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TESTICULAR AND GENOTOXIC EFFECTS INDUCED BY SUBCHRONIC ORAL ADMINISTRATION OF CHLORPYRIFOS IN JAPANESE QUAIL (*Coturnix japonica*)

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The aim of this study was to investigate the testicular and genotoxic effects of chlorpyrifos, an organophosphate insecticide, in adult male Japanese quail. For this purpose a total of 75 sexually mature birds were procured and randomly kept in five equal groups. Chlorpyrifos was given @ 0, 6, 8, 10 and 12 mg/kg BW orally for 36 days to experimental birds present in all groups (A-E). Adverse effects such as gasping, depression, watery droppings, decreased foam production, tremors and less frequency of mounting with pen mates were evident in a dose-dependent manner. Chlorpyrifos decreased feed intake, body weight and relative weight of testes, liver and kidneys as compared to control birds. Histopathological examination of seminiferous tubules of testes at higher concentration of chlorpyrifos (10 and 12 mg/kg BW) revealed less number of spermatogenic cell layers, increased number of degenerated spermatozoa and multinucleated giant cells. Furthermore, histopathological examination of liver tissues indicated extensive cytoplasmic vacuolation. Moreover, mild to moderate congestion with sporadic tubular epithelial cell necrosis was observed in kidneys. Significantly increased comet tail length confirmed DNA damage at higher concentration of chlorpyrifos in isolated lymphocyte and bone marrow cells. It was concluded that chlorpyrifos at 10 and 12 mg/kg BW rendered various clinical, pathological alterations in internal organs and was also involved in genotoxicity.

Keywords: Chlorpyrifos, quail, pathology, bone marrow, Comet assay, genotoxicity

INTRODUCTION

In the modern age, the farmers extensively use pesticides, insecticides and herbicides during pre and post-harvest of various food crops to save their crops from insects/pests. These synthetic compounds induce stress that contributes to different physical and biochemical disorders including decrease reproductive efficiency, poor erythropoiesis, oxidative disorder and genotoxic effects (Hussain *et al*., 2012; 2013). Severe stress in association to pesticides/herbicides induces serious risk to environment, animals, human beings and the birds living in same ecology (Hussain *et al*., 2011; Ahmad *et al*., 2012; Babar *et al*., 2012).

Pesticides are synthetic organic compounds that have played imperative role in home and public health management throughout the world (Mitra *et al*., 2011; Khan *et al*., 2012) and are a major source of public health hazard and deaths in developing countries (Saxena and Saxena, 2010; Tecles *et al*., 2013). Among different chemicals being employed in agriculture, the organophosphate insecticides are the major neurotoxic compounds. Extensive application of organophosphorus pesticides in agro-production sector, grain storage and public health management causes accumulation of the residues of these chemicals in different daily consume able food materials such as vegetables, cereal crops, natural water systems and act as the major sources of contact (Edwards *et al*., 2013). Organophosphorus pesticides result negative impact directly or indirectly on mammals, avian species and have a serious threats to ecosystem due to physicochemical alterations (Iqbal *et al*., 2012; Muhammad *et al*., 2012). Chlorpyrifos is an important member of organophosphate pesticides and is extensively used to control insects in cereal crops, grain storage, poultry houses to hold back termites, livestock and public health management (Moye and Pritsos, 2011).

Chlorpyrifos (O, O'-dithyl-O-3, 5, 6-trichloro-2-pyrydyl phosphorothionate) is usually metabolized in liver and its metabolites are excreted through the kidneys (Barr *et al*., 2005). Acute or sub chronic administration of chlorpyrifos enhances the generation of acetylcholine that potentiates cholinergic activity and marked decline in the production of acetylcholinesterase thus imposes neurotoxicity (Kousba *et al*., 2007). The neurotoxic effects produce nervous signs including gait abnormalities, decreased tail-pinch response in rats and mice (Wang *et al*., 2009; Tripathi and Srivastav, 2010). Hepatotoxic and nephrotoxic effects have also been reported at different concentrations of chlorpyrifos in rats (Mehta *et al*., 2008). Little information is available regarding the genotoxic and gonadotoxic effects of chlorpyrifos in avian species in the accessible literature. Therefore, in this study, we report gonatoxic and genotoxic effects induced by subchronic oral

administration of chlorpyrifos in multiple tissues of male Japanese quail (*Coturnix japonica*).

