocyte development in the female and male rat blood subsequent to *in vivo* chlorpyrifos exposure for 7 and 14 days.

Exposure Agent	Dosage (mg/kg BW)	Days of exposure	Frequency of BN cells (%)	Gender	Frequency of MN cells (%)
Control	0	07	0.18 ± 0.003^{B}	Female	05.10 ± 1.42^{C}
			0.15 ± 0.012^{B}	Male	04.94 ± 1.36^{C}
		14	0.16 ± 0.002^{B}	Female	09.25 ± 2.16^{C}
			NIL	Male	07.42 ± 2.42^{C}
Chlorpyrifos	03	07	0.10 ± 0.021^{B}	Female	46.92 ± 8.92^{B}
			0.11 ± 0.002^{B}	Male	59.83 ± 7.99^{A}
		14	0.15 ± 0.007^{B}	Female	48.46 ± 7.32^{B}
			0.48 ± 0.032^{AB}	Male	63.70 ± 6.73^{A}
	12	07	0.29 ± 0.045^{B}	Female	45.50 ± 7.47^{B}
			0.23 ± 0.031^{B}	Male	69.36 ± 8.51^{A}
		14	1.83 ± 0.182^{A}	Female	42.98 ± 6.09^{B}
			1.95 ± 0.126^{A}	Male	68.50 ± 4.97^{A}

All data are shown as average median values (n = 5) ± S.D.. $^{A-C}$ Letters indicate statistically significant differences between the various doses & the comparison was made between control and chlorpyrifos treated rats, LSD = P ≤ 0.05. BW = body weight; BN = Bi-nucleus lymphocytes; MN = Multi-nucleus lymphocytes.

