Impact of flubendiamide on hepatic structure and function in newborn domestic chicks

INTRODUCTION

The outburst of pesticide usage in agriculture has elevated the chances of toxicants entering the systems of non-target species, affecting their well-being (Zaller & Zaller, 2020; Kalyabina et al., 2021). Such contaminants (xenobiotics) are metabolized and eliminated from the organisms. The body uses multiple sites for toxicant metabolism, including the liver, intestinal wall, lungs, kidneys and plasma (Kieffer et al., 2016).

In vertebrates, these chemicals are primarily channeled to the liver for detoxification and clearance (Ozougwu, 2017). The liver is responsible for detoxifying and facilitating the excretion of xenobiotics by enzymatically converting lipid-soluble molecules to more water-soluble ones, which ensures their easy elimination (Grant, 1991) (Figure 6.1).

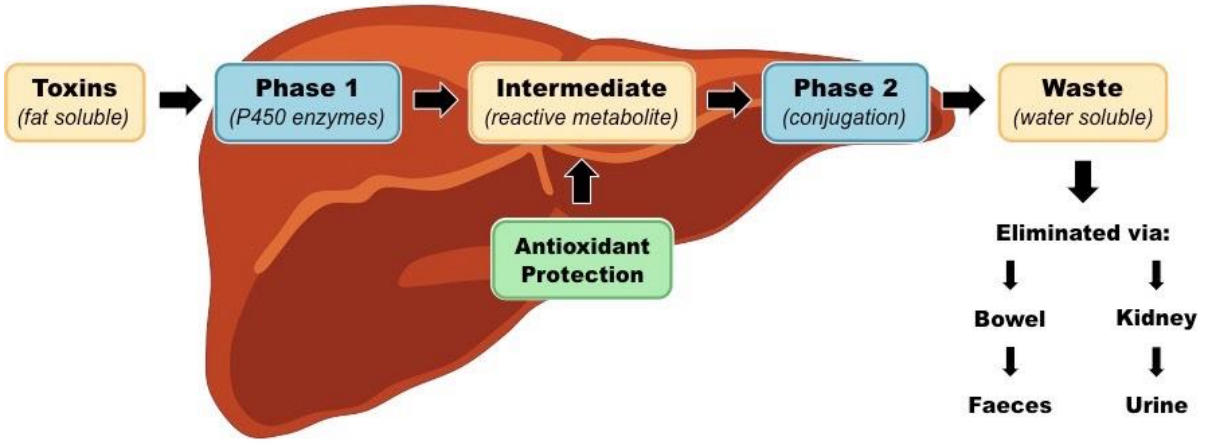

Figure 6.1: Drug detoxification in the body
(https://old-ib.bioninja.com.au/_Media/detoxification_med.jpeg)

Upon exposure to a toxicant, the organism's defense mechanism activates phase I and II enzymes comprising the CYPs system in the liver, oxidizing the compounds for further degradation. The cytochrome P450 subgroup is responsible for the majority of drug metabolism

(Zhao et al., 2021). Diamide insecticides, such as chlorantraniliprole and flubendiamide, are extensively utilized in agriculture and undergo metabolism in the liver via several cytochrome P450 enzymes.

CYP3A4 plays a crucial role in the process of converting these substances through oxidation, which helps in removing their harmful effects and eliminating them from the body (Guengerich, 2008; Zanger & Schwab, 2013). CYP1A1 and CYP1A2 have important functions in the process of oxidizing diamides, which enhances their ability to dissolve in water and facilitates their elimination from the body (Nebert & Dalton, 2006; Dong, 2023).

In addition, CYP2C19 plays a role in the breakdown of these pesticides by performing hydroxylation and demethylation (Pelkonen et al., 2008; Mao et al., 2019). Furthermore, CYP2D6 has a role in the breakdown of specific diamides, carrying out functions such as N-demethylation (Lynch & Price, 2007; Bempah & Donkor, 2011).

However, the process of detoxification generates free radicles and ROS, causing oxidative stress that is generally combated by elevated levels of the enzymatic and non-enzymatic antioxidant systems like SODs, Catalases and Glutathione reductases which serve as the indicators of stress and oxidative damage (Apel & Hirt, 2004; Schieber & Chandel, 2014; Ighodaro & Akinloye, 2018).

Although the mechanisms to reduce oxidative stress are foolproof, damage in liver tissues is still prominently seen when organisms are continuously exposed to chemicals such as insecticides (Abdollahi et al., 2004; Cataudella et al., 2012). Due to these insults, many liver marker enzymes have altered expression levels. Liver enzymes such as ALP, AST and ALT are commonly used to evaluate patients with various diseases. Elevated levels of ALP suggest biliary obstruction, while increased levels of AST and ALT indicate hepatic damage (Gotaro et al., 1980; Kew, 2000).

