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***Research Article** (Open Access)*

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

insecticides, including synthetic pyrethroids, organophosphates, organochlorines, abamectin, spinosad, indoxacarb, carbamates, and neonicotinoids. This is due to the fact that flubendiamide is more effective than these other insecticides and is presumed to be less toxic to non-target species (Troczka *et al.,* 2017). According to recent research, flubendiamide has been shown to have a negative influence on non-target organisms, such as the fruit fly (*Drosophila melanogaster*) and the Chinese tiger frog (*Hoplobatrachus chinensis*) (Sarkar *et al.,* 2014, 2017, 2018; Li *et al.,* 2014). Recent study showed that diamide effectuate harmful effects on the developing chick embryos even at very low concentration (Abbas *et al.,* 2018).

Poultry is an important, affordable and nutritious source of protein, frequently included in daily diets as eggs and white meat (Ghafoor, 2010). In Pakistan and India, rural communities commonly rear indigenous poultry breeds to satisfy their local meat consumption needs (Kumaresa, 2008). The significance of poultry in these regions underscores the necessity to ensure the safety of various agricultural practices and inputs, including the use of insecticides, which could potentially impact poultry and other non-target organisms. The molecular makeup, cellular structure, and anatomical properties of the chick embryo are quite similar to those of the human embryo (Stern, 2018). Due to its similarity, chick is an ideal model for investigating developmental mechanisms (Bellairs and Osmond, 1998). Current study aims to examine the teratogenic effects of flubendiamide on chick embryos (*Gallus domesticus*). By assessing morphometric changes in chick embryos exposed to flubendiamide compared to untreated controls, current research seeks to provide a comprehensive safety evaluation of flubendiamide, with a particular focus on its developmental toxicity. These findings are crucial for understanding the broader implications of flubendiamide use and ensuring its safe application in agriculture.

MATERIALS AND METHODS

2.1 Test substance

The insecticide flubendiamide of technical grade (CAS No. 272451-65-7) was acquired from Sigma-Aldrich Chemical Company located in St. Louis, MO, USA. Sigma-Aldrich (St. Louis, MO, USA) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India) were the suppliers of all common compounds.

2.2 Animal procurement and management

Fertilised eggs of domestic chicken were obtained from the Intensive Poultry Development Unit located in Vadodara, Gujarat, India. Before being incubated, eggs were cleaned with betadine (Povidone-iodine 10% w/v) and candled to reveal the air sac. The institutional animal ethics committee (IAEC; No. MSU-Z/IAEC04/10-2020) approved the protocols, and the studies were carried out in accordance with the guidelines established by the national regulatory authority for animal experiments, the Committee for Control and Supervision of Experiments on Animals (CCSEA).

2.3 Experimental design

A control group and a treatment group were randomly assigned to newly laid down eggs. Thirty eggs each were used in the control and treatment groups for each of the three trials. The eggshells were perforated with a sharp needle on day "0" of incubation in order to identify the air sacs using the candling procedure (Blankenship, 2003). A sterile BD 1 ml insulin syringe was used to administer the eggs in the air sac while laminar airflow was present. Flubendiamide at various doses of 5 µg,10 µg,15 µg, 20 µg and 25 µg in 50 µl of PBS was given to the treatment groups. PBS in an amount of 50 µl was given to the control group. Melted paraffin wax was used to close the opening right away, and it was then placed inside the incubator. During the incubation procedure, 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).

2.4 Mortality percentage

The embryos were isolated on day 10 of incubation and the mortal ones were separated and their number was recorded for both control and treated groups.

2.5 Morphometric analysis
A digital balance (SF-400) was used to measure the weight of the 10 days old embryos. A vernier calliper was utilised for determining characteristics such as crown-rump length, head diameter (anterior-posterior), eyes diameter, neck length, beak length, etc. A scale and compass were used to measure the fore and hind limbs of control and treated groups of embryos. Using a Sony SLT-A58K camera, morphological observations of embryos were directly captured.

2.6 Malformation frequency
Qualitative abnormalities such as ectopia cardis, omphalocele, microphthalmia, short upper beak, edema and hematoma formation, hydrocephaly, microcephaly and microphthalmia were noted.

2.7 X-Ray Analysis
The chicks of control and treated group were allowed to hatch out and were anesthetized using chloroform in a desiccator. The chicks were further subjected to X-Ray machine (BPL Medical Technologies, Bangalore, India). The X-Ray images were further checked for skeletal deformities.

