DECIPHERING THE MECHANISTIC DETAILS OF DEVELOPMENTAL ANOMALIES IN THE CHICK EMBRYOS EXPOSED TO SUBLETHAL DOSE OF TECHNICAL GRADE DIAMIDE INSECTICIDE

[Abstract Summary]

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Contents

Introduction

Flubendiamide, a phthalic acid diamide, is approved for use on over 200 crops, including soybeans, almonds, tobacco, peanuts, cotton, lettuce, tomatoes, watermelon and bell peppers. This insecticide targets caterpillars of lepidopteran pests by affecting calcium release channels, leading to paralysis in gut muscle fibers, cessation of larval feeding and eventual death due to starvation (Teixeira, 2013). However, concerns have been raised about the impact of flubendiamide on non-target organisms, such as fruit flies and Chinese tiger frogs (Li et al. 2014; Sarkar et al. 2018). In 2016, the United States Environmental Protection Agency (EPA) concluded that the continued use of flubendiamide could result in unreasonable adverse effects on the environment, including its biotic components. Studies suggest that flubendiamide is neurotoxic and can cause structural abnormalities in fruit flies. Additionally, residues of flubendiamide have been detected in human milk, raising concerns about potential health implications for both mothers and infants (Liu et al., 2022).

The chick embryo was selected for this study due to its similarity to the human embryo at molecular, cellular and anatomical levels, making it a valuable model for understanding developmental processes (Stern, 2018). Previous investigations have revealed significant developmental abnormalities in chick embryos exposed to flubendiamide. This study is aimed to assess the teratogenic potential of technical grade flubendiamide in developing chick embryos. Preliminary findings indicated that sublethal concentrations of flubendiamide administered to chick embryos resulted in eye malformations, inadequate blood vessel growth and incomplete ventral body wall closure.

The chorioallantoic membrane (CAM) is essential for forming new blood vessels, which provide oxygen and nutrients to developing tissues and facilitates gas exchange, nutrient transfer and waste elimination for the developing chick embryo (Ahmed et al., 2022). In chick embryo development, CAM angiogenesis begins with blood island formation on day 2, progressing to the emergence of primitive blood vessels and further vascularization by day 3. By day 4, the allantoic epithelium helps form the bilayered CAM. From days 6 to 9, the growth and branching of primitive blood vessels occur, with the vascular network maturing from days 10 to 14. Increased vascular density within the CAM is observed from days 15 to 18, reaching its final state by days 19 to 21 (Schmidt et al., 2019). Vascular endothelial growth factor (VEGF) and its receptor KDR (VEGFR-2) play pivotal roles in orchestrating angiogenesis,

initiating a cascade of molecular events critical for forming new blood vessels (Hiratsuka et al., 2005).

Further, the effect of flubendiamide on the formation of another essential organ, the eye, was assessed. Eye development in chick embryos begins as early as day 2 post-fertilization, involving a complex interplay of signaling molecules and transcription factors such as OTX2, PAX6 and SHH (Zuber et al., 2003; Zuber, 2010). Disruptions in these pathways can lead to congenital eye malformations like microphthalmia and anophthalmia, emphasizing the need for understanding the developmental toxicity of flubendiamide.

Pesticides are recognized as xenobiotics, which are essentially required to be cleared from the system via metabolic pathways. The liver's role in detoxifying and clearing xenobiotics involves multiple enzymatic pathways primarily mediated by cytochrome P450 enzymes (Zhao et al., 2021). Continuous exposure to pesticides can cause oxidative stress and damage liver tissues, leading to altered levels of liver marker enzymes like alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Elevated levels of these enzymes indicate hepatic damage and biliary obstruction (Abdollahi et al., 2004; Cataudella et al., 2012). Given the liver's critical role in detoxification, investigating the impact of flubendiamide on liver function and morphology during embryonic development is essential for understanding toxicity mechanisms and identifying biomarkers of exposure or adverse effects.

This study is aimed at elucidating the molecular mechanisms behind flubendiamide-induced reductions in CAM angiogenesis and its potential liver toxicity in developing chick embryos. By evaluating various enzymatic and non-enzymatic factors associated with detoxification and analyzing liver tissue structure, this research provides a comprehensive understanding of flubendiamide's developmental toxicity.

Chapter 1: *Hematological and Systemic Toxicity of in Ovo Flubendiamide Exposure in Newly Hatched Domestic Chicks*

The widespread application of pesticides has undoubtedly increased crop yields and decreased post-harvest losses; however, it has also had a substantial impact on non-target organisms and ecosystems (Al-Saleh, 1994). Flubendiamide, a novel pesticide, is efficacious against specific insect pests (Troczka et al., 2017). Li et al. (2014) and Sarkar et al. (2014, 2017, 2018) have reported that recent research has emphasized its interactions with non-target organisms, which has led to concerns regarding its broader ecological effects. This study examines the hematological effects of in ovo administration of flubendiamide on newly hatched chicks, disclosing significant findings.

