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

[Synopsis for Ph.D. in Zoology]

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INTRODUCTION

The Food and Agriculture Organization (FAO) has issued a communiqué highlighting the need to quadruple food production in developing countries to meet the demands of an expanding population that is projected to reach 9.1 billion by 2050 (Fischer, 2009). This heightened demand is reliant on the use of pesticides, without which more than half of the world's crops would be lost to pests, diseases, and weeds. Pesticides are crucial for safeguarding crops and increasing productivity per unit of land. Both conventional and organic farmers utilize them, although they may have different inclinations toward synthetic or natural sources, each with its own levels of toxicity (Popp et al., 2013). The introduction of pesticides into the market since the 1960s has led to a significant increase in the production of major crops. Weeds, pests, and diseases currently contribute to around 40 percent of potential crop loss globally each year, with thousands of species competing for food crops (Aktar, 2009). Additionally, transported crops face threats from insects, molds, and rodents during storage. Pesticides are known to play a crucial role in extending the lifespan of crops and reducing post-harvest losses, as well as decreasing the contamination of food by harmful microorganisms and toxins. However, while pesticides are designed to target specific pests, they can also affect non-target species, including humans, particularly those involved in pesticide production and agricultural work. The excessive use of pesticides, driven by the desire for quicker and greater results, poses significant risks to human health and the environment, as highlighted by studies (Rather et al., 2017).

Pesticides can be categorized in various ways, including their intended use, toxicity, mode of entry, mode of action, chemistry, and formulations. In terms of the pests they target, they fall into several classifications: herbicides, which control unwanted plant growth like weeds; rodenticides, which eliminate rodents such as rats and mice; bactericides, which combat bacterial infections; fungicides, for managing fungal infestations; and larvicides, which specifically target insect larvae (Lushchak et al., 2018). Insecticides are designed to eradicate insect populations. Given the tropical climate of the Indian continent, which heightens its vulnerability to insect infestations, the utilization of insecticides is particularly high compared to other types of pesticides (Aktar, 2009). One of the recent entrants in the insecticide market is Diamides. They exhibit highly effective specific target site activity, allowing them to control a wide range of pests. Additionally, they possess a favorable toxicity profile. These substances are synthetic ryanoids, specifically chlorantraniliprole, cyantraniliprole, and flubendiamide,

whereas Ryanodine is a natural insecticide found in *Ryania speciosa*. It binds to the ryanodine receptor and disrupts the flow of calcium by partially blocking the channel. Diamide insecticides also bind to these receptors, causing the calcium channel to stay open and resulting in an uncontrolled release of calcium stores. Since calcium is involved in various cellular processes, the inability to regulate calcium leads to lethargy, loss of appetite, and eventually death at the organismal level (Teixeira, 2013). Based on their chemical properties, diamides can be classified into two categories, Anthranilic Diamide, and Phthalic Diamide. Chlorantraniliprole and cyantraniliprole belong to the Anthranilic Diamide category, while flubendiamide belongs to the Phthalic Diamide category (Troczka et al., 2017). Flubendiamide (Fig. 1), an insecticide belonging to the phthalic acid diamide class, is commonly employed for the protection of crops such as rice, cotton, corn, grapes, and various other fruits and vegetables against lepidopteran pests. Its high specificity and minimal impact on non-target organisms have led to its widespread adoption as a replacement for organophosphate and organochlorine pesticides (Troczka et al., 2017). However, the extensive use of flubendiamide has raised concerns regarding environmental pollution. In response to its adverse environmental effects, the United States Environmental Protection Agency (EPA) issued a Notice of Intent to Cancel all remaining flubendiamide products in 2016 (Sun et al., 2018).

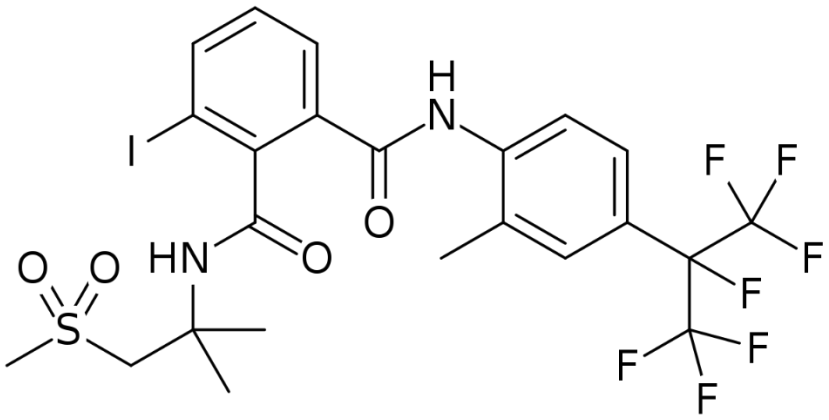


Fig. 1 Structural formula of flubendimaide

A recent study has demonstrated that diamides can induce harmful effects on developing avian embryos even at very low concentrations. These effects encompass a range of qualitative anomalies such as microcephaly, hydrocephaly, edematous swelling, hematoma formation, abnormal body coloration, microphthalmia, deformed beak, agnathia, micromelia, amelia, omphalocele, and ectopia cordis, which were observed in treated groups compared to untreated

groups (Abbas et al., 2018). Furthermore, investigations have revealed that flubendiamide leads to a significant decrease in fecundity in non-target dipteran insects like *Drosophila melanogaster* (Sarkar et al., 2017). Additionally, flubendiamide has been found to alter the visual and locomotory activities of *Drosophila melanogaster* (Sarkar et al., 2018). Recent studies have also raised concerns about the impact of flubendiamide on non-target organisms, such as the Chinese tiger frog (*Hoplobatrachus chinensis*) (Li et al., 2014). The detection of flubendiamide residues in human milk has raised concerns regarding potential implications for maternal and infant health (Liu et al., 2022). Consequently, conducting a safety assessment of flubendiamide to investigate its possible developmental toxicity is of paramount importance.

The chick embryo was chosen for this investigation due to its resemblance to the human embryo in terms of molecular, cellular, and anatomical characteristics. This makes it a great resource for comprehending developmental processes. Other advantageous factors to examine were the ease of maintenance and the ability for ex-utero development, which can potentially save the mother's life (Stern, 2018). The affordability of the eggs and their housing renders large-scale studies more feasible compared to other animal models, hence increasing accessibility to a wide range of facilities. In addition, eggs are readily accessible throughout the year in practically any location worldwide. They can also be obtained in precise numbers, which makes it easier to plan and schedule studies with greater effectiveness (Vergara, 2012). An initial study carried out in the laboratory uncovered significant developmental irregularities in the developing chick embryo, including eye deformities, insufficient blood vessel growth, and incomplete closure of the ventral body wall in the chick embryos. The chorioallantoic membrane (CAM) plays pivotal roles in facilitating essential physiological processes during the development of chick embryos (Ahmed et al., 2022). These include enabling efficient gas exchange, transferring nutrients, and eliminating waste products. This underscores the critical importance of angiogenesis, the formation of new blood vessels, in ensuring the proper functioning of vital physiological processes throughout embryonic development (Ahmed et al., 2022). Congenital defects affecting the eyes represent a significant portion of recorded congenital abnormalities worldwide, occurring at a frequency ranging from 0.36% to 4.7% of live births (Guarnera et al., 2024), highlighting the necessity for understanding the causative factors of such defects. The fetal liver, which is the primary location for xenobiotic metabolism, is particularly vulnerable to chemical damage during the developmental process. Thus, the study also investigated the effect of in-ovo administration of technical grade flubendiamide (500ppm - lowest observed effect concentration, LOEC) on the liver of newly hatched chicks.

