Toxicology Reports 13 (2024) 101697

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

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

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ARTICLE INFO

Handling editor: Prof. L.H. Lash.

Keywords:
Antioxidant
Chick embryo
Cytochromes
Flubendiamide
Hepatotoxicity
Pesticides

ABSTRACT

Pesticides have increased crop yield but severely impacted ecosystems and non-target organisms. Flubendiamide, a new generation pesticide, targets insect larvae but also affects non-target organisms. This study examines the effects of lowest observed effect concentration of technical grade flubendiamide (0.5 μg/μL) flubendiamide on chick liver, focusing on cytochrome P450 (CYP) enzyme expression, oxidative stress, and liver damage. Chick embryos treated with flubendiamide showed significant alterations in CYP mRNA and protein levels, indicating increased toxicant accumulation. Elevated CYP3A4, CYP1A1, CYP1A2, and CYP2C19 levels were noted, suggesting enhanced biotransformation and detoxification processes. However, increased oxidative byproducts led to oxidative stress, as evidenced by decreased glutathione (GSH) levels and elevated superoxide dismutase (SOD) and catalase activities. DCFDA staining confirmed increased hydrogen peroxide (H_2O_2) levels, indicating heightened reactive oxygen species (ROS). Liver function tests revealed significant increases in serum ALP, ALT, and AST levels, indicating acute liver damage. Histopathological analysis showed structural liver damage, including expanded sinusoidal spaces, impaired portal veins, and compromised hepatocyte architecture. These findings underscore flubendiamide's potential hepatotoxicity in non-target organisms, emphasizing the need for cautious pesticide use to minimize environmental impacts.

1. Introduction

The outburst of pesticide usage in agriculture has elevated the chances of toxicants entering systems of non-target species, affecting their well-being [1,2]. Such contaminants (xenobiotics) are metabolized and eliminated from organisms using multiple sites, including the liver, intestinal wall, lungs, kidneys, and plasma [3]. In vertebrates, these chemicals are primarily channeled to the liver for detoxification and clearance [4]. The liver detoxifies and facilitates the excretion of xenobiotics by enzymatically converting lipid-soluble molecules to more water-soluble ones, ensuring easy elimination [5].

Upon exposure to a toxicant, the organism's defense mechanism activates phase I and II enzymes, including the CYP system in the liver, oxidizing compounds for further degradation. The cytochrome P450 subgroup is responsible for most drug metabolism [6]. Diamide insecticides, such as chlorantraniliprole and flubendiamide, are extensively used in agriculture and undergo metabolism in the liver via several cytochrome P450 enzymes. CYP3A4 plays a crucial role in oxidizing these substances, aiding in their detoxification and elimination from the body [7,8]. CYP1A1 and CYP1A2 enhance the water solubility of diamides, facilitating their removal [9,10]. Additionally, CYP2C19 is involved in the breakdown of these pesticides through hydroxylation and demethylation [11,12], while CYP2D6 contributes to their metabolism via N-demethylation [13,14].

The detoxification process generates free radicals and reactive oxygen species (ROS), causing oxidative stress. Elevated levels of enzymatic and non-enzymatic antioxidants, such as superoxide dismutase (SOD), catalase, and glutathione reductase, are crucial indicators of oxidative stress and damage [15–17]. Despite these mechanisms, continuous exposure to insecticides can still cause significant liver tissue damage

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https://doi.org/10.1016/j.toxrep.2024.101697
Received 10 June 2024; Received in revised form 10 July 2024; Accepted 13 July 2024
Available online 15 July 2024

[18,19]. Liver marker enzymes like alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) are commonly used to assess liver function, with elevated levels indicating biliary obstruction or hepatic damage [20,21].

Flubendiamide, a new-generation insecticide, targets lepidopterans by binding to ryanodine receptors, leading to paralysis [22,23]. However, its potential hazards to non-target organisms necessitate toxicity evaluations. The chick embryo was selected for this study due to its similarity to the human embryo in molecular makeup, cellular structure, and anatomical features, making it valuable for examining developmental processes [24]. The process of liver development in chick embryos closely resembles that in mammalian embryos [25], achieving functionality by day fourteen [26]. 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.

This study hypothesizes that in ovo administration of a lowest observed effect concentration (LOEC) of flubendiamide elevates oxidative stress and induces liver damage in chick embryos by altering cytochrome P450 enzyme expression and reducing antioxidant defenses.

The aim of this study is to investigate the hepatotoxic effects of flubendiamide on newly hatched chicks by evaluating cytochrome P450 enzyme expression, oxidative stress levels, and liver damage markers.

2. Materials and methods

2.1. Animal procurement and maintenance

Fertilized Rhode Island Red (RIR) domestic chicken eggs were obtained from the Intensive Poultry Development Unit in Vadodara, Gujarat, India. Before incubation, the eggs were examined through candling to check the air sac and were sanitized using betadine (Povidone-iodine 10 % w/v). All experimental procedures strictly followed the guidelines set by the national regulatory authority for animal experimentation, the Committee for Control and Supervision of Experiments on Animals (CCSEA). These protocols were subjected to ethical scrutiny and received approval from the Institutional Animal Ethics Committee (IAEC; Approval No. MSU-Z/IAEC04/10–2020).