Mortality and Dosage Effects

Through probit and linear fit analysis, the study determined that mortality rates increased in a dose-dependent manner (Figure 1), with a median lethal dose (LD_{50}) of 1000 ppm. At the lowest observed effect concentration (LOEC) of 500 ppm, 75% of embryos survived, but they exhibited substantial physiological alterations. Substantial reductions in liver and body weights were observed in post-hatch analyses, suggesting the potential for hepatic toxicity (Table 1). These findings are consistent with prior research that has shown that pesticides can cause growth inhibition and hepatic dysfunction (López et al., 2007).

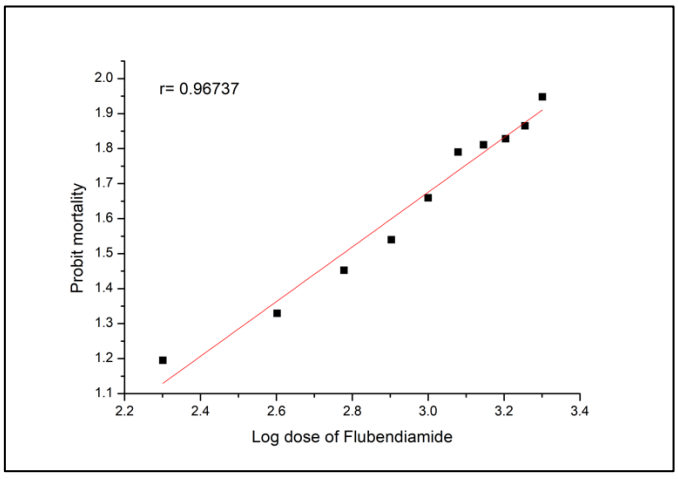

Figure 1: Median Effect Plot of Flubendiamide in chick embryos. Dose-range concentrations of 200 to 2000 ppm analysis of Flubendiamide in chick embryos. n=3 with 30 eggs per group per experiment.

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

Table 1 Liver and body weight of newborn chick. The values are presented as the mean ± standard error of the mean (SEM); n=6; ns indicates non-significant; * $p < 0.05$.

Hematological Alterations

The hematological analysis of flubendiamide-treated embryos revealed substantial decreases in albumin, globulin and total protein levels, which may be the result of liver dysfunction caused by oxidative stress and inflammation. This decrease in essential proteins is consistent with the results of other pesticide studies (López et al., 2007; Aktar et al., 2009) and suggests that liver function and immune response have been compromised.

Furthermore, the hemolytic and myelosuppressive effects of flubendiamide have likely contributed to the anemia that was observed in the treated embryos, as evidenced by significantly reduced hemoglobin levels and RBC counts (Figure 2). Other insecticides have caused comparable outcomes (Barna-Lloyd et al., 1991; Goel et al., 2006). The leucopenia and reduced lymphocyte counts observed in this study suggest a reduction in immunocompetence, which is consistent with previous findings on pesticide-induced immunotoxicity (Garg et al., 2004; Ojezele and Abatan, 2009; Gowri et al., 2010). The investigation additionally identified a reduction in platelet counts that was caused by oxidative stress induced by flubendiamide (Araujo et al., 2008). Conversely, an immune response to pesticide exposure was suggested by an increase in basophils, eosinophils and polymorphonuclear leukocytes, which could suggest allergic reactions or inflammation (Gupta, 2011; Kim et al., 2017).