HYPOTHESIS

Our hypothesis posits that administering flubendiamide at a sub-lethal dosage leads to the suppression of various signaling molecules, thereby disrupting the coordinated patterning essential for normal chick embryo development, ultimately leading to notable structural abnormalities.

The main aim of the investigation is to elucidate the underlying mechanisms behind the structural and functional abnormalities triggered by flubendiamide during the developmental stages of chick embryos.

This will be achieved through three parallel studies listed below, which can be treated as specific objectives.

1. To decipher the function of flubendiamide in the process of CAM (chorioallantoic membrane) angiogenesis in chick embryogenesis.
2. To unearth the role of flubendiamide in the domestic chick's eye development and patterning.
3. To elucidate the impact of flubendiamide on the liver of newborn chicks.

Egg procurement

Fertilized eggs of the Rhode Island Red (RIR) chicken breed were obtained from the Intensive Poultry Development Unit in Vadodara, Gujarat, India. Before incubation, the eggs underwent candling to assess the air sac and were then sterilized using betadine (Povidone-iodine 10% w/v). All procedures adhered to guidelines established by the national regulatory authority for animal experimentation, the CCSEA, and were approved by the institutional animal ethics committee (IAEC) under protocol number MSU-Z/IAEC04/10-2020. An automated incubator (Scientific Equipments Works, New Delhi, India) maintained a constant temperature of 37±0.5°C and relative humidity of 70-75% during the entire incubation period. Eggs were rotated on an hourly basis with their broader ends facing upward and inspected every two days using candling methods, with non-viable eggs being promptly removed (Fig. 2).

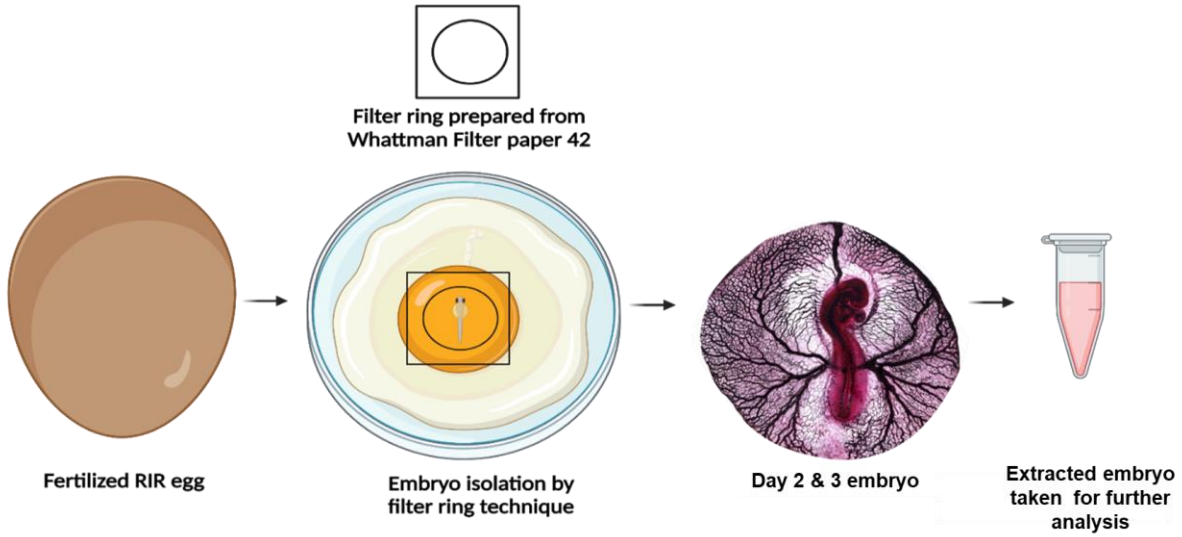
Fig. 2 Eggs inside incubator

Dosing of embryos

Using a sterile BD 1 ml insulin syringe under laminar airflow, the eggs were injected into the air cell, following the method described by Blankenship et al. (2003). The puncture site was promptly sealed with molten paraffin wax before placing the eggs in the incubator. Embryos from days 2, 3, and 4 were chosen for subsequent experimentation and analysis. The treatment groups were administered a dose of 500 ppm of flubendiamide (LOEC) in 50 µl of PBS, determined through a previously conducted dose range study. The control group received 50 µl of PBS.

Isolation of embryos

Embryos of days 2, 3, and 4 were selected for morphological examination. Day 2 and 3 embryos were isolated employing filter-ring techniques as outlined by Chapman et al. (2001), while day 4 embryos were gently scooped out using blunt-end forceps and a spatula (Fig. 3). Morphological assessments of day 2 and 3 embryos were conducted under a light microscope set to 4X magnification, with images captured using CatCam microscope cameras manufactured by Catalyst Biotech, in Maharashtra. Day 4 embryo observations were directly documented using a Sony SLT-A58K camera. The liver tissues of newborn chicks were also isolated after euthanasia for further study.



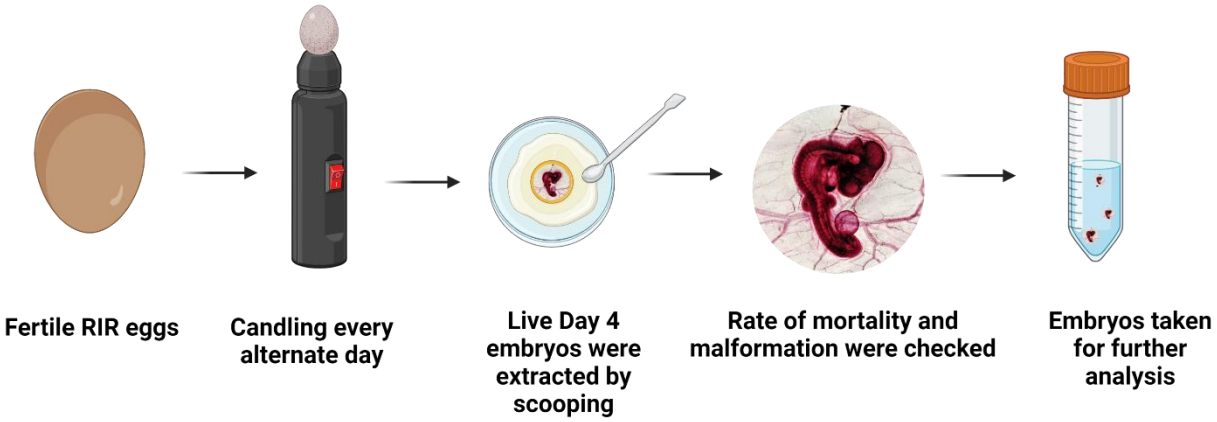


Fig. 3 Schematic representation of Filter Ring technique for Day 2, 3 and 4 embryo isolation

Vessel analysis of CAM

To measure morphological and geographical parameters of vascular networks, such as vessel density, number of vessel junctions, total vessel length, and lacunarity, images of the chorioallantoic membrane (CAM) from embryos on days 3 and 4 were taken and processed with AngioTool 0.6 software (RRID:SCR_016393) (Zudaire et al., 2011). The statistical analysis was conducted using Student's t-test to determine if there were any significant differences across the treatment groups with a 95% level of significance.

