



Toxicity evaluation of pesticide chlorpyrifos in male Japanese quails (*Coturnix japonica*)

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Abstract

In the current study, chlorpyrifos was used as a test chemical to evaluate its possible toxicological effect on birds. A total of 45 adult male Japanese quails were divided into five groups (A to E). Each group, containing 9 birds was further divided into 3 sub-groups (containing 3 birds each). Group A served as control, while all other groups and sub-groups were exposed to selected pesticide for different trial periods. Chlorpyrifos sub-lethal doses were orally administered daily at the rate of 3, 6, 9, and 12 mg/kg body weight per day to group B, C, D, and E, respectively. Birds were kept under observation for behavioral changes throughout the trial periods. Clinical signs, histological alterations, genotoxicity, and blood biochemical alterations were recorded after each 15-day trial. Mild to moderate clinical signs like staggering gait, tremors, diarrhea, dullness, less frequency of crowing, and decrease foam production were observed in group D and E throughout the study. The changes in the body weight gain and blood biochemical parameters among different groups at a given trial period were insignificant. The appearance of micronuclei in group E birds was more significant, indicating that nucleus damage was dose-dependent while to lesser extent duration-dependent. The comet assay showed significant dose- and duration-dependent DNA damage among various groups. In comparison with control group, extensive histological degenerative alterations in the liver, testes, and kidneys were observed in birds of group D and E, where mild to severe alteration like congestion, vacuolation, necrosis, apoptosis, karyopyknosis, extensive degeneration, and alteration in many cellular structures were noticeable.

Keywords Genotoxicity · Histopathology · Hematology · Non-targeted organisms

Introduction

Chlorpyrifos (CP) (IUPAC name, O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) is a broad spectrum, abundantly used chlorinated organophosphate insecticide (Solomon et al. 2014; Eaton et al., 2008) that provides broad spectrum protection to crops from insects. The use of CP was

allowed in the USA in 1965 for pest control. It is available in granular and flowable (liquid) formulations with many local names such as Dursban and Lorsban (Moore et al. 2014).

The discovery that organochloride pesticides such as dichlorodiphenyltrichloroethane, are highly persistent that can accumulate in food chains and severely affect the whole population of almost all species of wildlife, has led to ban and

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restrict in their uses (Kammon et al. 2010). Some studies have indicated that residues/metabolites of CP have caused soil, surface water, and groundwater contamination at many places around the globe. It has been found that CP is a persistent soil contaminant that stays active for long time (Moore et al., 2014). In spite of moderate persistence in the environment, many organophosphorus insecticides are toxic to non-targeted organisms, and their accumulations in invertebrates, fishes, and birds have been reported. However, owing to their high efficacy, rapid biodegradability, and comparative shorter persistence in the environment, this chemical has given preference over organochlorine pesticides (Varo et al. 2002). Among organophosphates, CP is an ideal insecticide and a substitute of much virulent organophosphorus insecticide, methamidophos (Zhou et al. 2007). However, it is a well-known fact that the extensive usage of any pesticide leads to environmental pollution (Costa et al. 2013). Water bodies are polluted through drainage from farms, drifted by the wind, illegal, and accidental release of a given contaminant (Aldenberg et al. 2002). The Environmental Protection Agency, USA, reports published in 2006 and 2014 reveals that residues of the hazardous chemicals are even present on food commodities if applied in agriculture (Karanth and Pope 2000).

Birds have unique role in ecosystem as they are vital for functional ecosystems and are good indicator of it. Birds are the part of food chain of many ecosystem as well as play important role in pollination, seed dispersal, restoration, and recolonization of disturbed ecosystem (Şekercioğlu et al. 2004). Avian population is on constant decline because of conversion of their natural habitat for agriculture purposes. The uses of pesticides to get more yield from agriculture further deteriorated birds population (Mitra et al. 2011). Pesticides enter into bird's body through contaminated food, water, and even direct dermal exposure. Therefore, birds have been found to be more at risk, because they could forage in pesticides-treated area (Kalender et al. 2010).

A number of experimental studies have reported that acute and chronic exposure of birds to pesticides including organophosphate has adverse effect or even kill the birds immediately. The adverse effects of pesticides on the birds are behavioral, reproductive, and developmental that retarded their growth. Endocrine disruption, neurological changes, gastrointestinal problems, immunological response deficit, oxidative stress, and pathological lesions in different tissues are the other alteration observed among different birds (Orris et al. 2000; Solomon et al. 2014). It has also been reported that pesticides like CP affect acetylcholinesterase activities leading to acetylcholine accumulation at nerve terminals and neuromuscular junctions which results in cholinergic overstimulation (Testai et al. 2010).

Birds have also nearly undetectable levels of paraoxonase 1 and are therefore, more sensitive to this chemical than mammals (Costa et al. 2013). Insecticides degrade into small fragment in the environment (air, water, and soil) and to

metabolites inside organism bodies. These fragments/metabolites have been found to be more toxic than their parental compounds that adversely affect even the non-targeted organisms. It has been reported that CP biologically transforms inside the animal bodies into chlorpyrifos-oxon, mainly in the liver which is the main site for detoxification of xenobiotics in animal's body. Different polymeric forms of cytochrome P450 (CYP) enzymes such as CYPs 2D6, 2B6, 3A5 and 3A4, and esterases present in liver cells participate in this bio-activation and detoxification process (Costa, 2006; Mutch and Williams 2006). The CYP 2B6 enzyme replaces the sulfur group with oxygen converting chlorpyrifos to chlorpyrifos-oxon (Costa 2006). This most toxic metabolite of chlorpyrifos is further detoxified into diethyl phosphate and 3,5,6-trichloro-2-pyridinol. However, even if CP is not converted to its oxon form, its hydrolysis results in the formation of 3,5,6-trichloro-2 pyridinol and diethyl thiophosphate which are the more toxic product of CP metabolism than oxon (Mehta et al. 2008).