New-generation insecticides, such as diamides, are among the recent pesticides used in contemporary agriculture. Flubendiamide is a phthalic acid diamide known to target lepidopterans by binding to ryanodine receptors, ultimately leading to paralysis (Tohnishi et al., 2005; Aghris et al., 2022). However, reports of insecticides being hazardous to non-target organisms necessitate evaluating their toxicity in other animals.

The chick embryo was selected for this study because it is similar to the human embryo in terms of molecular makeup, cellular structure and anatomical features. This similarity makes it a valuable resource for examining developmental processes (Stern, 2018). The current study investigated the liver toxicity of flubendiamide in newly hatched domestic chicks exposed to LOEC.

The process of liver development in chick embryos resembles that in mammalian embryos (Wong & Cavey, 1992). Between days 5 and 7 of development, the liver lobes begin to take shape, marking the onset of rapid growth for the chick embryo liver (Wong & Cavey, 1992). By day 14, the embryonic chick liver achieves functionality (Suksaweang et al., 2004).

Given the liver's pivotal role in detoxification, investigating the impact of pesticides on liver function and morphology during embryonic development is crucial for understanding toxicity mechanisms and identifying biomarkers of exposure or adverse effects. Hence, it has been hypothesized that in ovo administration of a sublethal concentration (500 ppm) of flubendiamide elevates oxidative stress, thereby affecting the structure and function of the fetal liver.

This study focused on evaluating various enzymatic and non-enzymatic factors associated with detoxification mechanisms. Additionally, an analysis of liver tissue structure was conducted to provide a comprehensive understanding of these processes.

MATERIALS AND METHODS

Fertilized Rhode Island Red chicken eggs were sourced from the Intensive Poultry Development Unit in Vadodara, Gujarat, India. Before incubation, the eggs were checked for air sacs using candling and sanitized with betadine.

Eggs were then placed in an automated incubator set to 37±0.5°C with 70-75% relative humidity, positioned broad end up and rotated hourly. Viability checks were performed every two days, with non-viable eggs removed. For the experiments, technical grade flubendiamide was obtained and the eggs were divided into control and treatment groups of 30 eggs each, with the experiment repeated three times.

On the first day of incubation, eggs were punctured and dosed with either flubendiamide in PBS or PBS alone. After incubation, the chicks were euthanized and liver tissues were collected for further analysis. All procedures carried out were ethically approved by the IAEC.

Expression levels of these cytochromes were assessed at both mRNA and protein levels using qRT-PCR and western blot analyses, respectively. To understand the liver's defense mechanism against oxidative stress, enzymatic antioxidants such as SOD, catalase, as well as non-enzymatic antioxidants like GSH, were evaluated. SOD activity was measured according to the protocol established by Marklund and Marklund (1974) while catalase activity was evaluated following Sinha's method (1972) GSH levels were assessed using the method described by Beutler and Gelbart (1986). Oxidative stress levels were quantitatively and qualitatively assessed using DCFDA staining, providing insight into the extent of oxidative damage induced by flubendiamide treatment.

Additionally, liver function tests were conducted to assess the impact of flubendiamide treatment on serum enzyme levels. Levels of ALP, ALT and AST were measured in the serum of treated chicks were estimated as per the manufacturer’s protocol (Reckon Diagnostics, Vadodara, Gujarat, India). Histopathological analysis was performed using hematoxylin and eosin staining to evaluate tissue damage inflicted by flubendiamide on the livers of newborn chicks, providing visual evidence of histological changes induced by flubendiamide exposure.

Data analysis was performed using Student's t-test, with significance set at $p \leq 0.05$.

RESULTS

Gene expression profile of cytochromes

The transcriptional activity of CYP1A1, CYP1A2, CYP2D6, CYP2C19 and CYP3A4 was assessed in the liver of newborn chicks across control and treated groups. Although the relative mRNA levels of CYTP1A1 were higher in the treated groups than controls, the difference was not statistically significant. Conversely, CYP1A2 and CYP2C19 showed significant upregulation by more than one and a half times compared to their respective controls ($p \leq 0.001$). Though CYP2D6 exhibited increased expression, it was not statistically significant. Notably, in the treated groups, CYP3A4 exhibited a more than threefold increase in expression ($p \leq 0.05$) (Figure 6.2; Table 6.1).