Molecular docking

AutoDock Tools 4.2.2 (RRID:SCR_011958) by Morris et al. (2009) was utilized to predict the binding energies of flubendiamide with proteins involved in CAM angiogenesis, eye development and apoptosis pathways: VEGFα (6MXR), CASPASE-3 (4QTX), BMP2 (2GOO), PI3K (1YI3), WNT7A (8TZO), SHH (3MVX), BMP7 (1LX5), CDH1 (3L6Y), FGF8 (2FDB), PAX6 (6PAX), OTX2 (model_03), and SOX2 (6WX9) (refer to Table 4). These protein structures were obtained from the Research Collaborator for Structural Bioinformatics Protein Data Bank (PDB) database. Subsequently, the protein data were formatted into PDB files after removing water and ligand molecules using BIOVIA Discovery Studio (DS) Visualizer v21.1.0.20298 (RRID:SCR_008398) by BIOVIA (San Diego, CA, USA) (Biovia, 2017). This software was also employed to predict protein structure and identify active sites essential for docking studies.

Gene expression

Total RNA was extracted using the TRIzol method, and its concentration was determined using the Qubit assay kit from Invitrogen, USA. Subsequently, 1µg of RNA from each group was utilized for cDNA synthesis through reverse transcriptase PCR. Real-time PCR was conducted utilizing the Roche single-colored multichannel detection system LC96. Primers targeting SHH, VEGF, KDR, CASPASE-3, WNT7A, BMP2, BMP6, AKT, PI3K, PCNA, RHOB, BMP4, BMP7, CDH1, CDH2, FGF8, OTX2, PAX6, SOX2, VIM, WNT11, CYTC, CYP1A1, CYP21A1, CYP3A4, CYP1B1 were employed (Table 1). The amplification reaction mixture comprised 5µl SYBR green, 1µl of specific primer set, 1µl of cDNA, and 3µl of nuclease-free water. The fold change in expression levels of various molecules between the groups was determined using the Livak and Schmittgen method (Livak and Schmittgen, 2001).

Gene	**Forward primer**	**Reverse primer**	**Accession no.**
18SrRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC	NR_003278.3
SHH	TGCTAGGGATCGGTGGATAG	ACAAGTCAGCCCAGAGGAGA	NM_204821
VEGF	CTCCACCATGCCAAGTGGTC	GCAGTAGCTGCGCTGATAGA	NM_205042.3
KDR	CGGACACCACGAATGCCAA	GCTCATCCTGCAGCGTTTTGTA	NM_001004368.2
CASPASE-3	AAAGATGGACCACGCTCAGG	TGACAGTCCGGTATCTCGGT	NM_204725
WNT7A	TATCGTCATCGGGGAAGGGT	GCTGCTTCTCTGCTACCCAC	NM_204292.3
BMP2	ATGTTGGACCTCTATCGCCTG	CCAAAACTTCTTCGTGGTGG	NM_001398170.1
BMP6	CCCCCTGAATGGACACATGAA	AGGATGACGTTGGAGTTGTCG	XM_040664958.2
AKT	CAGCCTGGGTCAAAGAAGTCA	ATGTACTCCCCTCGTTTGTGC	NM_001396387.1
PI3K	CCCCTGTGGTTAAACTGGGA	CCGTAAGGCAACATCCGAAGA	XM_040683928.2
PCNA	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC	NM_204170
RHOB	CAGCACATCCTTCCTTGGACA	TGCACAAATGCTGTGGTGAAC	NM_204909.2
BMP4	ATGTTGGACCTCTATCGCCTG	CCAAAACTTCTTCGTGGTGG	NM_205237.4
BMP7	ATCTGCCTACAAAATTGGTTCTC	TACTCACAGCGCATTCTCACTT	XM_040688362.2
CDH1	GAAGACAGCCAAGGGCCTG	TCTGGTACCCCTACCCTCTTG	XM_046925643.1
CDH2	AGCCCACGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC	XM_046910581.1
FGF8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT	NM_001012767.2
OTX2	CAACTACGAACTCCGCACCA	ATTCGAGGATCCGGGTACCAT	NM_204520
PAX6	AGCAAGGATACAGGTGTGGT	TGTGGGATCGGCTGGTAAAC	NM_001397301
SOX2	TGGTCAAGACGGAATCCAGC	GATCATGTCCCGAAGGTCCC	NM_205188.3
VIM	GACCAGCTGACCAACGACAA	GAGGCATTGTCAACATCCTGTC	NM_001048076.3
WNT11	GACCTGGGTATCGATGGGGA	GGCTTTCAAGACCTGTCTCC	NM_204784.1
CYTC	GCTGTATCCATCCGCCTACC	GTTTGTGTTCTCTCAGCAGCA	NM_001079478.2
CYP1A1	GAGCTGGATCAGACCATCGG	CTGGTTGATGAACACGCACG	NM_205147.2

CYP21A1	CACAGTTACCTGGCGTCCC	GCAGCCCCATAAGAGCTCAA	NM_001099358.2
CYP3A4	GTGGTGCTGTCAGGCTCTAT	AGGCTGCCTGCCATCATAAA	XM_046927350.1
CYP1B1	CTCATCAGGTATCCAAAAGTGCAG	GTGTGGGATGGTAACAGGCA	XM_040668785.2

Table 1 Primer sequences obtained from NCBI

Western blot

Embryos from the control and treatment groups of days 2, 3, and 4 were collected in lysis buffer containing protease inhibitor and centrifuged at 8000rpm for 20 minutes. Total protein content was determined using Bradford's method by analyzing 10% homogenate (Bradford, 1976). Subsequently, equal amounts of protein extract were subjected to electrophoresis on a 12% SDS-PAGE gel at 100 volts. The separated gel components were then transferred onto a PVDF membrane via semi-dry western blot transfer at 100mA for 20 minutes. This membrane was utilized for immunoblotting using specific antibodies. GAPDH served as the loading control for both groups. Blots were developed using Streptavidin-conjugated Alkaline Phosphatase (ALP) as the enzyme and BCIP-NBT as the substrate (Sigma-Aldrich, USA). Finally, the bands on the membrane were analyzed and densitometry measurements were quantified using ImageJ software.

Histological studies

Histological sections of embryos were rinsed in PBS and then immersed in 10% neutral buffered formalin for fixation. Tissues from control and treated embryos were processed and embedded in paraffin wax to prepare blocks. Longitudinal sections of the embryos, each 5µm thick, were sliced and subjected to staining with Harris' hematoxylin and eosin following the method described by Yu et al. (2015). The histological features of the tissue sections on slides were examined under a light microscope (Magnus, India) at 10X magnification, with images captured using Cat Cam equipment from Catalyst Biotech, India.

Statistical analysis

Statistical analysis was performed using Student's t-test in GraphPad Prism v8.0 software (RRID:SCR_002798) from GraphPad Software Inc., USA. Values were presented as mean ± standard error of the mean (SEM), and significance between the control and treated groups was determined when the p-value was equal to or less than 0.05.

