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Original Article

Ethoxysulfuron causes nuclear abnormalities in erythrocytes, DNA damage in some visceral organs, and oxidative stress in male Japanese quail

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Abstract
Ethoxysulfuron is frequently used as a post-emergent herbicide in different food crops to control broad leaf weeds. This study aimed to investigate the potential adverse effects of ethoxysulfuron herbicide on male Japanese quail. 48 adult male birds were divided into 4 groups and orally administered with varying doses of the herbicide for 45 days. Quail were exposed to four sub-lethal concentrations (0 mg/kg, 0.70 mg/kg/day, 0.90 mg/kg/day, and 1.25 mg/kg/day) of ethoxysulfuron starting from day 1 to 45 of trial and different samples from each quail were obtained at days 15, 30 and 45 of trial following exposure. Results exhibited that with increasing ethoxysulfuron concentrations different nuclear abnormalities in erythrocytes such as the formation of micronuclei, binucleated erythrocyte, notched nuclei, blabbed nuclei, lobbed nuclei, condensed nuclei, nuclear remnants and the erythrocytes without nuclei increased in treated birds. Results showed that exposure to herbicide led to genotoxicity, as evidenced by increased values of DNA damage in different visceral organs of the exposed quail compared to the control group. Various antioxidant enzymes and oxidative stress biomarkers were significantly reduced and escalated in the liver tissues of quail. Histological examination revealed prominent histoarchitectural changes in the liver (degeneration of hepatocytes and necrosis) and kidneys (degeneration of renal tubules, necrosis of renal tubules, and necrosis of renal tubular epithelial cells) of the exposed quail. In conclusion, this study demonstrates that exposure to ethoxysulfuron herbicide can cause significant morphological and nuclear abnormalities in erythrocytes, DNA damage, and histoarchitectural changes in different visceral organs of Japanese quail. These findings suggest that exposure to herbicides may pose a health risk to both humans and wildlife.

Keywords: Ethoxysulfuron, Japanese quail, Oxidative stress, DNA damage, Histopathology

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Introduction

In the present era, agriculture is the main source of food and livelihood for the world's population. The increasing demand for food has led to the use of various chemical agents like insecticides and herbicides to increase crop yield (Hussain et al., 2018; Ahmad et al., 2021; Hussain et al., 2022). However, the use of these chemicals has raised serious concerns about their effects on non-target organisms (Hussain et al., 2012; Hussain et al., 2014; Merdana et al., 2021). The use of insecticides and herbicides in modern agriculture and aquaculture has increased significantly in recent decades, resulting in an elevated exposure of wildlife to these chemicals (Ghaffar et al., 2020; Tahir et al., 2021; Mahmood et al., 2021; Ghaffar et al., 2021). In poultry industry, the Japanese quail has got significant importance and are frequently used as a source of protein and in biological research because of small size and lower risks of diseases (Nazir et al., 2022) Pesticides and different herbicides have been widely used in agriculture to protect crops from pests and diseases but their widespread use has resulted in environmental contamination and adverse effects on non-target organisms (Ghaffar et al., 2020; Namratha et al., 2021; Ghaffar et al., 2021; Ismail, 2022). Among the various classes of herbicides, sulfonylureas have gained significant attention due to their widespread use and toxicity concerns. Ethoxysulfuron (ES) is a widely used sulfonylurea herbicide that is known to have potential genotoxic and pathological effects on various organisms. Ethoxysulfuron is a post-emergent herbicide that is commonly used to control weeds in cereal crops. Pesticides are widely used to protect crops and increase agricultural productivity. However, their excessive use poses a significant threat to the environment, human and animal health. Different herbicides such as ethoxysulfuron, flucetosulfuron, penoxsulam, bispyribac sodium, cyhalofop butyl, fenoxaprop-p ethyl and glyphosate are widely used as post-emergence herbicides in agriculture (Namratha et al., 2021; Namratha et al., 2022). Ethoxysulfuron is commonly used in combination with other herbicides due to the broad-spectrum controlling of weeds and rapid rate of dispersion in soil (Sondhia, 2014; Raj and Syriac, 2015). It is known to inhibit acetolactate synthase (ALS) enzyme activity which is a key enzyme in the biosynthesis of branched-chain amino acids in plants. However, the toxicological effects of ES on non-target organisms, especially avian species are not well-documented. Japanese quail (*Coturnix coturnix japonica*) are widely used as a model organism for ecotoxicological studies due to their fast reproduction, easy handling, and small size.

