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RESEARCH ARTICLE

Exposure to Sub-Acute Concentrations of Glyphosate Induce Clinico-Hematological, Serum Biochemical and Genotoxic Damage in Adult Cockerels

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ABSTRACT

Current experimental study was aimed to find out the clinico-hematological, serum biochemical and DNA damage impacts of commonly used herbicide (glyphosate) on non-target adult cockerels. Therefore, a total of 25 cockerels were randomly placed in wire cages in five different groups each containing five birds. After seven days of acclimatization, glyphosate-based herbicide was administered to cockerels of groups (B-E) @ 50, 75, 100 and 125 mg/kg BW respectively except group A (control) for 45 days. Blood and serum was collected at 15, 30 and 45 day of the trial from each cockerel. The treated birds at higher doses indicated different clinical signs such as ruffled feather, dullness, tremors, anemic wattle and comb, depression and reduced frequency of crowing. Feed consumption and body mass was significantly ($P \leq 0.05$) lowered in cockerels exposed to higher doses of herbicide. The hematological parameters including red blood cell counts, hematocrit and hemoglobin was significantly lower in treated cockerels. Results on different biochemical parameters showed significantly lower quantity of total proteins and albumin while significantly higher concentrations of liver function tests (alanine aminotransferase, aspartate aminotransferase, alkaline phosphate), kidney function tests (urea and creatinine), cardiac biomarkers (CK-Mb, triglycerides, cholesterol) and oxidative stress parameter (malondialdehyde) of treated cockerels in dose and time dependent manner. Results indicate that the frequency of cells with tail DNA was significantly ($P \leq 0.001$) in exposed cockerels. The findings of the study suggested that long term exposure of glyphosate induces adverse clinico-hematological, serum biochemical and genotoxic effects on birds.

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INTRODUCTION

The pesticides and herbicides are extensively used in agriculture in response to increase demand of food due to growing population of the world. In spite of numerous benefits of these chemicals, their frequent and constant application has serious concerns to public health, marine and wildlife and the environment (Ghaffar *et al*., 2018a). Agriculture sector is the main and important source of variety of pesticides contaminations, those reach to adjacent water sources and their residues in different food crops lead to cause toxicological effects on exposed and non target species (Teixeira *et al*., 2018). Mostly, the farmers use pesticides and herbicides frequently and constantly without adopting the manufacture procedures and the

increase of volumes used have led to scenarios of constant and diffused environmental contamination (Riaz *et al*., 2018).

Glyphosate is one of the important members of broad spectrum herbicides and is frequently used in different cereal and food crops throughout the world to remove unwanted weeds (Pereira *et al*., 2018). For the last decade, the uses of herbicides have tremendously increased in vegetation control, agriculture and non-agricultural areas due to development of resistant of different crop varieties against glyphosate. Moreover, this herbicide is extensively used via aerial applications to remove unwanted weeds (Tarazona *et al*., 2017; Reno *et al*., 2018). The frequent and extensive applications of glyphosate in agriculture and public health have raised debate about its controversy toxicity (Blaylock, 2015). The toxicity of glyphosate formulations has been regularly monitored by different national and international agencies across the globe and the different studies have indicated relative low toxic effects in animals (Tarazona *et al*., 2017). More than 130 different commercial formulations of glyphosate herbicides are used in variety of food crops. It has been observed that a small quantity of pesticides reaches to the target pests and the huge part is spread in the environment. Reports are also available about the presence of glyphosate in surface water as a result of spraying over crops, rivers, streams, water flow and volatilization (Lopes *et al*., 2018; Reno *et al*., 2018). Earlier studies have shown that in glyphosate formulations some ingredients are highly toxic to non target species than glyphosate (Kim *et al*., 2013). Apart from the toxic effects of different agricultural pollutants at different populations and the possible recovery potential of target, non target and exposed species still remain unclear and is poorly understand because of constraints of investigation procedures. The toxic and deleterious effects of glyphosate depend upon different factors such as its formulations, concentrations and nature of salts (Piola *et al*., 2013; Reno *et al*., 2015). In published literature reports are available about the acute toxicity of glyphosate (Piola *et al*., 2013) but scanty information is available about its adverse effects on exposed organisms at very low concentrations. However, the toxic effects of glyphosate might be because of occurrence of sub lethal and complex changes like alteration in metabolic conditions, mitochondrial dysfunction, immunotoxicity, developmental toxicity, neurotoxicity and endocrine disruption (Bridi *et al*., 2017; Schimpf *et al*., 2017). Previously, in accessible published literature, no information is available regarding the adverse effects of glyphosate on male birds. Thus, this study for the first time indicate the adverse effects of glyphosate on behavior, blood and serum biochemical parameters of Lohman selected white leghorn male cockerels exposed to low concentration.