Vessel analysis of CAM

The CAM vascular networks were studied in 3- and 4-day embryos by analyzing the number of vessels, the length of the vessels, the vascular junctions, and the lacunarity. Treatment groups had significantly fewer blood vessels on days 3 and 4 compared to control embryos ($p < 0.001$) (Fig. 4). Embryos that were treated also exhibited a decrease in the density of vessels and the number of connections between them. On both days, treated embryos displayed a slight decrease in total vessel length. The spatial distribution of the CAM images is evaluated by the lacunarity, which counts the vacant spaces. In contrast to the control group, the treated group exhibited high lacunarity values (the number of empty spaces between blood vessels) (Fig. 5, $p < 0.001$). Examination of embryos from days 3 and 4 confirms the findings of the AngioTool study. According to Table 5, there was a significant decrease in vessel density in the treated groups as compared to the control group ($p < 0.001$). Significantly, both the overall length of the vessels and their connections decreased ($p < 0.001$). The treated groups exhibit a high mean lacunarity, indicating the presence of empty space (which is) likely caused by the absence of vessels due to the treatment (Fig. 5).

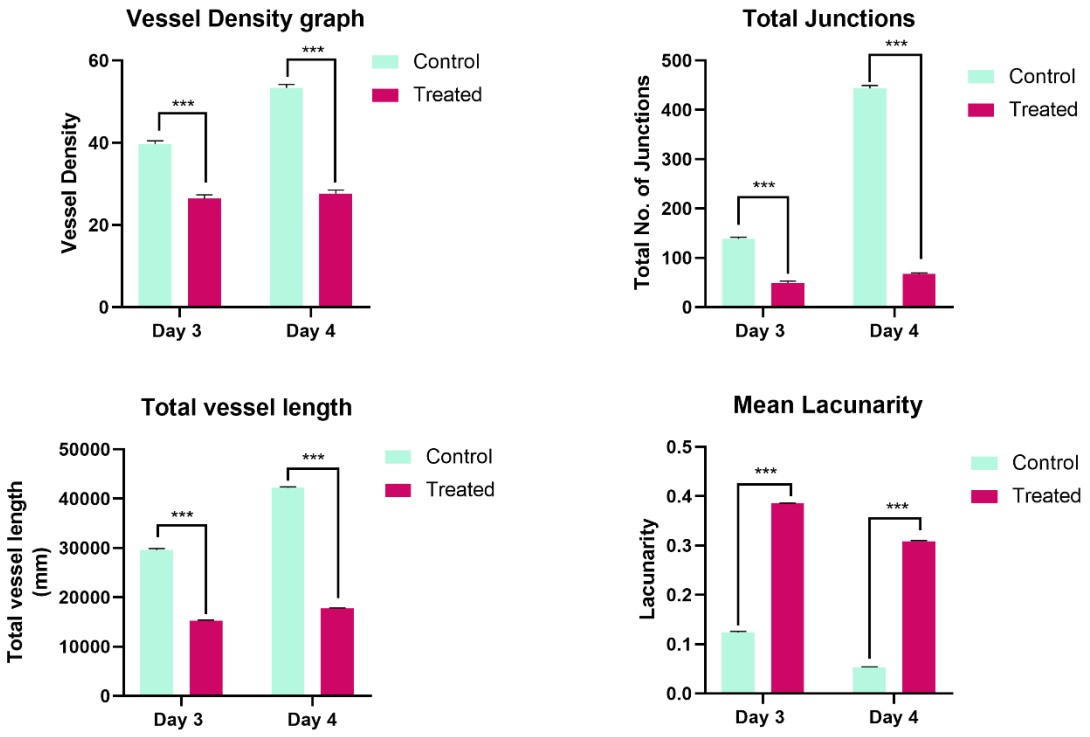


Fig. 4 Analysis of CAM angiogenesis in flubendiamide-treated day 3 and 4 embryos. n=6; ***p≤0.001.

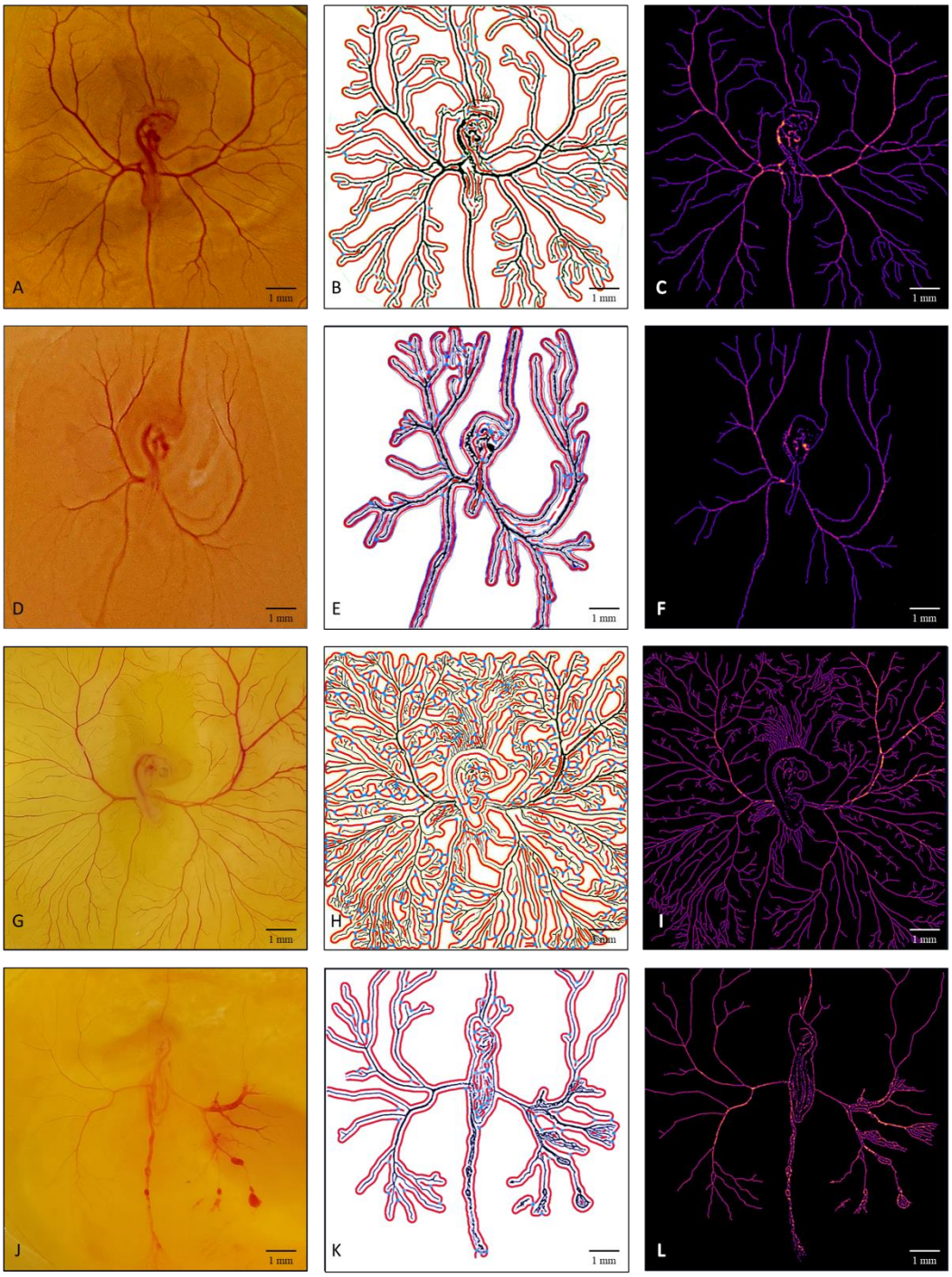


Fig. 5 Detection of vascular network architecture: **A, B, C**: Day 3 control embryo; **D, E, F**: Day 3 treated embryo; **G, H, I**: Day 4 control embryo; **J, K, L**: Day 4 treated embryo. Row 1: CAM images of the chick embryos Control and Treated; Row 2: Architectural layout of the vascular network (red lines) processed on AngioTool software, illustrating the junctions denoting vessel bifurcations (blue dots); Row 3: Skeletal image viewed on Fiji software.

