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Role of flubendiamide in the oculogenesis of developing chick embryo

INTRODUCTION

The rampant use of pesticides in the present agricultural era has caused deleterious effects on the non-target species (Kalyabina et al., 2021). Studies in the past have identified pesticides and insecticides as potential teratogens that lead to developmental anomalies in newborns (Uggini and Suresh, 2013; Sharma et al., 2018; Verma et al., 2021). With the emerging pesticide resistance among pests, new-generation pesticides are being introduced in the market with stronger efficacy to eradicate pest infestations.

One such new-generation insecticide is flubendiamide, which is a phthalic acid diamide that targets lepidopterans in more than 200 crop species (Tohnishi et al., 2005). It functions by impairing the muscle mobility of the pests by interacting with ryanodine receptors (Aghris et al., 2022). Concerns regarding the hazardous effects of this insecticide have recently been raised due to its unrestrained usage. The present study aimed to assess the teratogenic potential of technical grade flubendiamide in a developing chick embryo. Based on a previously performed dose range study, a sublethal concentration of flubendiamide was administered to the air cell of the egg at day zero post-fertilization. Subsequent observations revealed eye malformation, inadequate blood vessel growth, and incomplete ventral body wall closure in the chick embryos, with eye defect being the most predominant abnormality.

Numerous signaling molecules are responsible for the intricate process of eye development in chick embryos, which begins as early as day 2 after fertilization (Hamburger and Hamilton, 1951). This process is controlled by multiple signaling molecules. According to Saha et al. (1989), the process begins when the head ectoderm becomes responsive to signals from brain bulges, ultimately resulting in lens-forming bias. Zuber et al. (2003) and Zuber (2010) found that the specification of the anterior neural tube by OTX2, followed by the activation of PAX6, is essential for the specification of the eye field, which in turn triggers a cascade of transcription factors. Through the inhibition of PAX6 in the midline, SHH expression is therefore responsible

for playing a significant role in the process of dividing the single eye field into two lateral fields (Macdonald et al., 1995; Tétreault et al., 2009).

The development of the eye is controlled by several genes and signaling pathways, some of which include WNTs, BMPs, and FGFs. The WNT signaling pathway is absolutely necessary for the creation of optic vesicles and the differentiation of lenses. According to Ramón Martínez-Morales et al. (2004), the creation of the eye field requires the inhibition of WNT and BMP signaling by OTX2, which occurs at the anterior portion of the neural tube. According to Capowski et al. (2016), WNT7b is responsible for establishing the dorsoventral axis of the optic vesicle, while BMP4 in the overlaying surface ectoderm is responsible for inducing the lens placode. Additionally, the optic vesicle is responsible for the expression of BMP7, which is necessary for the formation of the optic cup, which further develops into the neuronal and pigmented retinas.

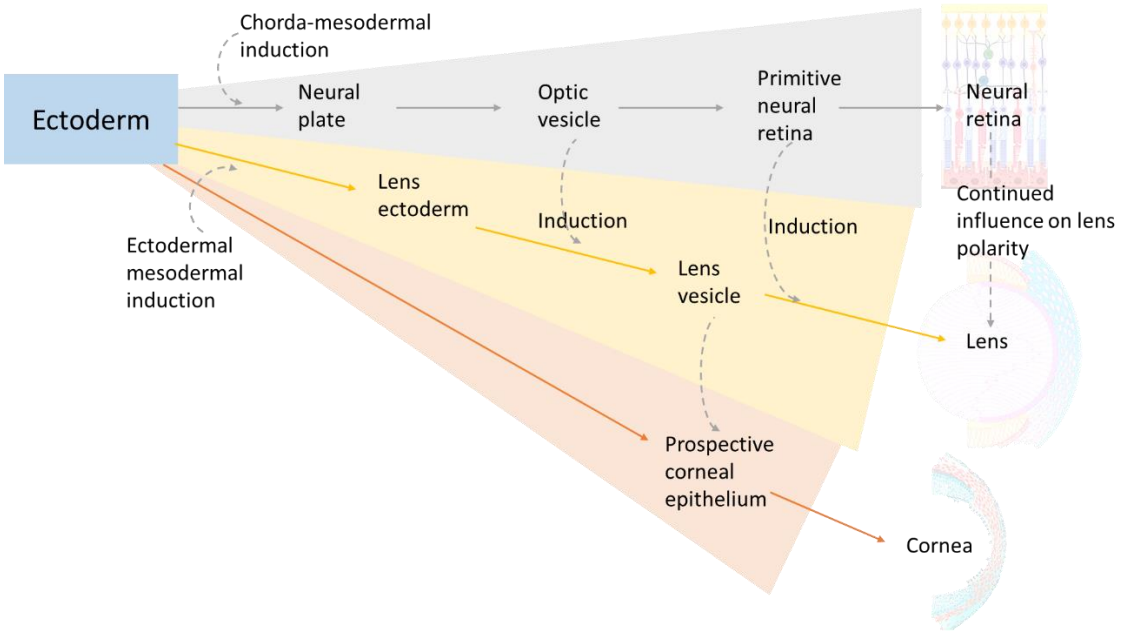


Figure 5.1: Regulatory Molecules in Eye Development
(Bibliowicz et al., 2011: Morris, 2011; Gestri et al., 2012)

Signals coming from the optic cup induce lens formation in the overlaying lens placode. These signals are responsible for the differentiation between the lens and the retina (Figure 5.1). Furuta and Hogan (1998), Esteve and Bovolenta (2006), and Perron and Harris (2000) all found that the production of BMP4, FGF8, and Delta by optic vesicle cells was responsible for the conversion of surface ectoderm into lens placode. The WNT/β-catenin pathway plays a vital

role in regulating the development of lens epithelial cells, while BMP7 plays a crucial role in the differentiation of lens fiber cells, which is essential for the creation of a clear lens (Hung et al., 2002). Research has shown that BMP4, which is located upstream of SOX2, has a role in lens differentiation as well as in the production of crystalline genes, which are an essential component of lens cell transparency (Furuta & Hogan, 1998; Wawersik et al., 1999; Kamachi et al., 2001) In the early stages of lens induction, FGF signaling is responsible for regulating the expression of Pax6 in lens placodes (Faber et al., 2001). Additionally, FGF8 is responsible for sustaining crystalline gene expression in the lens (Garcia et al., 2011; Reza et al., 2007). According to Yu et al. (2018), the expression of MSX2 by lens epithelial cells contributes to the differentiation of lens fiber cells.

It has been demonstrated that disruptions in these signaling pathways can result in congenital eye abnormalities. This has been demonstrated in animal models, where disrupted SHH and PAX6 signaling leads to microphthalmia or anophthalmia (Graw, 1996). Since congenital eye problems are among the most often reported congenital malformations worldwide, occurring between 0.36% and 4.7% of live births (Guarnera et al., 2024), it is of the utmost importance to comprehend the elements that lead to their occurrence. The purpose of this study is to test the hypothesis that administering sublethal amounts of flubendiamide to developing chick embryos can cause disruptions in the expression patterns of critical signaling molecules that are essential in early eye development. This disruption can result in congenital abnormalities such as microphthalmia and anophthalmia.

MATERIALS AND METHODS

Fertilized eggs of Rhode Island Red (RIR) chickens were sourced from the Intensive Poultry Development Unit in Vadodara, Gujarat, India, and disinfected with betadine before incubation. The incubation process was managed meticulously using an automated incubator set at 37±0.5°C with a humidity level between 70-75%. Eggs were positioned broad end up and rotated hourly to ensure even development. Non-viable eggs were promptly removed through regular candling.

For experiments, eggs were divided into control and treatment groups, each containing 30 eggs, with three repetitions. On the first day of incubation, the treatment group was administered 500 ppm of flubendiamide in 50 µl of PBS, while the control group received only PBS. Puncture sites were sealed with paraffin wax, and embryos were collected on days 2, 3, and 4 for further analysis.

Morphological studies involved isolating embryos using filter-ring techniques on days 2 and 3 and forceps and spatula on day 4. Observations were made with light microscopy and captured with specialized cameras. Molecular docking studies used AutoDock Tools to predict flubendiamide binding to key proteins involved in eye development and apoptosis. Protein structures were obtained from the PDB and validated using the SAVES server.

Gene expression analysis involved RNA extraction using the TRIzol method, cDNA synthesis, and qRT-PCR. Protein expression was analyzed through SDS-PAGE and Western blotting with specific antibodies. Immunohistochemistry used Cl. Caspase-3 antibodies to study embryos, while histological analysis involved fixing and staining sections of day 2 embryo heads.