MATERIALS AND METHODS

Birds and treatment: A total of 25 cockerels (Lohmann Selected White leghorn) free from any clinical ailments and having similar age (16 weeks) and body weight (1430 gm) were purchased from commercial poultry house. All the birds were placed under similar and standard laboratory conditions including temperature (27-28°C) and relative humidity (65-70%). All the birds were placed in experimental house under strict biosecurity and were vaccinated against various viral diseases (infectious bronchitis, Newcastle disease, infectious bursal disease and hydro pericardium syndrome). Clean drinking water and commercial poultry feed having 21-22% crude proteins was given to all the birds ad libitum. After one week of the acclimatization periods all the cockerels were randomly kept into five groups (A-E) in wire cages. Different concentrations of glyphosate were given to birds daily for 45 days through crop tube as follow: A (Control), B= (50mg/kg), C= (75mg/kg), D= (100 mg/kg) and E= (125mg/kg BW). All the birds were carefully monitored for any visible clinical and behavioral changes. The feed consumption and body weight was measured on daily basis.

Hematology and Serum Biochemistry: For hematological and serum biochemical investigations about 5 ml blood was collected from wing vein of each cockerels on day 15, 30 and 45 of the trial and kept separately in glass test tube containing anticoagulant (EDTA; 1 mg/ml). For serum separation all the blood samples were collected without an anticoagulant and were placed on ice (Ghaffar *et al*., 2018b). Different blood parameters (Total erythrocyte counts, hematocrit, hemoglobin concentration and total and differential leukocyte counts) were measured according to the previously described procedures (Hussain *et al*., 2017). Different serum biochemical parameters including aspartate aminotransferase (Cat# 12021150), alanine aminotransferase (Cat# 2022150), alkaline phosphatase (Cat# 12027150), urea (Cat# 10500400), creatinine (Cat# 389143), cardiac isoenzyme creatinine phosphokinase monobasic (Cat# 339333), cholesterol (Cat# 10150100), triglycerides (Cat # TR1697A) were measured commercial Kits with the help of chemistry analyzer (Ghaffar *et al*., 2018b).

Single cell gel electrophoresis (SCGE) or comet assay
Single cell gel electrophoresis (SCGE) or comet assay was conducted to determine the frequency of DNA damage according to the procedure of Singh *et al.* (1988) on blood lymphocyte. Briefly, lymphocytes were isolated from each bird at different experimental intervals. Microscopic smears of normal and low melting point agarose containing lymphocyte were kept in lysing buffer, neutralized with chilled tris buffer (pH 7.5) and finally stained with ethidium bromide. All the slides were immediately observed under fluorescent microscope. A total of 250 cells from each bird were examined and percentile rate of cells with tail DNA was recorded.

Statistical analysis: Data on feed consumption, weight, blood and serum biochemical observations were subjected to statistical analysis (M-stat statistical software). Means ± SE for different treatments were compared by Tukey's test with $P \leq 0.05$.

RESULTS

Physical Parameters: No mortality, behavioral and clinical ailments were observed in cockerels of control

group throughout the trial. However, the cockerels placed in group E showed different clinical signs like ruffled feather, anorexia, tremors, depression, sitting on their hock joint with closed eyes and less frequency of crowing. The mild to moderate similar clinical ailments were also evident in cockerels of group D. Severity of these ailments was increased in cockerels of group E with increased in time. The feed intake was significantly lower in cockerels of group E at day 15 as compared to cockerels of control group (A). However, significantly lower feed consumption was recorded in cockerels of groups D-E at 30 and 45 day of the trial in comparison to that of control group. The body mass of cockerels kept in group E at day 15 and in groups D-E at days 30 and 45 was significantly reduced when compared to cockerels of control group A (Table 1).