Molecular docking with flubendiamide

The docking analyses showed that flubendiamide binds favorably to important proteins involved in CAM angiogenic and apoptotic pathways, such as VEGFα, WNT7A, PI3K, BMP2, and CASPASE-3 (Fig 6). The high docking scores indicate that flubendiamide may have an impact on the functionality of these proteins and could potentially reduce the development of blood vessels in treated embryos.

Similarly, in the pathways involved in the development of the eye, flubendiamide had a high affinity for proteins such as BMP7, CDH1, FGF8, PAX6, OTX2, and SOX2 (Fig 6). This suggests that it may have an impact on the functioning of these proteins and lead to reduced eye development in treated embryos compared to the control group.

Fig. 6 Molecular docking 3D and 2D structure of flubendiamide with key molecules involved in CAM angiogenesis and oculogenesis.

Flubendiamide on the gene expression profile

The study comprehensively investigated the effects of flubendiamide on angiogenesis and oculogenesis pathways, as well as cytochrome gene expressions in developing chick embryos (Fig. 7). Analysis of angiogenesis-related transcripts on days 2, 3 and 4 revealed significant upregulation of CASPASE-3 and RHOB on day 3 in treated embryos, while day 4 showed downregulation of SHH, VEFGα, KDR, WNT7A, BMP2, BMP6 and PI3K. In oculogenesis, significant alterations were observed in CASPASE-3, BMP7, SHH, CDH2, FGF8, PAX6,

CDH1 and WNT11 transcripts across the three days, indicating disrupted eye development. Additionally, cytochrome gene analysis of newborn chick liver indicated increased differential expression of CYTC, CYP1A1, CYP21A1, CYP3A4 and CYP1B1, suggesting potential hepatic effects of flubendiamide exposure. These findings underscore the multi-faceted impact of flubendiamide on chick embryo development, implicating various molecular pathways and physiological processes.

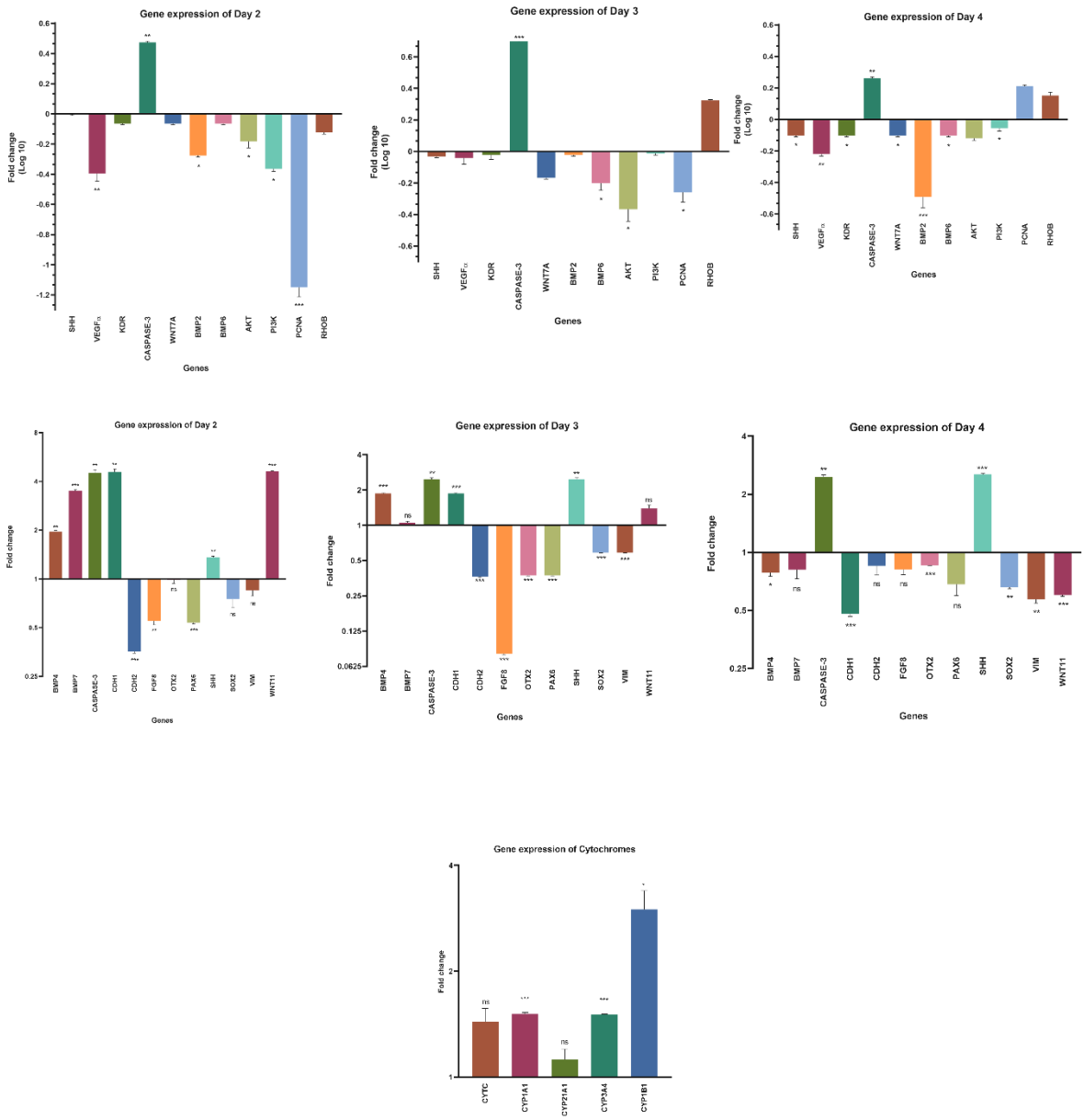


Fig. 7 Transcript levels of genes regulating eye development in flubendiamide treated day 2, 3 and 4 embryos for CAM angiogenesis and oculogenesis. Also, transcript levels of genes of cytochrome C and its variants in flubendiamide treated newborn chicks: Values are expressed in fold change (Mean ± SEM). Fold change values for control embryo is 1.0 for all the genes; n=3 with 30 eggs per group per experiment. *p≤0.05; **p≤0.01; ***p≤0.001.

