Research Article

Hematological and Systemic Toxicity of *in ovo* Flubendiamide Exposure in Newly Hatched Domestic Chicks

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ABSTRACT

Aim: This study examines the hematological effects of flubendiamide, a novel insecticide, on freshly hatched chicks at in-ovo injection. Given the extensive use of pesticides and concerns about its influence on non-target species, especially ecosystems, flubendiamide's potential side effects must be understood. **Materials and Methods:** After hatching, liver and body weights, hematological indices like albumin, globulin, total protein, hemoglobin, RBC count, white blood cell count and differential leukocyte count were measured in embryos exposed to 500 ppm flubendiamide. These analyses assessed flubendiamide-induced systemic toxicity, liver function, erythrocytopenia, immunocompetence and inflammatory responses. **Results:** Embryos treated to flubendiamide showed severe liver and body weight reductions post-hatching, indicating systemic poisoning. Though relative liver weight was maintained, hematological studies showed decreased albumin, globulin and total protein, suggesting liver dysfunction and protein production issues. Hemoglobin and RBC counts decreased, indicating flubendiamide-induced erythrocytopenia and lysis. Leucopenia and immunocompetence were shown by decreased total white blood cell count and lymphocytes, supporting pesticide toxicity research. Oxidative stress certainly decreased platelet counts and raised basophils, eosinophils and polymorphonuclear leukocytes, possibly due to acute inflammatory reactions and hypersensitivity. **Conclusion:** The study found that flubendiamide exposure in newly hatched chicks may cause immunological activation and tissue damage. These findings emphasize the need for pesticide caution to protect non-target creatures and ecosystems. Further research is needed to understand flubendiamide toxicity and establish safe and sustainable agricultural use solutions.

Keywords: Antioxidant, Chick embryo, cytochromes, Flubendiamide, Hematology, Pesticides.

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INTRODUCTION

The increased utilization of pesticides in agriculture has raised the likelihood of hazardous substances infiltrating the ecosystems of unintended species, so impacting their overall health and welfare.[1,2] Diamides are a type of modern insecticides that are commonly utilized in present-day agriculture. Flubendiamide is a kind of phthalic acid diamide that specifically affects lepidopterans by attaching to ryanodine receptors. This interaction ultimately results in paralysis followed by death in the affected organisms.[3,4] Recent findings have raised concerns about the impact of flubendiamide on non-target.[5-8] Nevertheless, the need to assess the toxicity of insecticides on non-target organisms in other animals arises due to reports of their potential harm.

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Hematological parameters can be used as sensitive indicators of systemic toxicity, organ dysfunction and immune system activation, which can provide valuable insights into the overall health of organisms.[9] It is essential to comprehend the hematological profile in order to evaluate the health status and physiological responses of organisms that have been exposed to environmental stressors, such as pesticides. Scientists can evaluate the impact of pesticides on vital physiological functions, such as the transportation of oxygen, immune response and tissue health, by examining changes in parameters like the number of red blood cells, levels of hemoglobin and counts of white blood cells.[9] Additionally, hematological analysis enables the early identification of adverse effects, which in turn enables the implementation of opportune interventions to reduce potential health risks. Therefore, it is imperative to examine the hematological effects of pesticides on non-target organisms in order to protect the health of the ecosystem and biodiversity. The importance of hematological profiling in ecotoxicological research has been underscored by research, which has emphasized its role in evaluating the sublethal effects of environmental contaminants on wildlife.[10]

The chick embryo was chosen for this study due to its molecular composition, cellular structure and anatomical features, which are comparable to those of the human embryo. It is a valuable resource for the examination of developmental processes as a result of this similarity.[11] Current study investigates the hematological consequences of *in ovo* flubendiamide administration to newly born chicks, with the objective of identifying potential systemic toxicity and organ-specific impairments. The objective of this study is to investigate the underlying processes of flubendiamide-induced changes in blood composition by evaluating various parameters including red and white blood cell counts, albumin, globulin and total protein levels, as well as body and liver weights. It is imperative to comprehend these effects in order to inform pesticide usage regulations, minimize environmental damage and ensure the health of the ecosystem and agricultural productivity.