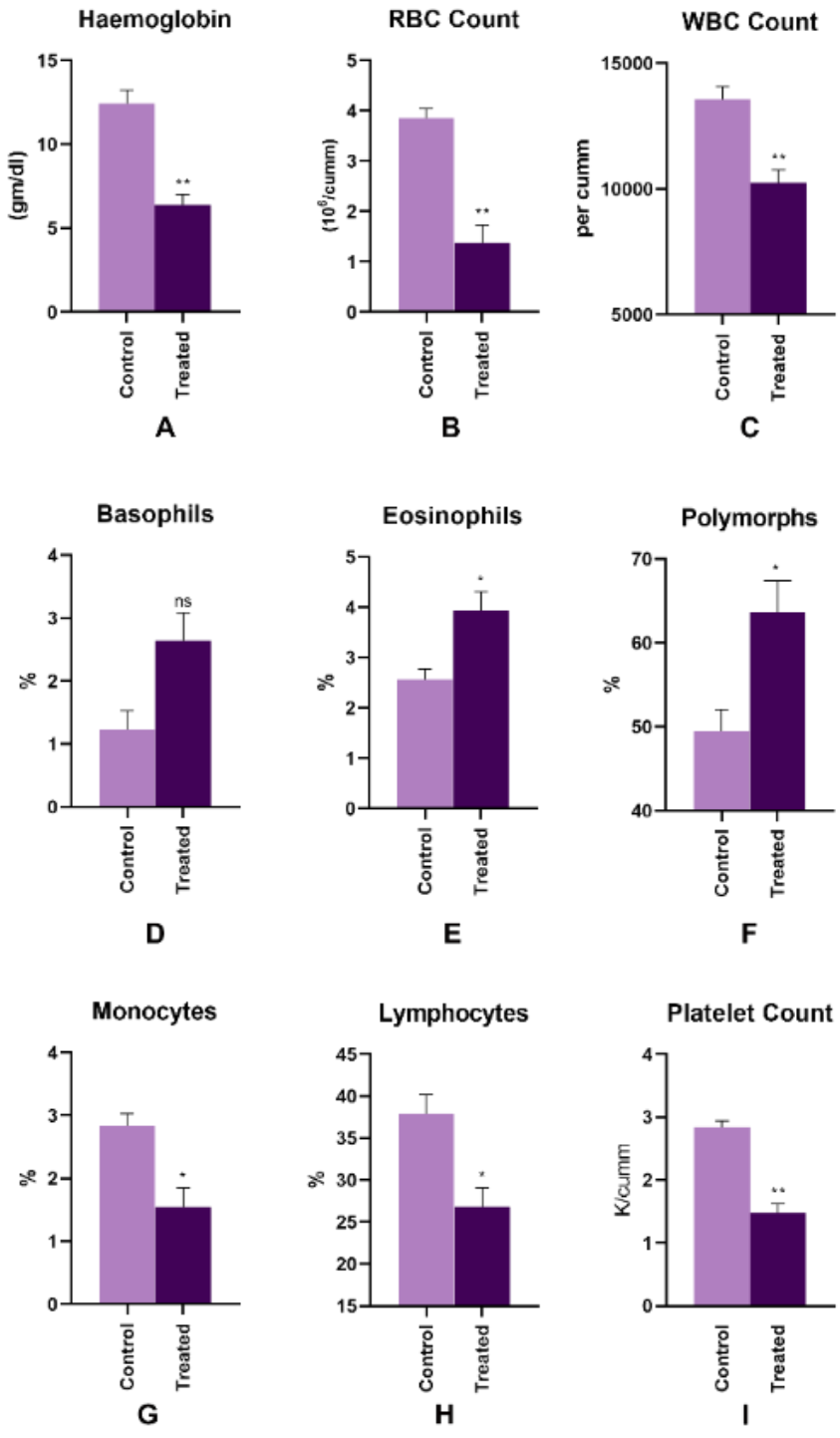

Figure 2: Blood cell counts of newborn chicks given flubendiamide during their embryonic development. The values are expressed as Mean ± SEM; n=3; ns indicates not significant; The values are stated as Mean ± SEM; with a sample size (n) of 3. Statistical significance was indicated as *p≤0.05 and **p≤0.01.

Morphological and Developmental Abnormalities

The X-ray examinations of the recently hatched chicks revealed a variety of skeletal abnormalities, such as malformed limb bones, kinked necks and abdominal congestion. These results indicate that flubendiamide induces substantial developmental defects by disrupting embryonic patterning and organogenesis. Other pesticides have been observed to have comparable teratogenic effects (Asmatullah et al., 1993; Anwar, 2003). Morphometric analysis verified substantial decreases in body weight, head diameter, crown-rump length and limb lengths, which are consistent with developmental abnormalities observed in investigations of other pesticides, such as malathion and cypermethrin (Nancy et al., 1994; Pourmirza, 2000; Uggini et al., 2010). Flubendiamide's teratogenic potential was further emphasized by qualitative defects, including microcephaly, hydrocephaly and limb malformations, which corroborated prior pesticide research (Pinakin et al., 2011).

Conclusions and Implications

The detrimental effect of flubendiamide on embryonic development is emphasized by the prevalence of abnormalities, including microphthalmia and hematomas. The potential hazards associated with pesticide exposure during critical developmental windows are further emphasized by prior research on dithiocarbamates and diamides (Van Steenis and Van Loghten, 1971; Kraggerud et al., 2010). The extensive hematological and morphological effects of flubendiamide on avian embryos highlighted by this study underscore the necessity of applying this pesticide with caution and following strict controls. The hepatotoxicity, immunotoxicity and developmental abnormalities that have been observed suggest that flubendiamide poses significant risks to non-target species, which could result in long-term ecological and health consequences. In order to guarantee the sustainable use of pesticides, it is imperative to conduct comprehensive risk assessments and implement regulatory measures that protect the health of humans and fauna in general.

Chapter 2: *Exposure to a sublethal dose of technical grade flubendiamide hampers angiogenesis in the chicken chorioallantoic membrane*

Flubendiamide, a phthalic acid diamide insecticide that is extensively employed, is applied to more than 200 commodities, such as soybeans, almonds, tobacco, peanuts, cotton, lettuce, tomatoes, watermelon and bell peppers (Teixeira, 2013). It effectively manages caterpillar infestations by disrupting calcium release channels, resulting in stomach muscle paralysis, ceasing feeding and death by starvation. Flubendiamide has raised concerns due to its adverse effects on non-target species, despite its agricultural benefits (Li et al. 2014; Sarkar et al. 2018). In 2016, the U.S. Environmental Protection Agency identified substantial environmental hazards associated with its use. Research has demonstrated its neurotoxic effects and potential to cause structural abnormalities in non-target organisms. Furthermore, the presence of flubendiamide residues in human milk has raised concerns regarding its potential impact on human health.