In experimental animals, the dermal or oral inhalation of CP has led to quick appearance of toxic symptoms from mild to severe such as salivation, nausea, headache, sweating, vomiting, diarrhea, blurred vision, muscle weakness, or paralysis, and even can lead to death due to inability of the lungs to cope with its toxicity (Wessels et al. 2003).

The decline of quail natural population from the last three to four decades in the South East Asian countries like Pakistan could be attributed to extensive use of pesticides like CP. The present study was therefore designed to assess possible toxicological effects of chlorpyrifos on quail's general health, physiology, body weight, blood, tissues, and DNA.

Materials and methods

Experimental design

The study was approved from the ethical committee of the University of Peshawar (No: 10/EC-16/Pharm), and the ARRIVE guidelines of in vivo animal studies were followed. A total of 45 sexually mature (adult) male Japanese quails, free of any apparent ailment, were procured from the local farm. The quails were divided into five groups (A to E) and reared in wooden wired bird cages for one-week acclimatization and 45-day experimental trial period under similar housing and management conditions. Each group, containing 9 birds, was further divided into 3 sub-groups (each containing 3 birds). Group A and its sub-groups served as a control, while the rest of the groups were exposed to selected pesticide intoxication. Each exposed group was given repeated doses of CP-mixed feed (poultry feed NO-3) for the entire trial periods. The lethal dose (LD₅₀) for the Japanese quail is about 15 mg at 94.5% purity (Celik et al. 2009). The CP

38.5% w/w sub-lethal doses were daily administered orally at the rate of 3, 6, 9, and 12 mg/kg body weight per day, to group B to E, respectively. The 45-day trial was subdivided into three trial periods of 15 days each. Birds were kept under observation for abnormal physical or behavioral changes throughout the trial periods.

Blood and tissues collection

Blood samples from birds of all groups were collected directly through the 3-mL disposable syringes from the jugular vein and were inserted into EDTA vacutainer tubes (light blue ATLAS-LABOVAC Italiano, K3EDTA, 3 mL). The blood was processed in 4 h for estimation of genotoxicity (comet and micronucleus assay) and 24 h for hematological parameters. For histopathological study, visceral organs (tissues) such as the kidneys, liver, and testes were collected and preserved in 10% neutral buffered formalin for a minimum of 72 h (Yadav et al. 2018). The remains of the scarified animals were disposed of according to the guidelines provided by the ethical committee of the University of Peshawar.

Body weight and hematology

Birds were weighed individually each on digital electronic balance at the start and end of each treatment period. While hematological parameters were estimated through automated hematology analyzer (Sysmex Corporation Japan, Model KX-21, Serial NO-8343).

Genotoxicity

Two types of assays were applied for DNA damage assessment, i.e., (i) micronucleus assay and (ii) comet assay.

Micronucleus assay

For the observation of micronuclei in quail's RBCs, the procedure of Yin et al. (2009) was followed. For the observation of micronuclei in the RBCs, thin smears were made from each blood sample on separate slides and air-dried at room temperature. The slides were then immersed in 10% Giemsa stain for 45–50 min. After rinsing with tap water or phosphate-buffered saline, slides were air-dried and observed under a light microscope (Olympus DP71, U-CMAD3 Japan, oil immersion lens, 100/1.25). A total of 1000 randomly selected erythrocytes per sample were examined for the presence of micronuclei in the RBCs.

Criteria for the identification of micronucleus were as follows:

1. Having no or slight connection with the main nucleus
2. Sharing the same color

3. Area smaller than one-third of the main nucleus

Calculation of micronucleus frequency was determined using the following equation:

$$\text{MN}\% = \frac{\text{Number of cells containig micronucleus}}{\text{total number of cells counted}} \times 100 \quad (1)$$

Comet assay (single cell gel electrophoresis)

The protocol of Siraj et al. (2018) was followed for the comet assay with minor modifications. First, single-layered slides were made ready by dipping in melted NMPA (normal melting point agarose) in the Coplin jar and immediately taken out to place in a tray until drying. The backside of the slide was cleaned from the agarose with the help of another slide. All these first layered slides were then kept in the slide box until further use.

In the second layer, a mixture of 75 μL of LMPA (1%, low melting point agarose) and 15 μL of blood was layered over the coated slides, using a micropipette. Coverslips were placed, and the slides were then immediately transferred to an ice pack or refrigerator for quick and complete solidification (5 to 10 min).

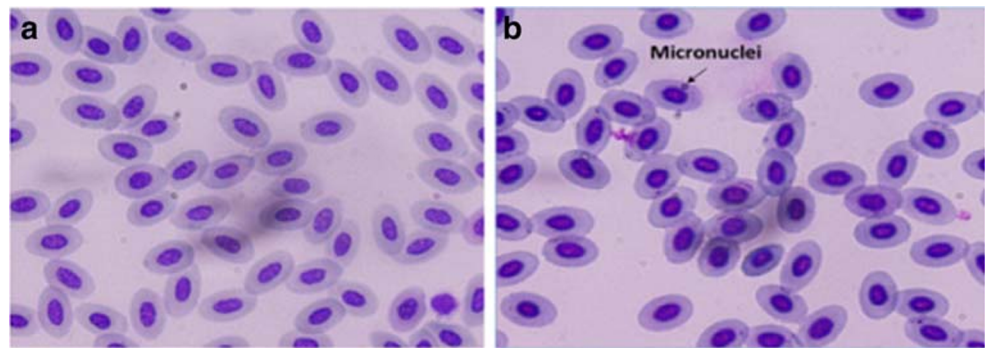
For the third layer formation, the coverslips were gently removed and a melted LMPA of about 85 μL was added to the slides by micropipette, and coverslips were placed again over it. The slides were then immediately transferred to an ice pack or refrigerator for quick and complete solidification (5 to 10 min).

For immersing the slides in the final lysing solution, the coverslips were gently removed, and the slides were slowly and gently placed in the freshly made final lysing solution in the glass tray. The tray was put in the refrigerator for about 2 h or overnight at $\sim 4^\circ\text{C}$.