Hematology: Various blood parameters such as total red blood cell counts (10^{12}/L), hemoglobin concentration (g/dl), hematocrit (%) and total white blood cell counts (10^{9}/L) of cockerels exposed to different concentrations of glyphosate were measured at day 15, 30 and 45 of the trial. The total erythrocyte counts were significantly lower in cockerels of group E at day 15 of the experiment. The total erythrocyte counts in cockerels of groups D-E at 30 and 45 day of experiment was significantly lower compared to cockerels of control group. At day 45 of the trial, values of erythrocyte were significantly ($P \leq 0.05$) lower in cockerels of group C (Table 2). Hemoglobin concentration was significantly lower in cockerels of groups D-E at all experimental days, while in cockerels of group C at 45 of the experiment. Hematocrit was significantly lower in cockerels of groups D-E when compared to cockerels of group A throughout the experiment. The total white blood cell count was significantly increased in cockerels of groups D-E at all experimental intervals, while at day 45 of the experiment in cockerels of group C in comparison to that of control group A. The results indicated that the response (increased or decreased) of different blood parameters was in treatment and time dependant manner. Frequency of blood lymphocyte with damaged DNA was significantly higher in cockerels of group D at day 45 of the trial. Similarly, percentile rate of blood lymphocyte with DNA damage (Fig. 1) was significantly increased in cockerels of groups D-E throughout the experiment in comparison to that of untreated control group (Table 2).

Serum Biochemistry: The results indicated that different serum parameters including liver function tests, kidney function tests, cardiac biomarkers and oxidative stress biomarkers were statistically different in herbicide exposed cockerels at various intervals of the experiment (Table 3). The quantity of serum total proteins and albumin was decreased significantly in cockerels of groups D-E at day 15, 30 and in groups C-E ad day 45 of the trial compared to cockerels of control group A (Table 3). The quantity of alanine aminotransferase at 15 and 30 day of the trial increased significantly in cockerels of groups D-E and at day 45 in cockerels of groups C-E. The quantity of alkaline phosphate significantly increased in cockerels of groups D-E at all experimental intervals as

Table 1: Feed intake (g/day/bird) and body weight (gm) of cockerels exposed to various doses of glyphosate

Parameter/Days	Glyphosate (mg/kg/day)				
	A(0.0)	B(50)	C(75)	D(100)	E(125)
Feed intake					
15	92.75±2.32	89.95±1.01	89.65±1.53	88.12±1.08	83.4±1.39*
30	98.42±2.74	93.4±1.8	91.7±2.06	87.87±1.22*	81.6±1.09*
45	103.8±1.14	99.2±1.1	97.92±1.78	87.92±2.01*	80.67±30.8*
Body weight					
15	1474.9±5.85	1469.9±4.41	1467.9±3.71	1466.5±2.1	1461.4±3.9*
30	1533.9±4.1	1526.3±3.6	1524.3±1.7	1522.9±2.3*	1496.9±7.5*
45	1556.6±5.4	1550.25±2.5	1544.4±4.9	1531.6±3.6*	1524.3±4.9*

The asterisk values in each row (mean±SE) differ significantly ($P \leq 0.05$) from control group.

Table 2: Hematological and genotoxic profile of cockerels exposed to various doses of glyphosate

