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Embryotoxic and Teratogenic Effects of Pyriproxyfen on Developing Chick Embryos (*Gallus domesticus*).

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KEYWORDS

Pyriproxyfen, Insect growth regulator, Embryotoxicity, Teratogenicity, Skeletal abnormalities, Chick embryo.

ABSTRACT:

Introduction: Insecticides are extensively used in modern agriculture to improve crop productivity; however, their widespread application has raised serious concerns regarding potential health risks to non-target organisms, particularly during sensitive stages such as embryonic development. Pyriproxyfen, a juvenile hormone analog insect growth regulator, is widely employed for controlling agricultural pests and disease vectors due to its high specificity and prolonged residual effectiveness. Despite its broad usage, information.

Objectives: The present study was designed to assess the embryotoxic, teratogenic, and skeletal effects of pyriproxyfen using the chick embryo (*Gallus domesticus*) as an experimental model.

Methods: Freshly fertilized chick eggs were incubated under standardized conditions and randomly allocated into control, low-dose (0.0025 µg/egg), and high-dose (0.005 µg/egg) groups. Pyriproxyfen was administered into the egg air sac on the 7th day of incubation, whereas control eggs received corn oil alone. Embryos were harvested on the 18th day of incubation and evaluated for mortality, body weight, morphometric measurements, gross morphological abnormalities, and skeletal development using Alizarin Red S staining. Statistical analyses were conducted using Student's *t*-test and the Mann–Whitney U test, with significance set at $p \leq 0.05$.

Results: Pyriproxyfen exposure produced a significant, dose-dependent increase in embryonic mortality, accompanied by pronounced reductions in body weight and morphometric parameters. Treated embryos displayed a range of teratogenic abnormalities, including limb deformities, beak defects, hematoma, subcutaneous hemorrhage, edema, and sparse body hair. Skeletal evaluation further revealed impaired cranial ossification, vertebral abnormalities, scoliosis, and limb skeletal defects, indicating disruption of normal osteogenic processes.

Conclusions: *In ovo* exposure to the pyriproxyfen-based insecticide Sumiprempt induced significant embryotoxic and teratogenic effects in chick embryos (*Gallus domesticus*), characterized by dose-dependent reductions in body weight, altered morphometric parameters, and multiple morphological abnormalities. These findings indicate potential developmental risks of pyriproxyfen formulations to non-target organisms and emphasize the need for careful regulation, controlled use, and further evaluation of chronic and low-dose exposures to support chemical health risk assessment.

1. Introduction

Insecticides are widely employed in modern agricultural practices to enhance crop productivity and to control insect pests, animal pests, and vectors of infectious diseases. Although they play a crucial role in integrated pest management strategies, the extensive and often indiscriminate use of insecticides has raised significant concerns regarding their detrimental effects on non-target organisms, including humans and wildlife. In India, insecticides constitute approximately 80% of total pesticide consumption, with an estimated annual usage of around 85,000 metric tons. Exposure to insecticidal compounds has been linked to teratogenic effects, morphological abnormalities, and a wide range of physiological and pathological disorders in various species. According to the World Health Organization (WHO), nearly 3 million cases of pesticide poisoning are

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reported worldwide each year, resulting in approximately 250,000 deaths, underscoring the severity of pesticide-related health risks [1].

Based on their chemical structure, toxicological properties, and mechanisms of action, insecticides are classified into several major groups, including organophosphates, carbamates, chlorinated hydrocarbons, neonicotinoids, synthetic pyrethroids, and insect growth regulators (IGRs) [2]. In recent decades, the use of insect growth regulators has increased markedly owing to their targeted mode of action, high efficacy at low application rates, and perceived selectivity toward insect developmental processes. IGRs disrupt normal growth, molting, and metamorphosis by interfering with hormonal regulation or chitin synthesis. However, accumulating evidence indicates that exposure to these compounds may pose significant developmental and ecological risks, particularly following chronic exposure or during sensitive early-life stages, raising concerns about their potential effects on non-target organisms.