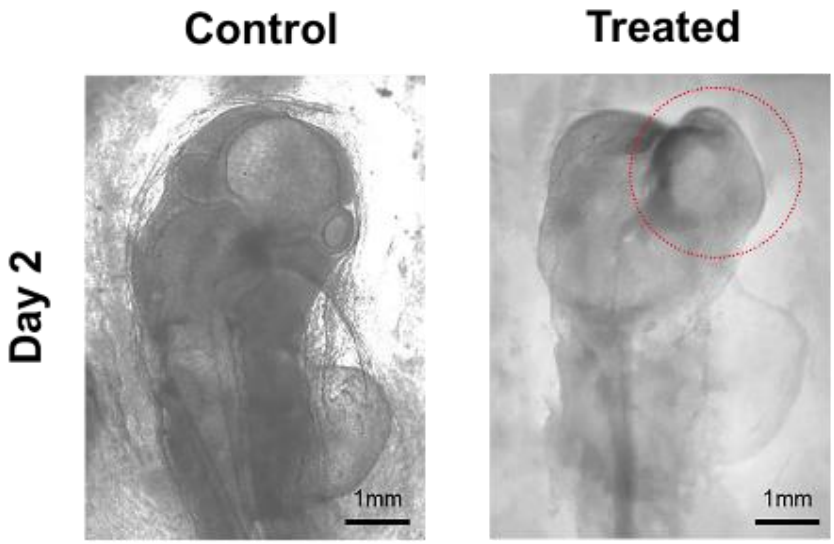
Skeletal staining of newly born chicks focused on the cranial region, revealing dysfunctional cartilage and bone growth in the face. The red stain, from a permanent calcium combination with alizarin red, indicated bone presence, while a blue hue indicated cartilage in embryos. Results showed the treated group was devoid of eyes.

Statistical analysis was performed using Student's t-test, with p-values ≤ 0.05 considered significant.

RESULTS

Gross morphological observations of eye development

The observations for days 2 and 3 were carried out using a light microscope and embryos were observed under the microscope at 4X magnification. The embryos of day 4 were viewed with the naked eye and the observations were recorded. The morphology of treated embryos on all days showed anomalies related to eye development. On day 2, the control embryos had a well-developed optic stalk and optic cup, whereas the treated embryos showed no optic cup and eye field formation (Figure 5.1). Anophthalmia (absence of eye) was observed on day 3 embryos from the treatment group. Distorted craniofacial features were seen in both days 2 and 3 of flubendiamide-treated embryos. Day 4 control embryos showed well-developed eye pigmentation. In contrast, the treated group showed lesser pigmentation in the eye and the eye size was also smaller, resembling the eye defect, microphthalmia (Figure 5.2).



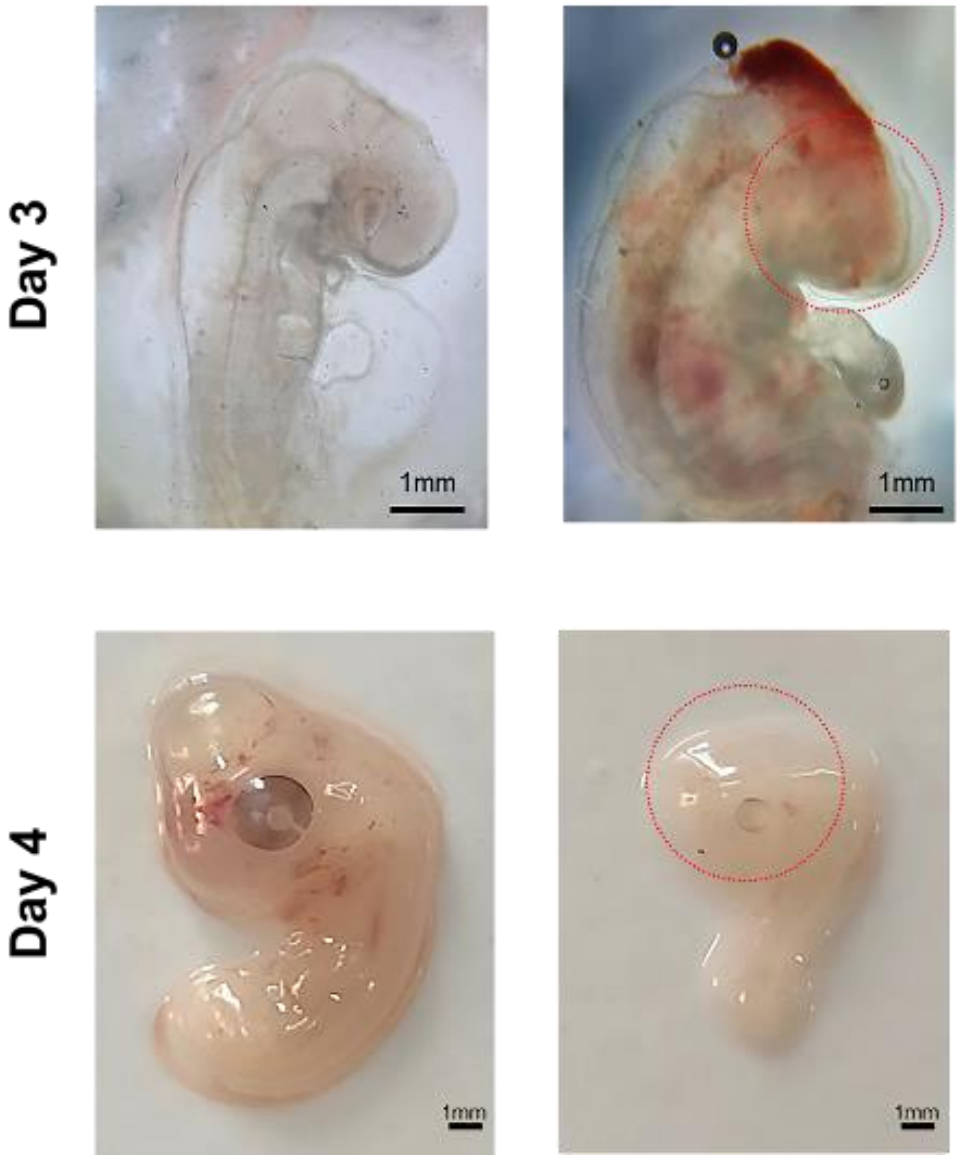


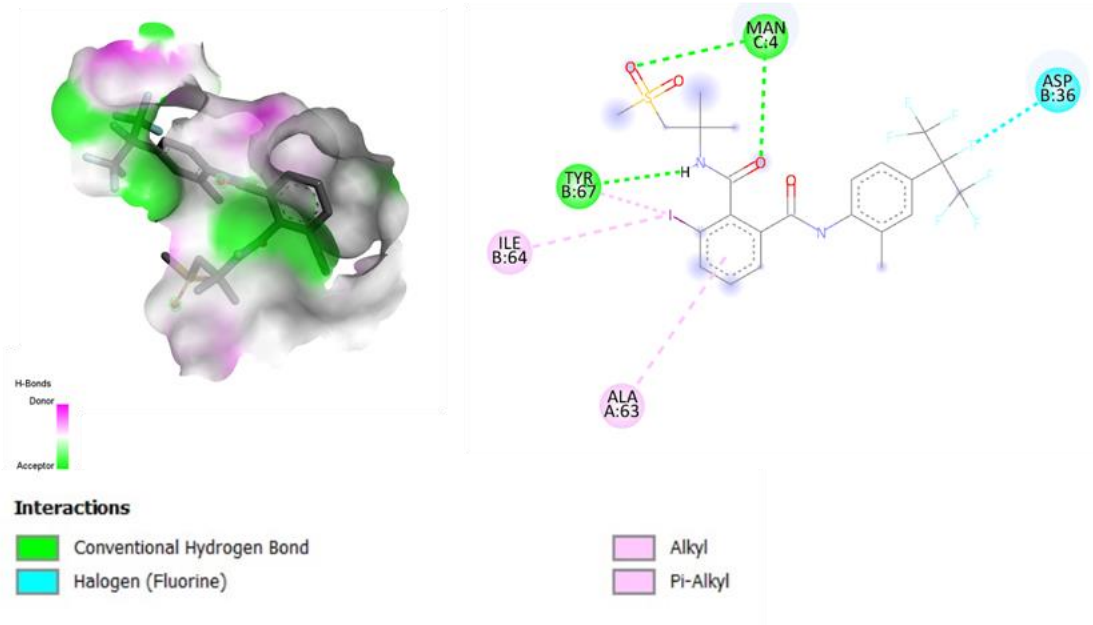
Figure 5.2: Gross morphology of head region of the flubendiamide-treated day 2, 3 and 4 embryos. Absence of optic cup and deformed craniofacial features can be seen in day 2 and 3 treated embryos. Improper eye development can be seen on day 4 of treated embryos. The deformity region is shown as red dotted line.

