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GENOTOXIC AND PATHOLOGICAL EFFECTS OF MALATHION IN MALE JAPANESE QUAIL (*Coturnix japonica*)

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In the present study, we examined the pathological and genotoxic effects of malathion in Japanese quail. For this purpose, 105 quail were randomly divided in seven equal groups (A-G) and were orally given technical grade malathion @ 0, 20, 40, 60, 80, 100 and 120 mg/kg/day for 51 days. Decrease in feed intake, body weight, seminiferous tubule diameter, serum testosterone and different hematological parameters such as leukocytes, erythrocytes, hematocrit and hemoglobin concentration started at day 17 in birds given malathion 100 and 120 mg/kg/day. Higher frequency of blebbed nucleated erythrocytes with 50 mg/kg/day, while micronucleus and binucleated erythrocytes with 75 mg/kg/day start appearing at day 34. Grossly, regression of testicles, swollen bursa and congestion in brain tissue was observed in groups given malathion @ 100 and 120 mg/kg/day. Histologically, lesser number of germinal layers in s*eminiferous* tubules with necrotic spermatozoa, vacuolar degeneration in bursa and *Purkinje* cell necrosis in brain tissues was observed. Moreover, quail given higher doses of malathion showed significant ($P \leq 0.05$) increase in blebbed and binucleated erythrocytes along with increased frequency of micronucleus. On the basis of these results, it can be concluded that malathion at different doses in food chain poses mutagenesis and gonado-toxic effects in birds.

Keywords: Malathion, pesticides, quail, histopathology, micronucleus

INTRODUCTION

Pesticides (insecticides, herbicides and fungicides) constitute the major source of potential environmental hazards when they become part of food chains (Hussain *et al.,* 2012). Long term exposure to these products causes countless abnormalities and reduces the life span of organisms (Hussain *et al.,* 2011; Hussain *et al.,* 2014). Environmental and ecological risk assessment is important in grain producing areas as wildlife species extensively inhabit in these ecosystems. Avian species have a distinctive place in ecosystem and constitute one of the diverse and evolutionary groups (Mitra *et al.,* 2011). Birds are the best indicators of early warning to environmental problems and threats. Healthy avian populations are the representative of a sound ecosystem (Sekereioglu *et al.,* 2004). Wild as well as migratory birds from different biodiversity are directly or indirectly exposed to lethal doses of pesticides, which are the major factor responsible for avian population decline worldwide.

It is difficult to establish a correct estimate of pesticide and insecticide persuaded deaths in birds. Birds may die away from the site of poisoning and their carcasses decompose quickly or may be eaten by the scavengers (Mitra *et al.,* 2011). Malathion (1,2-dicarbethoxyethyl) is a member of organophosphate pesticides (OPs) and is used over various food crops to control pests due to low environmental persistence. In addition, malathion is frequently used for mosquito eradication and control of ectoparasites infesting cattle, poultry, sheep and other domestic animals (Uzun *et al.,* 2009). This pesticide is frequently sprayed over various food crops including maize, cotton and wheat to control pests. Apart from domestic and wild mammals, birds living in the same ecosystems and searching feed in the field treated with malathion are also exposed to this compound (Mitra *et al.,* 2011). Organophosphate pesticides such as malathion are well known to arrest acetylcholinesterase enzyme expressing clinically muscular tremors and torticollis due to degenerative changes in brain tissue (Senger *et al.,* 2005). Degenerative as well as apoptotic changes have also been reported in testes (Maitra and Mitra, 2008; Uzun *et al.,* 2009) and bursa of Fabricius (Nain *et al.,* 2011). Previously, various studies have indicated that exposure to malathion (parathion) significantly reduces testosterone level in rats (Maitra and Mitra, 2008; Uzun *et al.,* 2009).

Blood cells reflect all physical and chemical changes in various organisms that are exposed to different groups of insecticides and pesticides (Ambali *et al.,* 2011). Micronuclei (MN) which are cytoplasmic chromatin containing bodies that materialize in cell cytoplasm like a small satellite nucleus, are considered as the best biomarker of DNA damage (Hussain *et al.,* 2014). Previously, different workers have used micronucleus test in visceral and blood cells such as bone marrow, ovary, liver, peripheral blood and fetal cells of different organisms (Hussain *et al.,* 2014).