Parameter/Days	Glyphosate (mg/kg/day)				
	A(0.0)	B(50)	C(75)	D(100)	E(125)
Erythrocyte counts (10^{12}/L)					
15	3.43±0.04	3.39±0.02	3.36±0.09	3.27±0.05	2.92±0.03*
30	3.43±0.09	3.29±0.06	3.28±0.08	2.97±0.03*	2.81±0.07*
45	3.45±0.08	3.28±0.03	3.13±0.02*	2.83±0.06*	2.72±0.03*
Hemoglobin concentration (g/dl)					
15	12.56±0.17	12.49±0.10	12.45±0.07	10.71±0.06*	10.28±0.22*
30	12.61±0.05	12.27±0.06	12.06±0.03	10.25±0.12*	9.95±0.17*
45	12.59±0.03	12.16±0.08	10.74±0.06*	10.12±0.04*	9.77±0.08*
Hematocrit (%)					
15	41.8±1.2	38.4±1.1	37.4±1.6	35.9±0.5*	35.5±0.4*
30	39.9±0.8	37.8±2.37	36.9±1.3	34.8±0.7*	34.6±0.3*
45	40.5±0.8	36.9±1.5	36.7±0.7	34.2±1.9*	34.1±0.4*
Leukocyte counts (10^{9}/L)					
20	9.95±0.12	10.01±0.06	10.21±0.08	10.93±0.15*	11.84±0.10*
40	9.93±0.16	10.21±0.05	10.35±0.03	11.94±0.20*	12.01±0.47*
60	9.97±0.05	10.32±0.03	10.99±0.02*	12.07±0.21*	12.32±0.26*
Lymphocyte with tail DNA (%)					
15	2.11±0.11	2.12±0.05	2.16±0.05	11.22±2.11*	15.39±3.18*
30	2.09±0.04	2.14±0.03	2.18±0.01	14.17±1.52*	18.33±2.441*
45	2.05±0.05	2.15±0.05	6.20±0.01*	17.23±2.12*	23.14±3.23*

The asterisk values in each row (mean±SE) differ significantly ($P \leq 0.05$) from control group.

Table 3: Serum biochemical profile of cockerels exposed to various doses of glyphosate

Parameters/days	Glyphosate (mg/kg/day)				
	A(0.0)	B(50)	C(75)	D(100)	E(125)
Total proteins (g/dl)					
15	3.12±0.02	3.06±0.01	3.03±0.04	2.83±0.04*	2.74±0.03*
30	3.14±0.02	3.01±0.04	2.95±0.02	2.76±0.04*	2.70±0.04*
45	3.13±0.03	2.97±0.04	2.76±0.04*	2.70±0.03*	2.62±0.02*
Albumin (g/dl)					
15	1.54±0.03	1.47±0.02	1.46±0.01	1.29±0.01*	1.26±0.01*
30	1.52±0.02	1.45±0.02	1.44±0.02	1.27±0.03*	1.23±0.02*
45	1.51±0.01	1.42±0.02	1.27±0.02*	1.23±0.01*	1.31±0.01*
Alanine aminotransferase (unit/L)					
15	6.25±0.23	6.57±0.13	6.75±0.07	7.73±0.19*	7.89±0.08*
30	6.45±0.60	6.61±0.15	6.70±0.08	8.22±0.09*	8.90±0.03*
45	6.30±0.11	6.84±0.05	7.97±0.04*	8.87±0.11*	9.29±0.13*
Aspartate aminotransferase (unit/L)					
15	186.32±7.13	191.32±1.75	193.9±3.39	199.1±3.94*	205.3±3.68*
30	186.72±3.94	193.62±1.50	198.25±3.10*	203.05±2.80*	215.3±4.67*
45	187.75±2.68	194.47±1.47	199.55±3.32*	215.9±6.96*	237.4±5.73*
Alkaline phosphate(unit/L)					
15	13.11±0.67	13.57±0.13	13.74±0.44	15.74±0.38*	16.24±0.22*
30	13.15±0.24	13.75±0.19	13.84±0.11	15.97±0.33*	16.45±0.40*
45	13.09±0.18	13.80±0.10	14.02±0.26	16.05±0.60*	17.45±0.43*
Urea (mg/dL)					
15	7.01±0.06	7.07±0.12	7.16±0.04	8.63±0.09*	8.95±0.04*
30	7.04±0.06	7.26±0.05	7.52±0.19	8.92±0.04*	9.13±0.11*
45	6.97±0.07	7.33±0.02	8.18±0.09*	9.25±0.23*	9.57±0.10*
Creatinine (mg/dL)					
15	1.20±0.02	1.22±0.03	1.25±0.01	1.49±0.03*	1.66±0.09*
30	1.21±0.02	1.27±0.02	1.30±0.01	1.59±0.03*	1.92±0.06*
45	1.19±0.02	1.29±0.02	1.53±0.01*	1.76±0.06*	2.09±0.10*
Cholesterol (mg/dL)					
15	90.37±1.59	92.17±1.62	92.62±1.52	99.85±1.51*	99.94±1.79*
30	91.55±1.48	93.62±1.45	95.17±1.14	102.6±1.90*	103.97±2.47*
45	91.5±1.11	94.1±1.86	95.47±1.25	105.3±1.62*	107.85±0.63*
Triglycerides (mg/dL)					
15	66.5±4.36	71.92±2.81	72.7±1.46	76.82±2.85*	79.57±2.8*
30	68.25±2.95	72.11±1.68	74.27±1.88	79.22±1.97*	85.5±1.81*
45	68.95±2.72	73.8±1.34	75.7±1.68*	85.82±2.52*	92.65±2.79*
CK-MB (unit/L)					
15	10.03±0.19	10.38±0.33	10.47±0.43	11.82±0.11*	12.44±0.21*
30	10.27±0.08	10.52±0.07	10.79±0.04	11.90±0.05*	13.67±0.80*
45	10.19±0.06	10.57±0.18	10.90±0.19	13.44±0.75*	14.31±0.17*
Malondialdehyde concentration (mg/dl)					
15	2.29±0.02	2.37±0.02	2.38±0.031	2.73±0.03*	2.83±0.04*
30	2.31±0.01	2.39±0.01	2.52±0.03	2.79±0.06*	3.09±0.07
45	2.32±0.02	2.52±0.04	2.69±0.03*	2.92±0.04*	3.25±0.05