Previous studies have reported the genotoxic effects of some herbicides on various organisms, including birds and fish. For instance, glyphosate exposure has been reported to induce DNA damage in the liver and blood cells of Japanese quail and freshwater fish (Ghaffar et al., 2021; Jabeen et al., 2021). Similarly, the herbicide atrazine has been demonstrated to cause genotoxicity in different avian species (Hussain et al., 2011; Hussain et al., 2012). However, the genotoxic effects of ES have not been studied in quail to date. Furthermore, several studies have reported the pathological effects of herbicides on different organs of birds (Hussain et al., 2012; Hussain et al., 2014). For instance, the herbicide atrazine has been shown to induce oxidative stress and pathological changes in the liver, kidney, and lung tissues of quail (Hussain et al., 2011; Hussain et al., 2012). Exposure to insecticides and herbicides is a major concern for both human and animal health. The widespread use of these chemicals in agricultural practices has led to their accumulation in the environment and food chain. Japanese quail, a widely used experimental model in toxicology studies, have been shown to be sensitive to the toxic effects of various environmental pollutants (Hussain et al., 2011; Hussain et al., 2012; Ahmed et al., 2021; Hussain et al., 2021). This study investigated the potentially toxic effects of ethoxysulfuron in adult male Japanese quail in terms of evaluation of nuclear abnormalities in erythrocytes, DNA damage in various visceral organs, and histoarchitectural changes.

Material and Methods

Study design

A total of 48 male Japanese quail with an average age of 5-6 weeks were obtained from the local market and randomly divided into four various groups for this trial. The birds were divided into four groups (A – D), with each group having 12 birds. The birds of one group (A) served as untreated control. The experimental quail had free access to normal drinking water and commercial poultry feed was offered early in the morning and late evening daily. After 07 days of acclimatization, the birds of groups (B-D) were orally administered with different doses of ethoxysulfuron (0.70mg/kg/day, 0.90mg/kg/day, 1.25mg/kg/day) on the basis of published literature through crop tube. The duration of the experiment was 45 days.

Collection of blood and nuclear abnormalities

Blood (4-5ml) from each quail was collected on days 15th, 30th, and 45th of the trial from the jugular vein under sterile conditions and placed in glass test tubes containing an anticoagulant and without an anticoagulant immediately after collection. Fresh blood films were prepared on glass microscopic slides from the blood of each quail without any anticoagulant. All the prepared slides were air-dried, fixed with methanol, and stained with field stains for evaluation of nuclear abnormalities in erythrocytes. The stained blood smears were examined using an oil immersion lens at a magnification of 1000X under a light microscope and nuclear abnormalities were recorded in accordance with the previous approach (Hussain et al., 2012; Hussain et al., 2014).

Histoarchitectural changes

The liver and kidneys were collected on days 15, 30, and 45th of the trial the birds were kept in each group after scarification. The liver and kidneys of each bird were immediately removed, washed with phosphate-buffered saline (PBS), and fixed in a 10% formalin solution for histological analysis. After a few days of fixation, all the tissues were processed for histological analysis. The sections were stained with hematoxylin, and eosin and were observed under a light microscope. The samples were then processed using a routine histological technique and sections of 5 µm thickness were stained with hematoxylin and eosin (H&E) for histological analysis (Hussain et al., 2014).

DNA damage

For estimation of DNA damage, the comet assay or single-cell gel electrophoresis was used according to the protocol described by earlier studies (Singh et al., 1988; Hussain et al., 2011) under alkaline conditions with some moderate modifications. Briefly, different tissues (liver, kidneys, and intestine) of control and treated birds were obtained at days 15, 30, and 45th of the trial washed and homogenized separately in chilled sterile normal saline solution of isolation of cells. The isolated cells were suspended separately from each tissue in low-melting point agarose and normal melting point agarose on microscopic glass slides. The prepared slides were then lysed in chilled lysing solution under alkaline conditions and then subjected to electrophoresis, neutralized, and stained with ethidium bromide. Finally, the slides were observed under a fluorescence microscope to determine the extent of DNA damage according to various previous studies (Akram et al., 2022a).

Oxidative stress and antioxidant profile

Different oxidative stress parameters including reactive oxygen species (ROS) Thiobarbituric acid reactive substances (TBARS) and antioxidative enzymes including the status of catalase (CAT), peroxidase (POD), and reduced glutathione (RGSH) were determined in homogenates of all the liver tissues of treated and control birds according to some modifications regarding estimation of ROS (Hayashi et al., 2007). TBARS (Iqbal et al., 1996), POD (Chance and Maehly, 1955), SOD (Kakkar et al., 1984) and RGSH (Jollow et al., 1974).