For the electrophoresis, the processed slides were placed in a horizontal gel box filled with freshly made electrophoresis buffer (pH > 13), until the covering of the slides. The slides were left in the buffer for about 20 min at $\sim 4^\circ\text{C}$ (in the refrigerator) in order to unwind the DNA and express the alkali-labile sites. The power supply was then adjusted to 300 mA current (25 V) for 25 min. The power supply was then turned on. Soon after the completion of electrophoresis, the slides were neutralized with neutralization buffer. The slides were shifted to cold ethanol (100%) for 20 min and then air-dried.

Before staining, the rehydration of slides was done in chilled distilled water for 30 min. About 70 μL acridine orange (20 $\mu\text{g}/\text{mL}$) was applied to each slide using a micropipette. The slides were left in the stain for about

Fig. 1 RBCs stained with Giemsa of the representative groups of birds. **a** Group A. **b** Group E (45 days exposure). An arrow indicating micronuclei within RBC



5 min. Edges and back of the slides were blotted away before observation under a fluorescent microscope. Fluorescent microscope (Nikon Eclipse 80 i), equipped with an excitation filter of 450–490 nm was used for observing the slides. The images of 100 randomly selected cells were taken at $\times 400$ in each slide. The comet tail length was measured as the degree of DNA damage. Cells having no tail were put in class 0, which means no tail and any damage. Cells having tail length up to 1.5 times the diameter of the comet nucleus were put in class 1. Those cells having tail length up to 2 times the diameter of the comet nucleus were put in class 2. Cells having a tail length up to 2.5 of the nucleus diameter were put in class 3. Cells having mostly total DNA in the tail were put in class 4 (maximum damage). A final and overall total comet score was obtained by summing up different comets in each class for 100 cells/sample (Collins, 2004).

Histopathology

After collection, the liver, kidneys, and testes were immediately transferred to 10% neutral buffered formalin, which were left over for a minimum of 72 h in the fixatives (Song et al. 2012). Conventional paraffin wax embedding method (Sadique et al. 2012) was adopted for the preparation of tissue slides.

After processing, staining, and drying, sections of the tissue were observed under the compound light microscope with a digital camera (Olympus DP71, U-CMAD3

Japan). Tissues were examined for microscopic lesions and structural alterations. Photomicrographs were obtained using different lenses of varied magnification power as per following details: scanning lens ($\times 4$), low power lens ($\times 10$), high power lens ($\times 40$), and eye piece having $\times 10$ magnification. Medium and higher resolution pictures were presented.

Statistical tests such as Student's *t* test (two-tailed) and ANOVA with Tukey's HSD post hoc were applied to determine the degree of significance of the data. For multiple comparisons, the SPSS version 21 was used.

Results and discussion

Morbidity, mortality, clinical signs, and behavioral alterations

In the present study, birds of group A (control) remained healthy and active, and did not show any clinical signs. No mortality occurred in any experimental group. Birds of groups B and C remain healthy and active like the control. However, from mild to moderate clinical signs like staggering gait, tremors, diarrhea, dullness, less frequency of crowing, and decrease foam production were observed in groups D and E throughout the study (Fig. 1). Al-Badrany and Mohammad (2007) also reported similar changes in chickens that were exposed to CP. Sánchez-Amate et al. (2001) reported linkages of all these signs with the

Table 1 Body weights (g) of control and exposed groups

Days	Groups				
	A	B	C	D	E
15	127.4 \pm 11.8	153.1 \pm 15.9	144.8 \pm 7.3	142.1 \pm 7.3	147.4 \pm 8.7
30	141.8 \pm 4.9	134.3 \pm 20.4	149.3 \pm 9.7	147.7 \pm 5.4	150.0 \pm 9.9
45	134.8 \pm 3.7	156.8 \pm 3.4	153.6 \pm 8.8	147.1 \pm 8.3	145.4 \pm 10.2

Values (Mean \pm SD) in each row have non-significant differences ($P > 0.05$) as compared with the control group. Group A served control, while groups B, C, D, and E were administered chlorpyrifos at 3, 6, 9, and 12 mg/kg bw/day, respectively, with different exposed periods

Table 2 Testes weights (g) of control and treated groups

Days	Groups				
	A	B	C	D	E
15	3.7 ± 1.2	4.6 ± 0.9	4.0 ± 0.4	3.3 ± 0.5	4.3 ± 1.9
30	4.3 ± 0.6	3.7 ± 0.1	3.4 ± 0.4	4.0 ± 0.9	3.5 ± 2.8
45	4.5 ± 0.9	4.1 ± 0.1	4.6 ± 0.4	3.6 ± 0.4	4.2 ± 0.6

Mean results with ± SD. Non-significant differences ($P > 0.05$) were observed in testes weight of all the treated and control groups

inhibition of cholinesterase. As mentioned above, CP converts to chlorpyrifos-oxon inside the body of living organisms. This oxon metabolite elicits AChE inhibition, leading to the acetylcholine build up in the cholinergic receptors, hence cause cholinergic toxicity (Eaton et al. 2008).

Thus, the clinical and behavioral signs observed in the current study could, therefore, be linked to the cholinergic toxicity caused by metabolites of the selected pesticide.

Body weight changes

The changes in the body weight (Table 1) in different groups at different time intervals were found insignificant, which was in accordance with the result of Joshi et al. (2007), in which also the body weight of rats did not show any significant change when exposed to different doses of CP. However, the current results were in not in agreement with some other studies (Barna-Lloyd et al. 1990; Ambali et al. 2010a), where the researchers have shown a dose-dependent decrease in body weight of mice and rats treated with CP.