CAM Angiogenesis

In this investigation, we focus on the impact of flubendiamide, an insecticide that may inhibit angiogenesis within the chick chorioallantoic membrane (CAM). Initial studies have revealed substantial developmental abnormalities in chick embryos exposed to flubendiamide, highlighting the potential risks associated with this chemical. Angiogenesis, the formation of new blood vessels, is crucial for delivering oxygen and nutrients to developing tissues (Ahmed et al., 2022). The CAM angiogenesis process in chick embryos begins with the formation of blood islands on day 2, followed by the emergence of primitive blood vessels on day 3. By day 4, the bilayered CAM is fully formed. Between days 6 and 9, these primitive blood vessels undergo significant growth and branching, with maturation occurring from days 10 to 14. A substantial increase in vascular density is observed from days 15 to 18, achieving its final state by days 19 to 21 (Schmidt et al., 2019). This intricate process is meticulously regulated by vascular endothelial growth factor (VEGF) and its receptor KDR (VEGFR-2) (Hiratsuka et al., 2005). This chapter delves into the mechanisms by which flubendiamide interferes with angiogenesis in the chick embryo, exploring the resulting morphological and developmental consequences. By understanding these effects, we aim to elucidate the broader implications of flubendiamide use and contribute to the development of safer agricultural practices.

Analysis of the CAM vasculature

The vascular networks of the CAM underwent significant alterations in embryos exposed to the lowest observed effect concentration (LOEC) of flubendiamide. The morphological and quantitative analyses revealed a decrease in vessel density, the number of vascular junctions, total vessel length and an increase in lacunarity in the treated groups compared to the controls (Figure 3). This suggests that the treated groups have fewer blood vessels and significantly more vacant spaces.

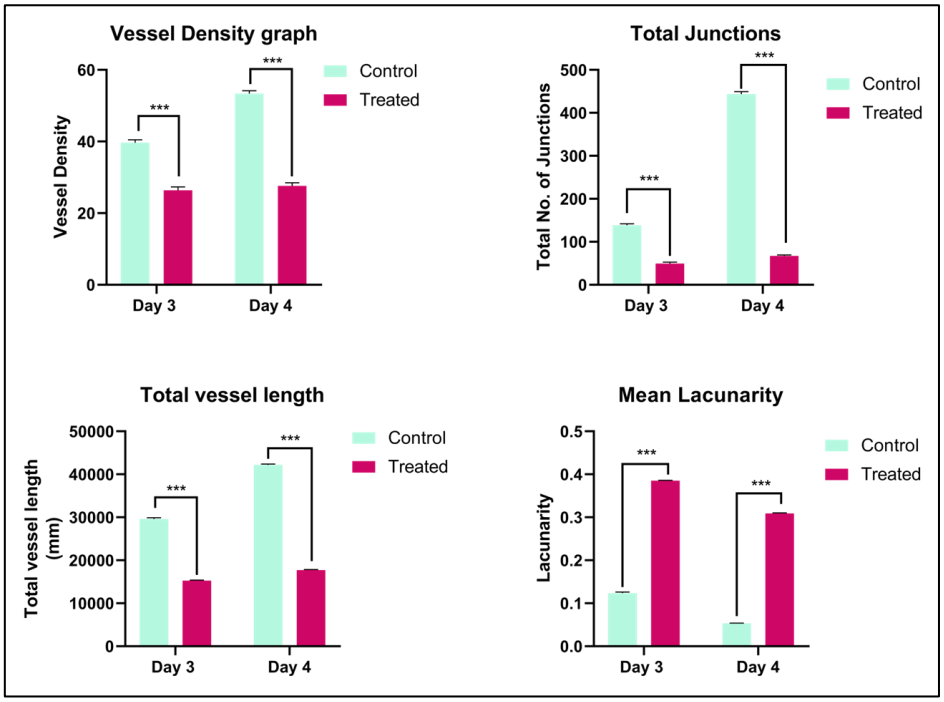

Figure 3: Analysis of CAM angiogenesis in flubendiamide-treated day 3 and 4 embryos. n=6; ***p≤0.001.

Molecular docking for CAM angiogenesis and apoptosis target protein

High binding affinities were observed for VEGFα, WNT7A, PI3K, BMP2 and CASPASE-3 in molecular docking analyses of flubendiamide with key proteins implicated in apoptotic and angiogenic pathways (Figure 4). These interactions indicate that flubendiamide may affect the functions of these proteins, thereby contributing to the reduction of blood vessel development in CAM.

Figure 4: Molecular docking 3D and 2D structure of flubendiamide with VEGFα (6MXR), CASPASE-3 (4QTX), BMP2 (2GOO), PI3K (1YI3), WNT7A (8TZO), SHH (3MVX)

Angiogenesis-related gene expression profile

The study also investigated the transcriptional status of genes including SHH, VEGFα, CASPASE-3, KDR, WNT7A, BMP2, BMP6, AKT, PI3K, PCNA and RHOB in control and treated embryos on days 2, 3 and 4. On the second day, the expression of CASPASE-3 was significantly increased, whereas PCNA was downregulated in the treated groups. The levels of VEGFα, BMP2, AKT and PI3K were marginally reduced. CASPASE-3 and RHOB were substantially upregulated on day 3, whereas VEGFα remained downregulated. The levels of BMP6, AKT and PCNA were also marginally reduced. SHH, VEGFα, KDR, WNT7A, BMP2, BMP6 and PI3K expressions were substantially downregulated by day 4, whereas CASPASE-3 remained elevated and PCNA and RHOB exhibited increased expression.

