

Summary

During the 1960s, India experienced a severe food shortage due to a fast-expanding population that exceeded the rate of agricultural output. India's agricultural landscape was altered with the introduction of the Green Revolution, which involved the adoption of high-yielding types of seeds, enhanced irrigation systems and improved farming practices (Patel, 2013). The implementation of this transformation had a substantial impact on crop productivity, namely wheat and rice, resulting in an excess of agricultural output. India attained food self-sufficiency and became a prominent food exporter due to enhanced agricultural output. The Green Revolution not only tackled urgent problems regarding food security but also established the groundwork for India's agricultural expansion, allowing it to sustain its growing population and make a contribution to global food security (Conway, 2012).

Pesticides were a crucial factor in the Green Revolution, especially in terms of increasing agricultural productivity and crop yields. From the mid-20th century onwards, there was a significant global shift of farming practices (Fischer, 2009). This involved the widespread adoption of high-yielding crop varieties, sophisticated irrigation methods and the use of chemical inputs such as fertilizers and pesticides. Like many other nations, India heavily depended on pesticides during the Green Revolution to combat illnesses and pests that threatened agricultural products (Hardin, 2008). Pesticides have proven to be a blessing in India, where agriculture is a significant industry that employs millions and provides food security for a large population by protecting crops from illnesses and insect infestations.

Pesticides helped farmers maintain constant agricultural production and avoid yield losses by shielding crops from harmful pests. Additionally, insecticides improved farmers' incomes and standard of living by lowering agricultural losses brought on by pests (Aktar et al., 2009). In a nation where agriculture plays a major role in the economy, the use of pesticides encouraged both the general economic growth of the country and the survival of rural communities. Furthermore, pesticides made it easier for contemporary farming techniques and technology to be adopted, which increased farming production and efficiency. They made it possible for farmers to use intensive farming methods and high-yield crop varieties without giving into the fear of pests (Patel, 2013). As a result, India was able to change from a country that was food

insecure to one that could feed its expanding population and even export excess products during the Green Revolution, thanks to impressive advances in agricultural output.

It is imperative to recognize the possible adverse consequences and ecological issues linked to the application of pesticides, such as contaminated soil and water, detrimental impacts on non-target creatures and the emergence of pesticide resistance in pests (Aktar et al., 2009). Sustainable pesticide management techniques and the advancement of integrated pest management systems are essential to minimize these hazards and maintain the advantages of pesticide use in agriculture.

As described by the FAO, pesticides are chemical substances employed to control, prevent, or eliminate pests, significantly enhancing crop yield by targeting pests that impede crop growth, storage, or processing (Fischer, 2009). However, the pervasive use of pesticides has led to their enduring presence in the environment, adversely affecting non-target organisms (Pathak et al., 2022). Studies have confirmed that exposure to pesticides can cause harmful effects, such as congenital malformations in non-target species (Kalliora et al., 2018). Despite this, the exact mechanisms underlying these effects remain incompletely understood. Flubendiamide is a new chemical family of substituted phthalic acid diamides with strong insecticidal properties (Trocza et al., 2017). Recent studies have raised concerns about flubendiamide's potential effects on non-target species (Li et al., 2014; Sarkar et al., 2014, 2017, 2018). As a result, this work aims to understand the basic mechanisms that cause structural defects in domestic chicks exposed to flubendiamide.

According to our research, flubendiamide can be potentially toxic when present in high concentrations, with an LD₅₀ of 1000 ppm. LOEC of 500 ppm was seen to cause decreases in liver and body weights in embryos after they hatch. The results of this study are consistent with earlier research that has demonstrated the harmful impact of pesticides on liver function and the inhibition of growth (López et al., 2007).

The hematological study showed notable reductions in albumin, globulin and total protein concentrations in embryos treated with flubendiamide, indicating possible liver impairment caused by oxidative stress and inflammation. The decrease in crucial proteins suggests a decline in liver function and weakened immune response, supporting previous research findings (López et al., 2007; Aktar et al., 2009).

The observed anemia, characterized by reduced hemoglobin levels and red blood cell count, is consistent with previous research on anemia caused by insecticides (Barna-Lloyd et al., 1991; Goel et al., 2006). This anemia is might have been caused by the hemolytic and myelosuppressive effects of flubendiamide. In addition, the presence of leucopenia and decreased lymphocyte counts indicates a weakened immune system and higher vulnerability to infections, which aligns with earlier studies on the immunotoxicity of pesticides (Garg et al., 2004; Ojezele and Abatan, 2009; Gowri et al., 2010).

Upon conducting morphometric analysis and X-ray scans on recently born chicks that were exposed to flubendiamide, notable skeletal abnormalities and developmental problems were observed. These included lower body weight, microcephaly (abnormally small head) and limb deformities. The results of this study confirm previous research on the harmful effects of pesticides (Asmatullah et al., 1993; Anwar, 2003). The study demonstrates that flubendiamide interferes with the growth and formation of embryos, resulting in notable abnormalities in development. During the morphological investigation, it was seen that exposure to 500 ppm of flubendiamide (LOEC) hindered the development of blood vessels and eyes in early chick embryos. Research has shown that prolonged exposure to pesticides impairs blood vessel function in mammals (Xiao-Ming et al., 2010) and high pesticide levels reduce blood vessel development and branching patterns (Priyadarshini et al., 2020).