2.2. Incubation

The automated incubator was calibrated to maintain a temperature of 37±0.5 °C and sustain a relative humidity of 70–75 % during the incubation phase (Scientific Equipments Works, New Delhi, India). Eggs were meticulously positioned with their broader ends facing upwards and underwent regular automated rotation every hour. Routine evaluations were performed via candling every two days to assess egg viability, with any non-viable eggs promptly removed from the incubator.

2.3. Experimental design

Technical grade flubendiamide insecticide (CAS No. 272451–65–7) was procured from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Freshly laid eggs were randomly divided into control and treatment groups. An earlier dose range study led to the selection of a lowest observed effect concentration of 0.5 μg/μL for subsequent experiments [27]. 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 (27 G X 0.5inch). Subsequently, eggs were carefully dosed within the air sac using a sterile BD 1 mL insulin syringe under laminar airflow [28]. The puncture site was promptly sealed with molten paraffin wax before transferring the eggs to the incubator. The treatment groups received a dosage of 0.5 μg/μL of flubendiamide dissolved in PBS, while the control group was administered PBS alone. For both groups, the dosage volume was kept constant at 50 μL. Subsequently, liver tissues and serum samples were collected from the newborn chicks following standard protocols for further experimentation and analysis.

2.4. Real-time PCR

Total RNA from the liver tissue of newborn chicks was extracted using the standard TRIzol method according to the manufacturer's instructions (Applied Biosystems, USA). Subsequently, 1 μg of total RNA was reverse transcribed into cDNA utilizing a one-step cDNA synthesis kit (Applied Biosystems, USA) following the manufacturer's guidelines. Primers for cytochromes were designed based on NCBI data (Table 1). Quantitative real-time PCR was carried out using a LightCycler 96 instrument (RRID:SCR_012155) (Roche Diagnostics, Switzerland). The experimental setup involved an initial incubation step at 95 °C for 3 min, followed by 35 amplification cycles. Each cycle comprised denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 20 s. Melt curve analysis was employed to verify the formation of specific PCR products. The obtained data were presented as mean Cq values normalized to 18 S rRNA levels (used as an endogenous control), and the fold change in expression was determined using the $2^{-\Delta\Delta Cq}$ method developed by Livak and Schmittgen (2001) [29].

2.5. Western blot

Liver homogenates from newborn chicks were lysed using a buffer containing 10 % protease inhibitor to extract total protein. The protein concentration of 10 % homogenates was determined using the Bradford method [30]. For separation, equal amounts of total protein were loaded onto a 12 % SDS-PAGE gel. Subsequently, the proteins were transferred onto PVDF membrane via semi-dry transfer at 100 mA for 20 min. The membrane was then cut and strips were individually probed with primary antibodies, including Mouse, anti-Cyp1a1 IgG Rabbit, anti-Cyp1a2 IgG Rabbit, anti-Cyp2d6 IgG Rabbit, anti-Cyp2c19 IgG Rabbit, anti-Cyp3a4 IgG Rabbit and anti-β-actin IgG Mouse. β-actin was used as the housekeeping control for all proteins. The membranes were incubated with biotinylated secondary polyclonal antibodies. For β-actin, antibodies generated in rabbits were used, while for Cyp1a1, Cyp1a2, Cyp2d6, Cyp2c19 and Cyp3a4, antibodies generated in goats were employed. The blot was developed using the BCIP-NBT system (Sigma-Aldrich, USA).

2.6. Estimation of reduced glutathione (GSH)

The GSH assay followed the methodology given by Anderson [31]. Liver tissue was homogenized in phosphate buffer (pH 7.4), and the assay system was prepared using a precipitating reagent (0.167 g glacial meta-phosphoric acid, 0.02 g EDTA, and 3.0 g NaCl dissolved in distilled water). After incubating the reaction system on ice for 10 min, it was centrifuged at 3000 rpm for 15 min at 4°C. Subsequently, 40 μL of the supernatant was mixed with 60 μL of Na_2HPO_4 and 3 μL of 5,5-dithio--bis-(2-nitrobenzoic acid) (DTNB), and the absorbance was measured at 412 nm (iMark Microplate reader, Bio-Rad Laboratories, Hercules, CA). The GSH content was determined using the slope from a standard curve, which was created with 50, 40, 30, 20, 10, and 0 μl of a 1 μg/μl GSH solution. The results were expressed as μg of GSH per mg of protein, with protein quantification performed using the Bradford assay [30].

2.7. Estimation of superoxide dismutase (SOD)

SOD activity was determined using the method outlined by Marklund and Marklund (1974) [32]. Three μL of liver homogenate was mixed with an assay solution comprising 50 μL of 0.2 M potassium phosphate buffer (pH 8.0) and 5 μL of pyrogallol in 0.5 N HCl. Enzyme activity was expressed as U/mL of assay mixture, with 1 U defined as the

Table 1
Primer sequences for cytochromes obtained from NCBI.