Flubendiamide on the protein expression profile

To verify the findings from qRT-PCR analysis, western blotting was employed to assess protein expression levels of key regulators in CAM angiogenesis, oculogenesis, and cytochromes (Fig 8). In CAM angiogenesis, treated embryos exhibited decreased expression of Vegfα, AKT, and PI3K across days 2, 3, and 4 compared to controls, with significant reductions observed on multiple days. Conversely, Cl. Caspase-3 expression showed a significant increase in treated embryos, aligning with qRT-PCR results. In oculogenesis, decreased expression of Cdh2 and Pax6 was noted in treated embryos, while Cl. Caspase-3 and Shh displayed notable increases, corroborating qRT-PCR findings. Similarly, western blot analysis of cytochromes confirmed elevated expression in treated embryos compared to controls, supporting qRT-PCR results. GAPDH and β-Actin served as internal controls for normalization during protein expression analysis.

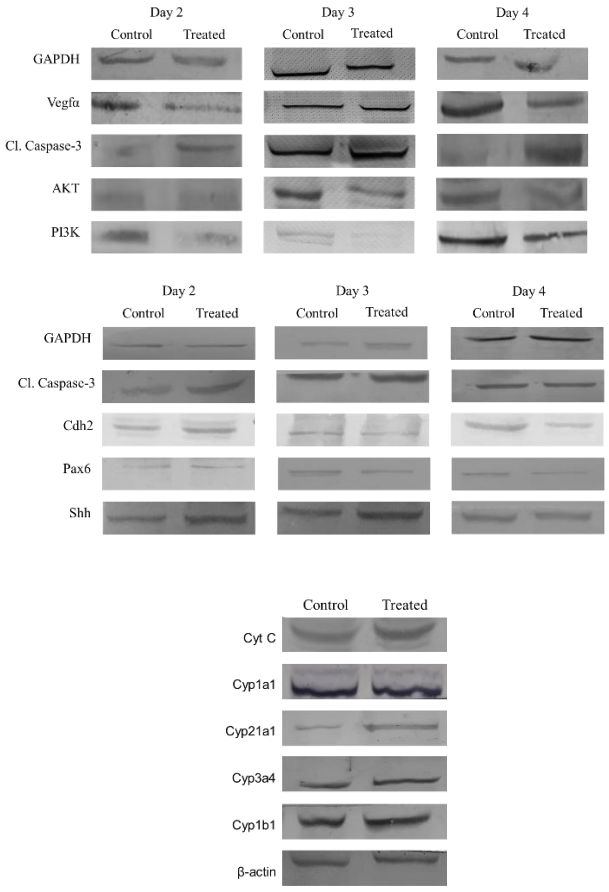


Fig. 8 Western Blot images showing comparative expression on days 2, 3 and 4 for CAM angiogenesis and oculogenesis. GAPDH was taken as loading control, n=3 with 30 eggs per group per day; *p≤0.05; **p≤0.01; ***p≤0.001. Also, the showing comparative expression on flubendiamide-treated newborn chicks. β-Actin was taken as loading control, n=3 with 30 eggs per group per day.

Histopathology analysis

Histopathological analysis of day 4 chick embryos' eyes revealed significant tissue damage induced by flubendiamide, as observed through differential staining with hematoxylin and eosin. Treated embryos exhibited impaired optic cup development, lacking clear differentiation between anterior and posterior cavities. Notably, the retinal pigment epithelium and neuronal retina were poorly defined and appeared disoriented. Moreover, the absence of a well-defined lens and cornea contrasted sharply with the control group's intact structures (Fig 9).

In the liver tissue of newborn chick, flubendiamide exposure led to notable abnormalities, as evidenced by differential staining with Hematoxylin and Eosin. Treated embryos displayed impaired portal veins, indistinct bile ducts, and disorganized sinusoids compared to controls. Signs of vasculitis, characterized by inflammatory cell infiltration in sinusoids, were evident, along with widespread inflammation and compromised hepatocyte architecture (Fig 10).

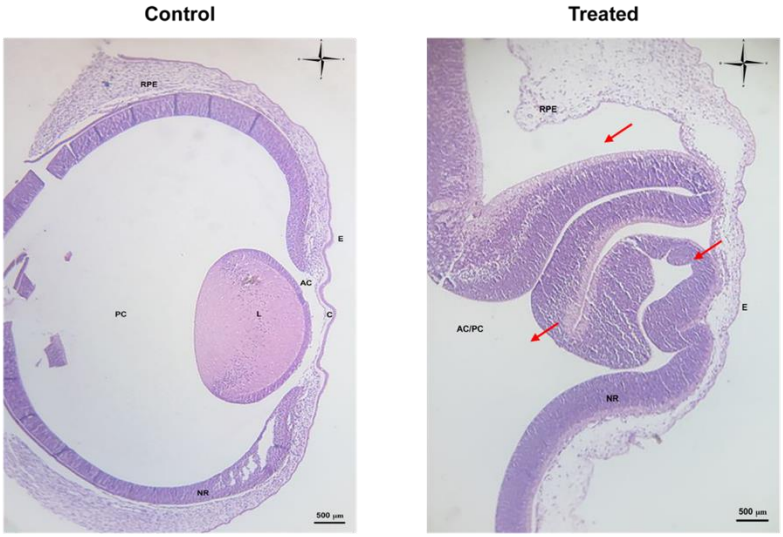


Fig. 9 Histological staining of day 4 embryos. **AC**: Anterior cavity, **C**: cornea, **E**: epithelium, **L**: lens, **NR**: neuronal retina, **PC**: posterior cavity, **RPE**: retinal pigmental epithelium. Deformities are indicated by red arrows. The scale bar represents 500 µm.