Molecular docking for eye forming protein

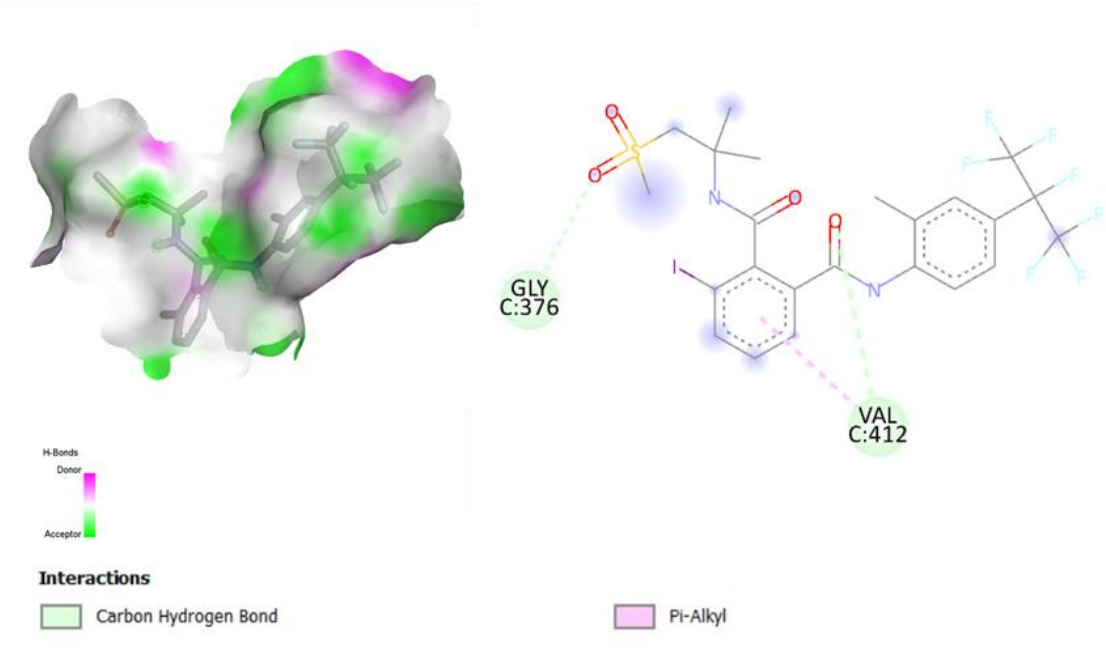
Docking analyses of flubendiamide with key proteins involved in eye development pathways displayed favorable docking scores: BMP7 (1LX5) exhibited a docking score of -7.1 Kcal/mol at the active site, in comparison, CDH1 (3L6Y) scored -8.1 Kcal/mol. Additionally, FGF8 (2FDB) showed a score of -7.9 Kcal/mol, PAX6 (6PAX) scored -8.0 Kcal/mol, OTX2

(model_03) scored -7.2 Kcal/mol and SOX2 (6WX9) scored -6.7 Kcal/mol (Figure 5.3). Analysis of the docking process also revealed the formation of various types of bonds between flubendiamide and these proteins (Table 5.1). The observed high binding affinity suggests that flubendiamide might affect the functionality of these proteins, potentially leading to reduced eye development in treated embryos compared to the control group.

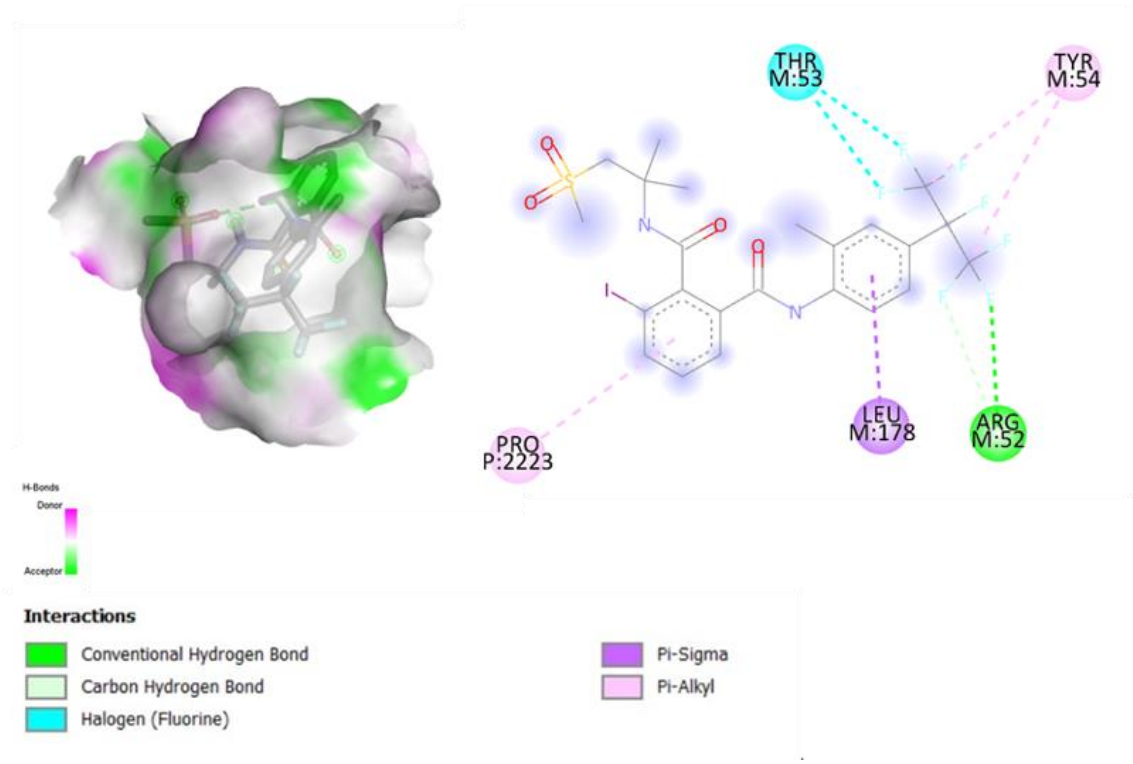
BMP7 (1LX5)
DOCKING SCORE = -7.1Kcal/mol



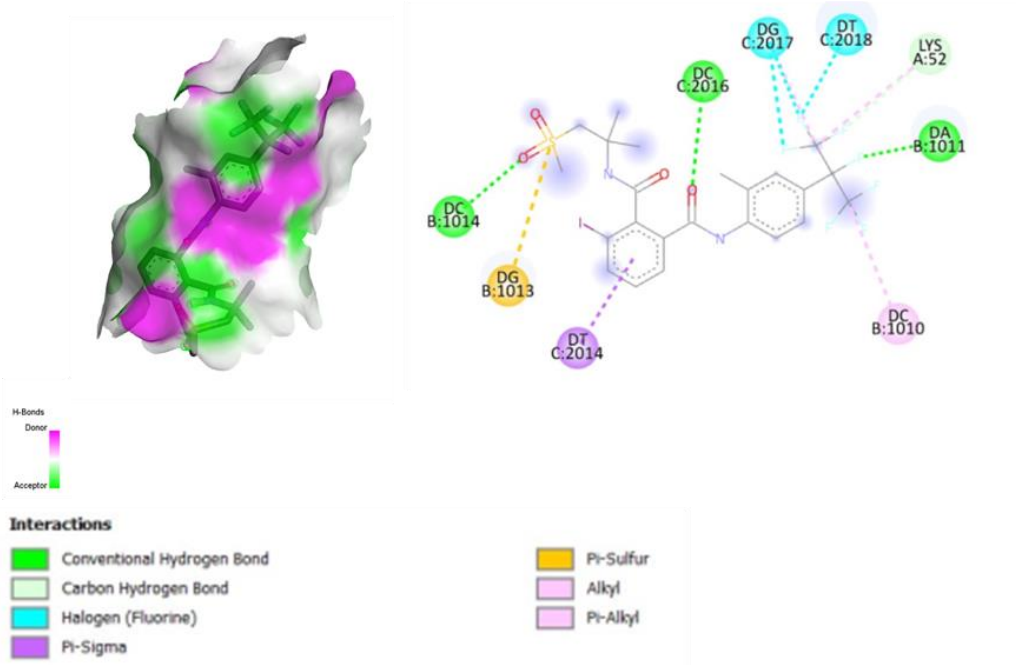
CDH1 (3L6Y)
DOCKING SCORE = -8.1Kcal/mol