Table 3 Hematological profile of control and treated groups

Parameter/days	Groups				
	A	B	C	D	E
RBCs (103/μL)					
15	2.9 ± 0.4	2.8 ± 0.1	2.8 ± 0.5	2.9 ± 0.4	3.1 ± 0.1
30	2.9 ± 0.6	3.1 ± 0.1	3.4 ± 0.3	3.4 ± 0.1	3.0 ± 0.4
45	3.6 ± 0.1	3.1 ± 0.7	3.5 ± 0.1	3.7 ± 0.4	3.7 ± 0.2
HB (g/dL)					
15	13.9 ± 2.2	13.9 ± 0.5	14.7 ± 1.5	15.4 ± 1.1	15.3 ± 0.3
30	14.8 ± 2.4	15.6 ± 0.4	16.5 ± 1.6	15.9 ± 0.9	15.4 ± 1.5
45	17.4 ± 0.7	14.2 ± 4.1	17.2 ± 0.2	18.3 ± 1.2	17.1 ± 0.1
HCT (%)					
15	43.4 ± 4.6	39.7 ± 9.4	43.3 ± 5.2	45.0 ± 4.6	48.1 ± 1.1
30	48.0 ± 9.0	49.6 ± 2.5	52.9 ± 5.1	52.3 ± 3.4	50.3 ± 5.4
45	53.7 ± 1.2	42.9 ± 8.5	52.2 ± 1.7	54.7 ± 3.0	53.0 ± 1.1
MCV (fl)					
15	149.0 ± 8.5	141.8 ± 35.9	156.8 ± 15.4	150.6 ± 4.8	154.0 ± 2.5
30	162.5 ± 4.9	159.4 ± 9.9	154.1 ± 2.8	153.9 ± 3.4	166.3 ± 8.8
45	147.5 ± 3.1	140.4 ± 4.1	146.9 ± 1.4	145.1 ± 9.4	143.3 ± 5.9
MCH (pg)					
15	47.7 ± 1.3	49.8 ± 1.4	53.3 ± 4.6	51.7 ± 4.7	48.5 ± 0.4
30	50.2 ± 2.8	50.2 ± 1.3	47.9 ± 1.5	46.9 ± 0.7	50.9 ± 2.2
45	47.8 ± 0.2	45.7 ± 2.8	48.4 ± 0.4	48.5 ± 2.2	46.4 ± 2.5
MCHC (g/dL)					
15	32.2 ± 2.6	36.7 ± 9.7	34.1 ± 2.6	34.2 ± 2.1	31.5 ± 0.2
30	30.8 ± 0.9	31.5 ± 1.2	31.1 ± 0.4	30.5 ± 0.2	30.6 ± 0.7
45	32.5 ± 0.8	32.6 ± 2.9	32.9 ± 0.5	33.4 ± 0.7	32.4 ± 0.4
PLT (× 10³/μL)					
15	18.7 ± 7.1	76.3 ± 39.7	66.0 ± 43.0	36.3 ± 20.4	22.5 ± 7.5
30	13.0 ± 19.9	15.0 ± 5.6	9.0 ± 1.7	10.0 ± 4.0	6.7 ± 3.7
45	3.7 ± 4.7	2.0 ± 0.0	3.7 ± 2.9	3.3 ± 2.1	2.0 ± 2.0
RDW(fl)					
15	36.8 ± 4.3	76.9 ± 49.8	82.5 ± 42.8	61.8 ± 33.9	37.3 ± 0.1
30	69.43 ± 9.0	97.7 ± 24.4	62.3 ± 10.0	66.8 ± 5.8	66.0 ± 10.9
45	34.3 ± 7.3	57.8 ± 0.0	30.3 ± 4.3	36.8 ± 0.0	36.1 ± 6.4

RBC, red blood cells; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelets; RDW, red blood cells distribution width

Statistical analysis of the hematological profile of the controlled and treated groups shows non-significant ($P > 0.05$) differences in all groups

Effect on testes weight

The mean testes weight with \pm SD of all the control and treated groups are given in Table 2. The relative mean weight of testes showed non-significant ($P > 0.05$) changes in all the treated groups throughout the trial period. Similar finding have been reported by Akhter et al. (2009), where the organ's weight was not affected by the exposure of CP at the rate of 9 mg kg/d (in rats). Although in chickens, a reduction in the relative weight of many organs including the testes and body weight has been observed but that was linked to the reduction of feed intake by them (Naraharisetti et al. 2009). The decrease in testicular weight observed in some studies was linked to the loss of spermatogenic elements and spermatozoa (Akhtar et al. 2009). The contradictions in observations mentioned above maybe due to the differences of amount intake, methods used, animal body weights, etc. In this study, a specified amount (dose) have been mixed with small amount of water and then with proper amount of feed, which may have reduced the chemical toxicity due to dilution, or the birds might have not consumed sufficient chemical-mixed feed because of taste aversion.

Effect on blood parameters

The results of the hematological profile of the control and treated groups are shown in Table 3. Variations in numerical values of some parameters were recorded. However, these variations were statistically insignificant ($P > 0.05$) when compared with control group. Also, the variations did not appear to be dose- or duration-dependent. However, Akhtar et al. (2009a) observed significant effects of CP on many blood parameters such as a reduction in RBCs, hemoglobin, hematocrit, and an increase in WBCs count. The dysfunction of the hematopoietic system was considered to be as the result of changes in hematological parameters as pointed out by Morowati (1998). Ambali et al. (2010b) demonstrated that changes in hemo-concentration (elevation in Hb, PCV, and RBCs count) in CP-treated mice was due to mild diarrhea. In another study rupturing of neutrophil observed was thought to be due to its phagocytic activity during xenobiotic intoxication, leading to a reduction in neutrophil counts (Savithri et al. 2010). The effect on blood parameters did not appear to remain the same in all studies, which may be because of the possible factors discussed above.