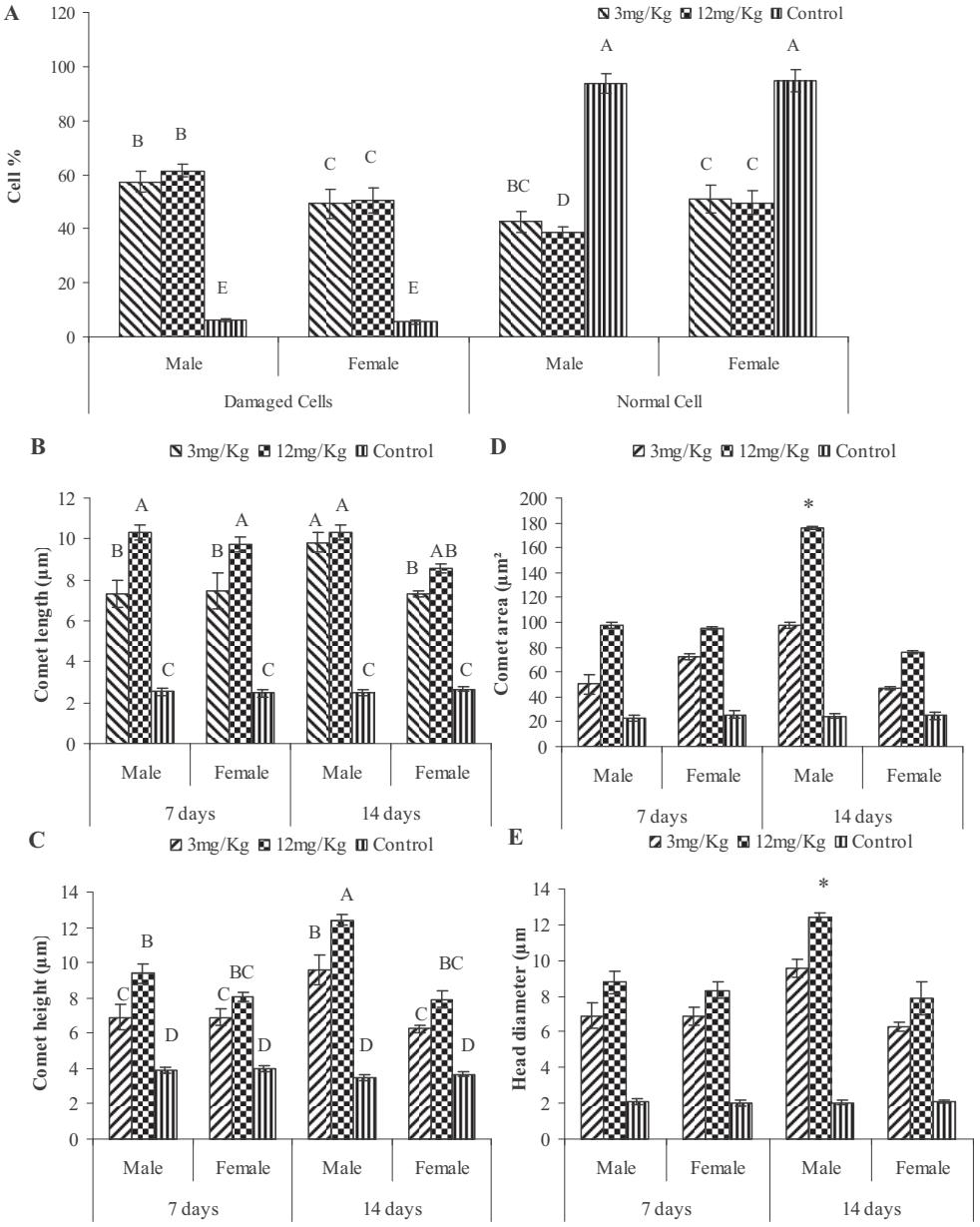

Fig. 2. Effects of *in vivo* 3 and 12 mg/kg BW chlorpyrifos exposure in male and female rats after 7 and 14 days of treatment: (A) Overall means of lymphocyte damage after exposure of two dose levels (B) Comet length (C) Comet height (D) Comet area (E) head diameter; in both genders after 7 and 14 days of treatment. Error bars represent standard deviations (S.D.). *($P \leq 0.05$) vs. control counterparts. A-E; similar alphabets do not differ significantly ($P \leq 0.05$).

appreciably more ($P \leq 0.05$) in both male and female rats after 14 days of 12 mg/kg BW CPF exposure as given in Fig. 3H. The OTM was significantly more in males rather than female subjects and the major blow was by high dose rate and prolonged dosage (Fig. 3I). The overall comet analysis showed a dose related increase in DNA damage and pronounced concern of CPF treatment in male rats.

DISCUSSION

The present study results indicate a significant increase in cytotoxic damage of blood lymphocytes associated

