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Materials and Methods

A variety of techniques were employed in various sets of experiments to accomplish the research's primary goals. For each goal, including dosing, candling, grouping eggs and sterilizing eggs, there are a few standard processes. For each experiment, a customized protocol was followed after these fundamental steps. The basic process involved dosing after obtaining the eggs. Based on a dose range analysis, the dosage of flubendiamide was chosen (Sarkar et al., 2017). The subsequent sections contain a description of the comprehensive technique and sample preparation for the studies.

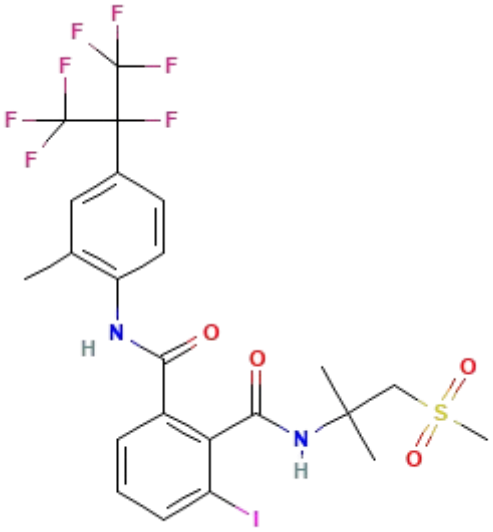
PROCUREMENT OF ANIMALS

Fertilized Rhode Island Red eggs were obtained from government-run intensive poultry, Vadodara unit. The fertilized eggs were candled to locate the air sac and disinfected with Povidone Iodine solution (Win-Medicare, New Delhi, India) and were maintained at 25°C till the time of dosing. All the protocols were approved by Committee for Control and Supervision of Experiments on Animals (CCSEA) and Institutional Animal Ethical Committee (IAEC) under protocol number IAEC No. MSU-Z/IAEC/09-2020.

TEST SUBSTANCE

The product, flubendiamide (CAS No. 272451-65-7) is an insecticide of technical grade that was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sigma-Aldrich (St. Louis, MO, USA) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India) were the suppliers of all standard chemicals.

Chemical Name	N2-[1,1-Dimethyl-2-(methylsulfonyl) ethyl]-3-iodo-N1-{2- methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}- 1,2-benzenedic
Molecular formula	$C_{23}H_{22}F_7IN_2O_4S$
CAS Number	272451-65-7
Molecular weight	682.39 g/mol
Manufacturer	Sigma-Aldrich Chemical Pvt Limited

Physical appearance	Powder solid
Storage condition	Store in dark and cool place (4°C)
Chemical structure	
Solubility	Water 300 µg/ml

INCUBATION OF EGGS

During incubation, the automated incubator was programmed to maintain a temperature of 37 ± 0.5°C and a relative humidity of 70–75% (Scientific equipments works, New Delhi, India). With their broader ends pointing upward, the thirty eggs in each of the treatment and control groups were automatically rotated once every hour (Figure 2.1). Every two days, the eggs were examined by candling and those that were found to be dead were taken out of the incubator.

Figure 2.1: Eggs inside incubator

EXPERIMENTAL DESIGN

A control group and a treatment group were randomly assigned to freshly laid eggs. Thirty eggs each were used in the control and treatment groups for each of the three replicates. The eggshells were perforated with a sharp needle on day "0" of incubation. A sterile BD 1 ml insulin syringe was used to administer the eggs in the air sac while laminar airflow was present (Blankenship et al., 2003). Melted paraffin wax was used to close the opening right away and it was then placed inside the incubator. Day 2 and 3 embryos were extracted utilizing filter-ring techniques (Chapman et al., 2001) (Figure 2.2). The embryos of Day 4 were scooped out using blunt end forceps and a spatula (Figure 2.3). Newborn chicks were used for morphological, hematological and hepatoxicity studies.