The asterisk values in each row (mean ±SE) differ significantly ($P \leq 0.05$) from control group.

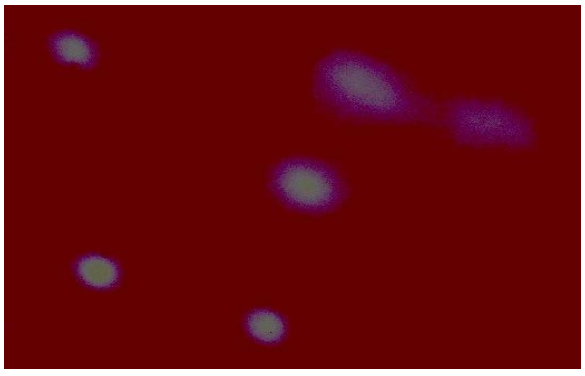

Fig. 1: Single cell gel electrophoresis or comet assay showing DNA damage fluorescing around the nuclei (making tail) of blood lymphocyte of cockerels following glyphosate exposure.

compared to cockerels of control group. The quantity of urea and creatinine was significantly higher in cockerels of groups D-E at 15 and 30 day of the trial while in groups C-E at day 45 as compared to control group. The level of cholesterol and cardiac isoenzyme creatinine phosphor-kinase monobasic (CK-Mb) increased significantly in cockerels of groups D-E at 15 and 30 day of experiment while in groups C-E at day 45 as compared to non treated cockerels. The concentrations of triglycerides and a lipid per oxidation product malondialdehyde increased significantly in cockerels of groups D-E at 15 and 30 day while in cockerels of groups C-E at day of the trial (Table 3).

DISCUSSION

It is well established that different pesticides, insecticides and herbicides are extensively used in agriculture to enhance the food production (Ghaffar *et al*., 2016). Among different herbicides, glyphosate (N-phosphonomethyl- glycine) is widely used herbicide across the globe to control unwanted weeds. It controls weeds via inhibition of a key enzyme (5-enolpyruvylshikimate-3- phosphate synthase) responsible for synthesis of aromatic amino acids in plants (Pereira *et al*., 2018). The investigation of deleterious effects of widely used glyphosate is of vital importance as different reports have indicated the presence of its residue in food and water. On the basis of previous adverse reports of adverse effects of glyphosate on different non-target