Insect growth regulators (IGRs) are a class of pesticides designed to disrupt normal insect development by interfering with chitin synthesis, molting, and metamorphosis. Pyriproxyfen [2-(1-methyl-2-(4-phenoxyphenoxy)ethoxy)] is a potent pyridine-based, aromatic, non-terpenoidal compound that effectively inhibits insect embryogenesis and prevents adult emergence. Acting as a juvenile hormone analog, pyriproxyfen exerts its effects through endocrine disruption, leading to dysregulation of hormonal signaling pathways essential for growth, development, and reproduction. Such interference with endocrine homeostasis has raised toxicological concerns due to the potential for adverse developmental and physiological effects not only in target insect species but also in non-target organisms [3-4].

The chick embryo (*Gallus domesticus*) is a well-established and highly sensitive experimental model for the assessment of chemically induced developmental toxicity. Its rapid and precisely characterized embryonic development, combined with ease of experimental manipulation and cost-effectiveness, makes it particularly suitable for evaluating embryotoxic and teratogenic effects of environmental contaminants. Despite the widespread application of pyriproxyfen, comprehensive and systematic studies examining its embryotoxic and developmental impacts using the chick embryo model remain scarce, highlighting a significant gap in current toxicological knowledge.

2. Objectives

The objective of the present study was to evaluate the embryotoxic, teratogenic, and skeletal effects of the pyriproxyfen-based insecticide Sumiprempt following in ovo exposure in chick embryos (*Gallus domesticus*). Specifically, the study aimed to assess dose-dependent impacts on embryonic mortality, body weight, morphometric parameters, gross morphological abnormalities, and skeletal development, in order to elucidate the potential developmental risks of pyriproxyfen formulations to non-target organisms and contribute to chemical health risk assessment.

3. Methods

Experimental Animals:

Freshly fertilized, zero-day-old eggs of *Gallus domesticus* were utilized in the present investigation. Eggs of uniform size and weight were selected to maintain experimental consistency. All experimental procedures were carried out in strict accordance with the guidelines approved by the Institutional Animal Ethics Committee (IAEC), IIS (Deemed to be University), Jaipur (IAEC Registration No. IAEC/2023/I/3).

Chemical:

The commercial insecticide pyriproxyfen (technical grade) was procured from Sigma-Aldrich. Pyriproxyfen [2-(1-methyl-2-(4-phenoxyphenoxy)ethoxy) pyridine; CAS No. 95737-68-1] is a juvenile hormone analog and insect growth regulator widely used for the control of agricultural pests and disease vectors. Corn oil was used as the vehicle for dose preparation.

Dose Preparation and Mode of Administration:

Pyriproxyfen was prepared in corn oil, and the experimental dose levels were determined based on preliminary dose-ranging studies. Fertilized eggs received a single administration of pyriproxyfen on the 7th day of incubation via the egg air sac at doses of 0.0025 µg/egg (low dose) and 0.005 µg/egg (high dose). Control eggs were treated with an equivalent volume of corn oil alone. The injection volume was standardized at 100 µL per egg across all experimental groups.

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Experimental Design:

A total of 180 freshly fertilized chicken eggs were obtained from a local hatchery in Ajmer, India. The eggs were disinfected using 70% alcohol and incubated horizontally on metal racks in a BOD incubator maintained at 37.5 °C with 70–75% relative humidity. Eggs were turned twice daily to ensure uniform embryonic development, and humidity levels were maintained by placing a water-filled beaker inside the incubator.

On the 7th day of incubation, eggs were candled to assess fertility, and 20 unfertilized eggs were discarded. The remaining 160 fertilized eggs were randomly assigned to three experimental groups (n = 40 per group): control (corn oil), low-dose pyriproxyfen (0.0025 µg/egg), and high-dose pyriproxyfen (0.005 µg/egg). All treatments were administered as a single injection into the egg air sac using a sterile insulin syringe.

The 7th day of incubation was selected for dosing as it coincides with the initiation of organogenesis and the development of the chorioallantoic membrane, allowing effective absorption of the test compound. Following administration, the injection sites were sealed with paraffin wax, eggs were appropriately labeled, and incubation was continued until the 18th day.

Embryo Collection:

On the 18th day of incubation, the eggs were carefully opened, and the embryos were gently removed and rinsed with 0.9% saline solution. Each embryo was weighed and systematically examined for gross morphological abnormalities. Morphometric parameters were measured, and representative embryos were documented through photography. Thereafter, the embryos were fixed in 95% ethanol in preparation for skeletal analysis.