Gene	Forward primer	Reverse primer	Accession no.
18SrRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC	NR_003278.3
CYP1A1	GAGCTGGATCAGACCATCGG	CTGGTTGATGAACACGCACG	NM_205147.2
CYP1A2	GCTGTATCCATCCGCCTACC	GTTTGTGTTCTCTCAGCAGCA	KR711986.1
CYP2D6	CTCATCAGGTATCCAAAAGTGCAG	GTGTGGGATGGTAACAGGCA	JX678711.1
CYP2C19	CACAGTTACCTGGCGTCCC	GCAGCCCCATAAGAGCTCAA	NM_000769.4
CYP3A4	GTGGTGCTGTCAGGCTCTAT	AGGCTGCCTGCCATCATAAA	XM_046927350.1

amount of enzyme necessary to achieve 50 % inhibition of pyrogallol auto-oxidation. Absorbance was measured at 420 nm.

2.8. Estimation of catalase

Catalase activity was assessed following the protocol described by Sinha [33]. Eight µL of the sample was combined with 50 µL of 0.2 M H_2O_2 and 80 µL of 0.01 M phosphate buffer (pH 7). After a 1-min incubation, 80 µL of dichromate acetic acid reagent was added, followed by heating for 10 min and cooling to room temperature. Absorbance was then measured at 570 nm. Results were reported as micromoles of H_2O_2 decomposed/min/mg protein.

2.9. Analysis for cytoplasmic oxidative stress

The cell-permeable reagent 2', 7'-dichlorofluorescein diacetate (DCFDA) measures ROS using fluorescence with specific excitation/emission wavelengths at 485 nm/535 nm. Liver sections from both control and treated groups were made into 5 µm slices using a cryostat microtome (Reichert-Jung Cryocut 1800, Leica Microsystems Inc., USA). The slides were then treated with DCFDA dye and incubated for 20 min in the dark. Subsequently, the samples were subjected to PBS washes and imaged using the LSM 710 Confocal Microscope (Carl Zeiss Microscopy, Jena, Germany, RRID:SCR_018063). The fluorescence intensities were determined using ImageJ Fiji software (RRID:SCR_003070).

2.10. Liver function tests

Serum levels of ALP, ALT, and AST are biomarkers used to detect early indications of acute liver damage. These enzyme levels were measured in serum samples according to the manufacturer's protocol (Reckon Diagnostics, Vadodara, Gujarat, India). ENZOPAK ALP utilizes a kinetic method following the guidelines of the German Society for Clinical Chemistry (GSCC). The assay system consisted of 100 µL of buffered solution (p-NPP 20 mmol/L, sodium chloride 500 mmol/L, buffer 800 mmol/L, pH 9.9 ± 0.5) and 2 µL sample. Readings are recorded at 30 s, 60 s, 90 s, and 120 s at 405 nm. The ALP activity (IU/L) is calculated using the formula ΔA/min × F, where F is 2713 (derived from the molar extinction coefficient for p-nitrophenol and the ratio of total assay volume to sample volume). ENZOPAK ALT adheres to the protocol endorsed by the IFCC. The assay system contained 100 µL of working reagent (NADH-Na_2 0.1 mmol/L, LDH 2000 U/L, buffer 50 mmol/L, L-alanine 200 mmol/L, and α-KG 10 mmol/L, pH 7.5 þ 0.1) and 10 µL of sample. After mixing, the assay system is incubated at 37°C for 60 s, and absorbance readings are taken every 30 s for 2 min at 340 nm. Subsequently, the enzyme activity is computed using the formula (IU/L) = ΔA/min × F, where F is set at 1746, derived from the millimolar extinction coefficient of NADH at 340 nm. ENZOPAK AST follows the procedure recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The assay system had a working solution (NADH-Na_2 0.1 mmol/L, LDH >1000 U/L, MDH >1000 U/L, buffer 50 mmol/L, L-aspartic acid 150 mmol/L, and α-KG 10 mmol/L, pH 8.0 + 0.1) of 100 µL and 10 µL of sample. After mixing, the assay system is incubated at 37 °C for 60 s, and absorbance is measured every 30 s for 2 min at 340 nm. The enzyme activity is calculated using the formula (IU/L) = ΔA/min × F, where F equals 1746 (based on the millimolar extinction coefficient of NADH at 340 nm).