DNA damage assessed trough micronucleus test

Table 4 indicates the mean frequencies of micronuclei in Japanese quail's 3000 RBCs (1000 RBCs/slide/sample). The highest mean frequencies of micronuclei were observed in group E administered with the highest dose. Table 4 also shows the detail of the mean values of micronuclei

Table 4 Micronuclei observed in Japanese quail's RBCs (1000 RBCs/slide/sample)

Days	Groups				
	A	B	C	D	E
15	0.6 \pm 0.5	2.0 \pm 1.0	2.3 \pm 0.6	2.3 \pm 1.1	2.6 \pm 0.6*
30	1.0 \pm 1.0	2.3 \pm 0.6	2.3 \pm 1.5	3.0 \pm 1.0	4.3 \pm 1.5*
45	1.7 \pm 1.5	3.0 \pm 1.0	3.0 \pm 1.0	4.0 \pm 1.0	6.3 \pm 1.5*

Mean results with \pm SD. Significant differences * $P < 0.05$ were observed in only birds of group E. While values in the control (A) and all other treated groups (B, C, and D) were non-significant ($P > 0.05$)

frequencies in each respective group. The mean frequencies were found to increase with the increase in dose and duration of exposure in all groups throughout the experiment. Mean frequency and significant results ($P < 0.05$) of micronuclei were observed for group E, administered with the highest dose throughout the experiment (Fig. 2). The increase in mean frequency of micronuclei was also observed in groups B–D. However, the values of all other groups except E were found statistically insignificant $P > 0.05$. This finding indicated that CP near to sub-lethal doses is genotoxic and causes permanent DNA damage in the target cells like RBCs that appears in the form of micronuclei (Fig. 1). Similar findings have been reported in an amphibian RBCs by exposure to CP (Yin et al., 2009).

DNA damage assessed through comet assay (15, 30 and 45 days' trial)

The mean comet tail length in the RBCs of the control and exposed birds are shown in Tables 5, 6, and 7. The tail length was manually measured as a degree of DNA damage. Table 5 shows non-significant DNA damage ($P > 0.05$) in the control and exposed groups, except from group E (highest dose). Significant ($P < 0.05$) DNA damage was only observed in birds of group E. Table 6 shows the results of the 30-day trial; significant DNA damage has been observed in all treated groups except group B (lowest dose), which was found non-significant ($P > 0.05$). Table 7 shows the results of the 45-day

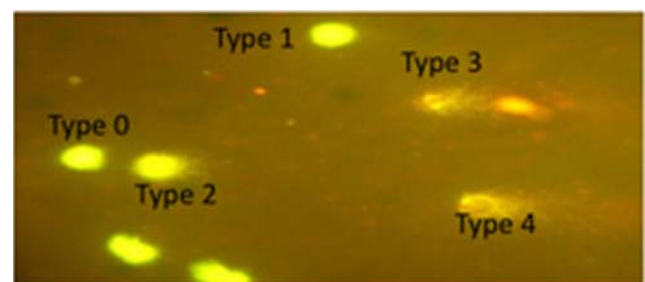


Fig. 2 Different classes of comets (0–4) indicating DNA damage in group E, (45 days)

Table 5 Total comet score (TCS) in RBCs of treated groups (100 cells/slide/sample) (15-day trial)

Groups	Class 0	Class 1	Class 2	Class 3	Class 4	TCS
A	90.0 ± 2.0	7.6 ± 2.1	1.6 ± 0.6	1.3 ± 1.5	0.0 ± 0.0	13.0 ± 2.6
B	87.3 ± 2.1	9.7 ± 2.5	2.6 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	15.0 ± 2.6
C	88.6 ± 2.3	8.7 ± 2.1	2.0 ± 1.0	0.6 ± 0.6	0.0 ± 0.0	14.6 ± 2.1
D	88.0 ± 2.0	9.3 ± 2.1	1.7 ± 0.6	0.6 ± 0.5	0.3 ± 0.6	15.6 ± 1.1
E	82.0 ± 2.0	12.6 ± 2.1	2.0 ± 1.0	2.3 ± 0.5	0.7 ± 0.6	25.3 ± 3.2*

Mean results with ± SD. Asterisk significantly differ ($P < 0.05$) than all other groups

trial; significant DNA damage was observed in all the treated groups. Highest significant DNA damages were found in groups D and E. The degree of damage was found to increase from B to E, as evident from the tables. It was, therefore, concluded from the above results that an increase in DNA damage was significantly dose- and duration-dependent. The micronucleus test has shown the highest number of micronuclei in group E at all duration, and the result was significant, while all other groups' results were non-significant. This means that nucleus damage, assessed through the micronucleus test was more dose-dependent and less duration-dependent. While the damage in DNA, assessed through comet assay was significantly increased with dose and duration as evident from Tables 5, 6, and 7 and Fig. 2.

Sodhi et al. (2008) and Celik et al. (2009) indicated that impairment in the synthesis of Ku protein because of ROS or nitrogenous species could lead to DNA strand breaks. Further, CP has been found to reduce antioxidant defense activities. Therefore, the oxy-radicals have been found to act as chemical nucleases on DNA, resulting in an ultimate DNA strand breakage. Similar findings have also been reported in isolated hepatocytes and brain cells of rats (Mehta et al. 2008) and mice (Rahman et al. 2002), while Hussain et al. (2011) also indicated that persistent exposure to xenobiotics is not only responsible for genomic aberrations in somatic cells but also cause adverse effects on reproduction.

Histopathological observations

Observations of the slides shown in Fig. 3 clearly indicates that CP has produced significant signs of toxicity in many organs of the Japanese quails, administered with highest doses (9 and 12 mg). Histopathology of the liver, kidney, and testes of the treated birds in group B did not exhibit any differences from the control at all time trials. However, very mild degeneration in different cellular structures was observed in the liver, kidneys, and testes of group C, at days 30 and 45 of the experiments. Repeated exposure to high doses (9 and 12 mg/kg body weight) of CP has produced many significant pathological and structural alterations. Histological degenerative changes in the liver of groups D and E included mild cytoplasmic vacuolation, biliary hyperplasia, mild congestion, extensive degeneration in hepatocyte's membranes and cellular structures with pyknotic nuclei, and intercellular spaces that have been observed (Fig. 3). Structural alterations observed in the kidney of groups D and E, included mild congestion, extensive presence of pyknotic nuclei in the tubular epithelial cells, degenerated tubules, glomeruli, and bowman capsules (Fig. 3). Similarly, degenerative changes in the testicular tubules include necrosis, apoptosis, karyopyknosis in the germinal layer of epithelium, degeneration in sustentacular cells of the seminiferous tubules, ruptured tubule membrane, decrease in the lumen and increase in the inter-seminiferous tubules space because of shrinkage, and increase interstitial