Morphological, Morphometric and Skeletal Assessment:

Embryos were assessed for mortality, body weight, and gross morphological abnormalities. Morphometric parameters, including crown–rump length, beak length, and neck length, were measured in centimetres. Endo skeletal development was evaluated using Alizarin Red S staining to visualize ossification patterns and identify skeletal anomalies. Photographic documentation was carried out using a Nikon D5200 digital camera.

Statistical Analysis:

All data are presented as mean ± standard error of the mean (SEM). Statistical comparisons between the control and treatment groups were performed using Student's *t*-test, whereas embryonic mortality data were analyzed using the nonparametric Mann–Whitney *U* test. Statistical analyses were carried out using IBM SPSS Statistics version 22, and results were considered statistically significant at $p \leq 0.05$.

4. Results

Embryonic mortality on day 18 was elevated in pyriproxyfen-treated groups relative to controls, with a clear dose-dependent increase (Table 1). Correspondingly, mean embryonic body weight was significantly lower in treated groups compared to controls, with the greatest reduction observed in the high-dose group, indicating dose-dependent growth inhibition.

Morphological and Morphometric Investigations

Morphological Anomalies

Embryos from both control and fenpropathrin-treated groups were examined on the 18th day of incubation for external morphological alterations. Control embryos exhibited normal development of the head, eyes, beak, feathers, and limbs, with no major teratological abnormalities (Figure 1A–B). Embryos in the control group (corn oil, n = 36) exhibited a very low incidence of morphological abnormalities, with only occasional beak defects (2.7%) and sparse body hair (5.5%). In contrast, embryos exposed to fenpropathrin showed a higher frequency of developmental anomalies, with effects increasing in a dose-dependent manner.

In the low-dose pyriproxyfen treated group (0.0025 µg/egg; n = 34), hematoma, subcutaneous hemorrhage, and sparse body hair were each observed in 5.8% of embryos, while beak defects and edema were noted in 2.9% of embryos. Limb deformities were the most prominent abnormality in this group, occurring in 17.6% of embryos.

In the high-dose pyriproxyfen treated group (0.005 µg/egg; n = 32), the incidence and severity of

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malformations were further elevated. Hematoma and subcutaneous haemorrhage were each observed in 6.2% of embryos, while beak defects and edema occurred in 6.2% of embryos. Sparse body hair was recorded in 9.3% of embryos, and ectopic visceral abnormalities were observed in 3.12% of embryos. Limb deformities were the most frequently observed malformation, affecting 21.8% of embryos. No cases of head enlargement, exencephaly, anophthalmia, or sacral hygroma were observed in any experimental group.

Overall, the data demonstrate a dose-dependent increase in the incidence and diversity of external morphological abnormalities following pyriproxyfen exposure during embryonic development.

Morphometric measurements

Fetal growth parameters measured on day 18 showed a dose-dependent reduction in pyriproxyfen-treated embryos compared with controls. Crown–rump length, beak length, and neck length were progressively decreased in the low- and high-dose groups, indicating significant growth retardation following pyriproxyfen exposure.

Endo-skeletal investigations

Skeletal malformations

Alizarin Red S staining was performed on 18-day-old chick embryos to assess endoskeletal development in control and pyriproxyfen-treated groups. In the control embryos, endoskeletal structures were well-developed, with extensive ossification observed in most cranial elements. The vertebral column was normally aligned, showing no lateral curvature. The sternum was enlarged and entirely cartilaginous. The pelvic girdle exhibited complete ossification of the ischium and ilium, while the pubis remained largely cartilaginous with partial mid-region ossification and slight curvature. Limb bones—including the humerus, radius, ulna, femur, tibiotarsus, carpometacarpus, and tarsometatarsus—were fully ossified except at the epiphyseal regions, and most phalanges displayed complete ossification.

The incidence of endoskeletal malformations observed in control and fenpropathrin-treated embryos is summarized in Table 4. Control embryos showed normal skeletal development.Skeletal examination revealed no major abnormalities in control embryos, except for occasional pubis malformation (2.7%) and limb malformations with incomplete digit ossification (5.5%).

