Appendix I: Publication from Thesis

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EMBRYONIC EXPOSURE TO FLUBENDIAMIDE INDUCES STRUCTURAL ABNORMALITIES IN DOMESTIC CHICKS (*GALLUS DOMESTICUS*)

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ABSTRACT
The current investigation aimed to evaluate the teratogenic impact of Flubendiamide on chick embryos (*Gallus domesticus*) and highlight its potential harm to non-target organisms, particularly focusing on liver susceptibility. Varied doses of technical grade Flubendiamide (0, 5, 10, 15, 20, and 25 µl/50µl, assigned to six experimental groups) were administered into the eggs' air sacs prior to incubation. Embryo were isolated on the 10th day of incubation. Results indicated a dose-dependent increase in mortality rates. Twelve morphometric parameters, including wet body weight, crown-rump length, anterior-posterior head diameter, eye diameter, beak length, neck length, humerus length, radius and ulna length, metacarpus length, femur length, fibula length, and metatarsus length, were measured and compared with a control group. Significant reductions were observed in all treated groups relative to the control. Additionally, qualitative abnormalities such as microcephaly, hydrocephaly, edematous swelling, hematoma, abnormal body coloration, microphthalmia, deformed beak, agnathia, micromelia, amelia, omphalocele, and ectopia cordis were noted in treated groups compared to controls. The study underscores the harmful effects of Flubendiamide on the development of avian embryos even at low dose concentrations, emphasizing the need for cautious pesticide use to mitigate adverse health impacts, particularly due to its potential effects on non-target organisms' health.

***Keywords:** Pesticides, Flubendiamide, Chick Embryo, Teratology, Morphometric Measurements*

INTRODUCTION
Pesticides are vital for sustaining the growing global population by safeguarding crops against weeds, pests, and diseases. Without them, over half of the world's agricultural produce would be at risk (Aktar, 2009). These chemical solutions are indispensable for prolonging the life of crops, minimizing losses after harvesting, and mitigating the presence of harmful microorganisms and toxins in food. The massive application of pesticides in modern agriculture has resulted in adverse impacts on species that are not intended to be affected by them (Rather *et al.,* 2017). Studies have demonstrated that a wide variety of pesticides and insecticides have the potential to serve as teratogens, resulting in congenital deformities (Kalliora *et al.,* 2018). In order to combat infestations in a more efficient manner, new-generation pesticides that have improved efficacy are being brought to the market. This is because pests are becoming more resistant to the chemicals that are now available.

Among the insecticides of the new generation is flubendiamide, which is a phthalic acid diamide. It is employed for the purpose of controlling lepidopteran pests in more than 200 different crop species (Troczka *et al.,* 2017). Flubendiamide is effective because it interferes with the mobility of pests' muscles through its interaction with ryanodine receptors, which ultimately results in the pests' paralysis and final death (Teixeira, 2013). However, there have been concerns raised over the potential dangers that could arise from its unrestricted use. Flubendiamide is a new class of insecticide that is anticipated to replace various older

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Article

Inhibition of Cyclooxygenase-2 Alters Craniofacial Patterning during Early Embryonic Development of Chick

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Abstract: A recent study from our lab revealed that the inhibition of cyclooxygenase-2 (COX-2) exclusively reduces the level of PGE_2 (Prostaglandin E_2) among prostanoids and hampers the normal development of several structures, strikingly the cranial vault, in chick embryos. In order to unearth the mechanism behind the deviant development of cranial features, the expression pattern of various factors that are known to influence cranial neural crest cell (CNCC) migration was checked in chick embryos after inhibiting COX-2 activity using etoricoxib. The compromised level of cell adhesion molecules and their upstream regulators, namely CDH1 (E-cadherin), CDH2 (N-cadherin), MSX1 (Msh homeobox 1), and TGF-β (Transforming growth factor beta), observed in the etoricoxib-treated embryos indicate that COX-2, through its downstream effector PGE_2, regulates the expression of these factors perhaps to aid the migration of CNCCs. The histological features and levels of FoxD3 (Forkhead box D3), as well as PCNA (Proliferating cell nuclear antigen), further consolidate the role of COX-2 in the migration and survival of CNCCs in developing embryos. The results of the current study indicate that COX-2 plays a pivotal role in orchestrating craniofacial structures perhaps by modulating CNCC proliferation and migration during the embryonic development of chicks.

Keywords: cranial neural crest cells; embryogenesis; development; cell migration

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1. Introduction

Craniofacial development involves the formation of cranial neural crest cells (CNCCs) via epithelial–mesenchymal transition (EMT), induction, delamination, and migration, followed by the morphogenesis of various organs of an organism [1]. The above-mentioned events are tightly regulated by several genes that coordinate for craniofacial formation and patterning [2]. CNCCs are clusters of multipotent cells and fate-restricted progenitors that can differentiate into a multitude of tissue types based on the molecular signals they receive [3]. Their precursors undergo EMT and migrate from the forebrain, midbrain, and rhombomeres of the hindbrain to populate at the pharyngeal arches and contribute to the patterning of head and face structures. Once CNCCs pass through the EMT process, they begin migration. During migration, they proliferate and increase the pool of cells. The whole process of CNCC migration and proliferation is governed by various signaling pathways such as Fgf (Fibroblast growth factors), Wnt (Wingless-related integration site), TGF-β, and BMP (Bone morphogenetic protein) [3,4]. When the migration or differentiation of CNCCs is disrupted, defects of descendant tissues occur, which result in craniofacial malformations, the most common birth defect in humans [5].