Angiogenesis-related protein expression profile

The gene expression results were confirmed by Western blot analysis. This showed that the protein levels of VEGFα, AKT and PI3K were reduced, while Cl. Caspase-3 levels increased in the treated embryos. Immunohistochemistry also supported these findings, indicating higher

apoptosis levels in the treated groups. The research posits that flubendiamide disrupts CAM angiogenesis by inhibiting critical signaling pathways. Impaired endothelial cell proliferation is indicated by decreased levels of VEGFα, KDR and PCNA. The efficacy of flubendiamide in targeting pro-angiogenic factors is evident in the reduced levels of SHH, AKT and PI3K. The downregulation of WNT7A, BMP2 and BMP6, in conjunction with the increase of RHOB and Cl. Caspase-3 suggests that endothelial cells experience increased apoptosis and inhibited sprouting, resulting in disrupted vascularization.

Conclusions and Implications

This research demonstrates that flubendiamide has a substantial impact on the development of chick embryos by disrupting CAM angiogenesis. The suppression of SHH, BMP2, BMP6 and WNT7A, as well as the upregulation of apoptosis-related factors, such as Cl. Caspase-3 and RHOB and the inhibition of VEGF-KDR signaling contribute to the disorganized and reduced formation of blood vessels. Further research is required to completely comprehend the molecular effects of flubendiamide on embryonic development, as these findings highlight the potential developmental toxicity of the drug. The findings have substantial implications for human and environmental health, particularly considering the presence of flubendiamide residues in human milk.

Chapter 3: *Embryonic flubendiamide exposure alters expression of OTX2 and other early regulators in domestic chick, leading to congenital eye defects*

Modern agriculture employs pesticides extensively to safeguard crops from diseases and pests. The potential impact of these chemicals on non-target species and the overall health of the environment has been a cause of concern due to their widespread use. Recently conducted research has demonstrated that neonates may develop developmental anomalies due to exposure to specific pesticides (Kalliora et al., 2018). The teratogenic potential of flubendiamide in chick embryos is the primary focus of this research, which explicitly examines eye malformations and the underlying molecular mechanisms. Flubendiamide is a new-generation pesticide intended to combat pest resistance with increased efficacy.

Gross morphological observations on eye development

The research uncovered substantial anomalies in eye development in embryos treated with flubendiamide during the early phases. Contrary to the well-developed structures observed in control embryos, treated embryos did not manifest the optic cup or eye field formation by the second day. Serious developmental issues, such as anophthalmia (the absence of the eye) and distorted craniofacial features, were evident in the treated group by the third day. The fetuses treated with flubendiamide exhibited reduced eye size (microphthalmia) and less pigmentation compared to the controls on the fourth day. The histopathological analysis of day four embryos revealed impaired optic cup development, evidenced by the absence of a well-defined retinal pigment epithelium and neuronal retina, inadequate differentiation between anterior and posterior eye cavities and the absence of lens and cornea formation.

Molecular docking for eye-forming protein

The results of molecular docking studies indicated that flubendiamide exhibited strong binding affinities with key proteins implicated in eye development, including BMP7, CDH1, FGF8, PAX6, OTX2 and SOX2 (Figure 5). High docking scores indicated that flubendiamide may potentially disrupt the functionality of these proteins, which could account for the developmental defects that have been observed.

Figure 5: Molecular docking 3D and 2D structure of flubendiamide with BMP7 (1LX5), CDH1 (3L6Y), FGF8 (2FDB), PAX6 (6PAX), OTX2 (model_03) and SOX2 (6WX9)

Eye formation-related gene expression profile

qRT-PCR analysis of gene expression demonstrated substantial modifications in the expression of critical genes. CASPASE-3, BMP7 and SHH were downregulated, while CDH1 and WNT11 were upregulated in the treated embryos on the second day. The downregulation of CDH2, FGF8, OTX2, PAX6, SOX2 and VIM was substantial by the third day, while CASPASE-3 and SHH were upregulated. BMP4, CDH1, OTX2, SOX2, VIM and WNT1 were downregulated in treated embryos on the fourth day.

Eye formation-related protein expression profile

The qRT-PCR findings were validated through Western blot analysis, which provided a complementary evaluation of the protein expression levels in the treated embryos. The qRT-PCR data was corroborated by the Western blot results, which demonstrated a substantial decrease in the expression of Cdh2 and Pax6 proteins. These proteins are essential for neural development and cell adhesion, respectively. This decrease is consistent with the transcriptional downregulation that was observed in the qRT-PCR results. Furthermore, the Western blot analysis demonstrated an increase in the levels of Shh, a critical signaling molecule in eye field splitting and Cl. Caspase-3, a marker of apoptosis, which is associated in embryonic development. These results suggest an upregulation at the protein level, which is in accordance with the elevated mRNA levels detected by qRT-PCR. These results collectively underscore substantial molecular modifications in the embryos that were treated, which may have implications for cellular processes, including adhesion, differentiation, apoptosis and signaling pathways.