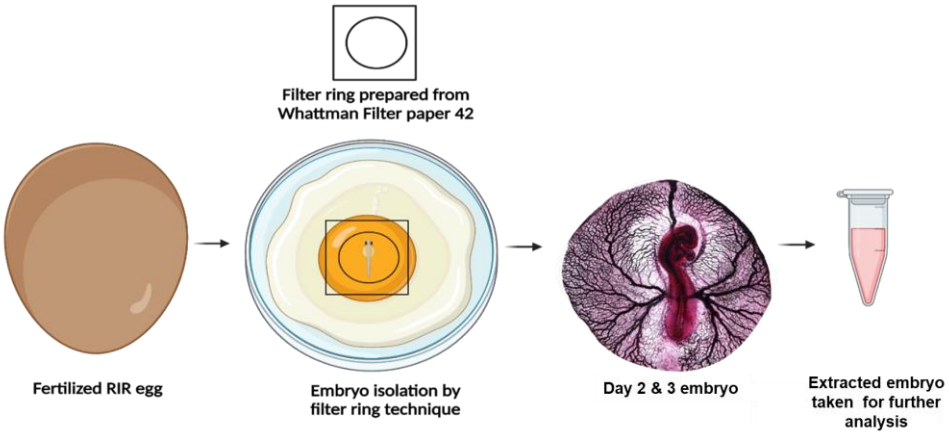


Figure 2.2: Schematic representation of Filter Ring technique for Day 2 & 3 embryo isolation

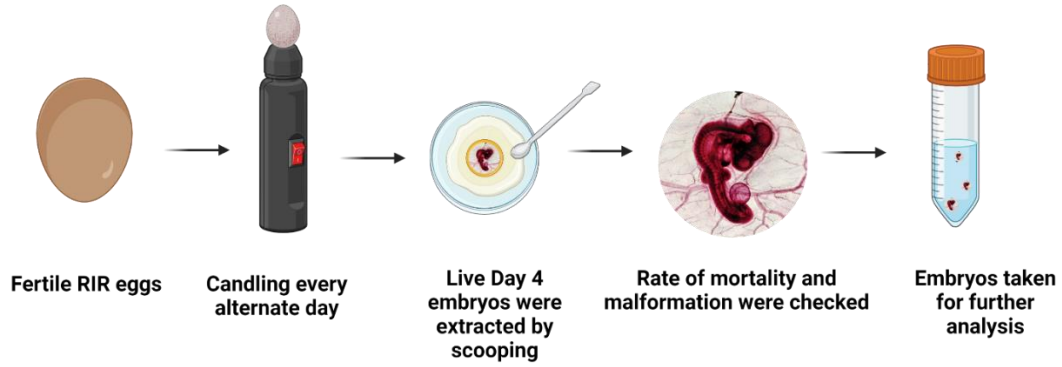


Figure 2.3: Schematic representation of Filter Ring technique for Day 4 embryo isolation

DOSE RANGE STUDY OF FLUBENDIAMIDE

Flubendiamide was prepared at 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 parts per milliliter in Phosphate Buffered Saline (PBS). On day zero, the eggs' air cells were dosed with a disposable 1 ml insulin syringe (BD Glide with TBL 31G- Insulin Syringe-U40). The other groups in the dose range trial received their appropriate doses of flubendiamide in PBS, while the control egg group received 50 µl of PBS. For every group, the dosage volume remained constant at 50 µl. After day 4 of incubation, the eggs' mortality rate was monitored. For each treatment group and the control group, a total of thirty eggs were collected. The median lethal dosage (LD_{50}) was calculated using OriginPro 8.5 software (RRID:SCR_014212) (OriginLab Corporation, Northampton, MA, USA) using probit analysis and linear fit analysis. It has been determined by meticulous analysis of embryos from days 2, 3 and 4 in all groups exposed to flubendiamide that 500 ppm is the concentration at which the lowest reported effect occurred. Consequently, in the ensuing trials, the treated group received 500 ppm of flubendiamide as the lowest observed effect concentration (LOEC), whilst the control group received 50 µl of PBS (Figure 2.4).

Figure 2.4: Dosing the eggs using sterile BD 1 ml insulin syringe

LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) OF FLUBENDIAMIDE

Combining LC-MS/MS, the presence of flubendiamide was checked in both the treated and control biological samples. On the second day, thirty embryos were gathered and the samples were extracted by homogenizing them in cold methanol. After that, the mixture was centrifuged, filtered and subjected to sonication. Acetonitrile-water (90:10) was used as the mobile phase in mass spectrometry, with an injection volume of 15 µl and a flow rate of 0.3 ml/min. To ensure there were no procedural errors, a standard solution containing 70 µg/ml flubendiamide was created. Using an ABSciex 3200 Q Trap machine in conjunction with an Eksigent Ekspert UltraLC 100 machine, the flubendiamide separation and data analysis were carried out. The software Analyst Pro (RRID:SCR_015785) (version 1.6.2) was used to analyze the data.