2.11. Histopathological study

Liver tissues were surgically excised from anaesthetized animals in both experimental groups and rinsed with normal saline. These tissues were then placed in 10 % neutral buffered formalin for fixation. Subsequently, the tissues from both control and treated groups were processed, and paraffin wax blocks were made. Transverse sections of the embryos, each 5 µm thick, were prepared and stained with Harris' Hematoxylin and Eosin [34]. The histological analysis of the tissue slides was conducted under a light microscope (Magnus, India) at 100X magnification, and images were captured using CatCam software (Catalyst Biotech, India). The alterations were evaluated using the scoring system [35]. Additionally, the extent of tissue damage was assessed semi-quantitatively using the histopathological alteration index (HAI) [36]. HAI values ranging from 0 to 10 represent normal organ functioning, while values from 11 to 20 signify slight alterations, and those from 21 to 50 indicate moderate alterations. Values between 50 and 100 suggest severe lesions, and values exceeding 100 indicate irreparable lesions in the organ [36].

2.12. Statistical analysis

Statistical analyses were conducted using Student's t-test in GraphPad Prism v8.0 software (RRID:SCR_002798) (GraphPad Software Inc., USA). The Student's t-test was selected to compare the means between the control and treated groups due to its robustness in determining significant differences in small sample sizes. Data were presented as Mean ± SEM, and statistical significance was defined as a p-value equal to or less than 0.05, indicating a less than 5 % probability that the observed differences occurred by chance.

3. Results

3.1. Upregulation of cytochrome P450 transcripts in treated liver

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 ($p \leq 0.001$). While CYP2D6 also exhibited increased expression, it was not statistically significant. Notably, in the treated groups, CYP3A4 exhibited a more than threefold increase in expression ($p \leq 0.05$) (Fig. 1; Supplementary Table 1).

3.2. Western blot confirms increased expression of cytochrome P450

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

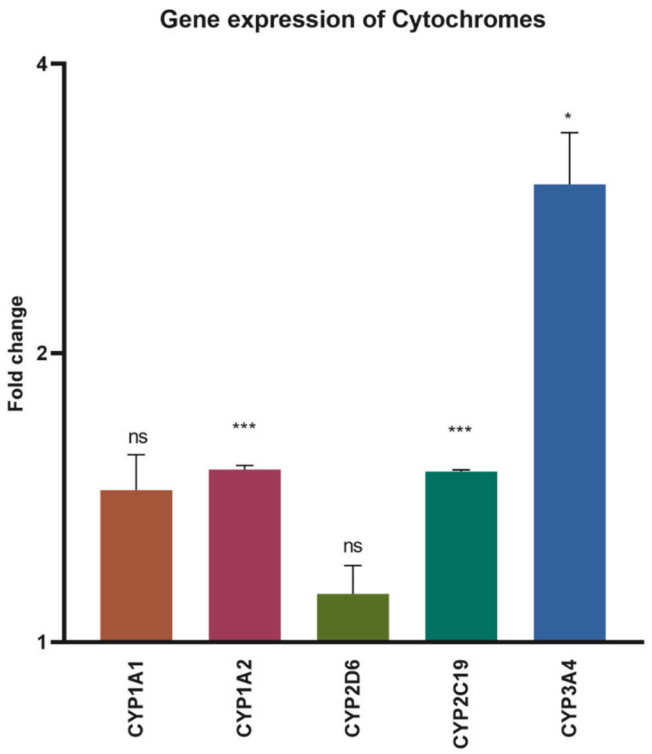

Fig. 1. Transcript levels of genes of cytochrome C and its variants in flubendiamide treated newborn chicks. Values are expressed in fold change (Mean ± SEM). Fold change values for control embryo is 1.0 for all the genes (n=3); ns = not significant; *p≤0.05; ***p≤0.001.

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 (Fig. 5; Supplementary Fig. 1; Supplementary Table 2). During the western blot analysis, β-Actin was utilized as an internal control for normalization.(Fig. 2)

3.3. Altered antioxidant enzymes and reduced glutathione levels in treated liver

The activity of SOD and catalase antioxidant enzymes notably increased in the treated group compared to the control (Fig. 3; Supplementary Table 3). The levels of reduced glutathione remained significantly reduced in the treated group (p≤0.001). Additionally, both SOD and catalase activities remained elevated in liver samples from the

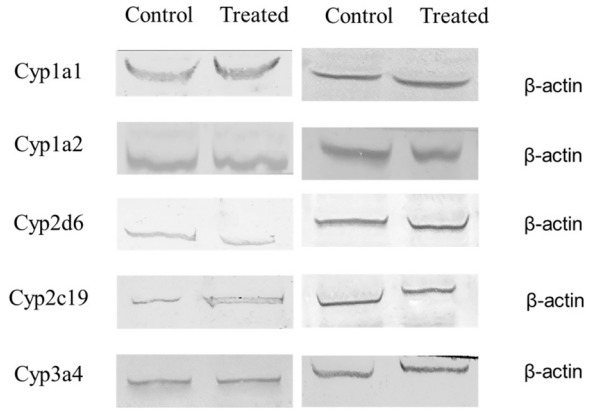

Fig. 2. Western blot images showing comparative expression on flubendiamide treated newborn chicks. β-Actin was taken as loading control, n=3 with 30 eggs per group per day.

treated group compared to the control (p≤0.001).