Assessment of apoptosis by immunohistochemistry

Immunohistochemistry analysis demonstrated that the head region was the primary site of Cl. Caspase-3 expression. This observation emphasizes the treatment's influence on apoptosis, with a particular emphasis on the cells in the head region of the neonatal chicks.

Histopathology analysis of eye

The optic cup development of treated embryos was significantly disorganized, as disclosed by histopathological examination. The structural defects caused by flubendiamide were evident in the fact that the treated embryos lacked distinct differentiation in retinal and lens structures. In particular, the severity of developmental disruption was emphasized by the absence of a well-

defined retinal pigment epithelium and neuronal retina, as well as the absence of lens and cornea formation.

Skeletal staining of the eye of newborn chicks

Skeletal staining was conducted on newly hatched chicks, specifically focusing on the cranial area (Figure 6). The staining results indicated impaired growth of cartilage and bone in the facial region. Alizarin red staining, which permanently binds with calcium, visually highlighted bones in red. Cartilage presence was indicated by a blue hue observed in the embryos. Overall staining observations suggested that the treated group lacked visible eyes.

Figure 6: The alcian blue and alizarin red staining of a day 21 newborn chick depicts the presence and absence of an eye. The whole chick staining is inset.

Conclusion and Implications

Flubendiamide exposure in chick embryos impairs early eye development, resulting in severe ocular defects, as demonstrated by this study. OTX2, PAX6, SHH and other signaling molecules are all involved in the complex process of eye development. Anomalies such as anophthalmia and microphthalmia are the result of flubendiamide's interference with these molecules, which disrupts critical developmental phases. The upregulation of apoptotic markers and the downregulation of critical developmental genes indicate a mechanism that involves impaired cellular migration and differentiation and increased apoptosis. The findings emphasize the necessity of additional research to elucidate the intricate molecular mechanisms, underscoring the potential risks of flubendiamide exposure during embryonic development.

Gaining an understanding of these pathways can assist in the mitigation of the risks associated with pesticide use and the development of safer agricultural practices. The findings underscore the necessity of assessing the environmental and health consequences of new-generation pesticides and advocate for caution in the use of flubendiamide. Additionally, additional research is necessary to develop strategies for safer pesticide use in agriculture and to gain a comprehensive understanding of the broader implications.

Chapter 4: *Embryonic exposure to flubendiamide induces hepatotoxicity in domestic chicks by altering drug-metabolizing enzymes, antioxidant status and liver function*

Although pesticides are essential for contemporary agriculture, their extensive application poses a threat to non-target organisms. The liver is responsible for the detoxification of xenobiotics in vertebrates, including domestic chicks, through the action of cytochrome P450 (CYP) enzymes. The hepatotoxic effects of the diamide insecticide flubendiamide on domestic chick embryos are investigated, with an emphasis on structural liver injury and oxidative stress.

Gene expression profile of cytochromes

The evaluation of gene expression in neonatal chick livers revealed that different cytochrome P450 enzymes exhibited distinct responses. Although the mRNA levels of CYP1A1 were elevated in the treated groups without statistical significance, CYP1A2 and CYP2C19 exhibited significant elevation. Despite the fact that CYP2D6 exhibited an increase in expression, it did not achieve statistical significance. In comparison to controls, the expression of CYP3A4 exhibited a significant increase of over threefold in the treated groups.

Protein Expression Profile of Cytochromes

In order to verify the results of the qRT-PCR, the protein levels of the cytochrome P450 enzymes were assessed using western blot analysis. The treated group consistently exhibited increased expression of all examined cytochromes (CYP1A1, CYP1A2, CYP2D6, CYP2C19 and CYP3A4) in comparison to the control group, as evidenced by the results. The conclusion that the treatment substantially affects the expression of these enzymes at both mRNA and protein levels in newborn chick livers is further substantiated by the alignment between gene expression and protein analysis.

Response to Oxidative Stress

Enzymatic antioxidants, including catalase and superoxide dismutase (SOD), as well as non-enzymatic antioxidants like reduced glutathione (GSH), are employed by organisms to mitigate oxidative stress caused by xenobiotic metabolisms. The activities of SOD and catalase were increased in flubendiamide-treated embryos, while GSH levels were substantially reduced, suggesting that oxidative stress was at an elevated level. The liver tissue's elevated hydrogen

peroxide (H_2O_2) levels were confirmed by the increased DCFDA fluorescence intensity, which indicated a significant degree of oxidative stress.

Liver function tests of newborn chicks

Elevated serum levels of liver enzymes, including alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), suggest liver injury. The flubendiamide-treated group exhibited substantial increases in these enzymes, which implies hepatic toxicity and impaired liver function, as the study observed (Table 2).