Persistent malathion exposure tends to accumulate in different fatty tissues of organisms as they move up the food chains (Mossalam *et al.,* 2011). The residual amounts of malathion has been detected in kidneys, blood, liver and intestine of animals and other food products (Mossalam *et al.,* 2011). However, scanty information is available about the pathological effects along with nuclear changes due to malathion in avian species. The present study shows experimentally induced mutagenic and pathological effects of malathion in different tissues of sexually mature male Japanese quail (*Coturnix japonica*).

MATERIALS AND METHODS

The present study was designed considering all the national legislation regarding protection of animal welfare and following the guidelines devised by the Advanced Studies and Research Board, University of Agriculture, Faisalabad, Pakistan.

Experimental design: A total of 105 sexually mature, 5-6 weeks old clinically healthy male Japanese quail with an average body weight of 98-108g were purchased from local hatchery. After one week of acclimatization, the birds were randomly divided into seven equal groups (A-G) and kept in wire cages under similar housing and management conditions. Basal feed (Olympia Feeds, Lahore Pakistan), i.e. corn soybean meal based feed without vitamins and minerals was offered twice daily and fresh clean water was provided *ad libitum* to birds. Malathion (95% technical grade) obtained from M/S Ali Akbar Enterprises, Pakistan was mixed in corn oil and administered via crop tubing to birds daily for 51 days. Birds of groups A to G were given malathion @ 0, 20, 40, 60, 80, 100 and 120 mg/kg/day, respectively. Birds were monitored twice a day for any apparent ailment. Feed intake and body weight were recorded on daily basis and average results were interpreted on days 17, 34 and 51.

***Protocols for pathological studies*:** Five randomly selected birds from each group were killed by cutting jugular veins on day 17, 34 and 51 of the experiment. Blood samples were collected with and without anticoagulant (EDTA; 1mg/ml). Erythrocyte and leukocyte counts were performed (Hussain *et al.,* 2014) while hemoglobin concentration and hematocrit values were determined (Ghaffar *et al.,* 2014). Thin blood smears were made from each bird for morphological observation of erythrocytes along with presence of micronuclei. A total of 1500 erythrocytes/bird were studied with the help of computer-assisted light microscope (Nikon, Tokyo, Japan) according to method described earlier (Hussain *et al.,* 2014). Serum was separated from blood without anticoagulant and stored at -20°C. Serum testosterone was determined using radioimmunoassay (RIA) kit. Visceral organs including testes, bursa and brain were removed and fixed in 10% neutral buffered formalin. About 4-5 µm thick tissue sections from these organs were cut and stained with hematoxylin and eosin to study histopathological changes (Islam *et al.,* 2013).

***Statistical analyses*:** Data obtained were subjected to statistical analysis (SAS, 2004) using analysis of variance (ANOVA) to find out any significant difference between different treatment groups. The group means were compared by Tukey's test with $P \leq 0.05$.

RESULTS

***Clinical and physical observation*:** Quail in group A (control) did not show any clinical signs throughout the course of the study. Severe clinical signs observed in high dose (100 and 120 mg/kg/day) groups decreased frequency of crowing, watery droppings, dullness, torticollis, tremors, ruffled feathers and salivation at days 34 and 51 of experiment. Mild degree of these clinical signs was observed in quail of groups D-E. Birds showed significantly decreased feed intake at all experimental days in groups D-G, except in group D at day 17 (Table 1). Body weight of birds in high dose groups (80 to 120 mg/kg/day) was significantly reduced throughout the experiment. However, malathion exposure also significantly decreased body weight in quail

Table 1. Feed intake and body weight of Japanese quail (*Coturnix japonica*) administered different levels of malathion.