3.4. Increased oxidative stress in flubendiamide-treated liver

The fluorescence intensity from DCFDA stained samples was higher in the flubendiamide-treated liver than in the control group (Fig. 4). 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 (Supplementary Table 4).

3.5. Elevated serum ALP, ALT, and AST levels in treated chicks

The evaluation of serum ALP, ALT, and AST levels revealed significant elevations in the treated group compared to the control group. ALP levels showed a marked increase (p≤0.001), while both ALT and AST levels also displayed statistically significant rises in the treated group (Fig. 5; Supplementary Table 3).

3.6. Histopathological changes in the liver of flubendiamide-treated chicks

Differential staining with Hematoxylin and Eosin was employed to evaluate tissue damage caused by flubendiamide in the livers of newborn chicks. Compared to the control group, treated embryos exhibited significant abnormalities such as impaired portal veins, indistinct bile ducts, and disorganized sinusoids. Additionally, signs of vasculitis, characterized by inflammatory cell infiltration in sinusoids, were evident. Inflammation was widespread throughout the liver tissue, including peribiliary inflammation with inflammatory cell infiltration into the portal vein. Structural abnormalities, such as distorted portal veins with loose boundaries indicating tissue integrity loss and visible gaps between cells, were also observed (Fig. 6). Furthermore, there was evidence of compromised hepatocyte architecture in the treated liver compared to the control group (Fig. 6). Histopathological alterations induced by flubendiamide administration were assessed using a scoring system developed by Hose and team [35]. Results showed that all animals in the control group received a score of 0, indicating no alterations. However, upon flubendiamide administration, the severity of alterations increased: 30 % of animals scored 1 (slight alterations), 60 % scored 2 (moderate alterations), and 20 % scored 3 (severe alterations) (Table 2). The severity of these alterations was further classified based on a classification system developed by Paulo and team [36], which ranges from 0 to 3, categorizing alterations from none to severe, with specific observed alterations listed for each stage (Table 3). Following the qualitative evaluation of histopathological alterations, a semi-quantitative assessment was conducted using the Hepatic Alteration Index (HAI). The control group exhibited no alterations, while the treated group showed moderate alterations with an HAI value of 53.73 ± 0.93 (Table 4).

4. Discussion

Pesticides have undoubtedly increased crop yields and reduced post-harvest losses. However, their rampant use has severely impacted ecosystems and non-target organisms [37]. Flubendiamide, a new-generation pesticide, specifically targets caterpillars of insects [38]. Recent findings have highlighted the adverse interactions of flubendiamide with non-target organisms [39, 40, 27, 41]. Organisms possess specific mechanisms for metabolizing and detoxifying xenobiotics or contaminants that enter their bodies [5]. The liver, being the primary site for xenobiotic metabolism, relies heavily on microsomal cytochrome P450 (CYP) enzymes, which play a central role in this detoxification process [42].

Diamide insecticides, such as chlorantraniliprole and flubendiamide, are widely used in agriculture and are metabolized in the liver by various cytochrome P450 enzymes. Key cytochromes involved in the

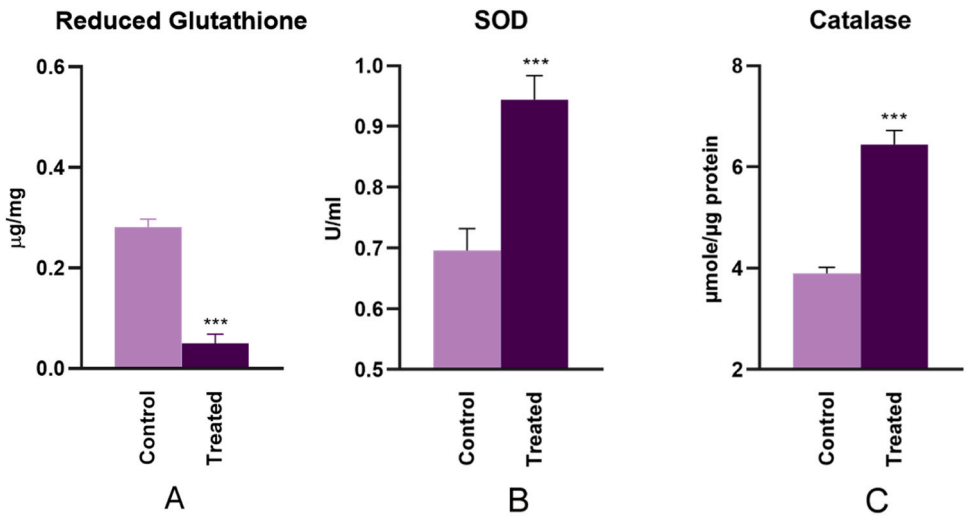

Fig. 3. Enzymatic and non-enzymatic antioxidant status in the liver of newborn chicks treated with flubendiamide. (A) Reduced glutathione content (B) Superoxide dismutase activity (C) Catalase activity. All values are expressed as Mean ± SEM. n=3 with 30 eggs per group per day; ***p≤0.001.