Attributes	**Control**	**Treated**
ALP levels (IU/L)	100 ± 0.60	130 ± 0.62***
ALT levels (IU/L)	3 ± 0.35	17.33 ± 0.30**
AST levels (IU/L)	5.33 ± 0.21	17 ± 0.23**
GSH levels (g/µL)	0.218 ± 0.16	0.502 ± 0.18***
SOD (U/ml)	0.70 ± 0.04	0.944 ± 0.04***
Catalase (µmol/µg protein)	3.9 ± 0.12	6.44 ± 0.29***

Table 2 Blood serum protein estimation on flubendiamide-treated newborn chicks. The values are expressed as Mean ± SEM; n=3; **p≤0.01; ***p≤0.001

Histological Examination of Liver Tissue

The histopathological examination of liver tissues in treated embryos revealed substantial structural abnormalities (Figure 7). These included pervasive inflammation, disorganized sinusoids, indistinct bile ducts and impaired portal veins. The presence of distorted portal veins compromised hepatocyte architecture and inflammatory cell infiltration underscored the extent of liver injury caused by flubendiamide.

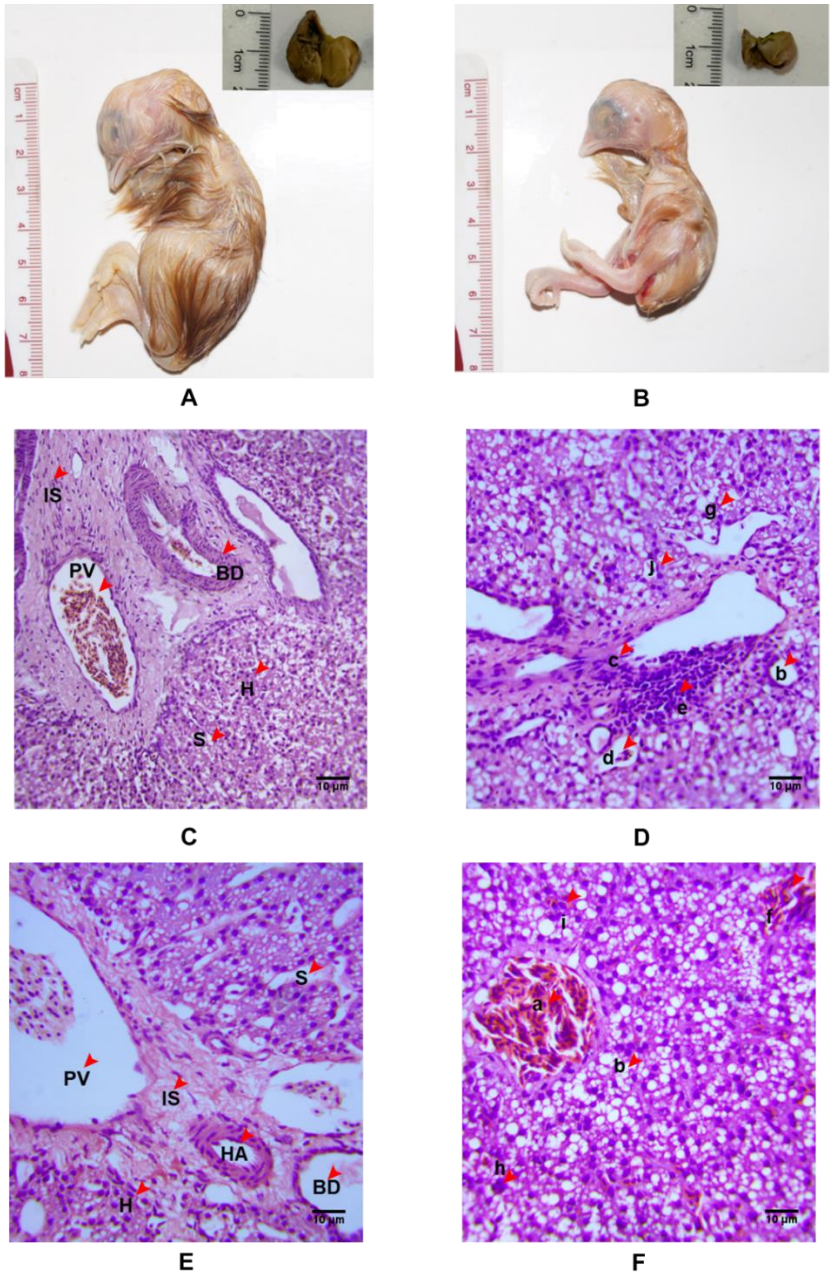

Figure 7: Histological section of flubendiamide-treated newborn chicken liver in comparison with the control group (H&E x40). The section shows hepatocyte (H), sinusoids (S), portal vein (PV), interlobular septum (IS) and a bile duct (BD). Control and treated chicks are labeled as A and B, respectively, with isolated liver insets. Liver sections 40X: C, E – control, D, F – treated. Deformities in the treated liver are shown as a) congested blood vessels, b) vacuolated cytoplasm, c) disrupted tissue integrity, d) disrupted bile duct, e) leukocyte infiltration, f) vasculitis, g) degeneration of cytoplasm, h) pyknotic nuclei, i) necrosis. n=3 with 30 eggs per group per day. Red arrows showing the mentioned sites.