Parameter/	Malathion (mg/kg/day)						
Days	0(A)	20(B)	40(C)	60(D)	80(E)	100(F)	120(F)
Feed intake (g)							
17	29.50±0.40	26.50±0.28	25.60±0.21	24.20±0.12	23.33±0.08	21.98±0.27*	18.00±0.40*
34	28.75±0.94	25.25±0.85	26.10±0.42	24.25±0.11	21.53±0.50*	19.33±0.29*	16.28±0.38*
51	28.43±0.37	26.18±0.51	25.13±0.20	24.50±0.09	20.67±0.54*	16.95±0.48*	13.13±0.60*
Body weight (g)							
17	133.07±0.33	132.12±0.21	129.80±0.20	128.52±0.21	127.32±0.08	123.77±0.28*	122.57±0.11*
34	133.87±0.23	130.12±0.38	128.47±0.44	126.57±0.38	126.60±0.49	121.47±1.21*	117.97±0.63*
51	134.20±0.89	129.17±0.54	127.82±0.20	127.07±0.29	122.45±0.55*	118.42±0.78*	115.00±1.59*

Values (mean ±SE) in rows bearing asterisk are significantly ($P \leq 0.05$) different from control group.

Table 2. Nuclear abnormalities in erythrocytes of male Japanese (*Coturnix japonica*) quail treated with malathion.

Parameter/	Malathion (mg/kg/day)						
Days	0(A)	20(B)	40(C)	60(D)	80(E)	100(F)	120(F)
Micronucleus (%)							
34	0.13±0.00	0.15±0.01	0.19±0.01	0.57±0.06*	1.40±0.09*	3.49±0.06*	4.54±0.05*
51	0.14±0.01	0.18±0.01	0.23±0.01	1.66±0.01*	2.49±0.02*	4.68±0.02*	5.79±0.09*
Binucleated erythrocytes (%)							
34	0.13±0.00	0.13±0.01	0.17±0.00	0.34±0.01*	0.41±0.01*	0.53±0.02*	0.61±0.01*
51	0.15±0.00	0.16±0.01	0.23±0.01	0.36±0.00*	0.54±0.00*	0.59±0.00*	0.63±0.00*
Blebbed nucleated erythrocytes (%)							
34	0.12±0.00	0.15±0.00	0.25±0.01*	0.29±0.01*	0.41±0.01*	0.57±0.03*	1.01±0.29*
51	0.21±0.01	0.27±0.00	0.34±0.01*	0.39±0.00*	0.87±0.02*	0.95±0.02*	1.40±0.19*

Values (mean ±SE) in rows bearing asterisk are significantly ($P \leq 0.05$) different from control group.

of 80 mg/kg/day dose group (Table 1).

***Nuclear abnormalities*:** Frequency of micronucleated (Fig. 1a) and binucleate erythrocytes (Fig. 1b) increased significantly in birds of groups D-F at all experimental days (Table 2). Frequency of blebbed nucleated erythrocytes (Fig. 1b) at days 34 and 51 in groups E-G increased significantly (Table 2).

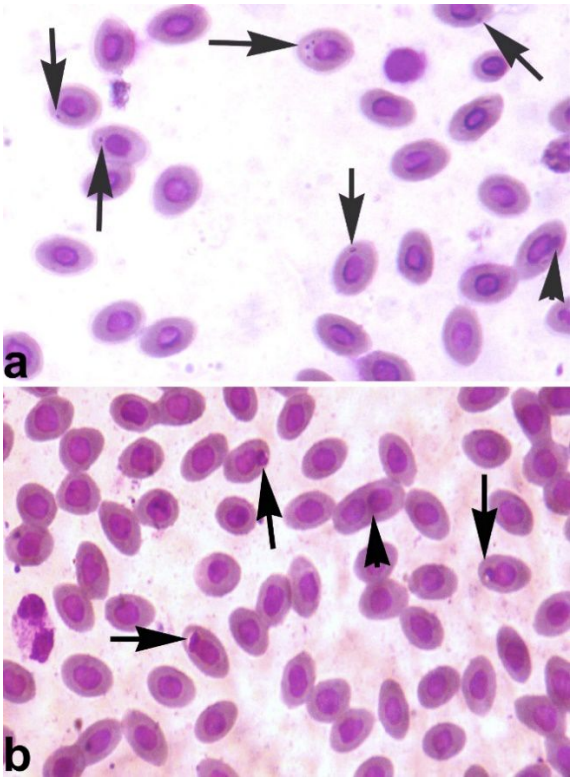

Figure 1. Blood smears of malathion treated quail (120 mg/kg/day) showing a: micronucleus (arrows) and blebbed nucleated erythrocytes (arrow head); b: micronucleus (arrows) and binucleated erythrocyte (arrow head). X1000. Geimsa stain.