RESULTS

3.1 Mortality percentage
The impact of varying doses of flubendiamide on embryo mortality of day 10 demonstrated a clear dose-dependent relationship between flubendiamide exposure and mortality percentage. The control group exhibited a baseline mortality rate of 3.67%. As the dose of flubendiamide increased, mortality percentages rose incrementally, indicating a heightened susceptibility of the embryos to the compound. Specifically, doses of 5 µg/50µl, 10 µg/50µl, 15 µg/50µl, 20 µg/50µl, and 25 µg/50µl corresponded to mortality rates of 7.05%, 15.67%, 18.45%, 21.33%, and 30.24%, respectively (Table 1).

Table 1: Mortality rate (%) of chick embryos treated with Flubendiamide (n=30 each group)

Treatment	Dose/egg (µg/50µl)	Frequency of mortality	Mortality (%)
Control	0	1	3.67
Flubendiamide (Treatment)	5	2	7.05
	10	4	15.67
	15	5	18.45
	20	6	21.33
	25	9	30.24

3.2 Morphometric analysis
A range of parameters, including wet body weight, head and eye diameter, neck length, humerus, radius, and ulna, metacarpus, femur, fibula, and metatarsus length, were measured for viable 10 days old chick embryos of control and treated groups. The measurements were recorded along with the mean and standard error of each parameter. The flubendiamide-treated chick embryos showed significant reductions in body weight, head diameter, crown-rump length, eyes diameter, neck length, beak length, and the length of the fore limbs (humerus, radius, and ulna, metacarpus) and rear limbs (femur, tibia and fibula, metatarsus) at all dose concentrations (Table 2).

Table 2: Morphometric parameters of control and Flubendiamide treated 10 days old chick embryos

Eggs Groups Parameters	Control	Flubendiamide treated (µg/50µl)				
		5	10	15	20	25
Wet body weight (g)	6.67 ± 0.03	5.49 ± 0.04***	4.52 ± 0.05***	3.32 ± 0.05***	2.57 ± 0.03***	2.02 ± 0.06***
Crown RL (cm)	6.12 ± 0.03	5.05 ± 0.04***	4.18 ± 0.04***	2.93 ± 0.05***	2.25 ± 0.03***	1.78 ± 0.06***
Head Diameter (cm)	2.82 ± 0.06	2.49 ± 0.06*	2.27 ± 0.06**	2.29 ± 0.07**	2.07 ± 0.06***	2.04 ± 0.07**
Eye diameter (cm)	2.48 ± 0.05	1.96 ± 0.05**	1.72 ± 0.05***	1.76 ± 0.06***	1.52 ± 0.05***	1.47 ± 0.06***
Beak length (cm)	2.88 ± 0.07	2.31 ± 0.07**	1.93 ± 0.07***	1.83 ± 0.08***	1.61 ± 0.07***	1.56 ± 0.08***
Neck length (cm)	2.62 ± 0.07	2.23 ± 0.07*	2.11 ± 0.07**	2.05 ± 0.07**	1.76 ± 0.07***	1.66 ± 0.07***
Humerus length (cm)	2.39 ± 0.06	2.06 ± 0.06*	1.84 ± 0.06**	1.81 ± 0.06**	1.45 ± 0.05***	1.27 ± 0.07***
Radius and ulna length (cm)	2.37 ± 0.06	2.07 ± 0.06*	1.75 ± 0.06**	1.67 ± 0.07**	1.34 ± 0.05***	1.22 ± 0.07***
Metacarpus length (cm)	2.38 ± 0.06	2.05 ± 0.06*	1.71 ± 0.06**	1.57 ± 0.07***	1.33 ± 0.06***	1.29 ± 0.07***
Femur length (cm)	3.01 ± 0.07	2.55 ± 0.07*	2.33 ± 0.07**	2.29 ± 0.08**	1.88 ± 0.06***	1.66 ± 0.08***
Fibula length (cm)	2.49 ± 0.05	2.09 ± 0.05**	1.86 ± 0.05***	1.78 ± 0.06***	1.41 ± 0.04***	1.23 ± 0.06***
Metatarsus length (cm)	2.67 ± 0.07	2.32 ± 0.07*	2.14 ± 0.07**	1.96 ± 0.08**	1.62 ± 0.07***	1.48 ± 0.08***

*Note: Values expressed as mean; n=3 with 30 eggs, *p≤0.05; **p≤0.01; ***p≤0.001*

3.3 Frequency of Malformation

By the tenth day of embryonic development, the treated groups showed certain abnormalities, while the control groups showed the regular form of normal chick embryos (Figure 1). There were no abnormalities in the embryos taken from the control group; they had all the normal features, such as the eyes, beak, legs, and ears. In the treated group, certain very concerning abnormalities were discovered, such as hematoma formation, abnormal body pigmentation, ectopia cardis, hydrocephaly, microcephaly, microphthalmia, extra-mass grow behind neck, meromelia, micrognathia, and meromelia (Table 3).