MORPHOLOGY ANALYSIS

The effects of flubendiamide administration were studied through phenotype analysis of developing embryos. Deformities related to the vascular system and oculogenesis were found to be the most recurrent. The abnormal phenotypes of flubendiamide treated embryos were further studied in detail and compared with control. Phenotypic deformities were documented with images captured using a camera (Nikon, USA) or a microscope (Leica DM2500, Germany).

ASSESSMENT OF THE BLOOD SERUM PROTEINS

The serum albumin was measured by commercially supplied biological kit Erba albumin kit (Cat. No. BLT00001). To obtain serum globulin levels, the value of serum albumin was subtracted from the total serum protein (Busher, 1990). The quantification of serum protein was accomplished using Bradford's assay (Bradford, 1976). Albumin, globulin and protein levels were calculated as g/dl blood sample.

HEMATOLOGICAL ESTIMATIONS

Blood samples were obtained from the brachial vein of newborn chicks using 2 ml disposable syringes rinsed with Ethylenediaminetetraacetic acid (EDTA) and then collected into vials rinsed with EDTA. Following collection, the samples were refrigerated and processed within 6 hours. The hematological parameters, including total erythrocyte (RBC) count, hemoglobin

(Hb) concentration, total leukocyte (WBC) count and differential leukocyte counts, were evaluated using a BC2300 hematology analyzer (Mindray Company, China).

ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT STATUS IN LIVER

Estimation of reduced glutathione (GSH)

The GSH assay followed the methodology given by Beutler and Gelbart (1986). 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 minutes, it was centrifuged at 3000 rpm for 15 minutes at 4ºC. Subsequently, 40 µL of the supernatant was mixed with 60 µL of Na_2HPO_4 and 3 µL of DTNB and the absorbance was measured at 412 nm (iMark Microplate reader, Bio-Rad Laboratories, Hercules, CA). The GSH content was calculated based on the slope obtained from a standard GSH graph and expressed as mg of GSH per mg of protein.

Estimation of SOD

SOD activity was determined using the method outlined by Marklund and Marklund (1974). 3 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 amount of enzyme necessary to achieve 50% inhibition of pyrogallol auto-oxidation. Absorbance was measured at 420 nm.

Estimation of catalase

Catalase activity was assessed following the protocol described by Sinha (1972). 8 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-minute incubation, 80 µL of dichromate acetic acid reagent was added, followed by heating for 10 minutes 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.

CYTOPLASMIC OXIDATIVE STRESS

The cell-permeable reagent 2', 7'-dichlorofluorescein diacetate (DCFDA) (Sigma Aldrich, Catalog Number: 6883) 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 minutes in the dark (Maurya & Vinayak, 2015). 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).

LIVER FUNCTION TESTS

ALP, ALT and AST are liver enzymes serving as biomarkers to detect early indications of acute liver damage. These enzymes were estimated as per 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-Na2 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 37ºC for 60 s and absorbance readings are taken every 30 s for 2 minutes 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-Na2 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 ºC 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).

VESSEL ANALYSIS OF CAM

AngioTool 0.6 software (RRID:SCR_016393) was used to measure the morphological and geographical parameters of vascular networks, such as vessel density, number of vessel junctions, total vessel length and lacunarity, through capturing pictures and analyzing the CAM of days 3 and 4 embryos (Zudaire et al., 2011). Student's t-test was used to evaluate the statistical analysis of significant differences between the treatment groups.

MOLECULAR DOCKING

The docking score is a crucial metric in molecular research, offering important information about the possible strength and affinity of binding interactions between a ligand and its target protein. Generally speaking, a higher negative docking score denotes greater binding stability, while a lower docking score represents a more favorable and stronger binding contact. To predict the binding energies of flubendiamide with proteins involved in the CAM angiogenesis, oculogenesis as well as apoptosis pathway: VEGFα (6MXR), CASPASE-3 (4QTX), BMP2 (2GOO), PI3K (1YI3), WNT7A (8TZO) and SHH (3MVX), BMP7 (1LX5), CDH1 (3L6Y), FGF8 (2FDB), PAX6 (6PAX), OTX2 (model_03) and SOX2 (6WX9) Auto Dock Tools 4.2.2 (RRID:SCR_011958) was utilized (Morris et al., 2009; Biovia, 2017; Waterhouse et al., 2018). The Protein Data Bank (PDB) database of the Research Collaborator for Structural Bioinformatics provided the protein structures used in this study. Following the extraction of water and ligand molecules, the protein data was formatted into PDB files using BIOVIA Discovery Studio (DS) Visualizer v21.1.0.20298 (RRID:SCR_008398) (BIOVIA, San Diego, CA, USA). Additionally, the same program was used to estimate the structure of the protein and locate active regions that were essential for docking investigations.