In contrast, pyriproxyfen-treated embryos exhibited a dose-dependent increase in skeletal anomalies. In the low-dose group, incomplete ossification of thoracic and caudal vertebrae was observed in 5.8% of embryos, while incomplete ossification of the ilium and ischium (8.8%), pygostyle and tail (11.7%), and limb malformations with incomplete digit ossification (11.7%) were also noted.

The high-dose group showed a further increase in both incidence and severity of skeletal defects, including skull abnormalities (3.12%), incomplete ossification of thoracic and caudal vertebrae (9.3%), ribs (3.12%), ilium and ischium (12.5%), pygostyle and tail (12.5%), pubis malformation (6.2%), and limb malformations with incomplete ossification of digits (15.6%). Overall, the findings demonstrate a clear dose-dependent disruption of skeletal development following fenpropathrin exposure.

Overall, the skeletal data presented in Table 4 demonstrate a dose-dependent increase in abnormalities affecting both the axial and appendicular skeleton following pyriproxyfen exposure.

5. Discussion

Insecticides are widely employed in agriculture and public health, but their extensive use has led to increased environmental contamination and potential health risks to non-target organisms. Exposure to insecticidal compounds during critical stages of embryonic development has been linked to embryotoxicity, growth retardation, and teratogenic effects. The present study evaluated the embryotoxic, teratogenic, and skeletal effects of pyriproxyfen, an insect growth regulator, using the chick embryo model, which is well-established for developmental toxicity assessment.

In this study, administration of pyriproxyfen on the 7th day of incubation caused a significant, dose-dependent increase in embryonic mortality, accompanied by reductions in body weight and morphometric parameters. These findings indicate that pyriproxyfen exerts lethal and growth-inhibiting effects during the organogenesis phase of embryonic development. Comparable dose-dependent increases in embryonic mortality and growth retardation have been reported with other insecticides,

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including dichlorvos, atrazine-based formulations, bendiocarb, malathion, endosulfan, and abamectin, underscoring the susceptibility of embryos to chemical exposure during early developmental stages [7–10].

The observed growth retardation in pyriproxyfen-treated embryos may result from impaired nutrient utilization, metabolic disruption, or altered cellular proliferation during embryogenesis. Similar reductions in embryonic growth have been reported following exposure to combined pesticide formulations and other insect growth regulators, suggesting that interference with embryonic metabolism and energy allocation is a key mechanism underlying insecticide-induced developmental toxicity [11–14]. The dose-dependent increase in mortality further corroborates previous findings of heightened lethality in chick embryos exposed to insecticides during mid-incubation [15,16].

The skeletal system was notably affected by pyriproxyfen exposure. Alizarin Red S staining revealed disruptions in ossification and skeletal patterning, particularly in the vertebral column, pelvic girdle, and limb bones. Treated embryos exhibited incomplete cranial ossification, vertebral deformities, scoliosis, malformed pelvic structures, and limb abnormalities, with severity increasing at higher doses. These results are consistent with earlier reports showing that insect growth regulators interfere with both endochondral and intramembranous ossification, processes essential for normal skeletal development [17–19].

Exposure on the 7th day of incubation represents a critical window for skeletal development, as mineralization of long bones begins shortly thereafter. Disruption during this sensitive period may therefore lead to permanent skeletal defects. Similar skeletal abnormalities, including vertebral malformations, reduced ossification, and limb deformities, have been observed following exposure to deltamethrin, quinalphos, and other insecticidal formulations in avian embryos [20–22].

In addition to skeletal defects, pyriproxyfen induced a spectrum of gross morphological abnormalities, including exencephaly, ectopic viscera, limb deformities, edema, hemorrhage, beak defects, and failure of yolk sac retraction. These malformations are consistent with insecticide-mediated disruption of neural development, vascular integrity, and tissue differentiation [27–30]. Similar teratogenic effects have been reported following exposure to cypermethrin, abamectin, and other insecticidal compounds, highlighting the teratogenic potential of these chemicals [28-29].

Overall, the findings demonstrate that pyriproxyfen induces significant embryotoxic, teratogenic, and skeletal abnormalities in chick embryos in a dose-dependent manner during organogenesis. These results underscore the developmental risks associated with exposure to insect growth regulators and emphasize the need for careful evaluation of insecticides to mitigate potential impacts on embryonic development and environmental safety.