Early chick embryos that were exposed to flubendiamide exhibited abnormalities in the blood vessels of the CAM and disruption in the establishment of the vascular network. The treated embryos showed symptoms such as bleeding, vessel breakdown and blood clotting. Morphometric and quantitative analyses indicated shortened blood vessels, lower vascular density and fewer vessel junctions, suggesting a decrease in capillary sprouting, which is crucial for blood vessel formation in the CAM network. Increased lacunarity in exposed embryos indicated more empty areas not filled by blood vessels, pointing to disorganized CAM vasculature due to reduced capillary sprouting.

Further computational docking studies revealed strong binding affinities between flubendiamide and several key proteins involved in angiogenesis, such as VEGF α , CASPASE-3, BMP2, PI3K, WNT7A and SHH. These findings indicated that flubendiamide could directly interact with and affect these angiogenic regulators. Subsequent transcript and protein level analyses confirmed differential expression patterns of these target molecules during the

developmental phase, highlighting impaired endothelial cell proliferation due to reduced VEGF α , KDR and PCNA expression and disrupted SHH signaling affecting the cytoskeleton of endothelial cells via the PI3K/AKT pathway (Hiratsuka et al., 2005; Strzalka & Ziemienowicz, 2011; Zavala et al., 2017; Salybekov et al., 2018; Lei et al., 2020).

The study also found that the initiation of blood vessel sprouting, driven by WNT7A signaling, was hindered by reduced WNT7A transcript levels in treated embryos. Additionally, BMP2 and BMP6, which maintain endothelial cell plasticity during sprouting, were significantly reduced, further supporting the negative impact of flubendiamide on CAM angiogenesis (Benn et al., 2017; Olsen et al., 2017). Increased expression of RHOB and Cl. Caspase-3 indicated heightened apoptosis, contributing to the disruption of CAM vascularization (Howe & Addison, 2012; Brentnall et al., 2013).

In an additional part of the study focusing on the eye development of domestic chicks, it was observed that flubendiamide exposure (LOEC) hampered eye formation in early embryos. The formation of eyes in chick embryos is a meticulously orchestrated process regulated by an intricate interplay of signaling molecules. The process starts when the head ectoderm receives signals from brain bulges, which creates an orientation that ultimately results in the creation of the lens (Saha et al., 1989). After that, the OTX2 protein determines the location of the anterior neural tube, while the activation of PAX6 inhibits this process. This leads to a series of transcription factors that are crucial for determining the eye region, ultimately resulting in the formation of a single eye field (Zuber et al., 2003; Zuber, 2010). The partitioning of the monocular visual field into two lateral visual fields is regulated by the expression of SHH, which suppresses the activity of PAX6 in the central area (Macdonald et al., 1995).

Histopathological examination on day 4 revealed impaired optic cup development, absence of lens and cornea and overall disorientation of early eye development. Alician blue alizarin red staining of newborn chicks exposed to flubendiamide also corroborated the histological study. This disruption was attributed to altered expression of key oculogenic proteins, including BMP7, CDH1, FGF8, PAX6, OTX2 and SOX2, as revealed by *in silico* docking experiments and subsequent transcript and protein level analyses. For instance, the robust affinity of flubendiamide for OTX2 suggested inhibition of its function, leading to a decline in OTX2 expression throughout the study frames.

Significant reductions in transcripts of SHH, PAX6, WNT11, CDH1, CDH2, BMP4, BMP7 and FGF8 were noted in treated embryos, correlating with observed ocular defects. Additionally, the study highlighted increased apoptosis markers, such as CASPASE-3, further impeding eye development and leading to congenital eye deformities.

Vertebrates predominantly metabolize and detoxify xenobiotics through the liver (Grant, 1991; Ozougwu, 2017). This study examines the hepatotoxic consequences of *in ovo* exposure to flubendiamide in recently born chicks. Notably, chick liver development closely resembles mammalian liver development (Wong & Cavey, 1992).

The significant increase in crucial cytochrome P450 enzymes, such as CYP1A1, CYP1A2, CYP2D6, CYP2C19 and CYP3A4, indicates a heightened metabolic reaction to the flubendiamide, resulting in elevated oxidative stress. This is supported by the heightened functioning of antioxidant enzymes like superoxide dismutase and catalase, along with the noteworthy reduction in levels of reduced glutathione, which suggests an augmented generation of reactive oxygen species. The increased fluorescence intensity found in DCFDA staining provides additional evidence of elevated amounts of H₂O₂, indicating significant oxidative stress in the liver tissue (Apel & Hirt, 2004; Schieber & Chandel, 2014; Ighodaro & Akinloye, 2018). The elevated levels of ALP, ALT and AST in the blood, along with the presence of liver inflammation and damage to the structure of liver cells, highlight the severity of liver damage caused by flubendiamide.

In conclusion, this study underscores the significant hepatotoxic effects of flubendiamide on newly hatched chicks, drawing attention to the broader ecological and health implications of pesticide use. The findings demonstrate that *in ovo* exposure to flubendiamide can cause profound liver damage, oxidative stress and disruptions in liver structure. The detrimental impact on liver function, as evidenced by altered cytochrome P450 enzyme activity and increased oxidative stress markers, highlights the need for stringent regulation and management of pesticide application.

Additionally, the observed hematological abnormalities and skeletal deformities in chicks emphasize the far-reaching consequences of pesticide exposure on overall development and health. The study calls for the advancement of integrated pest management systems and sustainable pesticide practices to mitigate these risks. As India continues to evolve its agricultural practices to ensure food security, it is imperative to balance productivity with

ecological and health considerations, ensuring that advancements in farming do not come at the expense of environmental and public health. This research not only contributes to the understanding of flubendiamide's toxicological profile but also provides a foundation for future studies aimed at developing safer and more sustainable agricultural practices.

OVERALL GRAPHICAL SUMMARY