Statistical analysis

The data obtained on nuclear abnormalities analysis, comet assay, and oxidative stress profile were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's posthoc test for determination of significant difference. P values less than 0.05 were considered statistically significant.

Results

Nuclear alterations in erythrocytes and genotoxicity

The results of the study revealed that exposure to ethoxysulfuron resulted in the development of nuclear abnormalities (Fig.1) in the erythrocytes of male Japanese quail. The micronuclei frequency increased significantly ($p < 0.05$) in the quail of groups given higher doses of herbicide as compared to the control group. The results of this study showed that exposure to ethoxysulfuron caused an increase in the percentage of erythrocytes with nuclear remnants in Japanese quail. The percentage of erythrocytes with nuclear remnants increased steadily over time, with the highest values observed at 45 days of exposure. The percentage of binuclear erythrocytes increased in a dose-dependent manner with ethoxysulfuron exposure. The highest percentage of binuclear erythrocytes was observed in birds exposed to the highest concentration of ethoxysulfuron. The results of the study indicate that exposure to ethoxysulfuron leads to a dose-dependent increase in nuclear abnormalities in erythrocytes in Japanese quail. The erythrocytes with condensed nucleus percentage increased significantly ($p<0.05$) with increasing concentration of ethoxysulfuron. The results of the present study suggest that exposure to ethoxysulfuron had a dose-dependent rise in the proportion of erythrocytic nuclei without cytoplasm in Japanese quail.

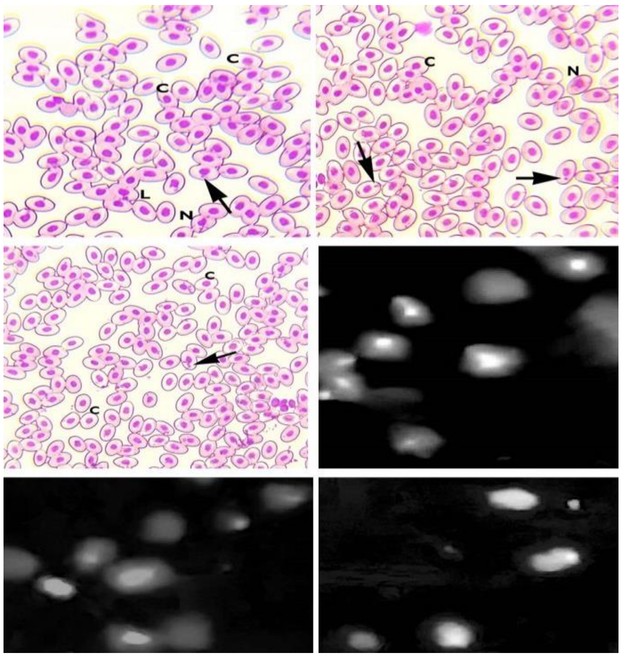

Figure-1. Photomicrograph showing nuclear ailments in erythrocytes Field stain A and B) and DNA damage (ethidium bromide stain) in some visceral organs of male Japanese quail treated with ethoxysulfuron.

At 15 days, the percentage of the erythrocytic nucleus without cytoplasm in quail exposed to higher concentrations of herbicides. Results showed that all exposed groups significantly increased the percentage of erythrocytes with lobbed nuclei with the increase in the dose of ethoxysulfuron as compared to the control group. Specifically, at day 45, the percentage of erythrocytes with lobbed nuclei in groups C, and D were significantly increased. The results revealed that the percentage of erythrocytes with blebbed nuclei was found to be significantly increased in a dose-dependent manner in quail compared to the control group. The highest increase was observed in the group treated with the highest concentration of ethoxysulfuron. The results on genotoxic levels recorded by the comet assay technique indicated the frequency of DNA damage in isolated cells of the liver was significantly escalated at day 45 of the experiment in quail of group C while at days 30 and 45th of the trial in Group D. The results showed that DNA damage was significantly increased in isolated cells of kidneys and intestine at days 30 and 45th of the study in quail of groups C and D (Fig. 2).