***Gross abnormalities and histology*:** Dose and time dependent changes were observed in different visceral organs of treated quail. At day 34 and 51 of the experiment smaller testes, swollen bursa and congestion in brain tissue were observed in birds given higher doses of malathion (100 and 120 mg/kg/day). However, shape of testes in all birds was apparently normal.

Histologically, the brain of birds in groups F and G exhibited severe degenerative changes in granular and pyramidal cells of the cerebrum as compared to the birds of control group, however, in some birds at day 51, brain tissue showed severe necrotic changes with hyperchromatic nuclei of the *Purkinje* cells of cerebellum and intact granular layer (Fig. 2). Mild to moderate congestive changes along with aggregation of mononuclear cells were observed. Pyknotic nuclei and degenerative cells in brain tissues were also observed at day 51 in quail treated with 100 and 120 mg/kg/day.

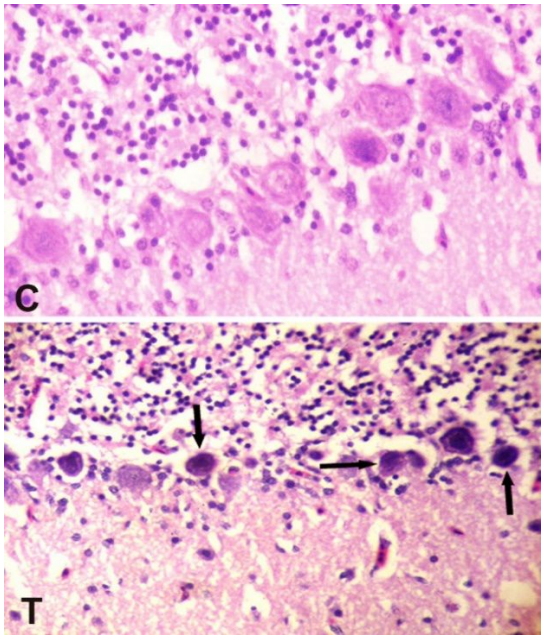

Table 3. Histo-morphometry of testes of Japanese quail (*Coturnix japonica*) administered different levels of malathion.

Parameter/	Malathion (mg/kg/day)						
Days	0(A)	20(B)	40(C)	60(D)	80(E)	100(F)	120(F)
Seminiferous tubule diameter (µm)							
17	381.25±3.11	368.75±1.37	354.50±3.42	364.75±2.6	365.25±2.05	361.75±1.25*	350.75±0.47*
34	378.75±1.10	361.00±1.35	359.75±2.56	361.00±2.41	353.75±1.31*	347.00±2.48*	338.25±1.54*
51	381.75±2.81	358.25±1.31	355.00±2.04	353.75±.01	348.25±1.54*	337.75±2.28*	298.50±12.1*
Epithelial height (µm)							
17	97.75±2.32	91.75±1.75	84.50±1.55	82.75±.85	80.75±.75	72.00±1.08*	66.50±1.19*
34	98.75±1.37	88.00±1.68	83.00±0.91	77.50±1.19	71.25±1.10*	63.75±2.59*	56.75±2.35*
51	98.75±0.85	89.25±.479	79.00±2.04	76.25±2.92	64.00±1.58*	55.50±2.95*	46.25±0.85*

Values (mean ±SE) in rows bearing asterisk are significantly ($P \leq 0.05$) different from control group.

Figure 2. Photomicrograph of brain (cerebellum) from control (C) and treated quail (T). Degenerated *Purkinje* cells (arrows) can be seen in brain of treated quail. X400. H & E.

Testes of birds in group G exhibited severe degenerative changes throughout the experiment when compared to the testes of birds in control group. At day 34, germinal epithelium of seminiferous tubules was thinner and disorganized. Lumen of most of the tubules were filled with eosinophilic proteinous fluid and showed abnormal spermatozoa (Fig. 3a).