MATERIALS AND METHODS

***Experimental outline*:** A total of 75 adult male Japanese quail without any ailment were procured from local market. The birds were given a corn and soybean meal based feed having 20% protein (Ahmad *et al*., 2013). Feed and water was provided twice a day *ad libitum* during the experiment. After five days of acclimatization, the birds were randomly divided into five equal groups, i.e., A-E. Chlorpyrifos (38.7% w/w; Dow Agrosciences, Pakistan) was mixed in corn oil and orally administered through crop tubing @ 0, 6, 8, 10 and 12 mg/kg BW, respectively, for 36 days. Birds were monitored twice daily for any apparent clinical signs and desirability for feed. The body weight and feed intake was recorded on weekly basis.

***Pathology procedures*:** Five birds were randomly selected from each group and killed by cutting their jugular vein on experimental days 12, 24 and 36 to collect different tissues for pathological examination. Blood was collected from each quail and lymphocytes were separated through centrifugation techniques. Bone marrow cells were isolated from the thigh bones. Visceral organs including liver, kidneys and testes were removed, weighed, fixed in 10% neutral buffered formalin (Song *et al*., 2012). Tissue specimens for histopathology were preserved in 10% neutral buffered formalin and processed by the routine method of dehydration and paraffin embedding techniques (Sharaf *et al*., 2013). Sections of 4-5µm thickness were cut and stained with hematoxylin and eosin (Sadique *et al*., 2012).

***Single cell preparation and comet assay*:** On each sampling, the blood samples were treated with anticoagulant (EDTA; 1mg/ml) and lymphocytes were separated by concentrated gradients using histopaque. Bone marrow cells were separated by dissecting femur head. The alkaline single cell gel electrophoresis was performed on isolated lymphocytes and bone marrow cells (Hussain *et al*., 2011; Azmat *et al*., 2012). Briefly, cells were mixed with 1.7% low melting agarose (LMA), then sand-witched between 0.5% normal melting agarose at the bottom and the 0.9% LMA upper layer. The slides were placed in chilled lysing buffer solution for 40 min and then electrophoresis was carried out in dark room. DNA migration was carried for 20 min at 23 V. Afterward slides were kept in neutralizing buffer, stained with ethidium bromide and observed under epifluorescent microscope (Leica DM RXA). The tail length (µm) of comets was measured by using a calibrated ocular micrometer (Hussain *et al*., 2011).

***Data Analysis*:** The data thus collected were subjected to statistical analysis using analysis of variance technique. The group means were compared by Duncan's Multiple Range tests.

RESULTS

***Physical observations*:** The birds of untreated group remained active, healthy and exhibited normal behavior throughout the experiment. They became alert at the time of feeding and watering. Various clinical signs such as restlessness, staggering gait, diarrhea, dullness and tremors were observed in quail receiving higher doses (10 and 12 mg/kg BW) of chlorpyrifos. Decreased frequency of crowing, mounting with pen mates and foam production was also observed at the higher levels of pesticide exposure. A moderate degree of these clinical signs were also observed in birds of group C (8 mg/kg BW).

***Feed intake, body weight and relative organ weight*:** Feed intake and body weight remained significantly lower in birds of group E at day 12 as compared to the untreated group. Feed intake and body weight at day 24 in groups D&E and at day 36 in groups C to E were significantly reduced (Table 1). The relative organ weight of the liver, kidneys and testes was significantly reduced at day 12 in group E, at day 24 in groups D&E and at day 36 in group C to E as compared to group A (Table 2).

***Necropsy and microscopic observations*:** Visual observation of the liver, kidneys and testes of quail in groups A to C

Table 1. Feed intake and body weight of Japanese quail administered different levels of chlorpyrifos

Parameter/ Days	Groups				
	A	B	C	D	E
Feed intake (g)					
12	24.75±2.06	22.80±1.04	20.47±1.11	20.02±1.07	19.80±0.04*
24	26.05±1.31	21.40±2.09	20.62±1.06	18.10±1.04*	17.32±0.8*
36	25.20±1.17	20.72±2.08	17.82±1.07*	16.22±.16*	14.72±0.08*
Body weight (g)					
12	131.67±2.22	128.04±1.01	127.22±1.9	126.30±1.04	122.00±1.33*
24	132.75±1.39	126.47±2.12	125.13±0.09	123.52±1.13*	120.52±1.23*
36	132.60±2.27	125.10±3.18	120.40±1.04*	121.02±0.20*	118.12±0.08*

Chlorpyrifos @ 0, 6, 8, 10 and 12 mg/kg BW was orally administered via crop tubes to group A, B, C, D, and E, respectively for 36 days. Values (mean±SE) in each row with asterisk differ significantly ($P<0.05$) than control group.