MATERIALS AND METHODS

Animal acquisition and ethical concerns

The fertilized domestic chickens' eggs of Rhode Island Red (RIR) were acquired from the Intensive Poultry Development Unit in Vadodara, Gujarat. Before being placed in an incubator, the eggs were disinfected with betadine (Povidone-iodine 10% w/v) and checked for the presence of air sacs using a method called candling. The experimental protocols adhered to rigorous rules established by the national regulatory authority for animal experimentation, the Committee for Control and Supervision of Experiments on Animals - CCSEA. The Institutional Animal Ethics Committee (IAEC) obtained approval for the protocols after conducting an ethical evaluation. The approval number is MSU-Z/IAEC04/10-2020.

Incubation

The automated incubator was calibrated to maintain a relative humidity of 70-75% and a temperature of 37±0.5°C during the incubation phase (Scientific Equipments Works, New Delhi, India). The broader edges of the eggs were positioned with precision, facing upwards and were rotated automatically every hour. Every two days, candling was employed to evaluate the viability of the eggs and any that were nonviable were promptly removed from the incubator.

Experimental design

Technical grade flubendiamide insecticide (CAS No. 272451-65-7) was secured from Sigma-Aldrich Chemical Company located in St. Louis, MO, USA. The freshly laid eggs were allocated into both treatment and control groups in a random manner. An earlier dose range study led to the selection of a lowest observed effect concentration (LOEC) of 25 µg/50 µL (500 ppm) for subsequent experiments.[7] Each experiment was replicated thrice, with 30 eggs allocated to each group. Using the candling method, air sacs were identified and on day "0" of incubation, eggshells were delicately punctured using a fine needle. Subsequently, eggs were carefully dosed within the air sac using a sterile BD 1 mL insulin syringe under laminar airflow.[12] The puncture site was immediately closed with liquefied paraffin wax prior to transferring the eggs to the incubator. The treatment groups received a dosage of 500 ppm of flubendiamide dissolved in PBS, whereas the control group was administered 50 µL of PBS. For both groups, the dosage volume remained constant at 50 µL. After euthanasia (carbon dioxide overdose), the body weight of newborn chicks as well as the isolated liver were weighed. Further, the isolated liver tissues were chosen for subsequent experimentation and analysis.

Assessment of the blood serum proteins

The Erba albumin kit of Catalog No. BLT00001 was utilized to measure serum albumin levels. Serum globulin levels were determined by subtracting the serum albumin value from the total serum protein.

Quantification of serum protein was accomplished by Bradford's test. The blood sample was analyzed to determine the amounts of albumin, globulin and total protein, which were expressed in grams per deciliter (g/dL).

Hematological Assessments

Disposable 2 mL syringes rinsed with Ethylenediaminetetraacetic Acid (EDTA) were used to collect blood samples from the brachial vein of newly hatched chicks. Subsequently, the samples were transferred into vials that had been washed with EDTA. Afterwards, the gathered samples were stored in a refrigerator and examined within a 6 hr period. Analyzing the hematological parameters, including the total erythrocyte count, Hemoglobin (Hb) concentration, total leukocyte count and differential leukocyte counts, was done using a BC2300 hematology analyzer (Mindray Company, China).

Statistical analysis

Data on hematological parameters were evaluated using the Student's *t*-test in the GraphPad Prism v8.0 software (RRID:SCR_002798) developed by GraphPad Software Inc., USA. The data were reported in the format of Mean±SEM. The significance of the difference between the control and treatment groups was established when the *p*-value was 0.05 or lower.

RESULTS

Estimations of body weight and liver weight

The embryos that were exposed to a concentration of 500 ppm of flubendiamide after hatching showed a statistically significant decrease in body weight compared to the control group ($p \leq 0.05$). An observable decrease in liver weight that is statistically significant was noted ($p \leq 0.05$). Nevertheless, there was no noticeable disparity detected in the liver's relative weight as seen in Table 1.

Table 1: Liver and body weight of newborn chick.

Parameter	Control	Treated
Body weight (g)	32.00±0.90	25.6±0.08*
Weight of liver (g)	1.021±0.08	0.848±0.08*
Relative liver weight (%)	3.19±0.24	3.31±0.32ns

The values are presented as the mean±Standard Error of the Mean (SEM); *n*=6; ns indicates non significant; * *p*≤0.05.