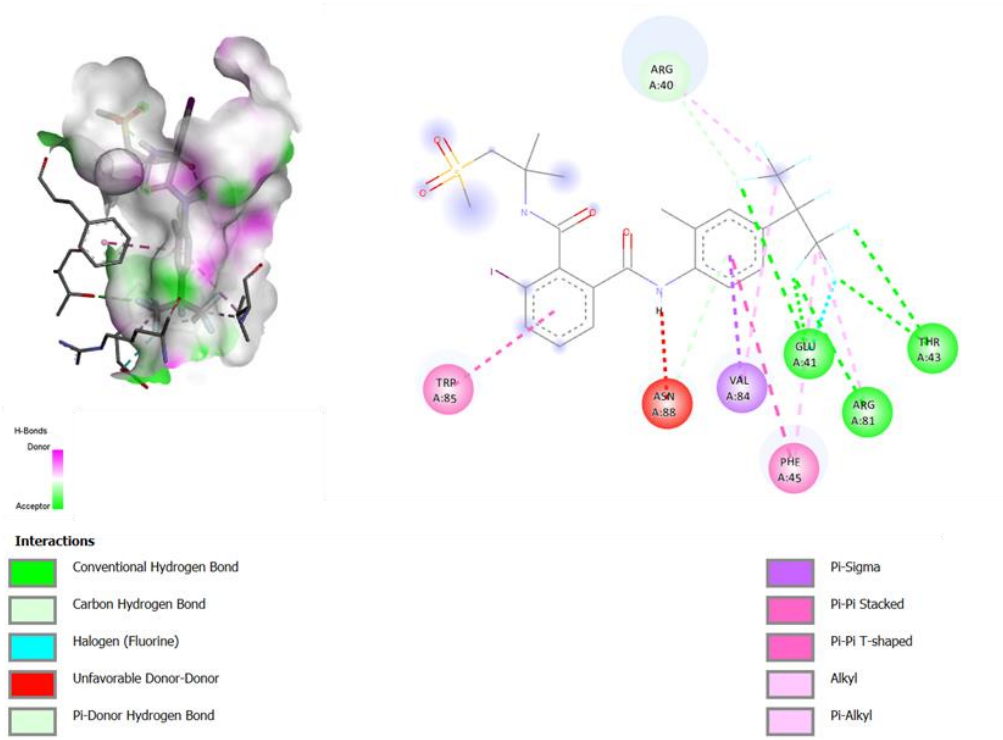
FGF8 (2FDB)
Docking score = -7.9Kcal/mol



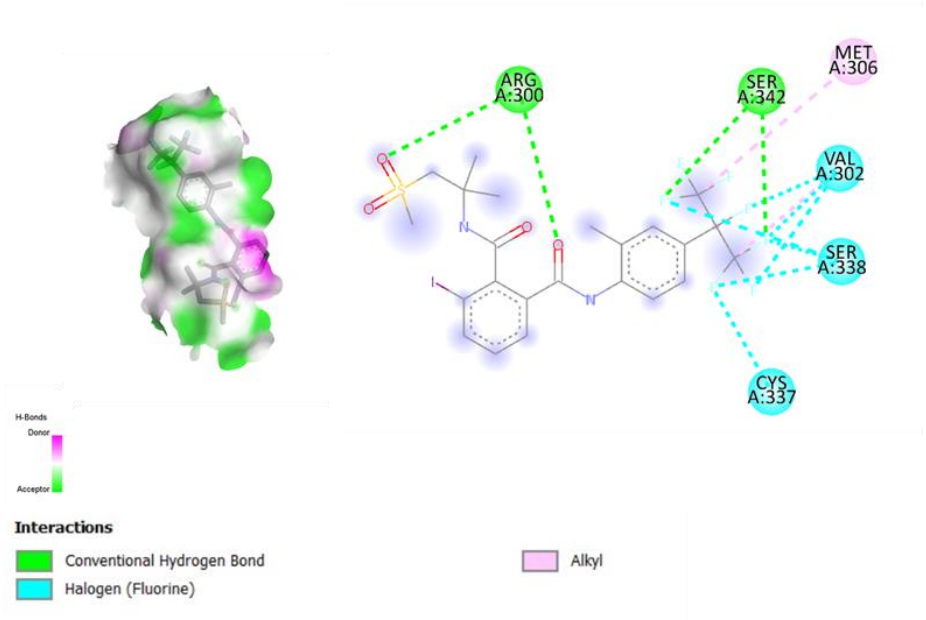
PAX6 (6PAX)
Docking score = -8.0Kcal/mol



OTX2 (model_03)
Docking score = -7.2Kcal/mol



SOX2 (6WX9)
Docking score = -6.7KcalL/mol



VIM (1GK4)
Docking score = -8.1Kcal/mol

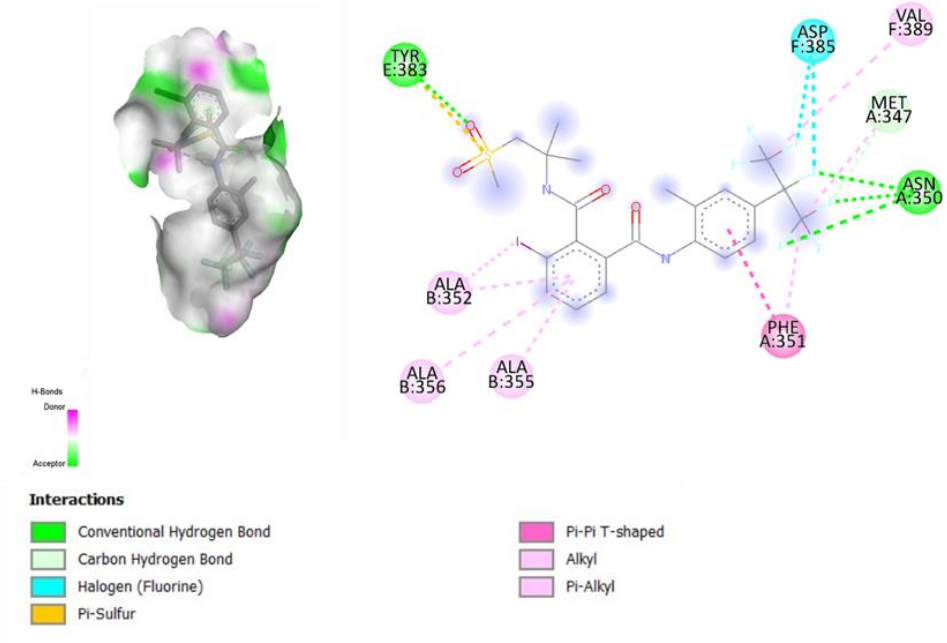
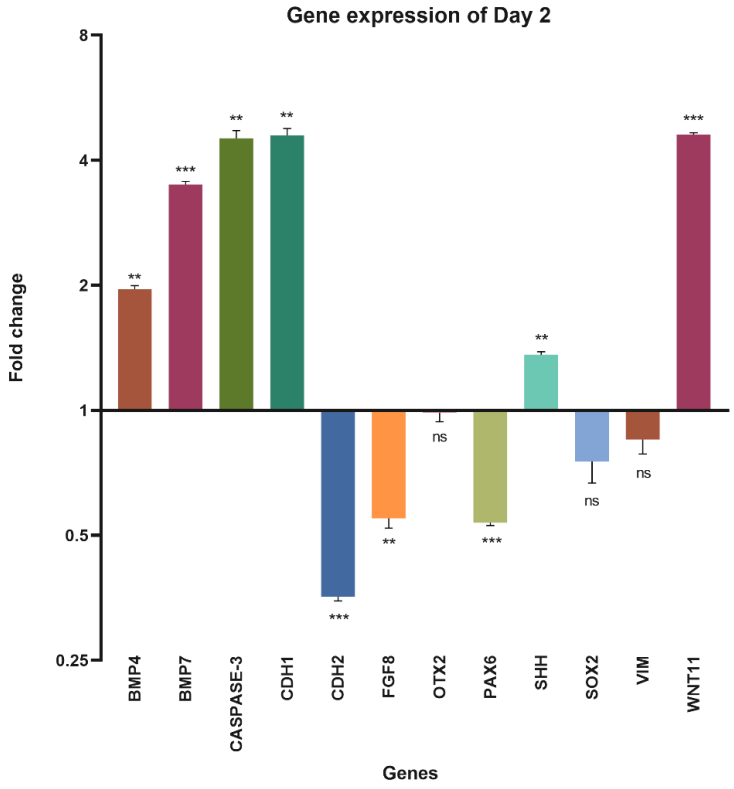


Figure 5.3: Molecular docking 3D and 2D structure of flubendiamide with BMP7 (1LX5), CDH1 (3L6Y), FGF8 (2FDB), PAX6 (6PAX), OTX2 (model_03) SOX2 (6WX9) and VIM (1GK4)