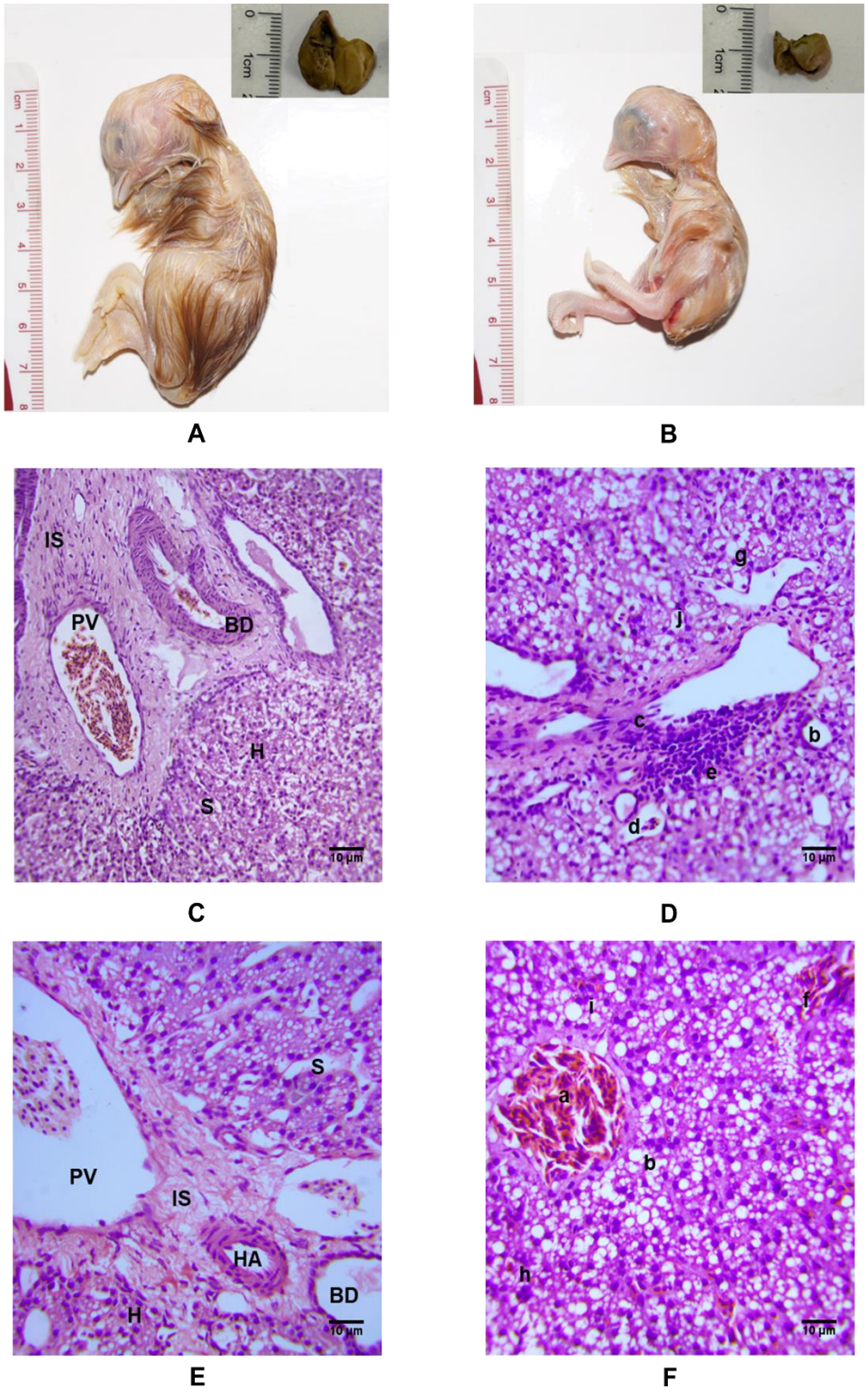


Fig. 10 Histological section of flubendiamide treated newborn chicken liver in comparison the control group (H&E x40). Section showing hepatocyte (H), sinusoids (S), portal vein (PV), interlobular septum (IS) and a bile duct (BD). Control and treated chicks are labelled as A and B, respectively, with 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.

Pesticides are essential in contemporary agriculture as they provide substantial benefits in safeguarding crops and increasing productivity. These chemicals are formulated to regulate, hinder, or eradicate pests that pose a threat to agricultural productivity (Tudi et al., 2021). Therefore, pesticides have a crucial role in reducing crop damage by specifically targeting insects, weeds, and viruses. This ensures a consistent food supply for an expanding worldwide population (Popp et al., 2013). The widespread use of pesticides has led to their widespread prevalence in the environment, unintentionally exposing non-target creatures to their harmful effects. Recent findings indicate that exposure to pesticides, particularly during pregnancy, carries risks for developing fetuses, potentially resulting in congenital disabilities (Kalliora et al., 2018). Nevertheless, the underlying cause of this phenomenon remains obscure. Recent findings have raised concerns about the potential impact of flubendiamide on species other than its intended targets (Li et al., 2014; Sarkar et al., 2014, 2017, 2018). Thus, the present study aims to uncover the fundamental mechanisms that cause structural defects in domestic chicks when they are exposed to flubendiamide.

In the morphological analysis, it was noted that exposure to 500 ppm of flubendiamide (LOEC) impeded the formation of blood vessels and eyes in early chick embryos. A recent study has shown that elevated levels of pesticides lead to a reduction in the development and branching pattern of blood vessels (Priyadarshini et al., 2020). Examination of the flubendiamide-exposed chorioallantoic membrane (CAM) vasculature through morphometric and quantitative techniques revealed shorter blood vessels, reduced vascular density, and a decrease in vessel junctions. These findings also highlight a decrease in capillary sprouting, which is crucial for establishing the vascular network system of the CAM. Moreover, the observed increase in lacunarity in embryos exposed to flubendiamide suggested a higher occurrence of empty spaces not occupied by blood vessels. Therefore, it became evident that exposure to flubendiamide caused a disruption in the vasculature of the CAM by preventing the development of capillary sprouts.

Computational docking of flubendiamide to the specific proteins involved in angiogenesis and oculogenesis cascades revealed strong binding affinities. These findings distinctly demonstrate the notable affinity of flubendiamide, suggesting its capability to directly interact with and influence these regulators of angiogenesis and oculogenesis.

Flubendiamide's impact on angiogenesis was assessed by studying key signaling molecules involved in blood vessel formation in chick embryos. CAM vascularization is governed by the sprouting of blood vessels facilitated by VEGFα, KDR, PCNA, and WNT7A transcripts, leading to the formation of a complete capillary network plexus (Hiratsuka et al., 2005; Karar et al., 2011; Strzalka et al., 2011; Zavala et al., 2017). Additionally, the SHH signaling pathway influences endothelial cell cytoskeleton via regulation of the PI3K/AKT pathway, while BMP2 and BMP6 maintain endothelial cell adaptability during sprouting (Pulkkinen et al., 2021; Salybekov et al., 2018). RHOB and Cl. Caspase-3 plays a crucial role in apoptosis regulation (Howe & Addison 2012; Brentnall et al., 2013). Reduced expression of VEGFα, KDR, and PCNA transcripts, as well as decreased VEGFα protein levels, indicated impaired endothelial cell proliferation. Flubendiamide also targeted pro-angiogenic factors such as SHH, AKT, and PI3K, leading to decreased expression levels. Furthermore, reduced levels of WNT7A, BMP2, and BMP6 transcripts suggested inhibition of endothelial cell sprouting and maintenance. RHOB and Cl. Caspase-3 expression levels increased, indicating inhibited proliferation and increased apoptosis, contributing to disrupted CAM vascularization.

The intricate process of eye development in chick embryos relies on a network of signaling molecules. Noggin initiates eye field formation by activating OTX2, but this activation is halted as PAX6 becomes active, specifying the eye field (Zuber et al., 2003; Tetreault et al., 2009). Flubendiamide's strong affinity with OTX2 suggests potential hindrance of its function, leading to decreased expression. SHH plays a role in splitting the single eye field into two by suppressing PAX6 expression, essential for eye field formation. Overexpression of SHH results in failure of eye field formation (Zuber et al., 2010). WNT11 overexpression promotes cohesion in eye field cells initially but may later disrupt cell migration, affecting eye field patterning (Cavodeassi et al., 2005). Downregulation of adhesion molecules CDH1 and CDH2 hinders proper cell migration for eye field formation (Laszo & Lele, 2022). Reduced BMP4 and BMP7 expression suggest impaired optic vesicle development, while decreased FGF8 levels correlate with the absence of lenses and distorted retinas (Vogel-Hopker et al., 2000). Flubendiamide downregulates these genes crucial for lens differentiation, potentially leading to congenital eye deformities. Decreased Vimentin levels indicate disrupted cellular integrity, while upregulation of CASPASE-3 leads to excessive cell death, impeding eye development and resulting in anophthalmia and microphthalmia (Arrindell & Desnues, 2023). Day 4 chick embryos histopathology to evaluate flubendiamide-induced damage to tissue architecture showed the optic cup disruption, with no obvious separation of the anterior and posterior

chambers. Importantly, in contrast to the clearly visible presence of these structures in the control group, treated embryos lacked any lens or cornea whatsoever. These results further substantiate that flubendiamide causes early eye development in chick embryos to become disoriented.