Estimations of serum proteins

Upon analysis of the results of serum albumin, globulin and total protein, it was seen that the embryos treated with 500 ppm of flubendiamide after hatching exhibited notable variance in comparison to the control group. The albumin content showed a significant decrease ($p \leq 0.001$), as did the globulin content ($p \leq 0.01$) and total protein content ($p \leq 0.001$) (Figure 1; Table 2).

Table 2: Blood serum protein estimation on flubendiamide treated newborn chick. The values are stated as Mean±SEM; with a sample size (n) of 3. Statistical significance was indicated as **$p \leq 0.01$ and ***$p \leq 0.001$.

Attributes	Control	Treated
Albumin (g/dL)	3.45±0.02	1.81±0.01***
Globulin (g/dL)	4.16±0.11	3.47±0.10**
Total Protein (g/dL)	7.61±0.10	5.29±0.09***

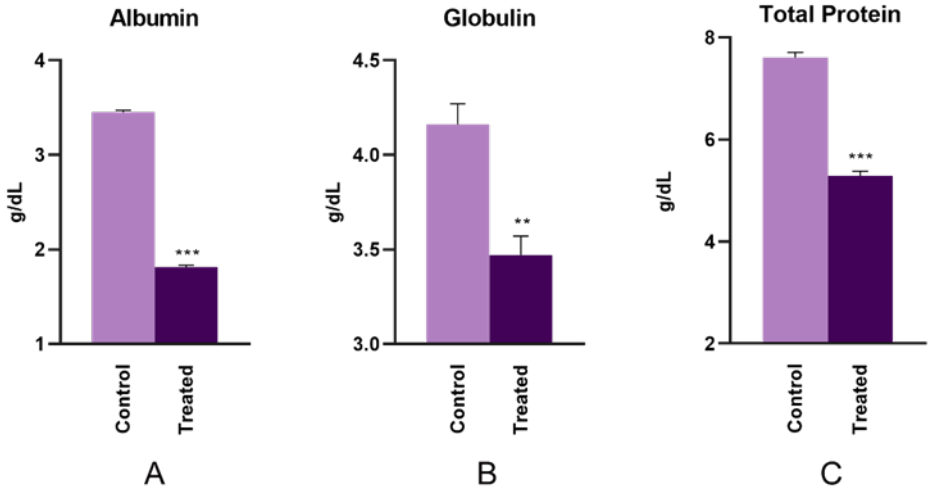

Figure 1: Blood serum protein estimation in liver of newborn chicks treated with Flubendiamide. (A) Albumin (B) Globulin (C) Total Protein. All values are presented as Mean±SEM. The sample size (n) was 3, with 30 eggs per group per day. Statistical significance was denoted as **$p \leq 0.01$ and ***$p \leq 0.001$.

Hematology profile of newborn chick

The blood analysis indicated a noteworthy reduction in hemoglobin levels in the treatment group compared to the control group ($p \leq 0.01$). Furthermore, the treated group showed a substantial reduction ($p \leq 0.01$) in both red and white blood cell counts when compared to the control group. In the differential leukocyte counts, there was an increase in eosinophils and polymorphs ($p \leq 0.05$), while basophils showed a non-significant increase. Conversely, lymphocyte and monocyte count decreased notably ($p \leq 0.05$). Furthermore, the platelet count exhibited a significant decrease ($p \leq 0.01$) as seen in Figure 2 and Table 3.