Eye formation-related gene expression profile

The transcriptional rate of various genes including BMP4, BMP7, CASPASE-3, CDH1, CDH2, FGF8, OTX2, PAX6, SHH, SOX2, VIM and WNT11 was assessed on days 2, 3 and 4 in both control and treated embryos. On day 2, CASPASE-3 and BMP7, along with SHH, showed more than a twofold decrease in mRNA expression compared to the control group ($p \leq 0.01$). CDH2, FGF8 and PAX6 were notably reduced in the treated group ($p \leq 0.001$), while OTX2, SOX2 and VIM exhibited non-significant downregulation. Conversely, CDH1 and WNT11 were markedly upregulated by over 4.5 folds ($p \leq 0.001$), with BMP2 showing around a twofold increase ($p \leq 0.01$). On day 3, CASPASE-3 and SHH displayed more than a twofold increase in expression ($p \leq 0.01$), while CDH2, FGF8, OTX2, PAX6, SOX2 and VIM were significantly decreased ($p \leq 0.01$) in the treated group compared to controls. BMP7 and WNT11 showed non-significant downregulation, while BMP4 and CDH1 remained increased in treated embryos ($p \leq 0.001$). On day 4, BMP4, CDH1, OTX2, SOX2, VIM and WNT1 expression levels were found significantly reduced in treated embryos. Although BMP7, CDH2, FGF8 and PAX6 showed decreased expression, the changes were statistically not significant. CASPASE-3 and SHH exhibited increased expression (Figure 5.4; Table 5.2).



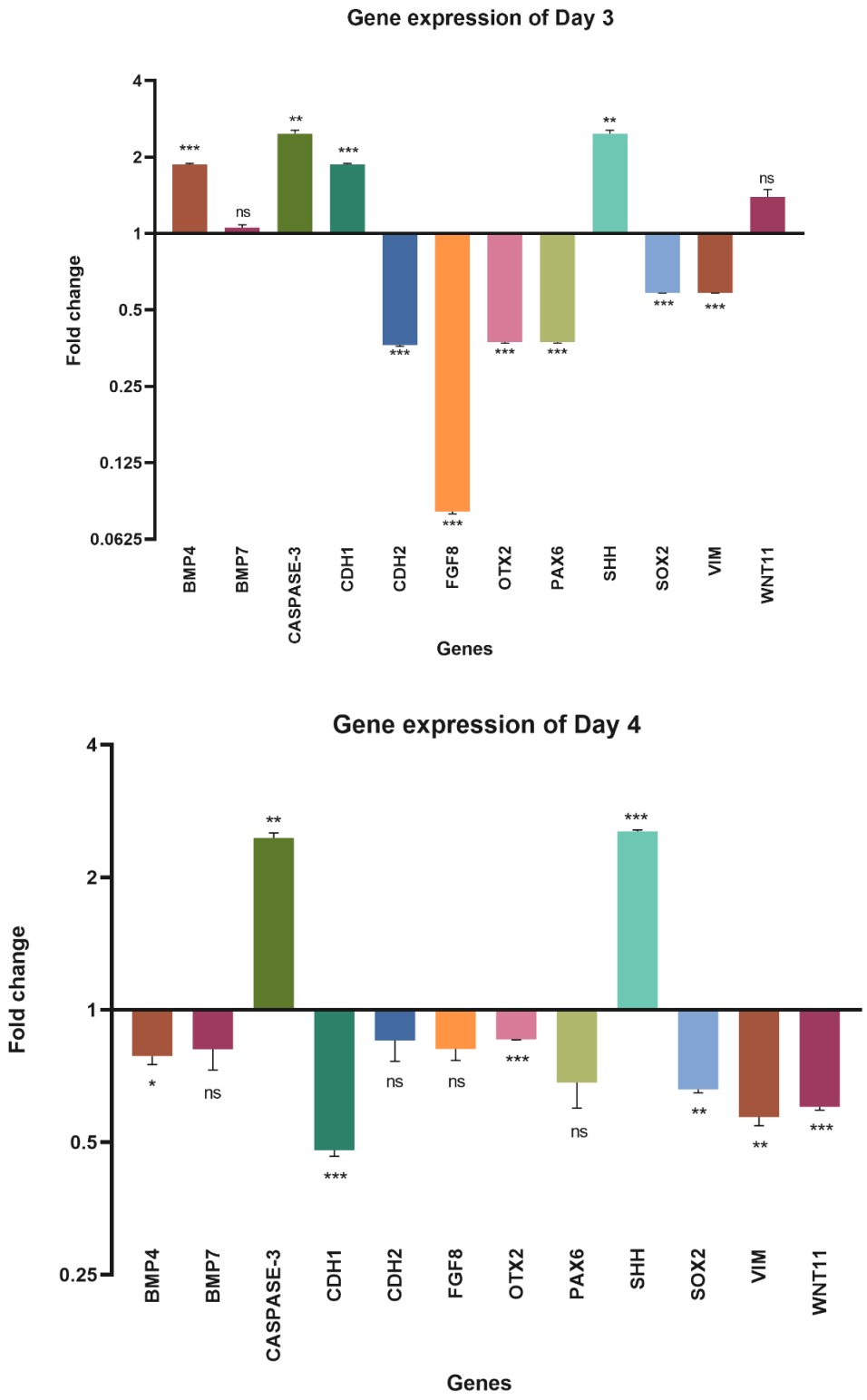
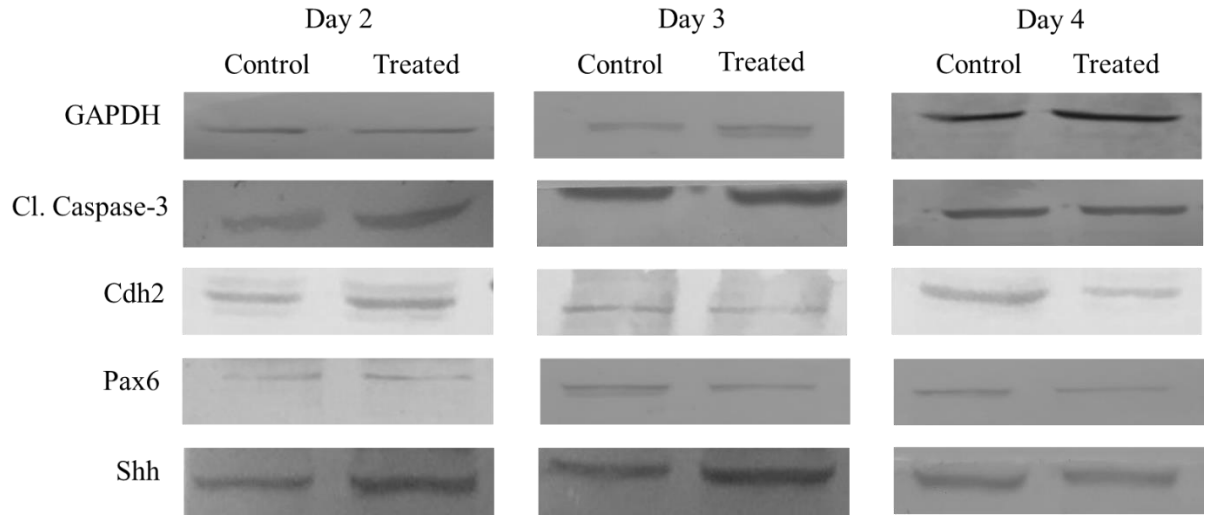
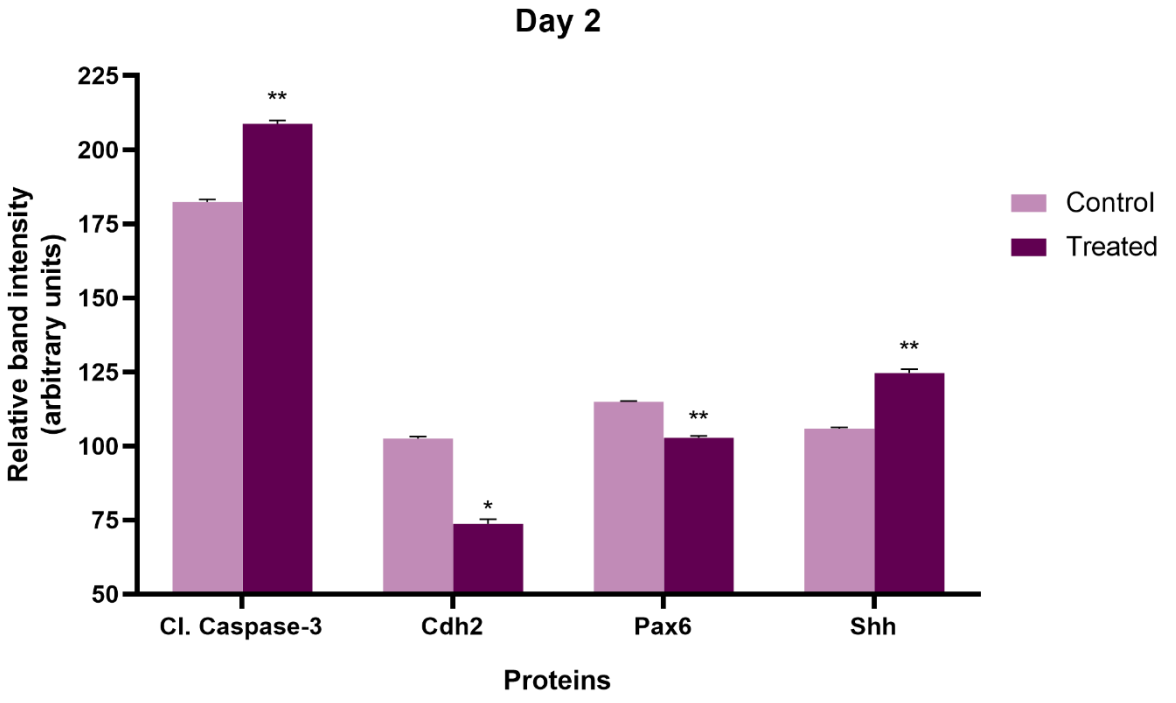