Table 2. Relative organ weight (% of body weight) of Japanese quail administered different levels of chlorpyrifos

Parameter/Days	Groups				
	A	B	C	D	E
Liver					
12	2.71±0.64	2.63±0.54	2.59±0.47	2.55±0.15	2.49±0.10*
24	2.65±0.64	2.59±0.70	2.55±0.16	2.33±0.11*	2.37±0.03*
36	2.78±0.17	2.52±0.64	2.48±0.32	2.28±0.14*	2.21±0.16*
Kidney					
12	0.82±0.15	0.67±0.06	0.66±0.09	0.64±0.06	0.58±0.04*
24	0.78±0.07	0.65±0.07	0.61±0.50*	0.58±0.25*	0.54±0.50*
36	0.84±0.23	0.63±0.75	0.59±0.23*	0.55±0.25*	0.52±0.26*
Testes					
12	2.81±0.01	2.57±0.03	2.47±0.04	2.40±0.02*	2.37±0.47*
24	2.89±0.20	2.48±0.08	2.41±0.47	2.30±0.06*	2.27±0.04*
36	3.09±0.25	2.44±0.01	2.39±0.01*	2.32±0.06*	2.21±0.17*

Chlorpyrifos @ 0, 6, 8, 10 and 12 mg/kg BW was orally administered via crop tubes to group A, B, C, D, and E, respectively for 36 days. Values (mean±SE) in each row with asterisk differ significantly ($P<0.05$) than control group.

Table 3. Mean tail length (µm) of comets produced in lymphocytes and bone marrow cells of Japanese quail administered different levels of chlorpyrifos

Parameter/Days	Groups				
	A	B	C	D	E
Lymphocyte					
12	2.93±0 .40	3.54±0.27	3.29±0.19	3.47±0.29	4.85±0.21*
24	3.03±0.43	3.71±0.26	4.03±0.18	5.15±0.33*	7.61±0.24*
36	2.70±0.21	3.76±0.16	4.07±0.19	6.19±0.33*	19.94±1.24*
Bone marrow cells					
12	2.34±0.25	2.47±0.28	3.49±0.26	3.55±0.39	5.92±0.15*
24	2.85±0.21	3.22±0.27	3.71±0.28	4.98±0.08*	6.95±0.50*
36	2.58±0.22	3.36±0.25	3.82±0.27	5.42±0.04*	16.63±1.25*

Chlorpyrifos @ 0, 6, 8, 10 and 12 mg/kg BW was orally administered via crop tubes to group A, B, C, D, and E, respectively for 36 days. Values (mean±SE) in each row with asterisk differ significantly ($P<0.05$) than control group.

determined that they were normal in color and consistency throughout the experiment. However, different liver tissues of birds in groups D and E were swollen, pale in color and friable in consistency. Kidneys of quail in these groups were also swollen and congested while testes were smaller in size as compared to testes of birds in group A.

Histological examination of testes of untreated birds exhibited all the layers of spermatogenic cells arranged in columns. Groups of filamentous spermatozoa were hanging in the clefts of the sustentecular cells of the seminiferous tubules (Fig. 1a). Testes of quail in group B did not differ from testes of quail in group A throughout the study. However, mild degenerative changes were observed in the primary spermatocytes in the testes of birds in group C. Extensive histological changes were observed in the testes of birds in groups D and E. In these groups, the seminiferous tubules of testes were lined only with 2-3 cell layers consisting of primary and secondary spermatocytes along with fewer of the round spermatids with pyknotic and hyperchromatic nuclei (Fig. 1b) at day 24. In some tubules, the lumen had multinucleated giant cells instead of sperms at day 36 in birds of group E (Fig. 1c).

Grossly livers in groups B and C did not show any morphological changes. However, histological examination of different liver tissues of birds in groups D&E exhibited extensive cytoplasmic vacuolation throughout the experiment. Most of these vacuoles were pushing the nucleus toward the periphery. Mild congestion along with billiary hyperplasia in group C was also observed at day 36 of the experiment. Kidneys showed extensive congestive changes with sporadic tubular epithelial cell necrosis in birds of groups D and E throughout the experiment.

***Comet assay*:** The comets were damaged DNA material fluorescing around the nuclei making a tail of different length. Mean tail length (µm) of isolated lymphocytes and bone marrow cells in different groups are presented in Table 3. Significantly ($P\leq0.05$) longer tail with more DNA fragments migrated along the current flow (Fig. 2) was observed in isolated cells in group E as compared to all other groups.