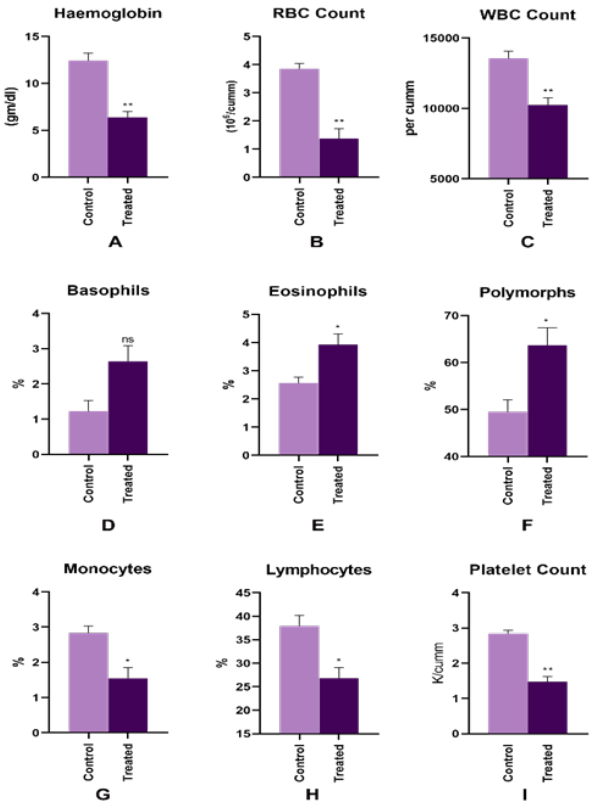

Figure 2: Blood cell counts of newborn chicks given flubendiamide during their embryonic development. The values are expressed as Mean±SEM; *n*=3; ns indicates not significant; The values are stated as Mean±SEM; with a sample size (n) of 3. Statistical significance was indicated as *$p \leq 0.05$ and **$p \leq 0.01$.

Table 3: Blood cell count of newborn chicks subjected to flubendiamide during their embryonic development. Data shown are expressed as Mean±SEM; *n* = 3; ns = not significant, *$p \leq 0.05$; **$p \leq 0.01$.

Parameters	Control	Treated
Hemoglobin (gm/dL)	12.43±0.81	6.38±0.64**
RBC Count (10^6/cumm)	3.85±0.24	1.38±0.35**
WBC Count (/cumm)	13560±2.27	10240±0.18**
Basophils (%)	1.23±0.31	2.64±0.44ns
Eosinophils (%)	2.57±0.17	3.93±0.38*
Polymorphs (%)	49.48±0.62	63.74±0.73*
Monocytes (%)	2.83±0.19	1.54±0.31*
Lymphocytes (%)	37.89±0.12	26.83±0.12*
Platelet Count (K/cumm)	2.84±0.14	1.48±0.14**

DISCUSSION

Pesticides have unquestionably enhanced crop yields and diminished post-harvest losses. Nevertheless, the ecosystem and non-target organisms have been significantly impacted by their widespread use.[15] Flubendiamide, a novel pesticide, is recognized for its ability to target insects specifically.[16] However, recent findings have highlighted the interaction of flubendiamide with non-target organisms.[5-8] This current study examines the hematological effects of *in ovo* administration of flubendiamide on newly hatched chicks, disclosing several significant findings.

In comparison to the control group, embryos exposed to 500 ppm of flubendiamide exhibited a statistically significant reduction in liver weight and reduced body weights post-hatching. However, the relative liver weight remained unaffected. This finding is critical as it suggests that while the liver may be experiencing toxic effects, its relative size does not change, indicating that the organ's functionality might be impaired without a corresponding change in size. The overall growth inhibition observed indicates that flubendiamide exposure may result in systemic toxicity that affects multiple organs, disrupts metabolic processes, or impairs nutrient absorption.[17,18]

The hematological study revealed a decrease in globulin, albumin and total protein levels in the flubendiamide-treated group, suggesting possible liver dysfunction and disturbed protein synthesis. Albumin, predominantly synthesized in the liver, is essential for the transportation of substances in the blood and the maintenance of osmotic pressure.[17] Similarly, globulins are crucial for immune responses. The observed decrease in these proteins suggests hepatotoxicity and compromised liver function, likely resulting from increased protein degradation or impaired synthesis due to oxidative stress and inflammation. These results align with prior research emphasizing the hepatotoxic potential of pesticides.[18]

Furthermore, a substantial decrease in hemoglobin levels and RBC count was observed, consistent with prior research documenting insecticide-induced anemia

caused by impaired RBC formation, destroyed RBCs and interference with hemoglobin biosynthesis.[19-21] The reduction in red blood cell count indicates that flubendiamide accumulates in cell membranes, resulting in erythrocytopenia and RBC lysis.[22] Additionally, leucopenia and diminished immunocompetence were observed, evidenced by decreased total white blood cell counts and lymphocytes, corroborating prior studies on insecticide toxicity.[23-25]

The administration of flubendiamide may lead to oxidative stress, resulting in a notable reduction in platelet levels within the treated group.[26] Conversely, an increase in basophils, eosinophils and polymorphonuclear leukocytes indicates an immunological response and the potential for allergic reactions or inflammation, as these cells are associated with acute inflammatory responses and hypersensitivity. These alterations in blood indicate significant activation of the immune system due to pesticide exposure, potentially leading to tissue damage and adverse health repercussions. This finding aligns with prior research on the effects of pesticide exposure.[27,28]

The strengths of this study include the comprehensive analysis of various hematological parameters, providing a detailed insight into the systemic effects of flubendiamide. The use of newly hatched chicks as a model allows for the observation of early developmental impacts, which is crucial for understanding the potential long-term consequences of pesticide exposure. Additionally, the study's methodology, including *in ovo* administration and subsequent hematological analysis, ensures that the findings are robust and reliable.