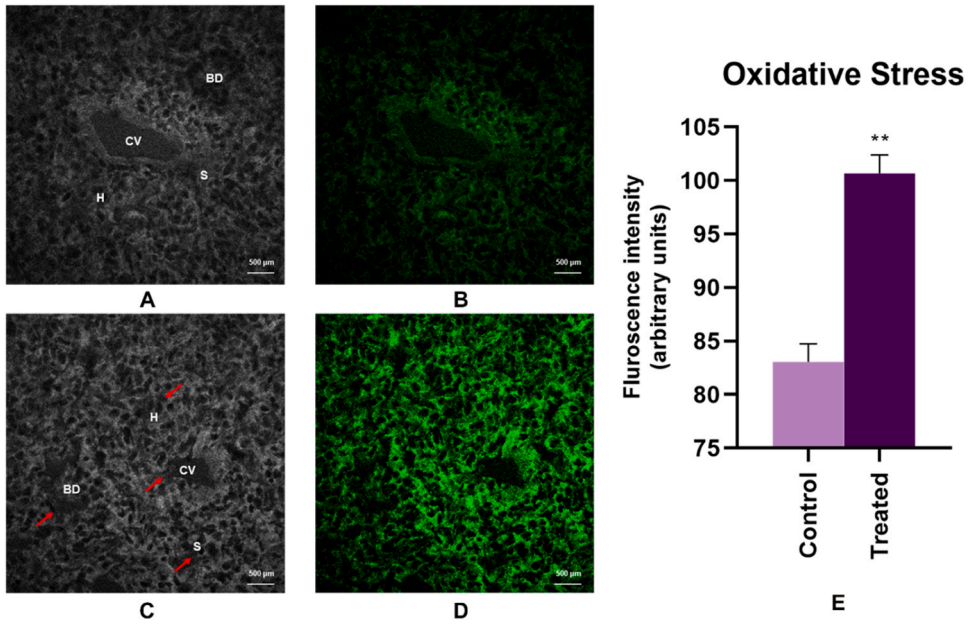

Fig. 4. DCFDA staining showing cytoplasmic ROS level in the liver of flubendiamide treated newborn chicks. A and C are control and treated group images of bright field respectively. B and D corresponds to DCFDA stained images of control and treated group respectively (scale bar in yellow: 50 µM); E denote graph showing statistical analysis of fluorescence intensity following DCFDA staining in control and treated groups. n=6, N=3; **p ≤0.01.

biotransformation of diamides include CYP1A1, CYP1A2, CYP2D6, CYP2C19, and CYP3A4. Among these, CYP3A4 plays a crucial role in the oxidation of these chemicals, assisting in their detoxification and promoting their elimination from the body [7,8]. CYP1A1 and CYP1A2 also play critical roles in oxidizing diamides, enhancing their water solubility and facilitating their removal from the body [9,10]. Additionally, CYP2C19 is involved in breaking down these pesticides through metabolic mechanisms such as hydroxylation and demethylation [11,12]. CYP2D6 contributes to the metabolism of some diamides by performing N-demethylation, although to a lesser degree [13,14]. The coordinated activity of various cytochrome P450 enzymes is essential for the efficient conversion and elimination of diamide insecticides from the organism, thereby reducing their potential harmful effects.

The present study revealed significant alterations in both mRNA and protein expression levels of major cytochromes involved in diamide biotransformation in the livers of flubendiamide-treated chicks. Both qRT-PCR and western blot analyses indicated elevated expression of CYPs at both mRNA and protein levels in the flubendiamide-treated groups. These increased levels of transcripts and proteins suggest an accumulation of the toxicant insecticide in the embryonic liver. The biotransformation of such toxicants leads to an increased production of oxidative byproducts [43], which need to be neutralized with the aid of antioxidants.

Antioxidants play a vital role in mitigating the detrimental impact of xenobiotic substances by counteracting reactive oxygen species produced during xenobiotic metabolism. The liver relies on enzymatic antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase, as well as non-enzymatic antioxidants like glutathione, to perform crucial functions in its defense mechanism [44–46]. Glutathione, in its reduced form (GSH), transfers a hydrogen atom to free radicals, converting itself to oxidized glutathione (GSSG). Glutathione reductase then regenerates GSH from GSSG under normal conditions.