GENE EXPRESSION STUDIES

The standard protocols of RNA isolation, RNA quantification and quality control were followed by cDNA synthesis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to analyze the expression pattern of a variety of genes.

- Embryos were collected and placed in a vial with TRIzol reagent (25 mg tissue per ml; Ambion, Life Science Technologies, USA).

- To remove cell debris and tissue chunks, the homogenized tissue was centrifuged at 8000g for 10 minutes at 4°C after being transferred to a new tube from a pre-cooled mortar and pestle.
- The supernatant was transferred to a fresh tube containing 0.2 ml chloroform, stirred occasionally and incubated for 20 minutes.
- After 15 minutes of centrifugation at 12,000 g at 4°C, the mixture separated into three distinct layers: a buffy interphase, a lower layer of pink-red phenol-chloroform and an upper layer of colorless water.
- A fresh tube was gently tilted at a 45° angle to receive the RNA-containing water phase. Isopropanol (0.5 ml) was added and the mixture was let to stand for 1 hour with occasional shaking every 15 minutes.
- A white gel-like RNA pellet settled to the bottom after 20 minutes of centrifugation at 12,000 g at 4°C.
- The supernatant was discarded and the pellet was resuspended in 1 ml of 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C.
- The supernatant was collected, the pellet was mixed with 100% ethanol, then centrifuged again at 7500g for 5 minutes at 4°C.
- The supernatant was discarded and the pellet was air-dried for 10-20 minutes.
- The pellet was resuspended in 20-50 µl of RNase-free water by pipetting up and down.

RNA Quality and Quantification

- Agarose gel electrophoresis was used to evaluate the purity of the extracted RNA.
- Following the manufacturer's procedure, the Qubit Assay System (Promega, USA) was used to quantify the RNA after it had been diluted 1:10.

cDNA Synthesis

- To make cDNA, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used with 1 µg of mRNA from every sample.

The reaction mixture included:

Materials	**Volume per vial (µl)**
10x RT Buffer	2.0
25xdNTP mix	0.8
10x RT Random Primer	2.0
RT enzyme	1.0
Nuclease-free water	4.2
RNA sample	10
Total volume	**20**

The thermal cycler (Bio-Rad, USA) was set to the following protocol:

Temperature	**Time (minutes)**
25°C	10
37°C	120
85°C	5
12°C	∞

- Prior to any future use, the synthesized cDNA was preserved at -20°C.

Semi-Quantitative PCR

- Gene expression study was conducted using synthesized cDNA by semi-quantitative PCR.

PCR reaction mixture included:

Reagents	**Volume per vial (µl)**
cDNA sample	1.0
5 µm Forward Primer	1.0
5 µm Reverse Primer	1.0
Nuclease-free water	2.0
2x PCR master mix	5.0
Total volume	**10**

- The following conditions were used for the polymerase chain reaction (PCR): 3 minutes at 95°C, followed by 35 cycles of 10 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. In a Thermal cycler T100 (Bio-Rad, USA), semi-quantitative polymerase chain reaction was carried out.
- In order to separate the PCR products, agarose gel electrophoresis was employed.

Quantitative PCR (qPCR)

- Quantitative PCR was employed to analyze gene expression levels in the LightCycler 96 (Roche Diagnostics, Switzerland) with specific primers.

The qPCR reaction mixture included:

Reagent	**Volume (µl)**
Forward primer (5µM)	0.5
Reverse primer (5µM)	0.5
cDNA template	1.0
Nuclease-free water	3.0
2x SYBR green master mix	5.0
Total	**10**

18s rRNA was used as the housekeeping control.