species, the current experimental study indicated the potential deleterious effects of sub lethal exposure this synthetic chemical on adult cockerels. Although no mortality of cockerels was observed during the experiment but results showed that glyphosate induced different remarkable behavioral alterations which include depression, dullness, tremors, ruffled feathers, anemic wattle and combs and decrease frequency of crowing. Previously, no reports are available about these behavioral changes in birds. These changes might be due to induction of oxidative stress and neuronal activities of glyphosate. The obtained results revealed remarkable decrease in feed consumption and body weight of cockerels exposed to increased concentrations of glyphosate. Lower feed intake and body weight at higher doses of glyphosate in broilers has been reported (Kubena *et al*., 1981). The less feed intake and body weight could poor feed utilization and taste aversion. Previously different clinical signs, behavioral changes, poor feed intake and significant lower body weight of birds have been reported due to other herbicides (Hussain *et al*., 2011; Hussain *et al*., 2014). In present experiment, the observed lower hematological values of total red blood cell population, hematocrit and hemoglobin suggesting anemia might be cause of toxic effects of glyphosate on hematopoietic tissues. The noticeable lower red blood values have also been reported due to pesticides in cockerels and commercial layers (Hussain *et al*., 2017; Ghaffar *et al*., 2018b). The lower values of hemoglobin in cockerels appeared to be from increased oxidation of methaemoglobin or it could be due to toxic stress by the herbicide. Less hematocrit appears to be due to decrease in size and destruction of red blood cells (Rahman *et al*., 2006). The significantly less values of blood parameters in exposed cockerels could be due to impaired functions of hematopoietic tissues or reduced hemopoietin synthesis (Hussain *et al*., 2014). In contrast to results of this study, the total white blood cell count decreased significantly in salvator merianae exposed to pesticide formulations containing cypermethrin, chlorpyrifos and glyphosate (Mestre *et al*., 2018). Higher values of white blood cells in this study clearly indicate the tissue damage and hypersensitivity of cockerels. In this study significantly increased frequency of lymphocytes with DNA damage (comet formation) has not been reported in cockerels in accessible literature. However, these findings might be due to induction of oxidative stress by glyphosate on blood forming tissues of birds resulting in overproduction of free radicals responsible for DNA damage. Previously, different reports are available about the mutagenic effects of various other herbicides such as atrazine and butachlor that induce oxidative stress and free radical injury in exposed animals leading to DNA damage (Hussain *et al.*, 2011; Hussain *et al.*, 2014; Ghaffar *et al.*, 2015).

Investigation and monitoring of blood biochemical changes are known as the best biomarkers and indicators of variety of physical stress induced by different compounds in different species (Ghaffar *et al*., 2016). In present study lower values of serum total proteins can be related to more utilization of proteins by the cockerels due to oxidative stress induced by glyhposate. Moreover, it is reported that the concentrations of serum total proteins decrease as a results of oxidation of amino acids ((Verma *et al*., 2015). In addition, the reduced serum total proteins and albumin in glyphosate treated cockerels could be due to less feed utilization, impaired tissue regeneration, abnormal detoxification and increased energy utilization during oxidative stress period. The poor values of these serum proteins are suggestive immunosuppressive (Nayak *et al*., 2004) nature of glyphosate in cockerels. Various other serum parameters such as alaninine aminotransferase, aspartate aminotransferase, alkaline phosphate, urea and creatinine were significantly increased in cockerels as increase in concentrations and time of the experiment. Previous studies have indicated increased concentrations of liver and kidney function tests in birds due to different herbicides including atarazine and butachlor has also been reported (Hussain *et al*., 2012; Hussain *et al*., 2014). The aspartate aminotransferase and alaninine aminotransferase enzymes are routinely used to determine the status of hepatocytes. The increased concentrations of these enzymes in glyphosate treated cockerels in this study are suggestive of damage to membranes of hepatocytes. Similar results have also been reported (Adejumo *et al*., 2015). The increased levels of urea and creatinine in cockerels might be due to abnormal mechanisms of filtration and injury to kidneys (El-Murr *et al*., 2015; Narra *et al*., 2017). The quantity of serum cholesterol, cardiac isoenzyme and triglycerides increased in glyphosate treated cockerels. Previously, no reports are available about the toxic effects of glyphosate on these parameters in birds. The increased concentrations of these serum parameters in birds due to different toxic compound have been reported (Ghaffar *et al*., 2018b). The higher values of serum cardiac isoenzyme, cholesterol and triglycerides might be due to toxic effects of glyphosate on cardiac tissues. In published literature no report is found about the induction of oxidative stress. Significantly higher concentrations of malondialdehyde (a lipid per oxidative stress parameter) can be related to induction of oxidative stress by glyphosate in cockerels. Moreover, lipid peroxidation in cockerels in this study might be due to increased generation of super oxides and free radicals by the glyphosate.

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