Xenobiotic metabolism takes place primarily in the liver, a location where microsomal cytochrome P450 (CYP) enzymes play a pivotal role (Grant, 1991; Ozougwu, 2017). The biotransformation of the investigated drug is significantly influenced by CYP enzymes, including CYTC, CYP1A1, CYP21A1, CYP3A4, and CYP1B1 genes, which contribute to the variability in drug pharmacokinetics and response (Zhao et al., 2021). The groups treated with flubendiamide exhibited heightened mRNA and protein expression of CYPs, as determined by qRT-PCR and western blot analyses. This finding may indicate that CYPs are potentially involved in the xenobiotic transformation of flubendiamide. Hematoxylin and Eosin differential staining demonstrated that flubendiamide caused liver damage in neonatal chicks, whereas the control group displayed no abnormalities. The treated group exhibited compromised portal veins and enlarged sinusoidal spaces, which may indicate a compensatory reaction to the increased demand for blood flow and structural changes brought about by flubendiamide. Furthermore, embryos that had been treated exhibited an absence of a distinct bile duct and manifested compromised hepatocyte architecture. Infiltration of cells into the portal vein and the development of hepatic lesions were additional indicators that flubendiamide altered structural integrity.

As a result of the profound environmental and public health ramifications of flubendiamide use, the results emphasize the critical necessity for regulatory oversight and comprehensive risk assessment. It is crucial to prioritize the ecological health of human and animal populations in agricultural landscapes by advocating for sustainable alternatives to flubendiamide pest management practices and enforcing rigorous protocols to reduce exposure to the chemical.

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Course Certificate

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Department: Department of Zoology

Paper Number	Course Title	Course Credits	Grade Earned	EGP
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I.	Research and Publication Ethics	2	A	18
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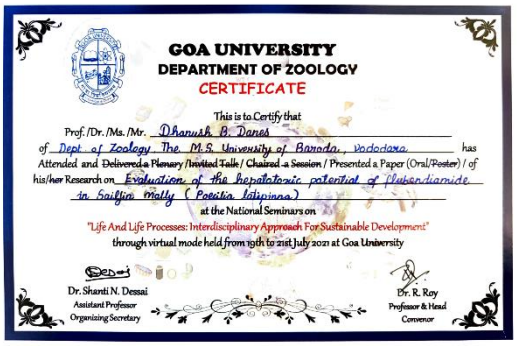

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This is to Certify that
Prof. /Dr. /Ms. /Mr. Dhanush. B. Danes
of Dept. of Zoology The M.S. University of Baroda., Vadodara has
Attended and ~~Delivered a Plenary / Invited Talk~~ / ~~Chaired a Session~~ / Presented a Paper (Oral/Poster) / of
his/her Research on Evaluation of the hepatotoxic potential of flubendiamide
in Saiffia molly (Poecilia latipinna)
at the National Seminars on
"Life And Life Processes: Interdisciplinary Approach For Sustainable Development"
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Certificate for Appreciation

This is to Certify that Mr. Dhanush B. Danes, Research Scholar, Zoology , The Maharaja Sayajirao University of Baroda has delivered Oral Presentation on the Title "Risk Assessment of a Commercial Insecticide - Flubendiamide in a Vertebrate Model of Ecotoxicology" during the National Conference on Advancement in Interdisciplinary Research (NCAIR-2021), Jointly organised by Amiruddaula Islamia Degree College, Lucknow, UP & Science Tech Institute, Lucknow, UP India on July 29-31, 2021.

Prof. R. K. Shukla, Conference Chair, University of Lucknow, Lucknow, UP
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CERTIFICATE

This is to certify that Dr./Mr./Ms. DHANUSH. B. DANES [RESEARCH SCHOLAR] has participated /presented a paper titled Investigating the impact of flubendiamide on hematoma in domestic chickens (Gallus domesticus) in the two - day International Seminar on 'Advanced Techniques in Biological Research' held at KKTM Government College, Pullut during 22nd and 23rd of November, 2023.

Dr. Shiji E. M.
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This is to certify that Prof./Dr./Ms./Mr. Dhanush. B. Danes
department of Zoology, The MS University of Baroda, Vadodara
has presented a paper titled Assessing the risk of a commercial insecticide
(Flubendiamide) in an Ecotoxicological Vertebrate Model
in the National Seminar on
Exploring the Diverse World of Insects organized by the Department of Zoology, Government Arts and Science college Elanthoor, sponsored by Directorate of Collegiate Education, Government of Kerala in 13th to 15th December 2023.

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Ms. HAVARNNISA M.
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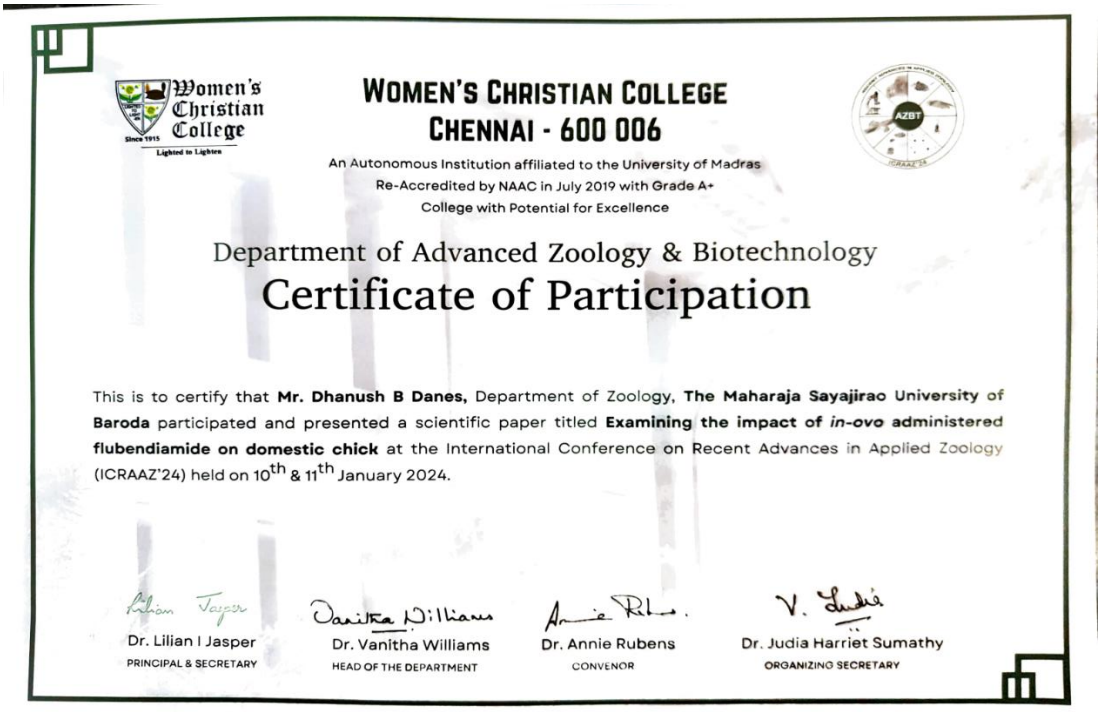

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