Figure 5.4: Transcript levels of genes regulating eye development in flubendiamide-treated day 2, 3 and 4 embryos: Values are expressed in fold change (Mean ± SEM). Fold change values for control embryo is 1.0 for all the genes; n=3 with 30 eggs per group per experiment. *p≤0.05; **p≤0.01; ***p≤0.001.

Eye formation-related protein expression profile

To confirm the findings of the qRT-PCR analysis, western blot was utilized to quantify the protein expression levels of key regulators involved in eye development. Immunoblot analysis indicated decreased Cdh2 and Pax6 expression in treated embryos across days 2, 3 and 4 compared to controls. Conversely, a notable increase in Cl. Caspase-3 and SHH expression was observed in treated embryos compared to their respective controls, which aligns with the qRT-PCR results (Figure 5.5; Table 5.3). GAPDH served as the internal control for normalization purposes during the analysis.





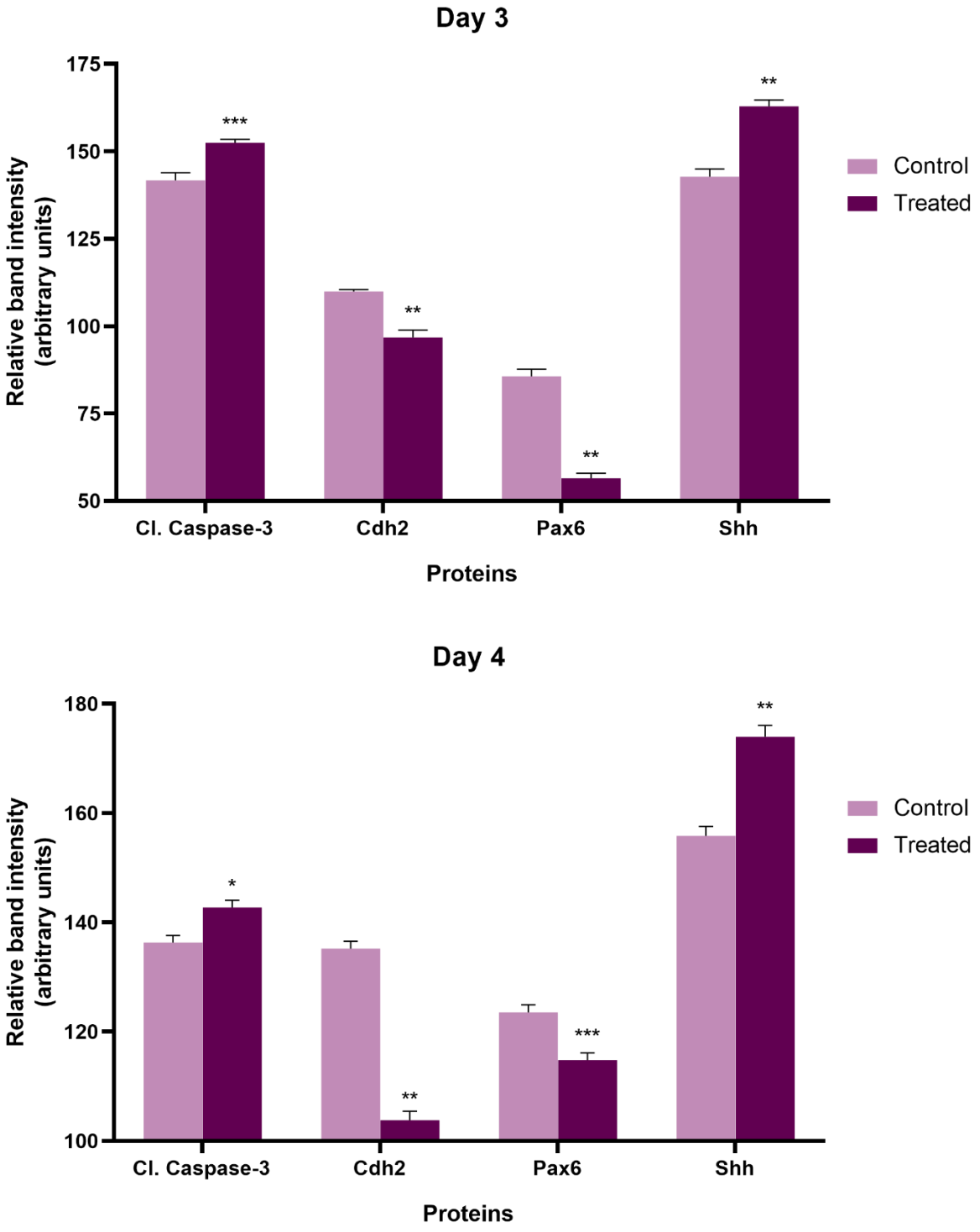


Figure 5.5: Western Blot images showing comparative expression on days 2, 3 and 4. GAPDH was taken as loading control, n=3 with 30 eggs per group per day; *p≤0.05; **p≤0.01; ***p≤0.001.

Assessment of apoptosis by immunohistochemistry

Both qRT-PCR and western blot analyses indicated increased levels of Cl. Caspase-3. Consequentially, immunohistochemistry was utilized on day 2 whole embryos to pinpoint the site of apoptosis. The staining revealed Cl. Caspase-3 expression primarily along the head region of the embryo. In the control group, cranial structures appeared normal without any observable blue staining. Conversely, the treated group displayed distorted cranial structures and malformed eyes with noticeable blue staining, indicative of Cl. Caspase-3 presence. These findings collectively suggest a heightened level of Cl. Caspase-3 in the treated group compared to the control (Figure 5.6).

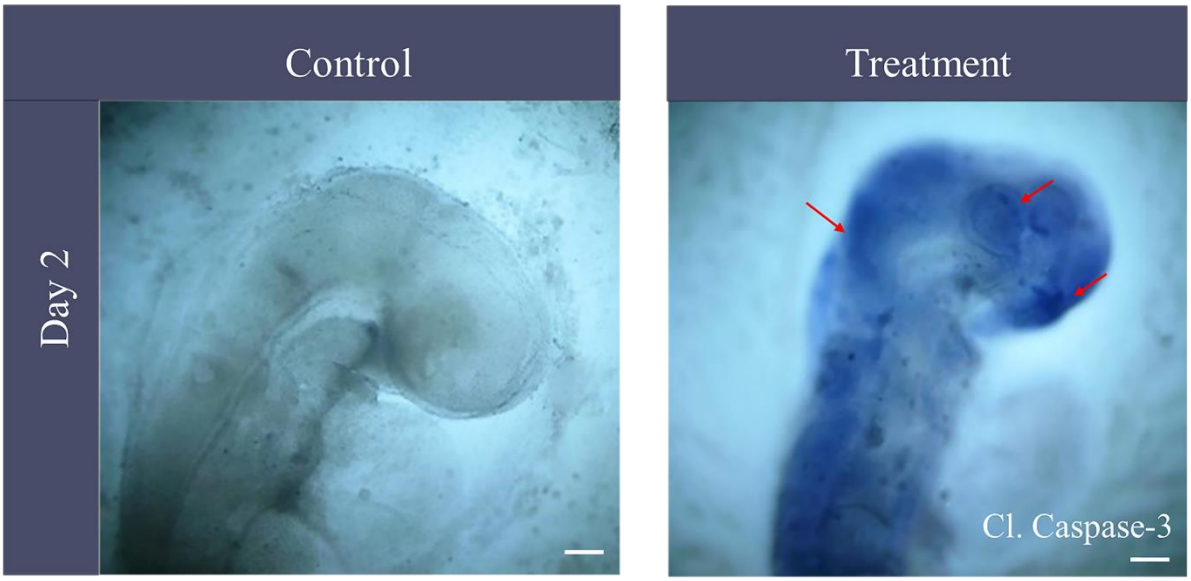


Figure 5.6: Immunohistochemical localization of Cl. Caspase-3 in a day 2 chick embryos. Blue-stained region indicates presence of Cl. Caspase-3 (shown by red arrow). Negative control for the primary antibody is shown as control. The scale bar represents 1 mm.