The qPCR program was:

<table>
<tr><th>Temperature</th><th colspan="2">Time (seconds)</th></tr>
<tr><td>95°C</td><td colspan="2">100s</td></tr>
<tr><td>95°C</td><td>10s</td><td rowspan="3">X 35 cycles</td></tr>
<tr><td>60°C</td><td>30s</td></tr>
<tr><td>72°C</td><td>30s</td></tr>
<tr><td>4°C</td><td colspan="2">∞</td></tr>
</table>

- Using the Livak and Schmittgen method (2001), the data was analyzed to determine fold change. The fold change values for each gene were then plotted on a logarithmic

scale. The control and treatment groups of embryos were compared using a Student's t-test to statistically analyze the data.

PROTEIN EXPRESSION STUDIES

- The protein expression was assessed using the western blot technique. The process involved various stages, including protein isolation, estimation, quantification and sample preparation, followed by SDS-PAGE.

Protein Isolation

- To isolate the total protein, embryos were treated with a lysis buffer.
- A mortar and pestle that had been pre-cooled was employed to homogenize the mixture.
- At 4°C, the homogenized mixture was centrifuged at 8000 rpm for 10 minutes.
- Total protein concentration was determined by Bradford's assay (Bradford, 1976) after the supernatant was collected.

Tris-Triton Lysis Buffer Composition

Reagent	**Concentration**
Tris base	50mM
NaCl	200mM
$CaCl_2$	10mM
Triton X-100	1%
Protease inhibitor (Sigma, USA)	1%
pH 7.5	

- A standard curve was constructed in the Bradford assay using the measured optical densities (OD) of known concentrations of BSA.
- A volume of 1 µl of the isolated protein was utilized to determine the protein concentration.
- The optical density (OD) was determined at a wavelength of 595 nm and the protein content was estimated using the slope values.

Bradford Assay Setup:

	Sample (µl)	Bradford Reagent (µl)	Water (µl)
BSA	1-10	200	9-0
Isolated Protein	0.5/1	200	9.5/9

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

- The protein samples were supplemented with a 5x sample buffer to achieve a final working concentration of 2 µg/µl.
- The solution was subjected to thermal treatment at a temperature of 80°C for a duration of 10 minutes using a water bath and thereafter preserved at a temperature of -20°C until it was employed.
- The SDS-PAGE assembly was established by utilizing two glass plates and spacers, which were sealed at the bottom with a 2% agarose gel.
- A 4% gel for stacking and a 12% gel for resolving were prepared and placed into the SDS-PAGE apparatus and allowed to polymerize for 30 minutes.
- The gel was inserted into a gel electrophoresis apparatus containing tank buffer. The experiment involved loading and resolving samples and a dual stain protein ladder (Bio-Rad, USA) at an electrical potential of 100 volts.
- Proteins that had been successfully resolved were subjected to staining with Coomassie Brilliant Blue in order to assess their quality.

Western Blot

- The resolved proteins on the gel were transferred to a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) using semidry transfer at a current of 100 mA for a duration of 20 minutes.
- To assess the efficiency of the transfer, the membrane was stained with Ponceau and rinsed with distilled water 3-4 times.
- Next, the membrane was blocked with a blocking solution for 1 hour at room temperature.
- The primary antibody was then added and left to incubate overnight at a temperature of 4°C.

- On the following day, any unbound primary antibody was eliminated through multiple washes.
- Subsequently, the secondary antibody was added and incubated for 1 hour at room temperature.
- To remove any unbound conjugates, the Streptavidin-ALP conjugate was added and washed.
- For color development, the substrate p-Nitrophenyl Phosphate (pNPP) was added.
- The color reaction was stopped by adding distilled water once the optimal color was achieved.
- Finally, high-resolution images were captured using a camera and the intensity of the bands was measured using ImageJ software.

IMMUNOHISTOCHEMISTRY

The localization of the protein of interest within specific tissue regions was assessed using immunolocalization in tissue sections. The embryos were washed in PBS and left to fix for an hour in a solution of 4% Paraformaldehyde (PFA) in PBS at room temperature. Embryos were immersed in OCT (Tissue-Tek®, SAKURA, USA) after being fixed, rinsed with PBS and then passed over a sucrose gradient. The cryostat microtome (Leica Biosystems, Germany) was used to create 8-10 µm thick tissue sections at -20°C. After that, the slices were set on slides that were not affected by frost and left at 37°C for an hour to eliminate the OCT from the tissue.