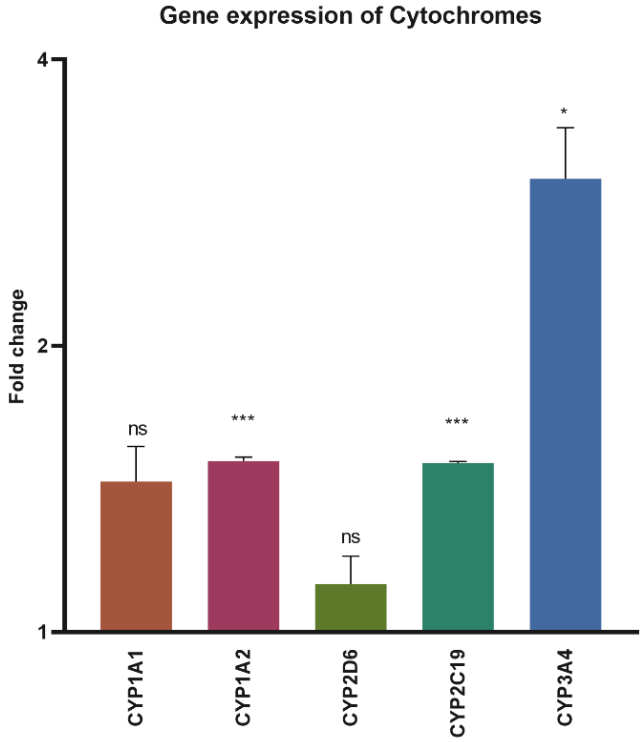

Figure 6.2: Transcript levels of genes of cytochrome C and its variants in flubendiamide-treated newborn chicks. Values are expressed in fold change (Mean ± SEM). Fold change values for the control embryo is 1.0 for all the genes (n=3); ns = not significant; *p≤0.05; ***p≤0.001.

Protein Expression Profile of Cytochromes

To validate the findings from the qRT-PCR analysis, the protein levels of the cytochromes were assessed using western blotting, followed by densitometric analysis of the bands. The results from the immunoblot analysis demonstrated elevated expression of all the cytochromes in the treated group compared to the control. The observation aligns with the results obtained from the qRT-PCR analysis, thus providing further support for the findings (Figure 6.3; Table 6.2). During the western blot analysis, β-Actin was utilized as an internal control for normalization.

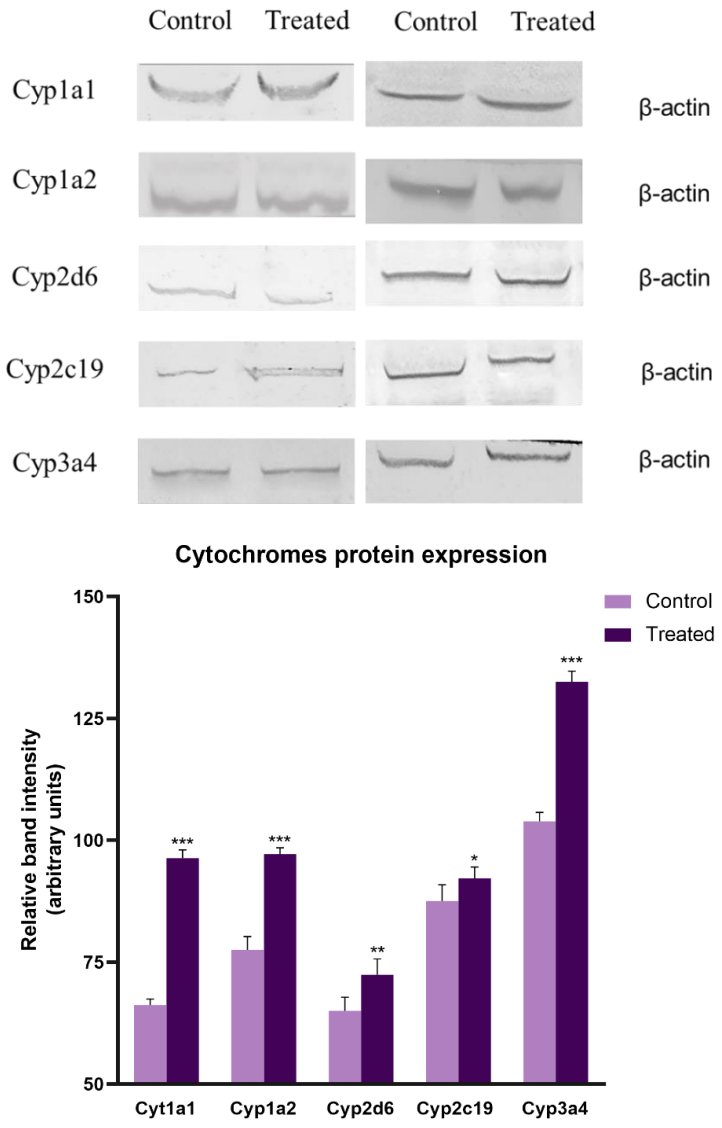

Figure 6.3: Western blot images showing comparative expression on flubendiamide-treated newborn chicks. β-Actin was taken as loading control, n=3 with 30 eggs per group per day; *p≤0.05, **p≤0.01, ***p≤0.001.

Enzymatic and non-enzymatic antioxidant status in liver

The activity of SOD and catalase antioxidant enzymes notably increased in the treated group compared to the control (Figure 6.4; Table 6.3). The levels of reduced glutathione remained significantly reduced in the treated group ($p \leq 0.001$). Additionally, both SOD and catalase activities remained elevated in liver samples from the treated group compared to the control ($p \leq 0.001$).