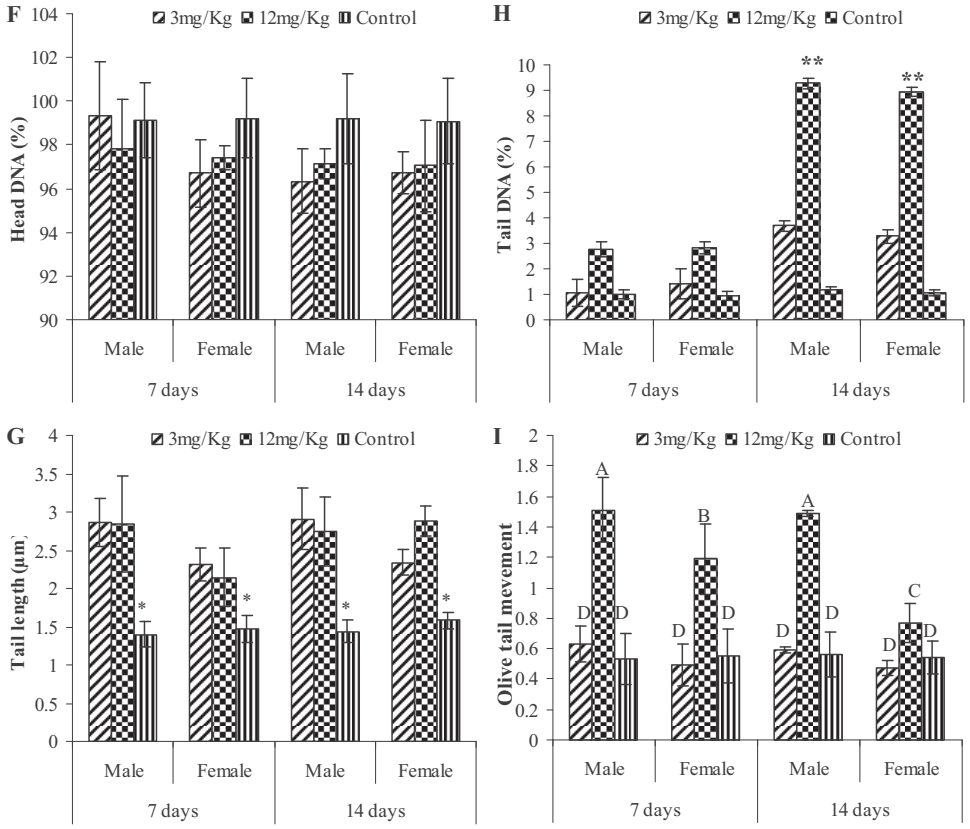

Fig. 3. Effects of *in vivo* 3 and 12 mg/kg BW chlorpyrifos exposure in male and female rats after 7 and 14 days of treatment: (F) Comet head DNA percentage (G) Comet tail length (H) Comet tail DNA percentage (I) Olive movement; in both genders after 7 and 14 days of treatment. Error bars represent standard deviations (S.D.). * ($P \leq 0.05$) vs. control counterparts. A-D & **; similar alphabets and signs do not differ significantly ($P \leq 0.05$).

with exposure to CPF via oral gavages. The genotoxic effects of pesticides / soil pollutants have been reported in several organisms, such as human (Bhalli *et al*., 2006) mice (Rahman *et al*., 2002), fish (Ali *et al*., 2009) and amphibians (Yin *et al*., 2009). In the present study, CPF expressed a dose-dependent effect on lymphocyte BN and MN frequency. Lymphocytes showed an unmitigated damage in males than that of females and this may be due to the fact that female lymphocytes are less sensitive to the oxidative stress-induced DNA injury. CPF cytogenetic toxicity has been detailed in previous studies both *in vitro* and *in vivo*. Amer *et al*. (1996) reported CPF induced chromosome aberrations in mouse spleen cell cultures and increased MN cells in rat bone marrow when treated with CPF (Muto *et al*., 1992). However, the information on the effect of multiple lower doses and its relationship with gender is missing or very less. In the cell, free radicals are produced by pesticides and persuade modification in oxygen free radical (OFR) scavenging enzymes (EL-Shenawy *et al*., 2011) however, Mendoza-Nuñez *et al*. (1999) reported non-significant DNA smash up in subjects with normal levels of antioxidants. It is noteworthy, that the augmented creation of MN lymphocytes in the current trial may be the reason of elevated OFR production caused by CPF. Along with MN assay, single cell gel electrophoreses is known to be an excellent and well-situated method of DNA damage. The alkaline Comet assay (pH > 13) can detect DNA damage, i.e. single / double strand breakage (Hartmann *et al*., 2003). Therefore, it put forward a considerable advantages over other cytogenetic methods like chromosome aberrations and micronucleus test because comet assay do not need the cells to be mitotically active (Buschini *et al*., 2003). This could be quoted as potential motive for extensive use of alkaline comet assay in the fields of genetic toxicology and environmental bio-monitoring (Lee and Steinert, 2003). With this

comet assay, our results showed a dose related response of lymphocyte damage at lower and even at higher doses of CPF. Comet length, height, area and head diameter was lower in females rather than males blood lymphocytes. The DNA damage in the comet development could have originated from DNA single strand breaks, double strand break, adduct formations, DNA–DNA and DNA–protein cross-links (Mitchelmore and Chipman, 1998). This could be attributed to the interface of pesticides or their metabolites with DNA (Fairbairn *et al*., 1995). The most plausible reason could be that, H_2O_2 levels remains high in males as compared to the females. Additionally, glutathione, an intracellular antioxidant, is higher in concentration in female hepatic mitochondria than in case of male (Viña *et al*., 2005). Another reason of amplified injury in male DNA may be due to the presence of androgens. Furthermore, this androgen enhances apoptotic damage, as stated by Ling *et al*. (2002) in human vascular epithelial cells those were cultured in serum-free conditions. There are studies those report enhanced hepatic mitochondrial antioxidant capacities via estrogen receptors causing lower H_2O_2 production along with increased antioxidant enzyme expression (Borrás *et al*., 2003). Collectively, these studies showed enhanced antioxidant capacity in females, suggesting a potential biological basis for gender differences in the contest of DNA damage.

There was a significant ($P \leq 0.05$) increase in tail DNA % and OTM clearly showing a higher DNA damage in males as compared to females. Increased number of comets in the presences of CPF can make males more compromising in animal/human health. DNA damage is considered a crucial mechanism in cancer development (Weinstein, 1988) and individuals showing high DNA damage and/or having cells with high spontaneous or induced DNA damage are more prone to cancers.

In conclusion, the study has exposed the consequences of CPF exposure on gender-related distinction of DNA damage. The DNA breakage was elevated in the males than that of females. It will be of immense interest to see how the data compare with epidemiological studies on different cancers caused by population. The results of this study may improve our understandings that how sex hormones interact in the presence of pesticides.

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