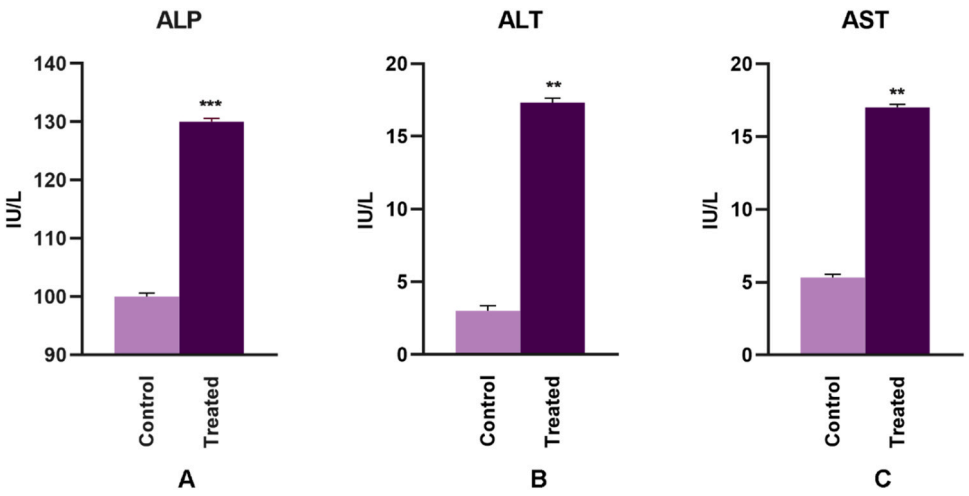

Fig. 5. Liver function enzymes in serum of flubendiamide-treated newborn chicks. (A) Alkaline phosphatase (ALP) activity (B) Alanine aminotransferase (ALT) activity (C) Aspartate aminotransaminase (AST) activity. All values are expressed as Mean ± SEM. n=3 with 30 eggs per group per day; **p≤0.01, ***p≤0.001.

However, if ROS formation exceeds the capacity for regenerating GSH, this leads to a depletion in GSH levels. The analysis of GSH in the flubendiamide-treated liver revealed a statistically significant decrease. Within the liver, reduced GSH levels result in ROS accumulation, which can induce lipid peroxidation, protein oxidation, and DNA damage [47]. These modifications have the potential to cause hepatocyte apoptosis or necrosis, resulting in liver injury.

Superoxide dismutase facilitates the conversion of the superoxide anion into hydrogen peroxide and oxygen, whereas catalase breaks down hydrogen peroxide into water and oxygen, thus reducing the risk of oxidative damage [44, 45, 48]. The heightened levels of catalase and SOD activity in the liver suggest that the organism is responding to elevated levels of oxidative stress, possibly induced by exposure to flubendiamide. Elevated SOD levels indicate increased conversion of superoxide to hydrogen peroxide, which needs to be managed by catalase action. However, DCFDA staining, which offers a quantitative and qualitative approach to assess oxidative stress levels [49], showed a notable increase in fluorescence intensity in the liver sections of treated newborn chicks. Thus, DCFDA staining verified elevated H_2O_2 levels in the liver tissue of the flubendiamide-treated chicks. These studies confirmed the incidence of elevated ROS production, which the organism could not adequately neutralize, leading to liver damage.

Healthy hepatocytes contain basal levels of metabolic enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Increased levels of these enzymes in the blood are indicators of acute liver damage [20,21]. Reports have shown that elevated serum levels of ALP, ALT, and AST are associated with liver damage induced by the release of free radicals through oxidative pathways mediated by the cytochrome P450 microsomal enzyme system during pesticide metabolism in the liver [50,51]. In the present study, liver function tests revealed a statistically significant increase in the levels of ALP, ALT, and AST enzymes in the serum of flubendiamide-treated chicks compared to the control group. This implies that flubendiamide can cause liver damage, leading to the release of these marker enzymes from hepatocytes into the blood serum, thus highlighting some pathophysiological conditions.

Drug metabolites, often free radicals, can initiate various chemical reactions leading to structural damage in the liver [52]. 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 and infiltration of cells in the portal vein, along with hepatic lesions, were observed in the treated liver, 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.

This study focused exclusively on newly hatched chicks, limiting the generalizability of the findings to other animal models and developmental stages. Additionally, the potential long-term effects of flubendiamide exposure were not investigated, restricting our understanding of its chronic toxicity. The findings of this study indicate that flubendiamide poses significant risks to non-target organisms due to its hepatotoxic effects. The observed oxidative stress and liver damage in chick embryos suggest that similar impacts could occur in other wildlife, potentially disrupting ecosystems. These results underscore the need for cautious use of flubendiamide in agriculture to minimize its environmental impact and protect biodiversity.

5. Conclusion

This study demonstrates that in ovo administration of flubendiamide at the lowest observed effect concentration (0.5 μg/μL) induces significant oxidative stress and liver damage in newly hatched chicks. The observed alterations in cytochrome P450 enzyme expression, antioxidant defense mechanisms, and liver histopathology provide compelling evidence of flubendiamide's hepatotoxic potential. These findings underscore the urgent need for rigorous toxicity evaluations of insecticides to protect non-target species and inform regulatory decisions to ensure human health and environmental safety. Future research should investigate the specific molecular mechanisms underlying these changes, extend studies to other animal models and developmental stages, and explore the long-term effects of flubendiamide exposure to gain a comprehensive understanding of its chronic toxicity. This will be essential for developing safer pest management strategies and minimizing the environmental and health risks associated with pesticide use.