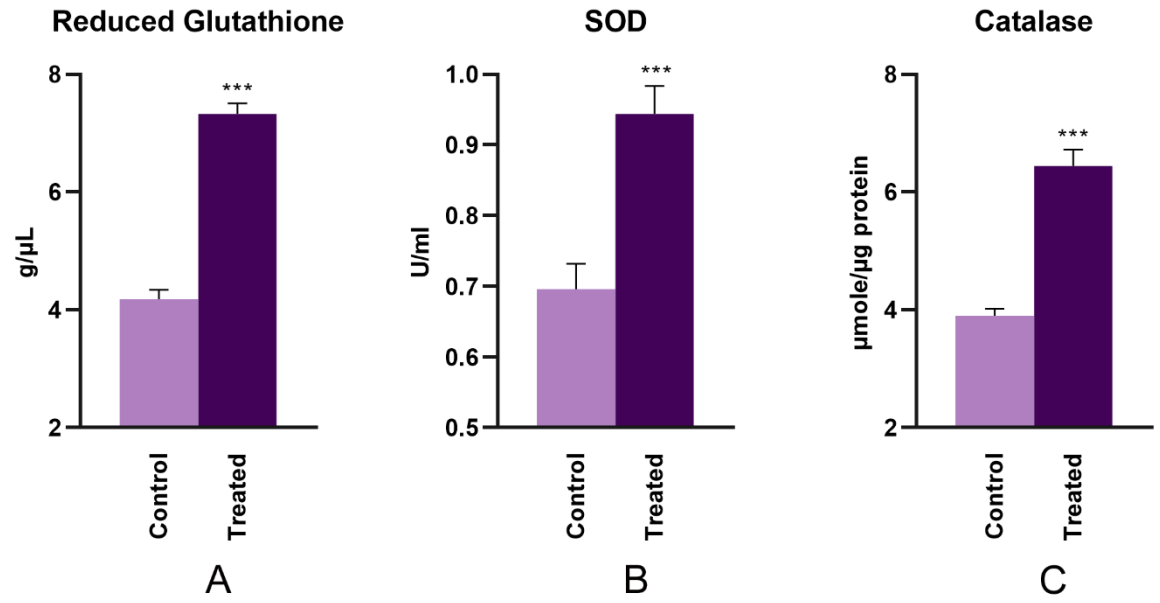

Figure 6.4: Enzymatic and non-enzymatic antioxidant status in the liver of newborn chicks treated with flubendiamide. (A) Reduced glutathione content (B) Superoxide dismutase activity (C) Catalase activity. All values are expressed as Mean ± SEM. n=3 with 30 eggs per group per day; ***$p \leq 0.001$.

Estimation of ROS levels in the liver

The fluorescence intensity from DCFDA stained samples was higher in the flubendiamide-treated liver than in the control group (Figure 6.5). This observation was substantiated by the quantification of fluorescence intensity, measured in arbitrary units, from the liver samples of the treated group, reinforcing the indication of increased oxidative stress in the flubendiamide-treated liver samples (Table 6.4).