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Table 1. Mortality rate (%) and weight of embryos at the 18th day of incubation in different groups

Experimental Group	Doses µg/egg (100µl)	No. of egg	No. of Live embryos	No of Dead embryos	Mortality rate (%)	Weight of embryos (g)
Control	0	40	36	4	10%	35.40±0.80
Treated 1 (low dose)	0.0025	40	34	6	15%	23.59±0.72*
Treated 2 (high dose)	0.05	40	32	8	20 %	21.93±1.25*

Data are presented as mean ± standard error (SE). Statistical significance relative to the control group was assessed at $p \leq 0.05$, using the Mann–Whitney *U* test for embryonic mortality and Student's *t*-test for body weight comparisons. All analyses were performed with IBM SPSS Statistics version 22.

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Table 2. Frequency percentage of morphological anomalies (%) observed at the 18thday of incubation in different groups.

Morphological Malformations	**Control (Corn oil) 100µl** (n = 36)	**Fenpropathrin**	
		Low dose 0.0025 µg per egg (n= 34)	**High dose 0.005 µg per egg** (n= 32)
Head enlargement	0%	0%	0%
Exencephaly	0%	0%	0%
Hematoma	0%	5.8% (2)	6.2% (2)
Anopthalmia	0%	0%	0%
Beak defects	2.7% (1)	2.9% (1)	6.2% (2)
Sparse body hair	5.5% (2)	5.8% (2)	9.3% (3)
Edema	0%	2.9% (1)	6.2% (2)
Ectopic visceral	0%	0%	3.12% (1)
Subcutaneous haemorrhage	0%	5.8% (2)	6.2% (2)
Sacral hygroma	0%	0%	0%
Limb deformities	0%	17.6% (6)	21.8% (7)

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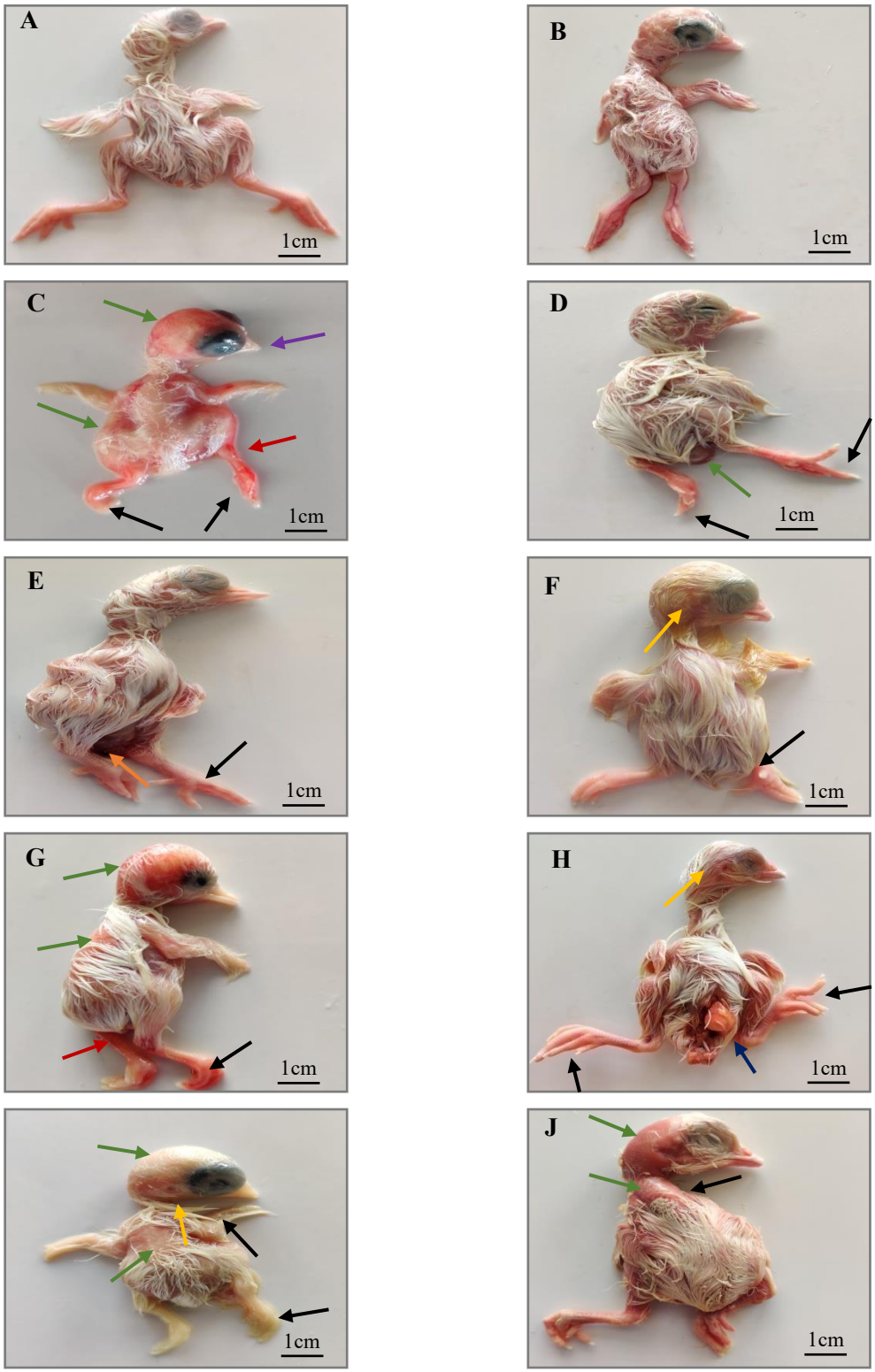