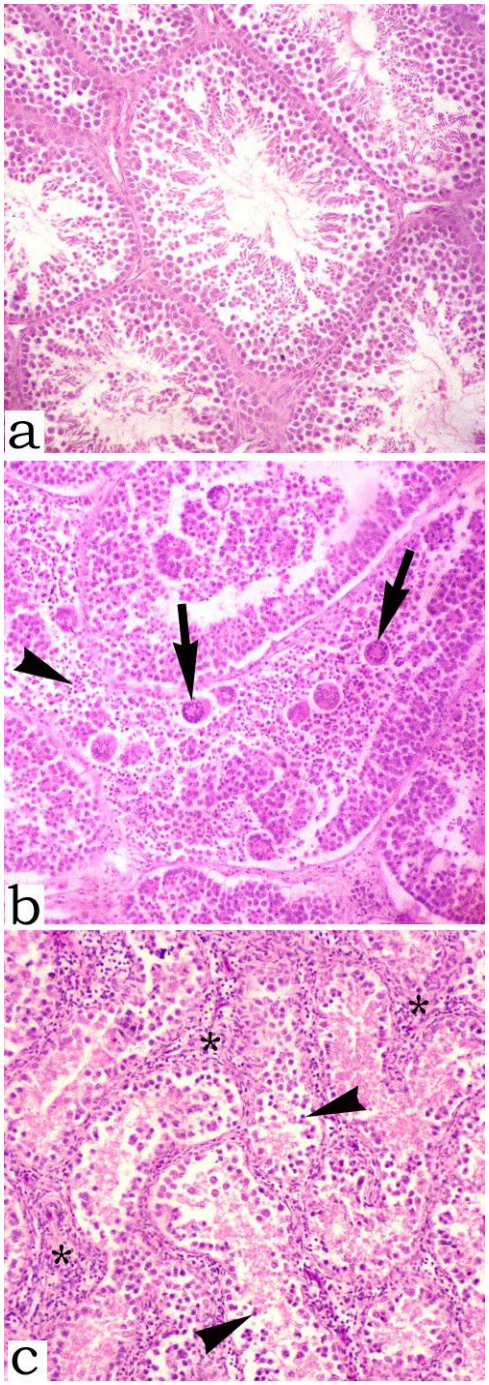

Figure 1. Testes of Japanese quail at experimental day 36: a) group A (control) showing normal process of spermatogenesis, primary spermatocytes, spermatogonia and spermatids, b) group D (chlorpyrifos; 10 mg/kg BW) showing pyknotic nuclei and reduced germinal layers (arrows) and c) group E treated (chlorpyrifos ; 12 mg/kg BW) showing pyknotic nuclei and multinucleated gain cell (arrows). (H&E stain) 200 X.

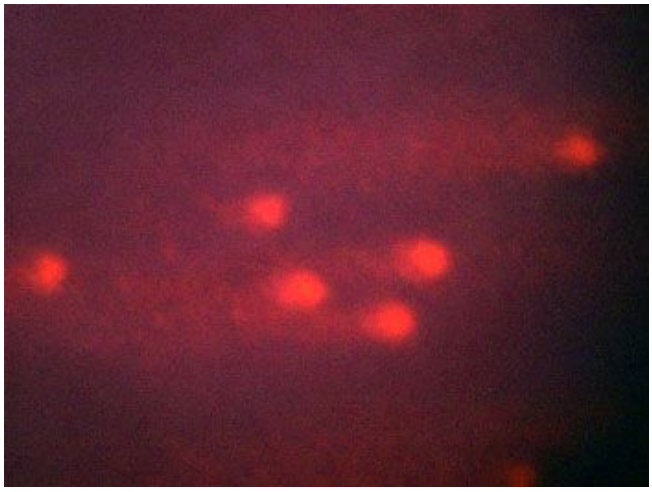

Figure 2. DNA damaged material of male Japanese quail (treated with 12 mg/kg BW chlorpyrifos) lymphocytes fluorescing around the nuclei making a ''tail'' of variable length along the electric field in a comet assay.

DISCUSSION

Pesticides are frequently used in agriculture for consumption of phytosanitary products and pests eradication and ultimately contaminate water resources through various processes, including spray drift, run-offs and leaching (Barranger *et al*., 2014). Persistent exposure to such chemical entities is not only responsible for genomic abrasions in the form of single or irreparable double DNA strand breaks in somatic cells, but also cause adverse effects upon reproduction (Hussain *et al*., 2011).