Table 6 Total comet score (TCS) in RBCs of treated groups (100 cells/slide/sample) (30-day trial)

Groups	Class 0	Class 1	Class 2	Class 3	Class 4	TCS
A	91.6 ± 4.2	7.3 ± 2.5	1.0 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	9.3 ± 5.8
B	84.7 ± 2.5	12.0 ± 2.0	2.3 ± 0.6	0.3 ± 0.6	0.0 ± 0.0	17.7 ± 1.5
C	87.3 ± 2.1	8.3 ± 2.3	2.0 ± 1.0	1.6 ± 0.6	0.7 ± 0.5	20.0 ± 1.7*
D	88.0 ± 1.0	7.6 ± 2.1	2.3 ± 1.1	1.3 ± 0.7	0.7 ± 1.1	19.0 ± 3.6**
E	83.0 ± 2.0	11.6 ± 3.2	2.7 ± 1.5	1.3 ± 0.6	1.3 ± 1.1	26.3 ± 1.5***

Mean results with ± SD. Each row with asterisk significantly differ

* $P \leq 0.018$,

** $P \leq 0.032$

*** $P \leq 0.001$

Table 7 Total comet score (TCS) in RBCs of treated groups (100 cells/slide/sample) (45-day trial)

Groups	Class 0	Class 1	Class 2	Class 3	Class 4	TCS
A	93.3 ± 2.1	5.0 ± 1.0	1.0 ± 1.0	0.7 ± 0.6	0.0 ± 0.0	9.0 ± 3.4
B	86.7 ± 1.1	9.7 ± 0.6	2.3 ± 0.6	0.6 ± 0.6	0.6 ± 0.6	19.7 ± 1.5*
C	83.0 ± 3.0	14.0 ± 2.0	1.3 ± 0.6	0.7 ± 0.6	1.0 ± 1.0	22.7 ± 6.0**
D	83.3 ± 1.5	12.3 ± 1.5	2.3 ± 1.1	1.0 ± 0.0	1.0 ± 1.0	24.0 ± 1.0***
E	81.0 ± 1.0	12.0 ± 1.0	3.3 ± 0.5	2.3 ± 1.1	1.7 ± 0.6	30.3 ± 3.1****

Mean results with ± SD. Each row with asterisk significantly differ

* $P \leq 0.025$

** $P \leq 0.005$

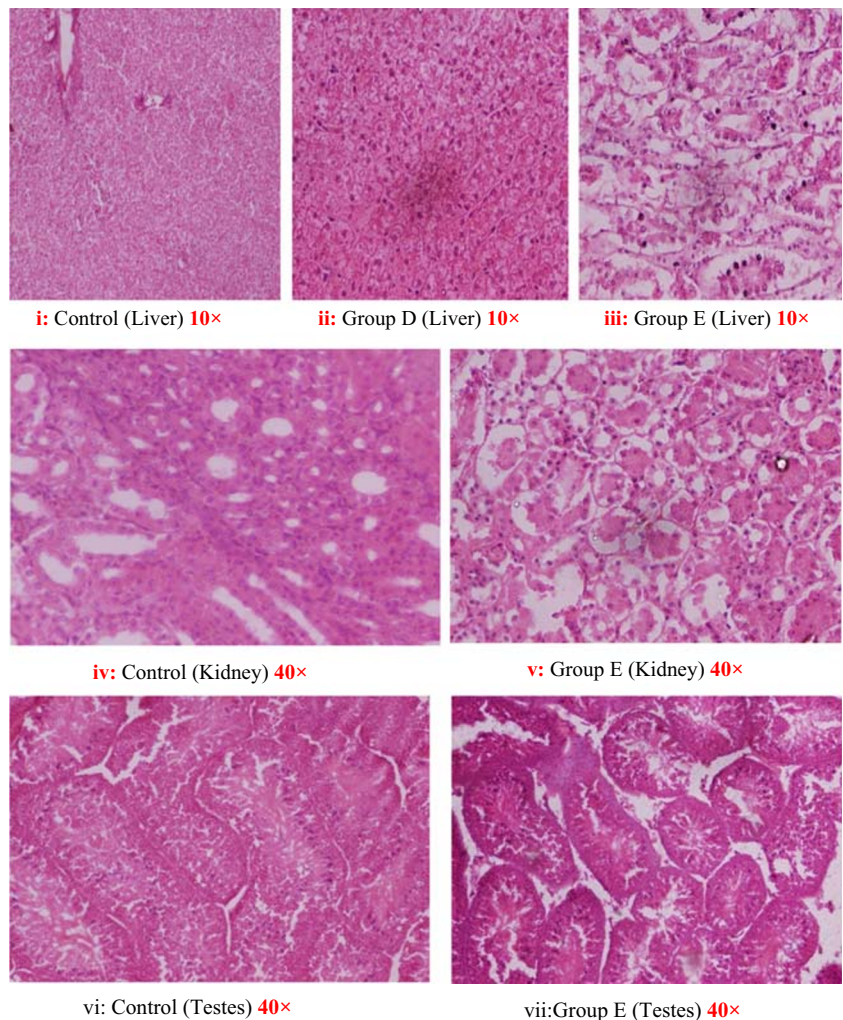
*** $P \leq 0.003$

**** $P \leq 0.0001$

spaces have been observed as well (Fig. 3). To conclude histological degenerative alterations in the liver, testes, and kidneys observed in birds of groups D and E, included histiocytosis (increase in number of Kupffer cells), mild congestion, vacuolation, and hepatic necrosis. The kidney renal

tubules showed extreme congestion, which leads to the complete damage of the lumen. Excessive abnormalities were presented by the epithelial cells in the glomeruli that results in high degree of hyalinization. Blood vessel degeneration was also prominent in the parenchyma layer. Thickened bowman