Histopathology analysis of eye

Differential staining with hematoxylin and eosin was utilized to assess the damaged tissue architecture caused by flubendiamide in day 4 chick embryos. Compared to controls, treated embryos showed impaired optic cup development, without clear differentiation between anterior and posterior cavities. Notably, the treated embryos lacked a well-defined retinal pigment epithelium and neuronal retina, with the epithelium appearing disoriented. Moreover, the lens and cornea were entirely absent, contrasting with the well-defined presence of these structures in the control group (Figure 5.7).

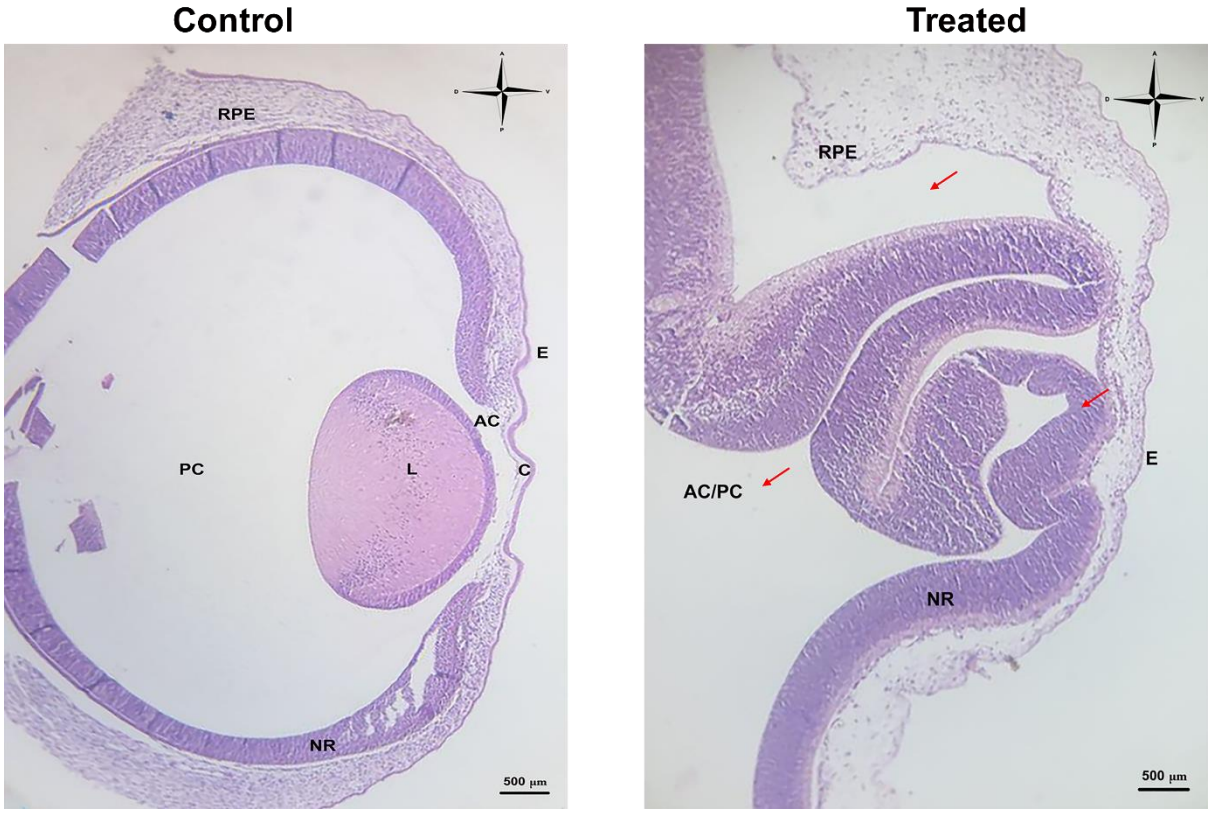


Figure 5.7: Histological staining of day 4 embryos. **AC**: anterior cavity, **C**: cornea, **E**: epithelium, **L**: lens, **NR**: neuronal retina, **PC**: posterior cavity, **RPE**: retinal pigmental epithelium. Deformities are indicated as red arrows. The scale bar represents 500 µm.

Skeletal staining of eye of newborn chick

Skeletal staining was performed on newly born chicks, with a particular focus on the cranial region. The results of this staining revealed dysfunctional cartilage and bone growth in the face region. Because of the creation of a permanent calcium combination with alizarin red, the red stain revealed the existence of bones to the naked eye. The existence of cartilage was shown by the formation of a blue hue in the embryos (Figure 5.8). The overall staining revealed that the treated group could not develop any eyes.

Figure 5.8: Alcian blue alizarin red staining of day 21 newborn chick depicts the presence and absence of eye. Whole chick staining inset.

DISCUSSION

Pesticides play a crucial role in modern agriculture, offering significant advantages in crop protection and yield enhancement. These chemical substances are designed to control, prevent, or eliminate pests that threaten agricultural productivity (Tudi et al., 2021). By effectively targeting insects, weeds and pathogens, pesticides help minimize crop damage, ensuring a steady food supply for a growing global population (Popp et al., 2013). However, the extensive utilization of pesticides has resulted in their pervasive presence in the environment, inadvertently exposing non-target organisms to their detrimental effects. Recent reports suggest that exposure to pesticides, particularly during pregnancy, poses risks to developing fetuses, potentially leading to congenital disabilities (Kalliora et al., 2018). However, the causative mechanism behind this remained elusive. The current study deciphers the underlying mechanisms responsible for improper eye formation in domestic chicks when exposed to flubendiamide.

In morphological analysis, it was observed that exposure to 500 ppm of flubendiamide (LOEC) hampered the development of eye formation in early chick embryos. Subsequent developmental stages revealed several ocular defects in treated embryos. Histopathological examination of day 4 chick embryos aimed to assess tissue architecture damage caused by flubendiamide unveiled impaired optic cup development, lacking clear differentiation between anterior and posterior cavities. Notably, both the lens and cornea were entirely absent in treated embryos, contrasting perfectly with the well-defined presence of these structures in the control group. These findings underscore the disorientation of early eye development in chick embryos induced by exposure to flubendiamide.

The development of the eye in chick embryos initiates as early as day 2, with the emergence of the optic stalk and optic cup. Signaling between the ectoderm and optic vesicles triggers the transformation of ectodermal cells into the lens placode, leading to the formation of the lens pit through invagination (Hilfer, 1983). By day 3, the lens vesicle is formed from the lens pit, followed by the development of the retina and cornea (Khan and Ackerman, 2023). Pigmentation of the eye occurs by day 4 (Hamburger and Hamilton, 1951). During the formation of the eye, diverse molecules play pivotal roles in coordinating processes like cell growth, movement, organization and programmed cell death (Khan and Ackerman, 2023). These processes are meticulously controlled and closely managed to ensure the successful development of the organ.

In silico docking experiments were conducted using Auto Dock Tools 4.2.2 software to analyze the interaction of flubendiamide with proteins involved in the oculogenic pathway. The proteins BMP7 (1LX5), CDH1 (3L6Y), FGF8 (2FDB), PAX6 (6PAX), OTX2(model_03) and SOX2 (6WX9) displayed high docking scores, indicating strong affinity for flubendiamide. The findings suggest the potential for direct interaction and influence of flubendiamide on these key regulators of oculogenesis. The target molecules identified underwent further screening through transcript and protein level analysis to observe their distinct expression patterns throughout the developmental phases.

The development of the eye in chick embryos is finely coordinated by a complex network of signaling molecules. It commences with the formation of the eye field in the anterior region of the neural tube within the head, where noggin triggers the activation of OTX2 (Zuber et al., 2003). However, this activation is subsequently halted as PAX6 becomes active. PAX6 assumes a crucial role in specifying the eye field by initiating a cascade of transcription factors (Zuber, 2010). The robust affinity between flubendiamide and OTX2, as evidenced by the docking score, suggests that it may have hindered the function of OTX2, resulting in a decline in its expression throughout the observed study frames.