Table 3: Frequency percentage of different anomalies observed in Flubendiamide treated 10 days old chick embryos.

Anomalies	**Treated Embryos (µg/50µl)**				
	5	**10**	**15**	**20**	**25**
Abnormal body coloration	0	24.12	27	54.72	88.34
Agnathia	0	0	0	46.37	67.23
Amelia	0	0	0	66.67	86.35
Ectopia cardis	0	0	0	13	23
Edema	0	6.47	12.75	27.43	35.33
Hematoma	50	64.65	75	89.31	95
Hydrocephaly	0	10	28	34	42
Meromelia	0	18.32	45	70	82
Micophthalmia	43	76	82	90	92
Microagnathia	0	26.33	48	54	62
Microcephaly	0	33.33	25	33.33	45.33
Micromelia	0	10.74	30	53.47	65.12
Omphalocele	7	23	65	70	89
Short beak	0	47.83	65	78	82
Swelling around eyes	0	3.87	9.80	13.73	25.89

Note: All values expressed in percentage (%).

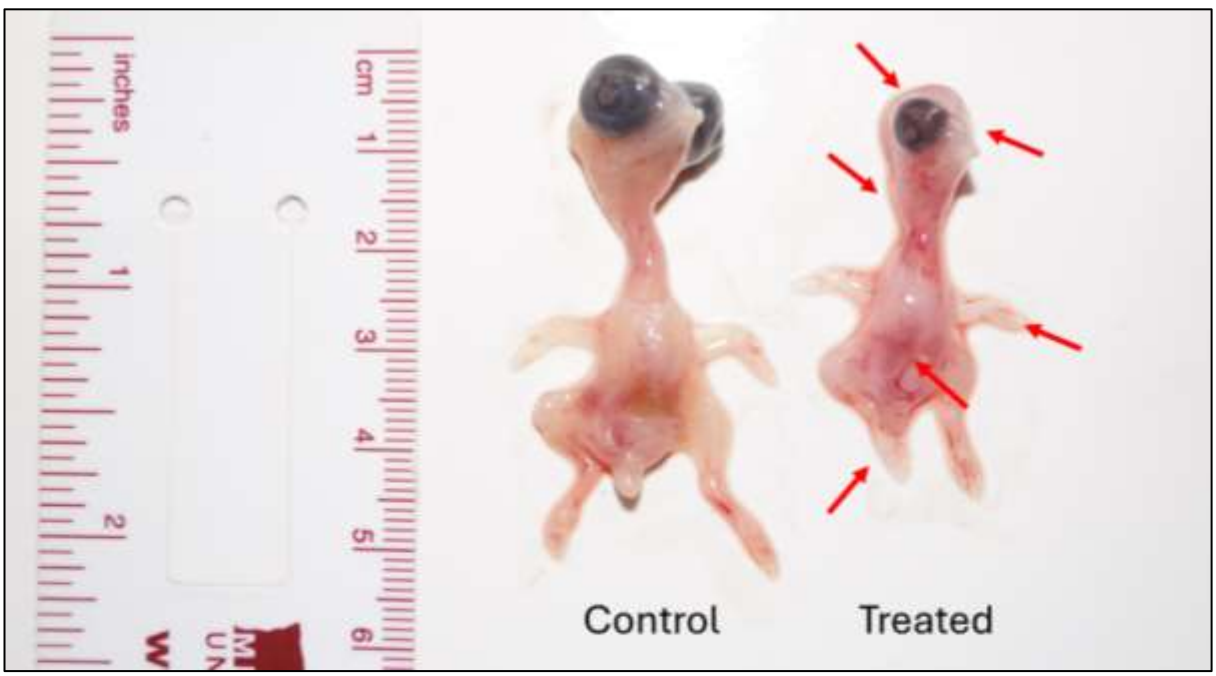

Figure 1: General morphology of embryos in control and flubendiamide-treated groups. The treated embryos exhibit several defects, including hematomas, abnormal body pigmentation, ectopia cordis, hydrocephaly, microcephaly, micrognathia, microphthalmia, an extra mass growing behind the neck, and meromelia. The regions of deformity are indicated with red arrows.