Fig. 3 Histo-micrograph of the liver, kidney, and testes of Japanese quails exposed to different concentrations of chlorpyrifos (i) Liver: control with normal hepatocytes and sinusoidal spaces. No vascular changes are found (H&E, × 100). (ii and iii) Liver (groups D and E): extensive degeneration in hepatocytes, sinusoidal spaces are increased, mild congestion (H&E, × 100). (iv) Kidney (control): tubular epithelium is intact with no cellular changes (H&E, × 400). (v) Kidney (group E): detachment of tubular epithelium from the basement membrane and extensive degeneration in the tubular epithelium (H&E, × 400). (vi) Testis (control): having normal structural details (H&E, × 400). (vii) Testis (group E): ruptured tubule membrane, decrease in the lumen of tubules and inter-seminiferous tubules space is increased (H&E, × 400)]



capsules were also observed, along with dilated urinary spaces along with rupture of bowman capsule, glomerular edema, extensive degeneration, and alteration in many cellular structures can be observed in the photographs. These alterations seemed to follow almost the same pattern as that previously reported by many investigators under the effect of different organophosphates. These alterations in cellular structures were the result of organophosphates ability to produce reactive oxygen species (Altuntas et al. 2002; Uzunhisarcikli et al., 2007), which are considered to be the major cellular source of producing oxidative stress leading to damage of membranes, lipids, carbohydrates and nucleic acids (Gawish et al. 2006). Increase in the lipid peroxidation process by organophosphates and adverse effects on membranes, cytoplasmic structures, and impairment in the biological mechanism was also confirmed by Akhtar et al. (2009) and Hussain et al. (2011).

Conclusions

In the present study, chlorpyrifos was orally fed to Japanese quails to assess its possible toxicological effect on birds. Based on the results of clinical signs, histological alterations, and genotoxicity, it was concluded that chlorpyrifos is a potentially toxic chemical that can induce different levels of toxicity to non-targeted organisms like birds and even in human as it is located on top of food chain. It is, therefore, recommended for the regulatory agencies of each state to raise awareness, make laws, and take regulatory measures regarding the safe use of this pesticide. States and regulatory agencies must ensure the avoidance of over, needless, and improper use of such pesticides.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

References

- Akhtar N, Srivastava MK, Raizada RB (2009) Assessment of chlorpyrifos toxicity on certain organs in rat, *Rattus norvegicus*. *J Environ Biol* 30(6):1047–1053
- Al-Badrany YA, Mohammad FK (2007) Effects of acute and repeated oral exposure to the organophosphate insecticide chlorpyrifos on open-field activity in chicks. *Toxicol Lett* 174(1):110–116
- Aldenberg T, Jaworska JS, Traas TP (2002) Normal species sensitivity distributions and probabilistic ecological risk assessment. Posthuma L, Suter GW II, Traas TP, editors. *Species sensitivity distributions in ecotoxicology*. Boca Raton (FL): Lewis. pp. 49–102
- Altuntas I, Delibas N, Demirci M, Kilinc I, Tamer N (2002) The effects of methidathion on lipid peroxidation and some liver enzymes: role of vitamins E and C. *Arch Toxicol* 76(8):470–473
- Ambali SF, Akanbi DO, Shittu M, Giwa A, Oladipo O, Ayo JO (2010a) Chlorpyrifos-induced clinical, haematological and biochemical changes in Swiss albino mice: mitigating effect by co-administration of vitamins C and E. *Life Science Journal* 3:37–44
- Ambali SF, Idris SB, Onukak C, Shittu MU, Ayo JO (2010b) Ameliorative effects of vitamin C on short-term sensorimotor and cognitive changes induced by acute chlorpyrifos exposure in Wistar rats. *Toxicol Ind Health* 9:547–558
- Barna-Lloyd T, Szabo JR, Davis NL (1990) Chlorpyrifos-methyl (Reldan R) rat subchronic dietary toxicity and recovery study. Unpublished Report TXT: K-046193-026 from Dow chemical, Texas, USA. Submitted to WHO by DowElanco, Indianapolis, USA
- Hematotoxic and hepatotoxic effects of dichlorvos at sublethal dosages in rats. *Environ Toxicol* 24(2):128–132
- Celik I, Yilmaz Z, Turkoglu V (2009) Hematotoxic and hepatotoxic effects of dichlorvos at sublethal dosages in rats. *Environ Toxicol* 24(2):128–132
- Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* 26:249–226
- Costa LG (2006) Current issues in organophosphate toxicology. *Clin Chim Acta* 366(1):1–13
- Costa LG, Giordano G, Cole TB, Marsillach J, Furlong CE (2013) Paraoxonase 1 (PON1) as a genetic determinant of susceptibility to organophosphate toxicity. *Toxicology* 307:115–122
- Eaton DL, Daroff RB, Autrup H, Bridges J, Buffler P, Costa LG, Neubert D (2008) Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit Rev Toxicol* 38(2): 1–125
- Gawish AM, Ahmed SK, Abdel-Mageed FA (2006) Studies on the effect of the pesticide diazinon and the metronidazole drug (Flagyl) on the histology of some body organs of the rat (*Rattus norvegicus*). *J Egv Ger Soc Zool* 50:183
- Hussain R, Mahmood F, Khan MZ, Khan A, Muhammad F (2011) Pathological and genotoxic effects of atrazine in male Japanese quail (*Coturnix japonica*). *Ecotoxicology* 20(1):1–8
- Joshi SC, Mathur R, Gulati T (2007) Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat. *Toxicol Ind Health* 23(7):439–444
- Kalender S, Uzun FG, Durak D, Demir F, Kalender Y (2010) Malathion-induced hepatotoxicity in rats: the effects of vitamins C and E. *Food Chem Toxicol* 48(2):633–638
- Kammon AM, Brar RS, Banga HS, Sodhi S (2010) Patho-biochemical studies on hepatotoxicity and nephrotoxicity on exposure to chlorpyrifos and imidacloprid in layer chickens. *Veterinarski Arhiv* 80(5): 663–672
- Karanth S, Pope C (2000) Carboxylesterase and A-esterase activities during maturation and aging: relationship to the toxicity of chlorpyrifos and parathion in rats. *Toxicol Sci* 58(2):282–289
- Mehta R, Verma RS, Srivastava N (2008) Chlorpyrifos-induced DNA damage in rat liver and brain. *Environ Mol Mutagen* 49(6):426–433
- Mitra A, Chatterjee C, Mandal FB (2011) Synthetic chemical pesticides and their effects on birds. *Res J Environ Toxicol* 5(2):81–96
- Moore DR, Teed RS, Greer CD, Solomon KR, Giesy JP (2014) Refined avian risk assessment for chlorpyrifos in the United States. In *Ecological Risk Assessment for Chlorpyrifos in Terrestrial and Aquatic Systems in the United States*, Springer International Publishing pp. 163–217
- Morowati M (1998) Inhalation toxicity studies of Thimet (Phorate) in the male swiss albino mouse, *Mus musculus*: II. Lung histopathology, pseudocholinesterase level and haematological studies. *Environ Pollut* 103(2):309–315
- Mutch E, Williams-Diazinon FM (2006) Chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. *Toxicology* 224(1):22–32
- Narahariseti SB, Aggarwal M, Ranganathan V, Sarkar SN, Kataria M, Malik JK (2009) Effects of simultaneous repeated exposure at high