After soaking the tissue sections in PBS- Triton (PBST) containing 10% goat serum and 1% BSA for 1 hour at room temperature, they were ready to be processed. The tissue sections were treated overnight at 4°C with primary antibodies that had been diluted in the blocking solution. Anti-Caspase 3 was the principal antibody utilized. After rinsing the plates with PBS several times, any extra antibodies were rinsed out. Later on, after rinsing the tissue slices with PBS, they were incubated with a secondary antibody for 1 hour at room temperature.

The sections were stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain, which was produced in PBS (1:1000) and then left to incubate for 10 minutes. After two or three washes with PBS, the slides were mounted at room temperature using Fluoroshield (Sigma-Aldrich, USA). The slices were imaged using a confocal laser scanning microscope (FV3000) and a Leica DM2500 fluorescent microscope (Leica, Germany and OLYMPUS, USA, respectively).

HEMATOXYLIN AND EOSIN (H&E) STAINING

Hematoxylin is a positive-charged stain that is dark bluish or violet in color and binds to negatively charged cell components, including DNA and RNA (Liu et al., 2015). Conversely, eosin is a reddish-pink stain that is negatively charged and bonds to positively charged cell structures. A complex known as hemalum is formed from oxidized hematoxylin and aluminum ions when a specimen is processed with these stains in combination. The DNA in the nuclei is bound by this dye-metal complex, which imparts a blue hue. Eosin Y, an aqueous solution, functions as a counterstain, resulting in the coloring of eosinophilic structures in red, pink, or orange tints.

Reagents	**Concentration**
Neutral buffered formalin (fixative)	10%
Xylene	-
Different grades of alcohol	70%, 80%, 95% and 100%
Harris Hematoxylin and eosin	-
DPX (mounting medium)	-

Procedure:

- The embryos were first excised to extract the desired tissue, which was then rinsed in PBS and fixated in a 10% Neutral buffered formalin (NBF) solution.
- Subsequently, the alcohol concentrations were progressively increased at room temperature to dehydrate the fixed tissue samples (50%, 70%, 90% and 100%).
- The tissue samples were immersed in xylene for 30 minutes and subsequently infiltrated with heated, molten paraffin wax for an hour.
- The tissues were solidified by separating them with metal blocks and subsequently sectioned to a thickness of 5 microns using a rotary microtome (Leica RM 2155).
- The sections were affixed to glass transparencies that had been previously coated with egg albumin.
- The sections were rehydrated, stained with Harris's hematoxylin and eosin for 10 minutes and subsequently washed under tap water to eradicate any remaining stain.
- Through a series of alcohol concentrations spanning from 30% to 90%, the slides were dehydrated and air-dried for a few seconds.

- Following dehydration, xylene was employed as a cleansing agent to eliminate any remaining wax for a duration of 10 minutes. The tissue sections that were cleared were subsequently permanently affixed with dibutylphthalate polystyrene xylene (DPX).
- Microphotographs were acquired using an EC3 Camera with LAS EZ software and the tissue architecture was visualized using a Leica DM2500 microscope.

ALCIAN BLUE AND ALIZARIN RED STAINING

In order to analyze structural deformities, the initial stage is to examine the skeletal architecture. Whole-mount staining enables the identification of changes in skeletal patterning by facilitating the assessment of structural elements in their appropriate locations. In order to stain cartilage and bone, respectively, alizarin red and alcian blue are employed in differential staining (Mcleod, 1980). A cationic dye, alcian blue, exhibits a significant affinity for sulfated glycosaminoglycans and glycoproteins that are primarily found in cartilage. To the contrary, alizarin red, an anionic dye, is able to bond to cationic metals such as calcium, resulting in its localization in bone.

The reagents used in the procedure are as follows:

Components	**Concentration (%)**
PFA	4
PBS	-
Acetone	-
Ethanol	-
Alcian blue 8GX (A5268, Sigma)	0.1
Alizarin red S (A5533, Sigma)	0.1
Potassium hydroxide (KOH)	1
Sodium borate	2
Glycerol	-

Procedure:

- Skeletal deformities were investigated by collecting control and treated embryos on day 10.
- The samples were initially isolated and subsequently rinsed in a chilled PBS solution to eliminate debris.
- The samples were stored in ethanol at 4°C for 48 hours after cleansing and subsequently in acetone for 2 hours.
- The samples were subsequently subjected to differential staining for 4 hours at room temperature in dark conditions using freshly prepared alcian blue and alizarin red stains.
- The stained specimens were rinsed in ethanol for 1 hour, washed under flowing tap water and subsequently cleared in a 1% KOH solution until the skeletons were visible.
- The samples were subsequently stored in 100% glycerol after the destaining procedure was conducted in a graded sequence of glycerol and 1% KOH.