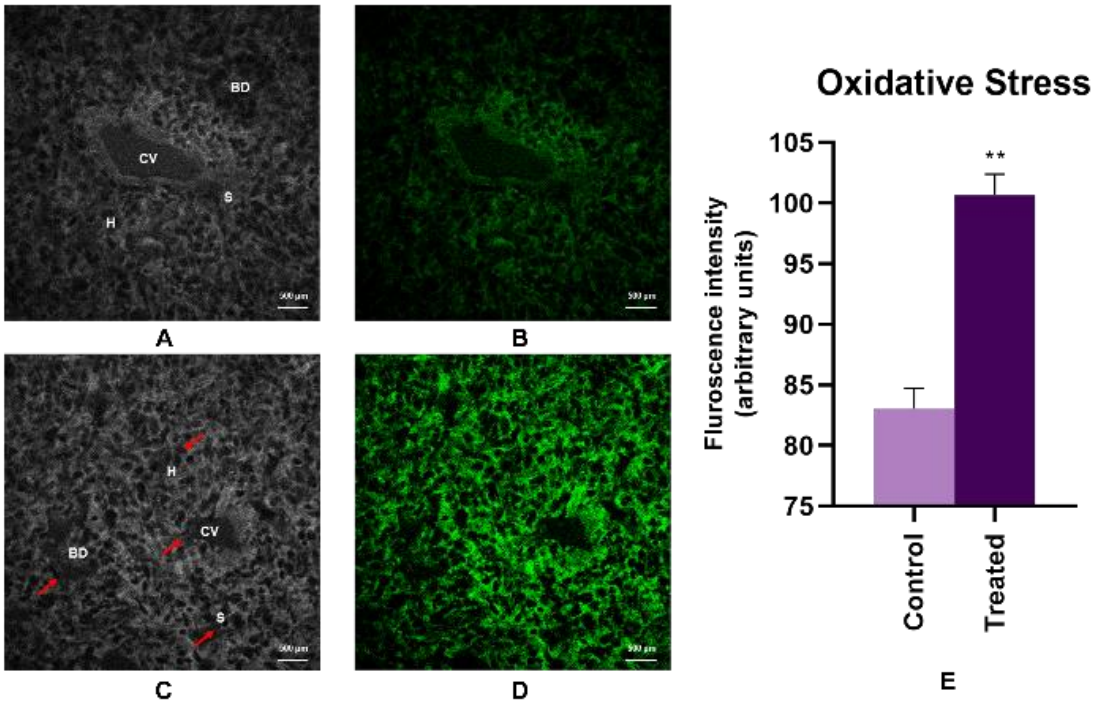

Figure 6.5: DCFDA staining showing cytoplasmic ROS level in the liver of flubendiamide-treated newborn chicks. A and C are control and treated group images of bright fields, respectively. B and D correspond to DCFDA stained images of the control and treated group, respectively (scale bar in yellow: 50 µM); E denotes a graph showing statistical analysis of fluorescence intensity following DCFDA staining in control and treated groups. n=6, N=3; **p ≤0.01

Liver function tests of newborn chicks

A comparison of the levels of ALP, ALT and AST in the treatment group with the control group found that the treated group had significantly higher levels of these values. It is worth noting that the levels of ALP exhibited a noteworthy rise ($p \leq 0.001$), while the levels of ALT and AST exhibited a statistically significant increase in the group that was treated (Figure 6.6 Table 6.5).

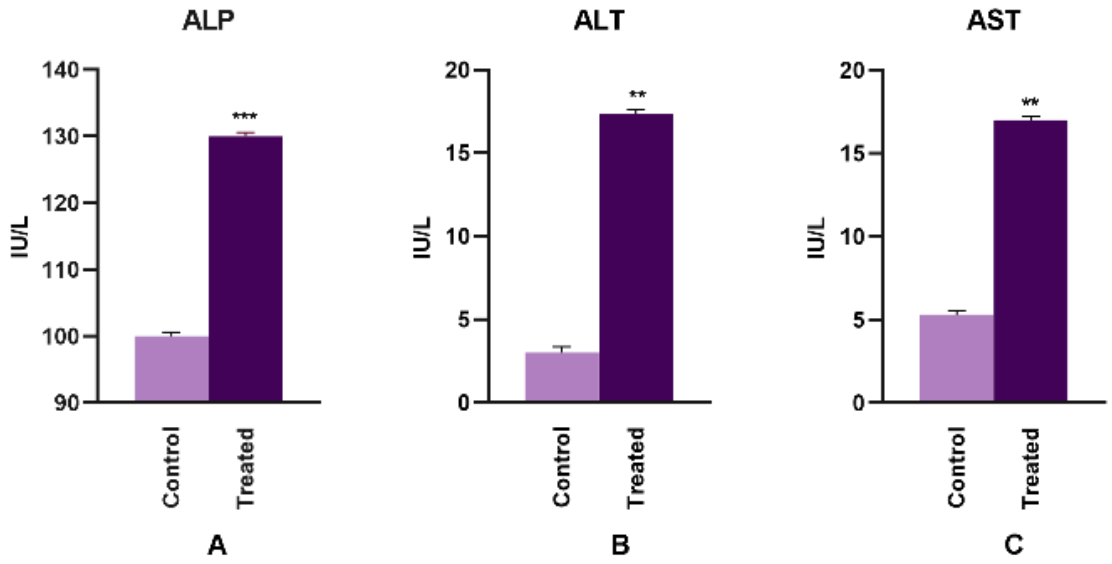

Figure 6.6: Liver function tests in the liver of newborn chicks treated with flubendiamide. (A) Alkaline phosphatase (ALP) activity (B) Alanine aminotransferase (ALT) activity (C) Aspartate aminotransaminase (AST) activity. All values are expressed as Mean ± SEM. n=3 with 30 eggs per group per day; **p≤0.01, ***p≤0.001.

Histological Examination of Liver Tissue

For the purpose of determining the extent of the tissue damage that flubendiamide induced in the livers of newborn chicks, a differential staining technique using H&E was utilized. The treated embryos displayed considerable abnormalities in comparison to the control group. These abnormalities included defective portal veins, indistinct bile ducts and disordered sinusoids.

In addition to this, there were obvious indications of vasculitis, which is characterized by the infiltration of inflammatory cells in each sinus. There was inflammation present across the entirety of the liver tissue, including inflammation in the peribiliary region, which was accompanied by inflammatory cell infiltration into the portal vein. Additionally, some

structural abnormalities were identified, like deformed portal veins with loose borders. This indicates that there was a loss of tissue integrity, leading to gaps between groups of cells (Figure 6.7). In addition, there was evidence of a damaged hepatocyte architecture in the liver group that was treated in comparison to the group that served as the control (Figure 6.7).

A scoring method proposed by Hose et al. (1996) was utilized to evaluate the histopathological modifications caused by the administration of flubendiamide. The findings demonstrated that every single animal in the control group was assigned a score of zero, which indicates that there were no changes. However, the degree of the abnormalities rose when flubendiamide was administered: thirty percent of the animals scored one, which indicated slight alterations; sixty percent scored two, which indicated moderate alterations; and twenty percent scored three, which indicated severe alterations (Table 6.6).