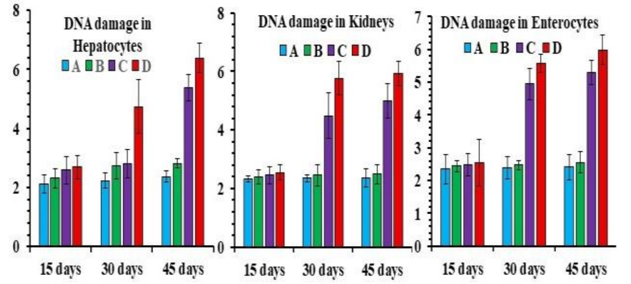

Figure-2. DNA damage in hepatocytes, kidneys, and enterocytes of Japanese quail exposed to different concentrations of ethoxysulfuron' P values less than 0.05

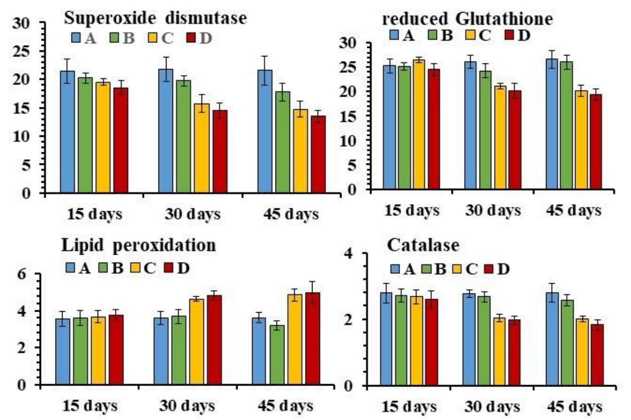

Figure-3. Oxidative stress and antioxidant enzymes status in the liver of quails. P values less than 0.05

Oxidative stress and histopathology

The results of oxidative stress and antioxidant enzymes in the liver of treated quail showed (Fig. 3) significantly increased contents of lipid peroxidation product and reactive oxygen species at days 30 and 45th of the trial. Grossly, the liver and kidneys of quail of groups A-B did not indicate any obvious pathological lesions and were normal in color and consistency. The liver and kidneys of quail of groups C and D at day 45 of the study moderate to severe changes like the liver was pale and fragile, consolidated while the kidneys were congested, swollen, and bulging from their sockets. Histological examination revealed prominent histoarchitectural changes in the liver (degeneration of hepatocytes and necrosis) and kidneys (degeneration of renal tubules, necrosis of renal tubules, and necrosis of renal tubular epithelial cells) of the exposed quail (Fig. 4). Different sections of kidneys of ethoxysulfuron treated quail exhibited atrophy of glomeruli, congestion and widening of urinary spaces at days 30 and 45th of the experiment at higher concentrations.

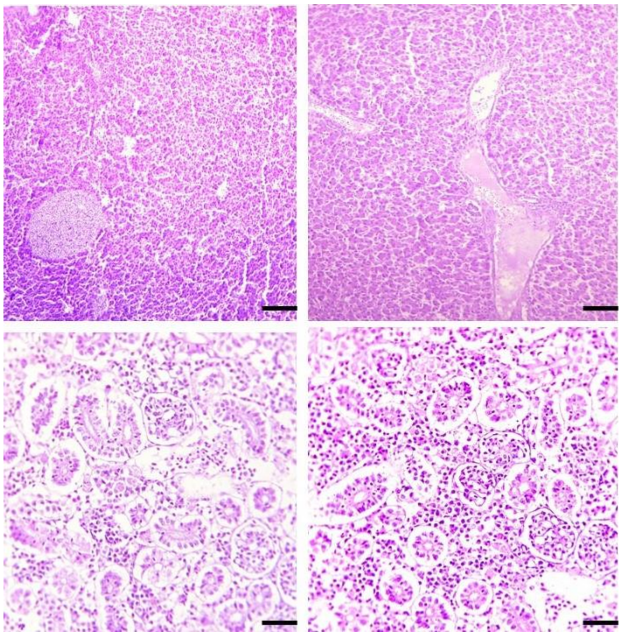

Figure-4. Photomicrograph exhibiting microscopic ailments in the liver (degeneration of hepatocytes, necrosis, atrophy of hepatocyte and edema) and kidneys (necrosis of renal tubular epithelial cells, widening of urinary spaces, inflammation and necrosis of renal tubules). H&E stain 400X.