3.4 X-Ray analysis

The chicks were allowed to hatch out on 21 day and their morphology was noted. The treated chicks showed multiple defects including overall reduction in size, crippled limbs and unsteady walk compared to the control group of chicks (Figure 2). The chicks were the analyzed in X-Ray machine where the flubendiamide treated chick showed defective skeletal structure with kinked neck, crippled limb bones, and edema in abdominal region indicating ventral body wall defect (Figure 3).

Figure 2: General morphology of 21-day hatched chicks in control and flubendiamide-treated groups. The treated chicks displayed multiple abnormalities, including a noticeable reduction in overall size, deformed limbs, and an unsteady gait, compared to the control group.

DISCUSSION

The Food and Agriculture Organization (FAO) defines pesticides as chemical agents used to control, prevent, or remove pests. They are widely used to increase crop output by targeting pests that impede crop development, storage, or processing (Fischer, 2009). However, the widespread use of pesticides has resulted in their persistent presence in the environment, unintentionally exposing non-target creatures to their hazardous effects (Rather *et al.,* 2017). According to scientific study, pesticide exposure can produce harmful effects in non-target creatures, such as congenital abnormalities (Kalliora *et al.,* 2018). Despite this, the particular processes underlying these impacts are not entirely understood.

Current study provides comprehensive insights into the teratogenic effects of flubendiamide on chick embryos, elucidating significant developmental anomalies and increased mortality rates associated with escalating doses. Mortality assessment on day 10 revealed a dose-dependent increase in mortality percentage. Morphometric analysis further substantiated these findings, demonstrating substantial reductions in various morphological parameters, including body weight, head diameter, crown-rump length, and limb lengths, across all dose concentrations in flubendiamide-treated embryos compared to controls.

These results are consistent with prior studies investigating the effects of other pesticides on embryonic development, such as malathion and cypermethrin, which also reported similar reductions in body weight and crown-rump length (Asmatullah *et al.,* 1993; Anwar, 2003). Additionally, the observed qualitative abnormalities in flubendiamide-treated embryos, including microcephaly, hydrocephaly, and limb deformities, align with findings from studies examining the teratogenic effects of other pesticide compounds, underscoring the broader implications of pesticide exposure on embryonic development (Nancy *et al.*, 1994; Pourmirza, 2000, Uggini *et al.,* 2010, Pinakin *et al.,* 2011).