In addition, the intensity of these alterations was further classified based on a classification system that was developed by Paulo et al. (2012). This classification system spans from 0 to 3, categorizing alterations from none to severe, with specific observable alterations given for each stage (Table 6.7).

The Hepatic Alteration Index (HAI) was utilized to carry out a semi-quantitative evaluation after the qualitative examination of histological abnormalities had been completed. The control group did not display any changes, whereas the treatment group displayed moderate changes, as indicated by the HAI value of 53.73 ± 0.93 (Table 6.8).

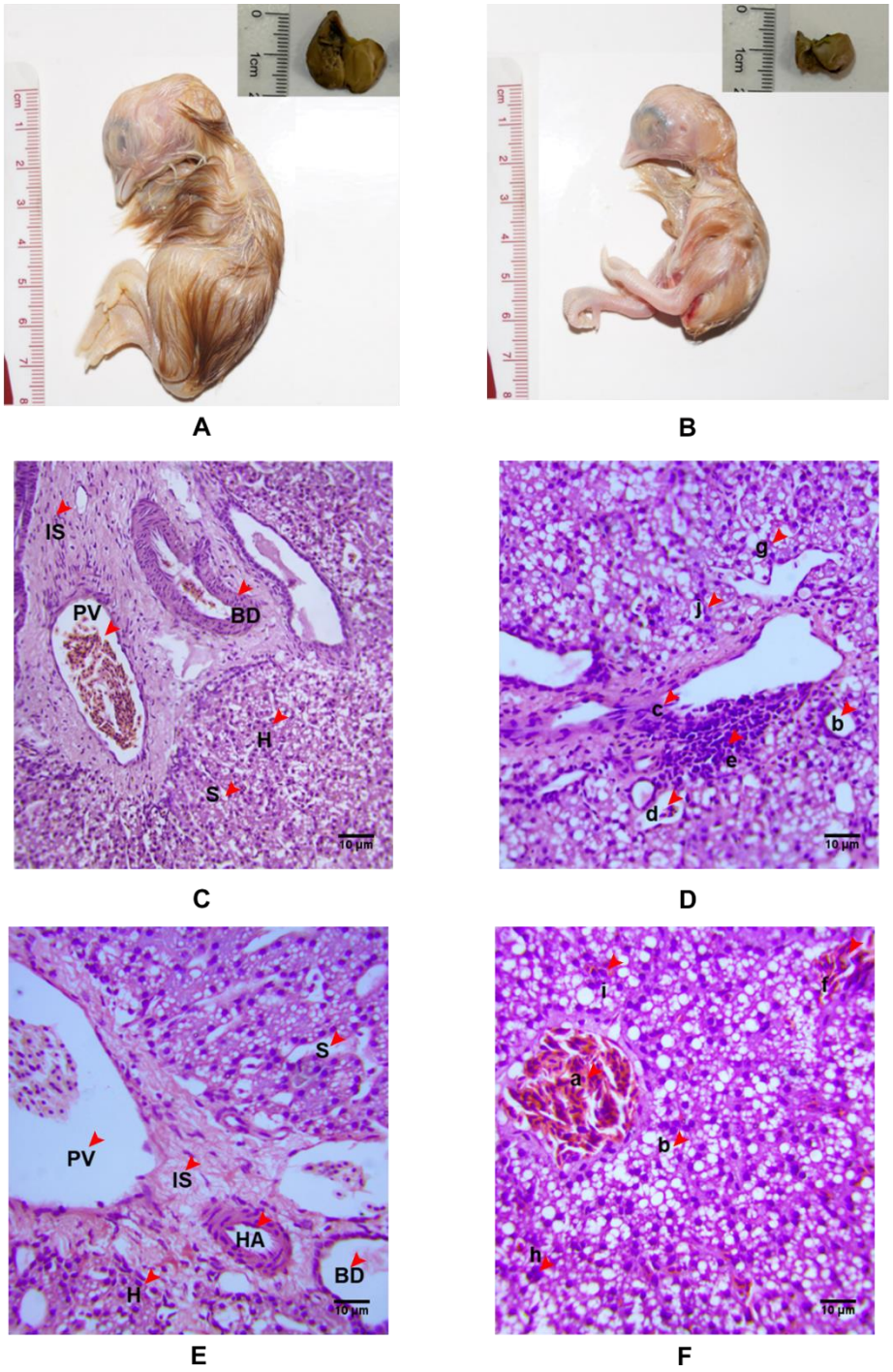

Figure 6.7: Histological section of flubendiamide-treated newborn chicken liver in comparison to the control group (H&E x40). The section shows hepatocyte (H), sinusoids (S), portal vein (PV), interlobular septum (IS) and a bile duct (BD). Control and treated chicks are labeled as A and B, respectively, 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.