At day 51, decreased number of spermatogenic cell layers with accumulation of admixture of degenerative and necrotic spermatids and spermatozoa along with proteinous materials were found in the lumen of the seminiferous tubules (Fig. 3b). Degenerative changes in the seminiferous tubules of birds treated with 100 mg/kg/day were also observed at day 51 (Fig. 3a). Diameter of seminiferous tubules and epithelium height decreased significantly at all experimental days at 100 to 120 mg/kg/day doses except at 80 mg/kg/day at day 17 (Table 3). No microscopic changes were observed in birds of groups A-E throughout the experiment. However, mild vacuolation in cells of bursa was observed in quail of group F at days 34 and 51 of the experiment. Severe vacuolar degeneration in bursa of birds kept in groups F-G at days 34 and 51 was observed.

***Haemato-biochemical parameters*:** Overall erythrocyte and leukocyte counts, haemoglobin concentration and haematocrit decreased significantly in high dose groups (100 to 120 mg/kg/day) at all experimental days when compared to control group. Leukocyte counts at days 34 and 51 and haemoglobin concentration at day 51 also decreased significantly (Table 4). On all experimental days, serum testosterone values in groups given 100 to 120 mg/kg/day of malathion were significantly decreased as compared to control group. Serum testosterone concentration decreased significantly at day 34 in birds treated with 80 mg/kg/day and at day 51 in birds treated with 75 and 100 mg/kg/day (Table 5).

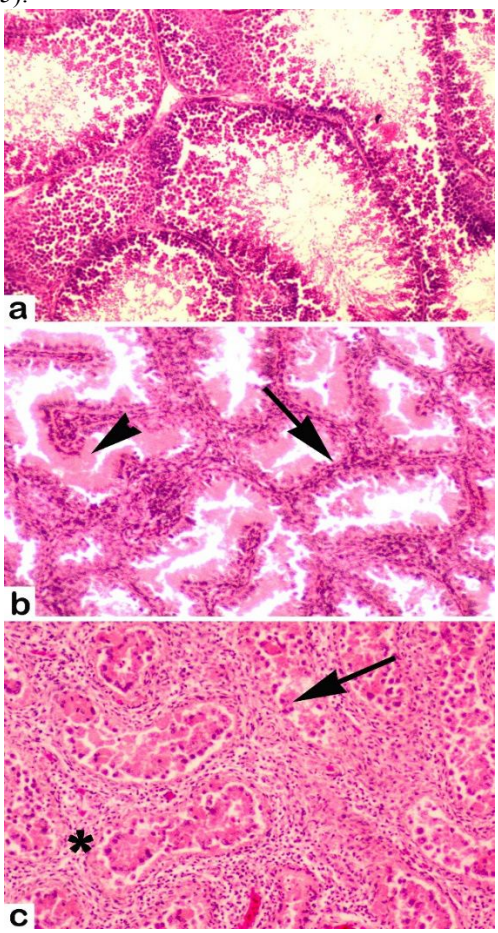

Figure 3. Testis of quail. a) All cells of spermatogenesis series in column fashion in the seminiferous tubules in control birds, b) lesser number of spermatogenic cell layers with necrotic spermatozoa (arrow) and cytoplasmic vacuolation (arrow head) and c) admixtures of dead spermatocytes and spermatids in the

Table 4. Hematological findings of the Japanese quail (*Coturnix japonica*) administered different levels of malathion.