CRediT authorship contribution statement

Dhanush Danes: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juhi Vaishnav:** Writing – review & editing, Validation, Investigation, Data

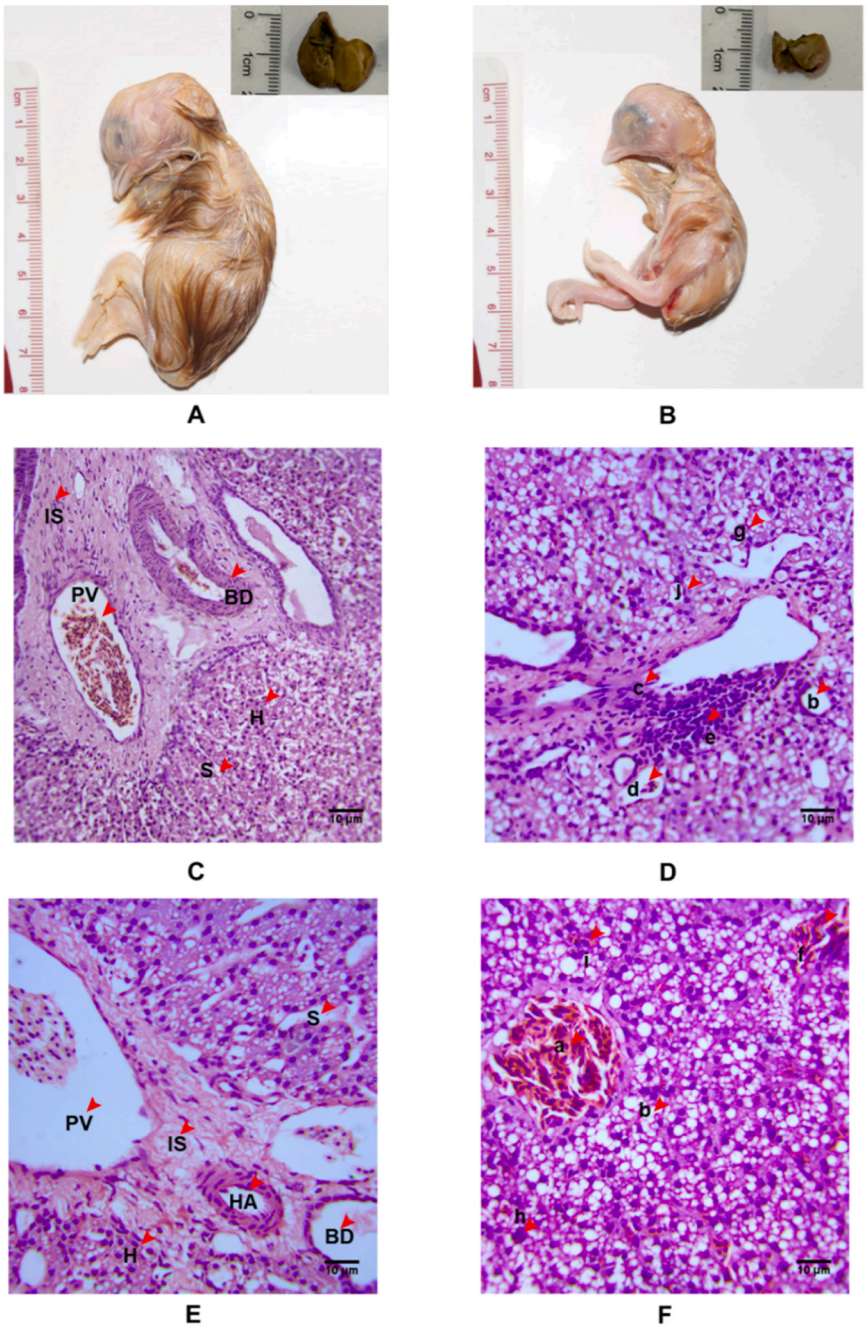

Fig. 6. Histological section of flubendiamide treated newborn chicken liver in comparison the control group (H&E x40). Section showing hepatocyte (H), sinusoids (S), portal vein (PV), interlobular septum (IS) and a bile duct (BD). Control and treated chicks are labelled as A and B respectively, with 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. The grading scores for histopathological alterations are mentioned in Table 3.

Table 2
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	100 %	0 %
1	Slight alteration	0 %	30 %
2	Moderate alteration	0 %	60 %
3	Severe alteration	0 %	20 %

curation. **Lakshmi Pillai:** Writing – review & editing, Data curation. **Anjali Singh:** Writing – review & editing, Data curation. **Suresh Balakrishnan:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 3
Classification of the severity of histopathological changes observed in the liver of newborn chick.

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 4
Histopathological alteration index (HAI) observed in liver of newborn chick.

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

Data Availability

Data will be made available on request.

Acknowledgement

This work was supported by DBT-BUILDER-Cat III (Grant number: BT/INF/22/SP41403/2021) and Gujarat State Biotechnology Mission (GSBTM) Gandhinagar, India, for financial assistance (Grant number: GSBTM/JD(R&D)/618/21–22/1224, Date: 28/12/2021).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101697.

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