DISCUSSION

Pesticides undoubtedly increased crop yield and reduced post-harvest losses. However, the rampant use of pesticides severely impacted the ecosystem and non-target organisms (Al-Saleh, 1994). Flubendiamide is a new-generation pesticide known to target caterpillars of insects (Troczka et al., 2017). Recent findings highlight the interaction of flubendiamide on non-target organisms (Li et al., 2014; Sarkar et al., 2014, 2017, 2018). Organisms have specific mechanisms for metabolizing and detoxifying contaminants or xenobiotics that enter their body (Grant, 1991). The liver is the primary site for xenobiotic metabolism, with microsomal cytochrome P450 (CYP) enzymes playing a central role in this process (Esteves et al., 2021). Diamide insecticides, such as chlorantraniliprole and flubendiamide, are widely used in agriculture and are broken down in the liver by different cytochrome P450 enzymes. The key cytochromes involved in diamide biotransformation include CYP1A1, CYP1A2, CYP2D6, CYP2C19 and CYP3A4. Out of them, CYP3A4 plays a crucial role in the oxidation of these chemicals, assisting in their detoxification and promoting their elimination from the body (Guengerich, 2008; Zanger & Schwab, 2013). CYP1A1 and CYP1A2 play a critical role in oxidizing diamides, which enhance their ability to dissolve in water and facilitate their removal from the body (Nebert & Dalton, 2006; Dong, 2023). In addition, CYP2C19 plays a role in breaking down these pesticides through metabolic mechanisms such as hydroxylation and demethylation (Pelkonen et al., 2008; Mao et al., 2019). CYP2D6 also plays a role in the metabolism of some diamides by performing N-demethylation, although to a lesser degree (Lynch & Price, 2007; Bempah & Donkor, 2011). The coordinated activity of various cytochrome P450 enzymes is essential for the efficient conversion and elimination of diamide insecticides from the organism, thus reducing their possible harmful consequences. The present study revealed that in the livers of flubendiamide-treated chicks, both mRNA and protein expression levels of major cytochromes involved in diamide biotransformation were significantly altered. Both qRT-PCR and western blot analyses indicated elevated expression of CYPs at both mRNA and protein levels in the flubendiamide-treated groups. The increased levels of transcripts and proteins suggest an increased accumulation of the toxicant insecticide in the embryonic liver. The biotransformation of such toxicants leads to an increased production of oxidative byproducts (Conde de la Rosa et al., 2022), which need to be curbed down with the aid of antioxidants.

Antioxidants play a vital role in reducing the detrimental impact of xenobiotic substances by counteracting reactive oxygen species produced during xenobiotic metabolism. The liver relies on enzymatic antioxidants, such as SOD and catalase, as well as non-enzymatic antioxidants like glutathione, to perform crucial functions in its defense mechanism (Matés et al., 1999; Valko et al., 2006; Sies, 2017). Glutathione, in its reduced form, transfers a hydrogen atom to free radicals, resulting in its conversion to oxidized glutathione (GSSG). Glutathione reductase is responsible for converting GSSG back to GSH in the usual manner. Nevertheless, if the formation of ROS exceeds the capability for regenerating GSH, it ultimately leads to a depletion in levels of GSH. The analysis of GSH in the flubendiamide-treated liver revealed a statistically significant decrease. Within the liver, the reduction of GSH leads to the buildup of ROS, which can induce lipid peroxidation, protein oxidation and DNA damage (Lu, 2013). These modifications have the potential to cause hepatocyte apoptosis or necrosis, eventually resulting in liver injury.

SOD facilitates the conversion of the superoxide anion into hydrogen peroxide and oxygen, whereas catalase breaks down hydrogen peroxide into water and oxygen, hence reducing the risk of oxidative damage (Matés et al., 1999; Borković et al., 2005; Valko et al., 2006). The heightened levels of catalase and SOD activity in the liver suggest that the organism is reacting to elevated levels of oxidative stress, possibly induced by exposure to flubendiamide. The elevated SOD levels indicate an increased conversion of superoxide to hydrogen peroxide, which needs to be worked upon by catalase action. However, the DCFDA staining, which offers a quantitative and qualitative approach to assess oxidative stress levels (Mitov et al., 2016), showed a notable increase in fluorescence intensity in the liver sections of treated newborn chicks. Thus, the DCFDA staining verified elevated levels of H_2O_2 in the liver tissue of the flubendiamide-treated chicks. The studies corroborated the incidence of elevated ROS production, which the organism could not compensate for, further leading to liver damage.

Healthy hepatocytes contain basal levels of key marker enzymes of liver damage like alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase and increased levels indicate acute liver damage (Gotaro et al., 1980; Kew, 2000). According to a report by Manna et al. (2004), elevated levels of serum enzymes such as ALP, ALT and AST are linked to liver damage induced by the release of free radicals through esoteric and oxidative pathways mediated by the cytochrome P450 microsomal enzyme system during the metabolism of

pesticide in the liver (Linklater & Higgs, 2016). The liver function tests revealed that the levels of ALP, ALT and AST enzymes in the serum of chicks treated with flubendiamide displayed a significant statistical variance compared to the control group. All the enzymes seemed to increase in the treated group. This implies that flubendiamide can cause an insult to the fetal liver, leading to the release of marker enzymes from the hepatocytes into the blood serum, thus emphasizing some pathophysiological conditions.