Parameter/	Malathion (mg/kg/day)						
Days	0(A)	20(B)	40(C)	60(D)	80(E)	100(F)	120(F)
Erythrocyte counts (10^{12}/L)							
17	4.76±0.03	4.68±0.01	4.64±0.01	4.67±0.03	4.59±0.01	4.50±0.02	4.26±0.01*
34	4.74±0.02	4.61±0.01	4.58±0.01	4.61±0.02	4.54±0.02	4.44±0.06	4.21±0.03*
51	4.78±0.03	4.61±0.06	4.50±0.01	4.55±0.01	4.49±0.02	4.24±0.06*	4.04±0.02*
Leukocyte counts (10^{9}/L)							
17	37.97±0.11	36.65±0.06	35.27±0.04	34.80±0.04	33.75±0.06	33.60±0.04	31.30±0.09*
34	37.58±0.68	36.90±0.24	34.20±0.41	34.15±0.32	30.75±0.29*	27.55±0.26*	25.80±0.17*
51	38.55±0.45	36.60±0.88	35.05±0.30	34.62±0.32	28.47±0.23*	25.80±0.26*	24.22±0.15*
Hemoglobin concentration (g/dl)							
17	14.8±0.19	14.35±0.11	13.57±0.07	13.45±0.06	13.30±0.13	12.90±0.10	10.57±0.33*
34	13.65±0.09	12.92±0.08	12.55±0.16	12.32±0.04	11.92±0.18	10.62±0.25*	9.50±0.22*
51	13.22±0.13	12.52±0.06	11.99±0.11	11.83±0.01	10.74±0.14*	10.01±0.30*	8.95±0.08*
Hematocrit (%)							
17	41.67±0.27	39.67±0.07	39.02±0.39	38.22±0.08	37.82±0.04	33.20±0.16*	32.75±0.06*
34	40.92±0.31	38.30±0.09	38.02±0.19	37.35±0.18	36.97±0.12	33.12±0.10*	32.09±0.07*
51	41.75±0.06	37.70±0.19	37.37±0.38	35.93±0.13	35.76±0.10	31.52±0.11*	28.37±0.51*

Values (mean ±SE) in rows bearing asterisk are significantly ($P < 0.05$) different from control group.

lumen of the seminiferous tubules (arrow) of treated quail (120 mg/kg/day). X200. H & E.

Table 5. Serum testosterone concentration (ng/ml) in malathion administered male Japanese (*Coturnix japonica*) quail.

Malathion	Experimental days		
(mg/kg/day)	17	34	51
0(A)	4.03±0.06	4.05±0.01	4.07±0.01
20(B)	4.01±0.03	3.85±0.03	3.85±0.02
40(C)	3.84±0.01	3.80±0.01	3.77±0.01
60(D)	3.82±0.01	3.75±0.00	3.70±0.00*
80(E)	3.77±0.01	3.75±0.02*	3.70±0.01*
100(F)	3.76±0.01*	3.71±0.01*	3.57±0.02*
120(G)	3.71±0.02*	3.67±0.02*	3.39±0.02*

Values (mean ± SE) in rows bearing asterisk are significantly ($P \leq 0.05$) different from control group.

DISCUSSION

Malathion is an important member of OPs and is extensively used in agro-production, grain storage, ectoparasite control in livestock and public health management (Falicia *et al.,* 2011). Birds/animals and human beings living in same ecosystem are directly or indirectly at the risk of exposure. Acute and chronic toxicity of malathion in mammals is well established both under field and experimental conditions (Uzun *et al.,* 2009; Moore *et al.,* 2009), but scanty information about its gonado- and genotoxic effects in birds is available (Maitra and Mitra, 2008; Suresh *et al.,* 2009).

Clinical signs such as torticollis, salivation, decreased crowing, less foam production and shivering/tremors could be due to muscarinic action of malathion, inhibition of acetylcholinesterase and testicular toxicity (Maitra and Mitra, 2008; Kalipci *et al.,* 2010). Similar nervous signs have been reported in mammals, birds, fish and invertebrates after prolonged exposure of commercially available OPs (Lasram *et al.,* 2008; Sodhi *et al.,* 2008). Feed intake and body weight of birds exposed to higher level of malathion were significantly reduced which reflects the taste aversion and toxic effects. Previously it has been reported that OPs are responsible for decreased feed intake and weight gain in mouse and rats (Uzunhisarcikli *et al.,* 2008).

The hematological changes in present study could due to decreased hemopoietin production, reduced erythropoiesis, impaired proliferation of haemopoietic progenitor cells and/or direct RBC lysis (Salih, 2010; Saleemi *et al.,* 2014; Ali *et al.,* 2014). The decrease in leukocyte counts in quail has already been reported (Nain *et al.,* 2011). In accessible literature no report could be found about the effect of malathion on erythrocyte counts, haematocrit and haemoglobin concentration in birds. Previously, no report is available about the increased frequency of micronuclei and cytopathogenic effects of malathion in birds as far as our knowledge is concerned. These cellular changes as well as DNA damage could be due to intracellular generation of reactive oxygen and nitrogenous species (Sodhi *et al.,* 2008; Hussain *et al.,* 2014). Nuclear blebbing and fragmentation observed in the present study could be due to over generation of caspase activated DNase which is responsible for the cleavage of cytoskeletal (gelsolin, fodrin and vimentin) and nuclear proteins (Lim *et al.,* 2009). Moreover, the cellular alterations such as binucleated erythrocytes and lobed nuclei could be due to aneuploidy. Similar

observations have also been reported in rats (Farrag and Shalby, 2007; Koç *et al.,* 2008).