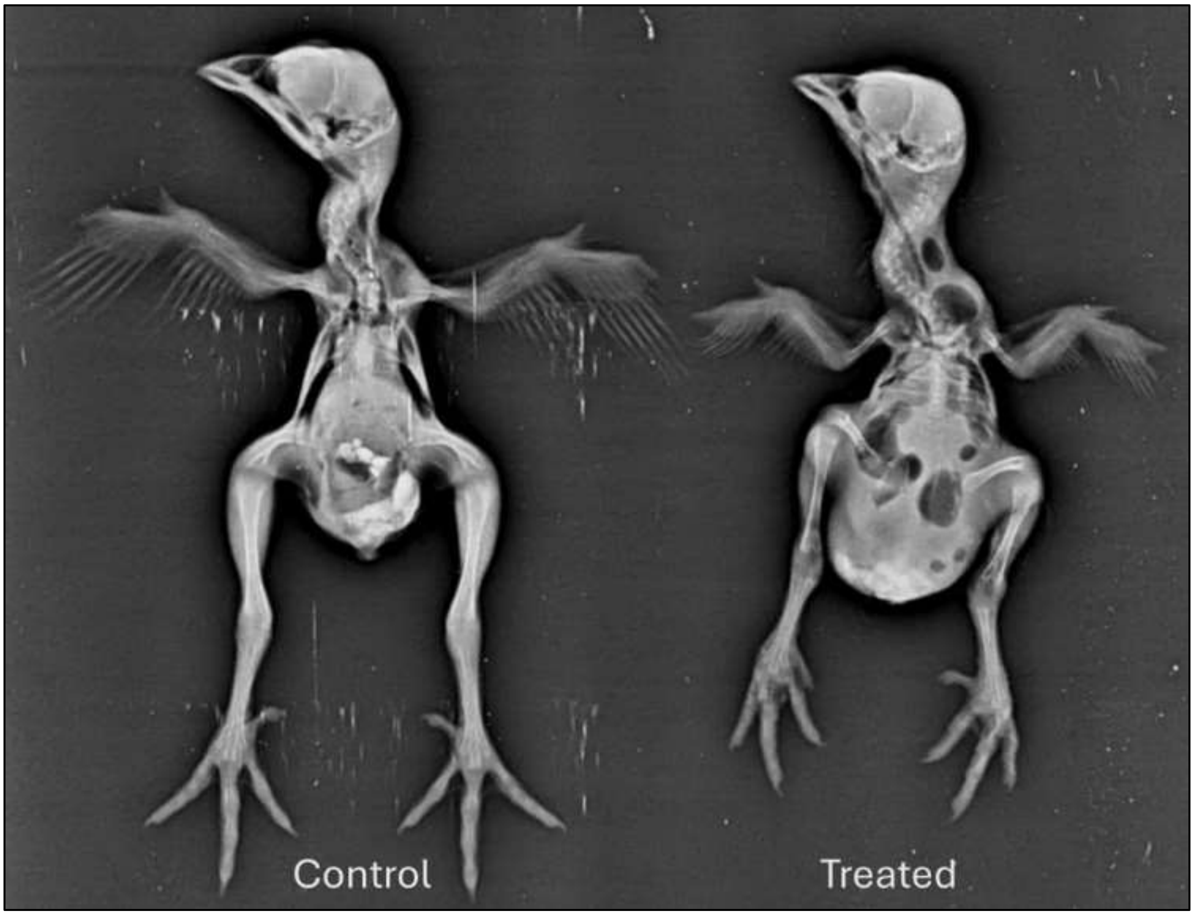

Figure 3: X-ray analysis of chicks in control and flubendiamide treated group. The flubendiamide-treated chicks exhibited defective skeletal structures, including a kinked neck, deformed limb bones, and abdominal edema.

The frequency of malformations observed in flubendiamide-treated embryos, ranging from hematomas to microphthalmia, corroborates previous research on pesticide-induced teratogenic changes, emphasizing the detrimental impact of pesticide exposure on embryonic morphology. Notably, the skeletal deformities and abnormalities observed in this study align with findings from prior investigations into the teratogenic effects of dithiocarbamates and diamides, further highlighting the potential risks associated with pesticide exposure during embryonic development (Van Steenis and Van Loghten, 1971; Kraggerud *et al.,* 2010). In contrast, control embryos exhibited normal morphology consistent with established descriptions, underscoring the specificity of the observed abnormalities in flubendiamide-treated embryos.
X-ray analysis of hatched chicks provided further insights into the structural defects induced by flubendiamide exposure, revealing multiple abnormalities including kinked necks, crippled limb bones, and

abdominal edema indicative of ventral body wall defects. These findings underscore the systemic impact of flubendiamide on skeletal development and overall morphology, implicating potential disruptions in embryonic patterning and organogenesis. Importantly, the observed defects in flubendiamide-treated chicks highlight the need for stringent regulations and cautious application of pesticides to mitigate adverse health impacts on both wildlife and human populations.
The present study contributes to our understanding of the teratogenic effects of pesticides, specifically flubendiamide, on embryonic development in avian species. The findings underscore the importance of further research to elucidate the molecular mechanisms underlying these effects and inform the development of safer pesticide use practices. Ultimately, comprehensive understanding of the impacts of pesticide exposure on embryonic development is essential for safeguarding both environmental and human health.

CONCLUSION

The present study contributes to the expanding evidence on the teratogenicity of pesticides, specifically Flubendiamide, highlighting its significant adverse effects on the embryonic development of chick embryos. This research demonstrates a clear dose-dependent increase in mortality and developmental anomalies, paralleling findings in other species exposed to similar chemicals. The observed reductions in morphometric parameters and the prevalence of severe abnormalities such as microcephaly, hydrocephaly, and skeletal deformities underscore the toxicity of Flubendiamide. These results align with previous studies on various pesticides, reinforcing the need for stringent regulatory measures and careful application to mitigate potential health risks to non-target organisms, including humans. Continued research is imperative to unravel the molecular mechanisms of Flubendiamide's teratogenic effects and to develop safer pesticide usage practices.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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