The drug metabolites, often the free radicals, can initiate various chemical reactions leading to structural damage in the liver (Kaplowitz, 2004). Differential staining with hematoxylin and eosin revealed tissue damage inflicted by flubendiamide on the livers of newborn chicks. In contrast, the control group showed no abnormalities, with distinct boundaries in the portal vein and narrow sinusoidal spaces indicating normal conditions. The more expansive sinusoidal space observed in the treated liver might be a compensatory response to increased blood flow demand due to the insult caused by flubendiamide. Compared to the control group, treated embryos exhibited impaired portal veins and lacked a clearly defined bile duct. Additionally, compromised hepatocyte architecture was observed in the treated liver. Cell infiltration was observed in the portal vein of the treated liver, along with hepatic lesions, indicating structural integrity alterations induced by flubendiamide. These results highlight the possible adverse effects of flubendiamide on non-target organisms due to increased oxidative stress, emphasizing the importance of its cautious usage to minimize adverse environmental impacts.

TABLES

Table 6.1: Transcript level expression of genes of cytochrome C and its variants in the liver tissue of flubendiamide-treated newborn chicks. Fold changes are expressed as Mean ± SEM. The fold change values for the control embryo are 1.0 for all the genes; n=3 with 30 eggs per group per experiment, ns = not significant, *p≤0.05; ***p≤0.001

Gene	Cytochrome fold change
CYP1A1	1.440 ± 0.13^{ns}
CYP1A2	$1.510 \pm 0.02^{***}$
CYP2D6	1.123 ± 0.08^{ns}
CYP2C19	$1.511 \pm 0.01^{***}$
CYP3A4	$3.001 \pm 0.40^{*}$

Table 6.2: Spot densitometry analysis of the western blot bands on the liver tissue of newborn chicks. The values are expressed as Mean ± SEM; n=3 with 30 eggs per group per experiment; *p≤0.05; **p≤0.01; ***p≤0.001

Protein	Relative band intensity in arbitrary units	
	Control	Treated
Cyp1a1 (58 kDa)	66.183 ± 0.72	$96.321 \pm 0.99^{***}$
Cyp1a2 (56 kDa)	77.511 ± 1.58	$97.126 \pm 0.76^{***}$
Cyp2d6 (55 kDa)	65.015 ± 1.61	$72.438 \pm 1.86^{**}$
Cyp2c19 (55 kDa)	87.562 ± 1.92	$92.248 \pm 1.32^{*}$
Cyp3a4 (57 kDa)	103.872 ± 1.08	$132.494 \pm 1.28^{***}$

Table 6.3: Antioxidants estimation on flubendiamide-treated newborn chick. The values are expressed as Mean ± SEM; n=3; ***p≤0.001

Attributes	**Control**	**Treated**
GSH levels (µg/µL)	4.18 ± 0.16	7.326 ± 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 6.4: Statistical analysis of fluorescence intensity following DCFDA staining in Control, flubendiamide-treated liver tissues of newborn chicks. n=6, N=3; **p≤0.01.

Groups	**Fluorescence intensity**
Control	83.052 ± 0.68
Treated	100.673 ± 0.71**

Table 6.5: Liver function estimation on flubendiamide-treated newborn chick. The values are expressed as Mean ± SEM; n=3; **p≤0.01; ***p≤0.001

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**

Table 6.6: Histopathological alterations in flubendiamide-treated liver of newborn chick classified using scores from 0 to 3 according to Hose et al. (1996). Data from dead animals were excluded.

Score	Stage	No. of animals showing alterations (frequency in %)	
		Control	Treated
0	No alteration	10/10	0/10
1	Slight alteration	0/10	3/10 (30%)
2	Moderate alteration	0/10	6/10 (60%)
3	Severe alteration	0/10	2/10 (20%)

Table 6.7: Classification of the severity of histopathological changes observed in the liver of newborn chick. Modified from Paulo *et al.* (2012).

Score	Stage with HAI values in parenthesis	Alterations
0	No alteration	-
1	Slight alteration	Congested blood vessels Vacuolated cytoplasm Disrupted tissue integrity Irregular nuclei Leukocyte infiltration
2	Moderate alteration	Vasculitis Degeneration of cytoplasm Pyknotic nuclei Peribiliary inflammation
3	Severe alteration	Focal necrosis Tumor

Table 6.8: Histopathological alteration index (HAI) observed in the liver of newborn chick.

Group	HAI (mean ± SEM)	Effect
Control	0	No alteration
Treated	53.73 ± 0.93	Moderate alteration

GRAPHICAL SUMMARY