It has been documented that SHH, an essential signaling molecule, is involved in patterning and splitting of the single eye field into two by suppressing the expression of PAX6 in the midline (Cavodeassi et al., 2019). The PAX6 initiates eye field formation and induction. These two are the primary molecules that orchestrate the whole process of eye formation (Baker et al., 2018). Studies have shown that SHH downregulates PAX6, resulting in the disruption of optic cup development, apoptosis of lens cells and arrested eye development (Yamamoto et al., 2004). When the SHH is overexpressed during the early days of development i.e., day 2, 3 and 4, we see that PAX6 is getting suppressed to the level that eye field fails to form ultimately.

WNT 11, a gene involved in early development was seen to get overexpressed on day 2 and 3 which is known to promote cohesion in the eye field cells (Cavodeassi et al., 2005), however their overexpression subsequently might have led to failure in cell migration affecting the patterning of eye field. Conversely, CDH1, an adhesion molecule essential for proper eye field formation, gradually decreases in expression, hindering cranial neural crest cell migration required for eye field formation (Niessen et al., 2011). Additionally, the downregulation of

CDH2, which facilitates cellular migration, persists throughout embryo development, indicating flawed cellular migration patterns (László and Lele, 2022). On the other hand, BMP4 and BMP7 are pivotal in optic vesicle formation and lens induction, with BMP7 peaking during chondrogenesis and ossification (Furuta and Hogan, 1998; Cagen and Reh, 2010). Reduced BMP4 and BMP7 transcript expression in treated embryos on days 2, 3 and 4 suggests impaired optic vesicle development. Furthermore, FGF8, critical in neural retina and lens initiation and differentiation, shows decreased transcript levels on all selected days, correlating with the absence of lenses and distorted retinas in treated embryos (Vogel-Höpker et al., 2000).

Moreover, OTX2 and PAX6 are present in the head ectoderm prior to the formation of the lens. Meanwhile, SOX2 is induced in the lens placode by BMP4, which is secreted from the optic vesicle. The co-expression of PAX6 and SOX2 in these cells marks the initiation of lens differentiation and triggers the activation of crystallin genes (Reza et al., 2007). The downregulation of all these genes underscores flubendiamide's potential to form congenital eye deformities.

It is reported that Vimentin plays a critical role in attachment, migration and cell signaling by preserving cellular integrity and stabilizing cytoskeleton interactions (Arrindell and Desnues, 2023). The decreased levels of VIM transcript observed on all selected days align with the disorganized optic features observed in the treated embryos. Furthermore, the marker for apoptosis, CASPASE-3 (Porter and Jänicke, 1999), was found to be upregulated with flubendiamide treatment on days 2, 3 and 4. This upregulation resulted in a higher frequency of cell death and apoptosis than necessary, impeding the overall process of eye development during early stages and leading to occurrences of anophthalmia and microphthalmia at later stages of development.

In conclusion, this study aimed to understand how pesticides like flubendiamide hinder vital processes of embryonic development and pose health hazards to humans, with congenital eye defects being one of the most prevalent consequences of flubendiamide exposure. Our findings suggest that OTX2 and other early regulators are disrupted, which in turn alters the expression patterns of other associated molecules, affecting the overall process of eye development. Further research is needed to elucidate the finer details of the molecular mechanisms involved, and later stages of development should be targeted to provide a more comprehensive understanding of the effects of flubendiamide on a developing embryo.

TABLE

Table 5.1: Molecular docking study of flubendiamide PDB IDs

Compound	PDB ID	Bond	Amino Acid Interactions	Docking Score Kcal/mol
Flubendiamide	BMP7 (1LX5)	Conventional Hydrogen Bond Halogen (Fluorine) Carbon Hydrogen Bond Alkyl Pi-Alkyl	TYR67, ASP36, ALA63, ILE64	-7.1
	CDH1 (3L6Y)	Conventional Hydrogen Bond Pi-Alkyl	GLY376, VAL412	-8.1
	FGF8 (2FDB)	Conventional Hydrogen Bond Halogen (Fluorine) Carbon Hydrogen Bond Pi-Alkyl Pi-Sigma	ARG52, PRO2223, TYR54, THR53, LEU178	-7.9
	PAX6 (6PAX)	Conventional Hydrogen Bond Pi-Sulfur Halogen (Flurine) Carbon Hydrogen Bond Pi-Alkyl Pi-Sigma Alkyl	LYS52	-8.0
	OTX2 (model_03)	Conventional Hydrogen Bond Pi-Sigma Halogen (Fluorine) Carbon Hydrogen Bond Pi-Alkyl Pi-Pi Stacked Alkyl Pi-Pi T-shaped Unfavourable Donor-Donor	ARG40, THR43, ARG81, GLU41, PHE45, VAL84, ASN88, TRP85	-7.2

		Pi-Donor Hydrogen Bond		
	SOX2 (6WX9)	Conventional Hydrogen Bond Halogen (Fluorine) Alkyl	CYS337, ARG300, SER342, MET306, VAL302, SER338	-6.7

BMP7 (1LX5), CDH1 (3L6Y), FGF8 (2FDB), PAX6 (6PAX), OTX2 (model_03) and SOX2 (6WX9) their bonding, amino acid interactions and docking score.

Table 5.2: Transcript level expression of genes involved in eye development in flubendiamide-treated day 2, day 3 and day 4 embryos. Fold changes are expressed as Mean ± SEM. Fold change values for the control embryo is 1.0 for all the genes; n=3 with 30 eggs per group per experiment, ns = not significant, *p≤0.05; **p≤0.01; ***p≤0.001

Gene	**Fold change Day 2**	**Fold change Day 3**	**Fold change Day 4**
BMP4	$1.957 \pm 0.04^{**}$	$1.871 \pm 0.01^{***}$	$0.785 \pm 0.03^{*}$
BMP7	$3.498 \pm 0.06^{***}$	1.054 ± 0.03^{ns}	0.812 ± 0.08^{ns}
CASPASE-3	$4.519 \pm 0.20^{**}$	$2.470 \pm 0.08^{**}$	$2.454 \pm 0.07^{**}$
CDH1	$4.590 \pm 0.18^{**}$	$1.871 \pm 0.01^{***}$	$0.480 \pm 0.02^{***}$
CDH2	$0.356 \pm 0.01^{***}$	$0.364 \pm 0.01^{***}$	0.851 ± 0.09^{ns}
FGF8	$0.549 \pm 0.02^{**}$	$0.080 \pm 0.01^{***}$	0.815 ± 0.05^{ns}
OTX2	0.987 ± 0.049^{ns}	$0.374 \pm 0.01^{***}$	$0.856 \pm 0.01^{***}$
PAX6	$0.536 \pm 0.01^{***}$	$0.374 \pm 0.01^{***}$	0.683 ± 0.09^{ns}
SHH	$1.362 \pm 0.02^{**}$	$2.470 \pm 0.08^{**}$	$2.539 \pm 0.02^{***}$
SOX2	0.753 ± 0.09^{ns}	$0.584 \pm 0.01^{***}$	$0.659 \pm 0.01^{**}$
VIM	0.850 ± 0.07^{ns}	$0.584 \pm 0.01^{***}$	$0.570 \pm 0.03^{**}$
WNT11	$4.615 \pm 0.04^{***}$	1.391 ± 0.10^{ns}	$0.602 \pm 0.01^{***}$

Table 5.3: Spot densitometry analysis of the western blot bands on day 2, 3 and 4 embryos. 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	Band intensity in arbitrary units	
	Control	**Treated**
Day 2		
Cl. Caspase-3	182.29 ± 0.53	208.77 ± 0.63**
Cdh2	102.51 ± 0.38	73.75 ± 0.90*
Pax6	114.90 ± 0.14	102.77 ± 0.39**
Shh	105.81 ± 0.31	124.61 ± 0.73**
GAPDH	145.39 ± 0.60	134.43 ± 0.52
Day 3		
Cl. Caspase-3	141.72 ± 0.91	152.49 ± 0.40***
Cdh2	109.92 ± 0.22	96.72 ± 0.89**
Pax6	85.61 ± 0.840	56.48 ± 0.58**
Shh	142.75 ± 0.90	162.90 ± 0.74**
GAPDH	153.28 ± 0.36	152.20 ± 0.40
Day 4		
Cl. Caspase-3	136.32 ± 0.52	142.71 ± 0.57*
Cdh2	135.20 ± 0.55	103.79 ± 0.67**
Pax6	123.54 ± 0.57	114.78 ± 0.56***
Shh	155.84 ± 0.71	173.97 ± 0.85**
GAPDH	137.52 ± 0.53	131.16 ± 0.83

GRAPHICAL SUMMARY