In present study, various clinical signs including staggering gait, tremors, diarrhea, dullness, less frequency of crowing and decrease foam production were observed. In this regards, Al-Badrany and Mohammad (2007) reported that chickens exposed to chlorpyrifos exhibit similar changes which could be due to cholinesterase inhibition (Sanchez-Amate *et al*., 2001). A significant decrease in feed intake and body weight was recorded in this study. Lower body weight could be due to reduced feed intake and represents taste aversion (Moye and Pritsos, 2011). Reduction in relative weight of testes, liver and kidneys of birds exposed to chlorpyrifos could also be related reduced feed intake ultimately leading to reduced body weight and relative organ weight in chickens (Naraharisetti *et al*., 2009).

Microscopic examination of testes revealed reduced number of spermatogenic cell layers, marked degeneration and necrotic changes in spermatocytes, round spermatids and spermatozoa. The lumens of seminiferous tubules were not only filled with mixture of the degenerated spermatocytes but also with large number of multinucleated giant cells and necrotic cells. Interstitial areas had mononuclear cells, as

well as, fibroblast infiltration. However, the presence of multinucleated giant cells and recruitment of mononuclear cells in the testes in present study could be due to recognition of necrotic debris (Molecular DAMPS) through inflammasome over surface patrolling macrophages thus activation of intracytoplasmic caspase I and generation of IL-1α and IL-33. Moreover, the production of cytokines was recognized through IL-1 α receptors and switch on the cytokines genes for further recruitment of responders. Previously, similar degenerative and necrotic changes with variable intensity in testes of rats (Uzunhisarcikli *et al*., 2007) due to organophosphate pesticides have been reported. These pathological alterations in testicular tissue could also be due to the oxidative stress and generation of free radicals particularly reactive oxygen and nitrogen species responsible for lipid peroxidation of biological membranes (Hussain *et al*., 2014).

In the present study, liver tissues in birds receiving higher levels of chlorpyrifos exhibited mild congestion, extensive cytoplasmic vacuolation and pyknotic nuclei. Degenerative changes of variable degree in liver with the treatment of organophosphate have been reported in rats (Mehta *et al*., 2008; Tripathi and Srivastav, 2010), birds (Chishti *et al*., 1993) and fish (Kunjamma *et al*., 2008). Such degenerative changes could be due to hepatolysis or obvious deficiency of liver glycogen resulting in fatty degeneration, necrosis and fibrosis in advanced stages (Hussain *et al*., 2011).

In present study, the isolated bone marrow cells and blood lymphocytes showed DNA damage with significantly increased comet tail length at higher levels of chlorpyrifos. The genotoxic effects observed in this study could be due to DNA strand break as a result of reactive oxygen and nitrogenous species generated through lipid peroxidation process in cells responsible injury to the thymine strands of DNA or impaired synthesis of ku protein (Sodhi *et al*., 2008; Campos-Pereira *et al*., 2012). Little information is available in accessible literature about DNA damage effects of chlorpyrifos in avian species. In our study comet assay revealed significantly increased DNA strand breaks in bone marrow and blood lymphocytes in birds exposed to chlorpyrifos can be related to oxidative stress induction. Moreover, chlorpyrifos appears to lower the activities of antioxidant defenses and hence the oxyradicals produced by chlorpyrifos could act as chemical nucleases for DNA, ultimately causing DNA strand breakage. However, similar findings have been reported in isolated hepatocytes and brain cells of rats (Mehta *et al*., 2008) and mice (Rahman *et al*., 2002). Exposing species to genotoxicants may result in irreversible and reversible DNA abnormalities such as DNA base modifications, single or double-strand breaks and DNA adducts. These nuclear abnormalities can be rapidly defeated by DNA repair mechanisms (Mateuca *et al*., 2006). Moreover, irreversible changes in DNA can result in anxiety during cell division ultimately leading to aneuploidy and variations in nuclear DNA contents.

***Conclusion*:** The results of present study suggested that the sub-chronic exposure of chlorpyrifos in male Japanese quail induces clinical signs and histological changes in various visceral organs showed cellular toxicity and mutagenic effects. The comet assay used in present study is the most reliable, rapid, easier and useful techniques for routine biomonitoring programs to evaluate the pollutant-induced stress syndrome and to assess the genotoxic potential of different environmental pollutants.

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