- levels of arsenic and malathion on hepatic drug-biotransforming enzymes in broiler chickens. *Environ Toxicol Pharmacol* 28(2): 213–218
- Orris P, Chary LK, Perry K, Asbury J (2000) Persistent organic pollutants and human health. World Federation of Public Health Associations, Washington, DC
- Rahman MF, Mahboob M, Danadevi K, Banu BS, Grover P (2002) Assessment of genotoxic effects of chlorpyrifos and acephate by the comet assay in mice leucocytes. *Mutat Res Genet Toxicol Environ Mutagen* 516(1):139–147
- Sadique U Chaudhry ZI, Rana MY, Anjum AA, Sajid, AMushtaq M (2012) Pathogenesis and immunohistochemical studies of caprine pleuropneumonia in experimentally infected goats. *Pak Vet J* 32(3): 427–431
- Sánchez-Amate MC, Flores P, Sánchez-Santed F (2001) Effects of chlorpyrifos in the plus-maze model of anxiety. *Behav Pharmacol* 12(4): 285–292
- Savithri Y, Sekhar PR, Doss RJ (2010) Changes in hematological profiles of albino rats under chlorpyrifos toxicity. *Int J Pharm Bio Sci* 1:1–7
- Şekercioğlu ÇH, Daily GC, Ehrlich PR (2004) Ecosystem consequences of bird declines. *PNAS* 101(52):18042–18047. <https://doi.org/10.1073/pnas.0408049101>
- Siraj M, Khisroon M, Khan A, Zaidi F, Ullah A, Rahman G (2018) Bio-monitoring of tissue accumulation and genotoxic effect of heavy metals in *Cyprinus carpio* from river Kabul Khyber Pakhtunkhwa Pakistan. *Bull Environ Contam Toxicol* 73(3):115–120
- Sodhi S, Sharma A, Brar APS, Brar RS (2008) Effect of α tocopherol and selenium on antioxidant status, lipid peroxidation and hepatopathy induced by malathion in chicks. *Pestic Biochem Physiol* 90(2):82–86
- Solomon KR, Williams WM, Mackay D, Purdy J, Giddings JM, Giesy JP (2014) Properties and uses of chlorpyrifos in the United States. In *Ecological Risk Assessment for Chlorpyrifos in Terrestrial and Aquatic Systems in the United States*. Springer International Publishing pp. 13–34
- Song CH, Ku SK, Jang HS, Kye EY, Yun SH, Jang KH, Kwon YS (2012) Eyelid squamous cell carcinomains. *Pak Vet J* 32:474–476
- Testai E, Buratti FM, Consiglio ED (2010) Chlorpyrifos. In: Krieger RI, Doull J, van Hemmen JJ, Hodgson E, Maibach HI, Ritter L, Ross J, Slikker W (eds) *Handbook of pesticide toxicology*, vol 2. Elsevier, Burlington, pp 1505–1526. <https://doi.org/10.1016/b978-0-12-374367-1.00070-7>
- Uzunhisarcikli M, Kalender Y, Dirican K, Kalender S, Ogutcu A, Buyukkomurc F (2007) Acute, subacute and subchronic administration of methyl parathion-induced testicular damage in male rats and protective role of vitamins C and E. *Pestic Biochem Physiol* 87(2): 115–122
- Varo I, Serrano R, Pitarch E, Amat F, Lopez FJ, Navarro JC (2002) Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish. *Arch Environ Contam Toxicol* 42(2):229–235
- Wessels D, Barr DB, Mendola P (2003) Use of biomarkers to indicate exposure of children to organophosphate pesticides: implications for a longitudinal study of children's environmental health. *Environ Health Perspect* 111(16):1939–1946
- Yadav B, Niyogi D, Tripathi KK, Singh GK, Yadav MK (2018) Pathomorphological effects in broiler birds induced with sub-acute chlorpyrifos toxicity and its amelioration with vitamin E and selenium. *J Pharmacogn Phytochem* 7(2):1877–1882
- Yin X, Zhu G, Li XB, Liu S (2009) Genotoxicity evaluation of chlorpyrifos to amphibian Chinese toad (amphibian: Anura) by comet assay and micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 680(1):2–6
- Zhou SP, Duan CQ, Hui FU, Chen YH, Wang XH, Yu ZF (2007) Toxicity assessment for chlorpyrifos-contaminated soil with three different earthworm test methods. *J Environ Sci* 19(7):854–858

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