However, the study also has limitations. One limitation is the lack of long-term observation, which would provide a more complete picture of the chronic effects of flubendiamide. Moreover, while the study demonstrates significant hematological changes, it does not delve into the molecular mechanisms underlying these changes, which would be essential for developing targeted mitigation strategies. Future research should focus on long-term impacts and explore the biochemical pathways affected by flubendiamide exposure to fully understand its implications on non-target organisms.

In conclusion, this study underscores the necessity of exercising caution when administering flubendiamide to reduce negative environmental effects on non-target organisms. The observed hematological changes, including reduced protein levels, anemia and immune system activation, highlight the potential for significant health repercussions. Understanding these impacts is crucial for developing safer pesticide use practices and protecting non-target species from unintended harm.

CONCLUSION

To summarize, present study offers important information about the impact of flubendiamide on the blood-related aspects of freshly hatched chicks when they are exposed *in ovo*. Our findings demonstrate significant adverse impacts, including reduced body and liver weights and altered hematological parameters such as decreased albumin, globulin and total protein levels, indicating potential liver dysfunction and compromised protein synthesis likely due to oxidative stress and inflammation. The observed reductions in hemoglobin levels and RBC count suggest that flubendiamide may induce anemia by interfering with red blood cell formation and integrity. Additionally, notable decreases in white blood cell counts and lymphocytes, coupled with increased basophils, eosinophils and polymorphonuclear leukocytes, indicate immunotoxic effects, an immune response and potential allergic reactions. The significant decrease in platelet counts further underscores oxidative stress as a contributing factor. These hematological changes highlight the broader ecological risks posed by flubendiamide, emphasizing the need for cautious use to prevent adverse effects on non-target organisms.

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SB conceptualized and designed the study. DD and JV performed material preparation, data collection and analysis. DD wrote the first draft of the manuscript and all authors commented on previous versions. All authors read and approved the final manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures strictly followed the guidelines set by the national regulatory authority for

animal experimentation, the CCSEA. These protocols were subjected to ethical scrutiny and received approval from the Institutional Animal Ethics Committee (protocol number: MSU-Z/IAEC04/10-2020).

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with ethical scrutiny and received approval from the Institutional Animal Ethics Committee (IAEC; Approval No. MSU-Z/IAEC04/10-2020).

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CCSEA: Committee for Control and Supervision of Experiments on Animals; **IAEC:** Institute Animal Ethics Committee; **SEM:** Standard error mean; ppm: parts per million; **RBC:** Red blood cell; **WBC:** White blood cell; **LOEC:** Lowest observed effect concentration; **EDTA:** Ethylenediaminetetraacetic acid; **Hb:** Hemoglobin; **NS:** Not significant.

SUMMARY

This study investigates the hematological effects of *in ovo* flubendiamide exposure on newly hatched chicks, considering the widespread use of pesticides and their potential impact on non-target species. Embryos were exposed to 500 ppm of flubendiamide and post-hatching assessments revealed significant reductions in liver and body weights, indicating systemic toxicity. While relative liver weight remained unchanged, hematological analyses showed decreased levels of albumin, globulin and total protein, suggesting potential liver dysfunction and impaired protein synthesis. Furthermore, reductions in hemoglobin levels and RBC count implied flubendiamide-induced erythrocytopenia and RBC lysis. Leucopenia and diminished immunocompetence were observed through decreased total white blood cell count and lymphocytes, supporting previous research on insecticide toxicity. Increased basophils, eosinophils and polymorphonuclear leukocytes indicated an immunological response, possibly due to acute inflammatory reactions and hypersensitivity. These findings underscore significant immune activation and potential tissue injury from flubendiamide exposure in chicks, emphasizing the need for cautious pesticide use to mitigate adverse effects on non-target organisms and ecosystems. Further research is essential to understand flubendiamide toxicity mechanisms and develop strategies for its safe and sustainable agricultural application.

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