Discussion

High consumption of herbicides in agriculture and inadequate disposal cause contamination in soil, groundwater, lakes, rivers, and the environment (Islam et al., 2018; Namratha et al., 2022). Birds, animals, and human beings are all affected by the toxic effects of herbicides (Hussain et al., 2011). The percentage of erythrocytes with micronuclei increased in a dose-dependent manner. These findings indicate that ethoxysulfuron has a genotoxic potential and can cause DNA damage in erythrocytes. The study highlights the need for careful use of this herbicide to prevent adverse effects on avian species and the environment. These results suggest that ethoxysulfuron exposure may lead to morphological and nuclear abnormalities in erythrocytes in Japanese quail at a dose of 1.25mg/kg/day) and duration of exposure (15-45 days). These findings suggest that ethoxysulfuron may harm the erythrocytes of Japanese quail, which could have implications for the health of these birds and potentially for other wildlife and humans exposed to this herbicide. Results showed that exposure to herbicide led to genotoxicity, as evidenced by increased values of DNA damage in different visceral organs of the exposed quail compared to the control group. Various antioxidant enzymes and oxidative stress biomarkers were significantly reduced and escalated in the liver tissues of quail. Histological examination revealed prominent histoarchitectural changes in the liver (degeneration of hepatocytes and necrosis) and kidneys (degeneration of renal tubules, necrosis of renal tubules, and necrosis of renal tubular epithelial cells) of the exposed quail. In the current study, birds exposed to greater herbicide doses had a considerably higher frequency of micro nucleated, lobed, and notched erythrocytes. As far as we know, there has never been a report on the prevalence of micronuclei and the cytopathogenic effects of butachlor in birds. Increased production of intracellular reactive oxygen and nitrogenous species may be the cause of these cellular changes (Sodhi et al., 2008; Campos-Pereira et al., 2012; Mohammed et al., 2021). Micro nucleated and lobed erythrocytes in present research work could be due to over generation of caspase-activated DNase which is responsible for the cleavage of cytoskeletal (gelsolin, fodrin, and vimentin), nuclear proteins, oxidative stress to mitochondrion and aneuploidy. it is well documented that exposure to different toxicants in various organisms causes the generation of a higher number of free radicals (ROS) due to natural detoxifying mechanisms in all animals. These morphological changes might also be caused by oxidative stress to the mitochondria, which could result in cytochrome c potentially leaving the voltage-dependent anion channels of the mitochondria's outer membrane. Additionally, it triggers apoptotic changes like the cleavage of cytoskeleton proteins like gelsolin and fodrin and increased caspase production (Behera et al., 2023; Newman and Shadel, 2023). Evaluation of antioxidant enzymes like superoxide dismutase, catalase, reduced glutathione, and oxidative stress parameters like lipid peroxidation and reactive oxygen species are known as the top biomarkers of inflammatory response and useful tools in preventing tissues damage caused by free radicals (Hussain et al., 2019; Meli et al., 2020; Akram et al., 2021; Akram et al., 2022b) and screening of toxic effects of toxicants (Ju-Wook et al., 2019; Naseer et al., 2020; Naseem et al., 2022). Free radicals are produced in stress conditions which cause the dysfunction of cell and mitochondria membranes and their structural anomalies (Liu et al., 2014; Kiran et al., 2022). The process of lipid peroxidation is initiated with the production of ROS, which leads to irregularities in cellular membranes and the production of TBARS (Raza et al., 2022; Li et al., 2022). Therefore, depletion and improper balancing of antioxidant enzymes in the current study could be due to increased contents of oxidative stress parameters in quails. The determination of nucleic acid concentration is also considered a biomarker for the evaluation of apoptosis (Miladinović et al., 2021). In the current study DNA damage was found in Japanese quail due to ethoxysulfuron. This damage could be due to genotoxicity because ROS leads to DNA strand breaks where oxyradicals function as chemical nucleases on DNA which ultimately leads to DNA strands breakout. In another study, it is reported that DNA damage affects the survival, reproduction, and growth of avian species because it makes them less resistant to changes in the environment (de Faria et al., 2018; Aziz et al., 2022). Several studies support our findings that quails suffer from DNA damage by many other toxicants (Sengul et al., 2008; Eshak et al., 2013; Suljevic et al., 2019; Pan et al., 2022). Histologically degenerative changes have been observed in the liver and kidney of Japanese quail, an enlargement of the liver with fat accumulation in the cytoplasm, necrosis and decrease in spermatogenesis, presence of necrotic spermatids in normal spermatids and decreased number of spermatids in the seminiferous tubules respectively. This could be due to the effects of ethoxysulfuron on estradiol and testosterone which further leads to abnormal secretion of follicle stimulated hormone or reproductive disorders. Previously, no data was reported about the histopathological changes in quails due to ethoxysulfuron and other toxicant (Kılıç et al., 2022). However, it is suggested that several other herbicides are responsible for causing toxic effects in the liver and kidneys of Japanese quail (Ali et al., 2016; Faryadi and Sheikhahmadi, 2017; Kou et al., 2020; Nohara et al., 2022).

Conclusions

The results showed significant alterations in these parameters, indicating the potential cytotoxic and genotoxic effects of this insecticide-herbicide mixture on Japanese quail. These findings emphasize the need for further studies on the toxicological effects of environmental pollutants on animals and humans.

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Hussain R: Designed the experiment, did data analysis and approved final draft of article