Based on studies involving a wide array of model organisms, it can be construed that the molecular organizers of CNCC migration are conserved across various classes of vertebrates [4,6,7]. The canonical Wnt/β-catenin signaling pathway is reported to play a major role in the formation and progression of CNCCs, as it influences both delamination and migration by interacting with BMP4 and TGF-β, respectively [8–11]. Delamination is a collective effort orchestrated by downstream targets of Wnt3A and BMP4 signaling.

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Investigating the Impact of Flubendiamide on hematoma in Domestic Chickens (*Gallus domesticus*)

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Abstract

Pesticides have gained notoriety as pollutants that pose significant health risks to humans. Research reports have indicated that pesticides can lead to congenital birth defects, but their specific causes remain elusive. Exposure to such toxic chemicals has been linked to various health complications, including the formation of haematomas. In this study, we focused on understanding the impacts of a pesticide Flubendiamide, a phthalic acid diamide, on chick embryo development. A dose range study was carried out, where flubendiamide of 10µL/50µg to100µL/50µg was injected into eggs, followed by incubations at optimal temperature of 37.5°C ± 0.5°C and humidity conditions 70-75% with regular rotations every 1.5 hours to ensure consistent exposure. From the results, 25µg/50µL of flubendiamide dissolved in PBS was chosen as the Least observed effect concentration (LOEC). Further, the final concentrations of flubendiamide in treated samples in comparison to control were detected using an LC-MS/MS. Based on our preliminary morphological observations of the haematomas developed in the treated samples, we hypothesized that flubendiamide, at sub-lethal concentrations, alters the expression patterns of the essential signalling molecules such as VEGF, Cleaved caspase-3, AKT and KDR involved in angiogenesis processes, leading to anomalies in blood vessel development. Mortality and malformation rates in the 2nd and 4th days of embryonic development were also analysed in combination with gene and protein expression studies. Results indicated altered expression pattern of VEGF, Cleaved caspase-3, AKT, KDR in the embryos under study, which in turn suggests that sub-lethal dose of Flubendiamide could disrupt the blood vessel formation in a developing chick embryo by targeting VEGF signalling cascade. The study's findings could raise concerns about the potential environmental impact of Flubendiamide and its effects on non-target organisms. It might also prompt further investigation into the safety of the chemical for higher organisms' growth and development.

Keywords: Pesticides, flubendiamide, haematoma, angiogenesis

INTRODUCTION

The environment, comprising biotic and abiotic elements, witnesses constant interactions between living organisms and surrounding conditions. Pollution stands as a leading factor causing environmental deterioration. Among pollutants, pesticides, designed for pest control, emerge as a concerning group of environmental contaminants. Despite their role in safeguarding plants and humans from pests, pesticides often affect non-target species, even at low doses, spreading beyond intended areas.

Research has highlighted the teratogenic effects of various pesticide compounds on animal embryonic development, contributing to concerns about congenital birth defects in babies when pregnant women are exposed to these substances (Kalliora *et al.,* 2018). Pesticides, including herbicides, insecticides, bactericides, insect repellents, microbicides, fungicides, and animal repellents, have been associated with such congenital anomalies (Garces *et al.,* 2020).

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"Examining the impact of in ovo administered flubendiamide on domestic chick"

4.6

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Pesticides are widely used to control specific pest populations, but their harmful effects on non-target organisms have been an increasing problem. Hence, new generation of pesticides are developed to target specific pests and minimize non target interactions. Flubendiamide is one such insecticide that operates by targeting specific calcium channels in insects. The current study was conceptualized to assess the safety of commercially used flubendiamide on domestic chick (*Gallus gallus domesticus*). To determine Least observed effect concentration (LOEC), a dose range of 10 to 100 µg/50 µL flubendiamide in PBS was injected into air sacs of the eggs using BD 1 ml insulin syringe. The eggs were incubated at 37.5°C ± 0.5°C, 70-75% humidity and rotated every 1.5 hours to ensure consistent exposure. For further experiments, 25 µg/50 µL of flubendiamide was chosen as the LOEC. After 21 days of incubation, liver function of newly hatched chicks of the control and treated groups (n=30) were evaluated for antioxidants like reduced glutathione, glutathione peroxidase, superoxide dismutase, catalase, alkaline phosphatase, aspartate transaminase and alanine transaminase. The gene expression and protein expression analyses were carried out by PCR and western blot, respectively and liver damage by histopathology. The results revealed significant differences in both the enzymatic and non-enzymatic antioxidants between the control and treated groups, indicating the impact of flubendiamide administered *in ovo*. Gene expression and protein expression studies showed elevated expression pattern of hepatic drug metabolism indicators, including CytC, CYP3A4, CYP1B1, CYP21A1, and CYP1A1. Furthermore, the histological analysis of liver tissue revealed visible physical damage in the treated group confirming the toxic effects of flubendiamide on non-target organisms. These findings underscore the potential harm posed by flubendiamide to unintended organisms, emphasizing the need for judicious use of flubendiamide to mitigate adverse environmental consequences.

Keywords: Pesticides, Flubendiamide, Cytochromes, Antioxidant