Conclusion and Implications

This study elucidates the substantial hepatotoxic effects of flubendiamide exposure on domestic chick embryos, as demonstrated by the extensive histological abnormalities observed in liver tissues, pronounced upregulation of CYP enzymes, heightened oxidative stress and elevated liver enzyme levels. These results emphasize the urgent necessity of diligent pesticide management practices and stringent regulatory measures to reduce the environmental and health hazards associated with flubendiamide and similar pesticides. The implications extend to the necessity of implementing integrated pest management strategies that prioritize sustainability and biodiversity conservation while ensuring food security. To further our comprehension of the mechanisms of pesticide-induced hepatotoxicity and to create safer alternatives that protect both human health and the environment, it is imperative that we continue to conduct research. In order to effectively address these challenges and promote sustainable agricultural practices worldwide, collaborative initiatives that involve scientists, policymakers and agricultural stakeholders are essential.

Study Highlights

- **Pesticide Concerns:** The increased use of pesticides in agriculture raises concerns about the infiltration of hazardous substances into ecosystems, which can impact the health and welfare of non-target species.
- **Flubendiamide:** A modern insecticide from the phthalic acid diamide group, specifically targeting lepidopterans by binding to ryanodine receptors, leading to paralysis and death.
- **Non-Target Organisms**: Recent studies indicate the potential harm of flubendiamide on non-target organisms, necessitating an evaluation of its toxicity.
- **Systemic Toxicity**: Flubendiamide exposure results in systemic toxicity, affecting growth, metabolic processes and nutrient absorption. Exposure to sublethal concentrations of flubendiamide resulted in eye malformations, inadequate blood vessel growth and incomplete ventral body wall closure in chick embryos.
- **Chick Embryo Model for Developmental Studies**: The chick embryo, resembling the human embryo, offers insights into developmental processes. Preliminary findings suggested developmental abnormalities in chick embryos exposed to flubendiamide.
- **CAM Angiogenesis Process**: CAM angiogenesis initiates with blood island formation, progressing to primitive vessel emergence and further vascularization. VEGF and KDR play pivotal roles in orchestrating angiogenesis, which is crucial for tissue growth and homeostasis.
- **Study Design and Findings**:
  - Dose Range Study: LD_{50} of flubendiamide was determined, with 500 ppm selected for further studies.
  - CAM Vascular Analysis: Flubendiamide exposure led to reduced vessel density, junctions and length, along with increased lacunarity.
  - Molecular Docking: Flubendiamide showed strong binding affinity to proteins associated with angiogenesis and apoptosis pathways.
  - Gene Expression Profile: Downregulation of angiogenesis-related genes like VEGFα, KDR and SHH and upregulation of apoptosis-related genes like CASPASE-3 and RHOB were observed.
  - Protein Expression Analysis: Reduced expression of Vegfα, AKT and PI3K, along with increased Cl. Caspase-3 expression validated gene expression findings.

- Apoptosis Assessment: Immunohistochemistry confirmed elevated Cl. Caspase-3 levels in treated embryos, indicating increased apoptosis.

- **Morphological Observations:** Description of observed eye malformations, inadequate blood vessel growth and incomplete ventral body wall closure in treated chick embryos.
- **Molecular Docking Analysis:** Examination of the interaction between flubendiamide and key proteins involved in eye development pathways. Highlighting the strong affinity between flubendiamide and essential signaling molecules.
- **Alteration of Gene Expression Patterns:** Findings indicating changes in the expression patterns of crucial signaling molecules such as OTX2, PAX6 and SHH. Implications of these alterations on normal eye development.
- **Histopathological Examination:** Confirmation of impaired optic cup development and absence of essential eye structures in treated embryos. Insights into the tissue architecture damage caused by flubendiamide exposure.
- **Increased Apoptosis:** Detection of heightened levels of apoptotic marker CASPASE-3 in treated embryos. Implications of increased cell death on the occurrence of congenital eye defects.

- **Cytocrome P450 Enzyme Upregulation**: Exposure to flubendiamide led to significant upregulation of key cytochrome P450 enzymes involved in pesticide metabolism, indicating an enhanced metabolic response to the pesticide.

- **Oxidative Stress**: Elevated levels of antioxidant enzymes (SOD and catalase) and reduced glutathione depletion suggested increased production of reactive oxygen species (ROS) and oxidative stress in the liver due to flubendiamide exposure.

- **DCFDA Staining**: Increased fluorescence intensity in DCFDA staining confirmed elevated H_2O_2 levels, further indicating substantial oxidative stress within the liver tissue.

- **Liver Function Tests**: Elevated serum levels of liver marker enzymes (ALP, ALT and AST) indicated acute liver damage induced by flubendiamide exposure.

- **Histological Examination**: Histopathological analysis revealed significant abnormalities in liver tissue, including impaired portal veins, inflammation, compromised hepatocyte architecture and structural integrity alterations induced by flubendiamide.

- **Liver Dysfunction**: Reduced serum protein levels indicate liver dysfunction, aligning with previous research on the hepatotoxic potential of pesticides.
- **Anemia and Immunotoxicity**: Flubendiamide causes anemia and reduced immunocompetence, corroborating earlier studies on insecticide toxicity.

Implications: Pesticides like flubendiamide can disrupt crucial developmental processes, particularly CAM angiogenesis and oculogenesis and affect the liver's metabolizing potential, posing potential health risks. Further research is needed to fully understand the molecular mechanisms underlying flubendiamide's effects on embryonic development.

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