STATISTICAL ANALYSIS

GraphPad Prism v8.0 (RRID:SCR_002798) from GraphPad Software Inc., USA, was employed to conduct the statistical analysis. The significance of the differences between the control and treated groups was determined at a P value of ≤ 0.05 using the Student's t-test. The results were presented as the mean ± standard error of the mean.

SEQUENCES OF PRIMERS USED IN THE STUDY

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
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

LIST OF ANTIBODIES USED IN THE STUDY

Primary antibody	Host	Dilution used	Catalogue no
β-actin	Mouse	1:1000	A2228 (Sigma- Merck)
Cl. Caspase-3	Rabbits	1:1000	PC679 (Sigma- Merck)
Akt	Rabbits	1:1000	SAB5600126 (Sigma- Merck)
Vegfα	Mouse	1:1000	SAB1402390 (Sigma- Merck)
Pi3k	Mouse	1:1000	AMAB91513 (Sigma- Merck)
GAPDH	Mouse	1:1000	G8795-25UL (Sigma- Merck)
Cdh2 (N-cadherin)	Mouse	1:1000	MA1-91128 (Invitrogen)
Pax6	Mouse	1:1000	AMAB91372-100UL (Sigma- Merck)
Shh	Mouse	1:500	MA5-17173 (Invitrogen)
Cyp1a1	Mouse	1:1000	AB1258 (Sigma- Merck)
Cyp1a2	Mouse	1:1000	AB1248 (Sigma- Merck)
Cyp2d6	Rabbit	1:1000	AV41675 (Sigma- Merck)
Cyp2c19	Rabbit	1:1000	SAB2100520 (Sigma- Merck)
Cyp3a4	Mouse	1:1000	SAB5300118 (Sigma- Merck)

GENERAL CHEMICAL PREPARATIONS:

Phosphate Buffered Saline (PBS)

Reagent	Concentration
NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
NaH_2PO_4	1.8 mM
pH 7.4	

Tris Buffered Saline (TBS)

Reagent	Concentration
Tris base	50 mM
NaCl	150 mM
pH 7.5	

Sample buffer (5X)

Reagent	Concentration
Tris base	250 mM
SDS	10%
Glycerol	50%
Bromophenol blue	0.5%
β-mercaptoethanol	Added freshly to make 100 mM final concentration
pH 6.5	

Tank buffer

Reagent	Concentration
Tris base	25 mM
Glycine	250 mM
SDS	10%

Gel stock (30%)

Reagent	Concentration
Acrylamide	29%
Bis-acrylamide	1%
Solution kept in the dark, overnight at room temperature and filtered before use	

Resolving gel buffer (12%; 5 ml)

Reagent	Volume (ml)
30% Gel stock	2
Double distilled Water	1.6
1.5 M Tris Cl (pH 8.8)	1.3
10% SDS	0.05
10% APS (freshly prepared)	0.05
TEMED	0.003
APS and TEMED were added just before pouring gel in PAGE assembly	

Stacking gel buffer (4%; 3 ml)

Reagent	Volume (ml)
30% Gel stock	0.4
Double distilled Water	1.8
1.5 M Tris Cl (pH 6.8)	0.75
10% SDS	0.03
10% APS (freshly prepared)	0.02
TEMED	0.002
APS and TEMED were added just before pouring gel in PAGE assembly	

Buffers for Western blot

Semi-dry transfer buffer

Reagent	Concentration
Tris base	48 mM
Glycine	39 mM
pH 9.2 and stored at 4°C	
Methanol (freshly added)	10%

Antibody dilution buffer

Reagent	Concentration
BSA in TBS-T	2%
Sodium Azide in TBST-T	0.02%

Substrate solution:

Reagent	Concentration
Tris base	0.1 M
NaCl	0.1 M
$MgCl_2$	0.05 M
pH 9.5	
BCIP	9.4 mg/ml
NBT	18.75 mg/ml
Stored in dark	

RNA loading dye

Reagent	Volume (µl)
500 mM EDTA	8
40% Formaldehyde	72
Glycerol	200
Formamide	30
2.5% Bromophenol Blue	30
10X TBE	400