Severe degenerative changes in the granular and pyramidal cells of the cerebrum, gliosis and necrosis of Purkinje cells along with infiltration of mononuclear cells in present study could be due to release of intracellular DAMPS (HsP, neuropeptides and N formal peptides) and extracellular DAMPS (hyloroune and biglycan). Moreover, these tissue changes could be overproduction of IL-1α and IL-33 from dead cells which resultantly lead to transcription of cytokine genes. These pathological changes could also be due to cholinergic toxicity caused by the inhibition of cholinesterase enzyme. The neurotoxin effects of OPs have previously been documented (Senger *et al.,* 2005; Kalipci *et al.,* 2010). Unfortunately, no reports could be found in accessible literature about the histopathological effects of malathion in brain tissues. Histologically, testes of quail given higher doses of malathion exhibited severe degenerative changes including reduced number of spermatogenic layers with accumulation of admixture of degenerative and necrotic spermatids and spermatozoa. The seminiferous tubule diameter, epithelial height and serum testosterone was significantly reduced indicating decreased process of steroidogenesis. Previously a few reports are available about the adverse impacts of organophosphorus pesticide in birds (Auon *et al.,* 2014).

It is likely that these effects of malathion and other OPs relate, at least in part, to their ability to cross the blood–testis barrier (Uzunhisarcikli *et al.,* 2007) after which they induce oxidative stress and lipid peroxidation that damages the biological membranes in the testes. This in turn may cause degeneration of the spermatogenic and Leydig cells, which disrupts spermatogenesis and reduces sperm counts (Uzun *et al.,* 2009). This idea is supported by our findings that sub acute exposure to malathion induced histopathological changes in the seminiferous tubules, namely, necrosis and edema in the seminiferous tubules and interstitial tissue. The sperm themselves may also be damaged by the oxidative effects of OPs, which affect the activities of mitochondrial enzymes and the structure of the microtubules in the sperm. This in turn reduces their motility. The fact that reactive oxygen species may contribute to infertility caused by defective sperm function has been reported previously (Latchoumycandane *et al.,* 2002). Another mechanism through which OPs can affect male reproductive function is the damage of DNA (Sarabia *et al.,* 2009). Increases in abnormal sperm counts and the disruption of spermatogenesis are important indicators of genetic damage in pesticide-exposed mammals (Burruel *et al.,* 2000). DNA damage may also reduce sperm motility. OPs may also affect male reproductive function by decreasing FSH, LH and testosterone levels, as significant alterations in concentration of these hormones levels have been reported in several animal spices after exposure pesticides (Maitra and Mitra, 2008). Extensive use of pesticides in agro-ecosystem results in long lasting and major negative impact on livestock, poultry, wildlife, water contamination and is serious threat to biodiversity due to physicochemical changes (Hussain *et al.,* 2011). Field studies demonstrated that in addition to direct toxicity, insecticides, pesticides and other pollutants can lead to changes in community structure of an ecosystem by altering nervous system, direct damage to cells and organs of immune system, causing malfunction, tremors and death (Mitra *et al.,* 2011; Sharaf *et al.,* 2013). Due to prolonged and indiscriminate use of pesticides different avian populations in an ecosystem could encounter many serious problems which originate from unsustainable agricultural practices.

***Conclusion*:** From the findings of the present study it can be concluded that pesticides cause serious pathological changes during reproductive stages of birds. Alteration of feeding behavior and chances of reproductive failure in birds due to increased and persistent exposure to pesticides reduces the ability of these birds to maintain healthy populations in ecosystem. Furthermore, it will more help to assess the toxicity of malathion in newly hatched quail or nestling birds since these may be far more sensitive than adults birds.

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