Figure. (1). Photographs of 18-day-old chick embryos from the control group (A, B), the low-dose (0.0025 µg per egg) pyriproxyfen-treated group (C, D, E, F), and the high-dose (0.005 µg per egg) pyriproxyfen-treated

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group (G, H, I, J), administered on embryonic day 7. A and B show normal morphology. C showing a beak defect, such as a short beak (purple arrow), sparse body hair on the head and lower body (green arrow), discoloration of the limbs (dark red arrow), and limb deformities, including deformed hindlimbs (black arrows). D displays subcutaneous hemorrhage (orange arrow) and limb deformities, such as clinodactyly in the left hindlimb and a crooked right hindlimb (black arrows). E illustrates subcutaneous hemorrhage (orange arrow) and limb deformities, including flexed hindlimbs (black arrow). F highlights a hematoma under the eye (yellow arrow) and a limb deformity, shortened hindlimb (black arrow). G shows sparse body hair on the head and lower body (green arrows), discoloration of the limbs (dark red arrow), and a limb deformity, such as clinodactyly (black arrow). H presents a hematoma under the eye (yellow arrow), ectopia visceral (dark blue arrow), and a limb deformity, including flexed hindlimbs (black arrows). I showing growth retardation along with malformations, such as sparse body hair on the head and lower body (green arrows), a hematoma under the eye (yellow arrow), and deformed limbs (black arrows). J displays sparse body hair on the head, neck, and limb deformity such as absence of forelimbs.

Table 3. Morphometric measurements of 18 ED chick embryos after treatment with fenpropathrin at 7th day of Incubation.

Data are represented as Mean± S.E. Statistical difference from the control : * significant at p≤0.05, by

	Groups		
Fetal growth parameters	**Control**	**Treated 1** **Low dose** **(0.0025µg per egg)**	**Treated 2** **High dose** **(0.005µgper egg)**
Crown rump length (cm)	9.82±0.11	9.45±0.16	9.20±0.13
Beak length (cm)	1.68±0.14	1.23±0.05	0.93±0.02
Neck length (cm)	1.94±0.12	1.53±0.14	1.46±0.18

Student's t test. using IBM SPSS statistics 22 software

Table 4 Endo-skeletal malformations of 18th day old chick embryos in different groups (percentage %).

Skeletal Malformations	**Control (Corn oil) 100µl** (n = 36)	**Fenpropathrin** **0.0075 µg per egg** (n=34)	**0.015 µg per egg** (n=32)
Skull abnormality	0%	0%	3.12% (1)
Incomplete ossification of cervical vertebrae	0%	0%	0%
Incomplete ossification of thoracic vertebrae	0%	5.8% (2)	9.3% (3)
Scoliosis	0%	0%	0%

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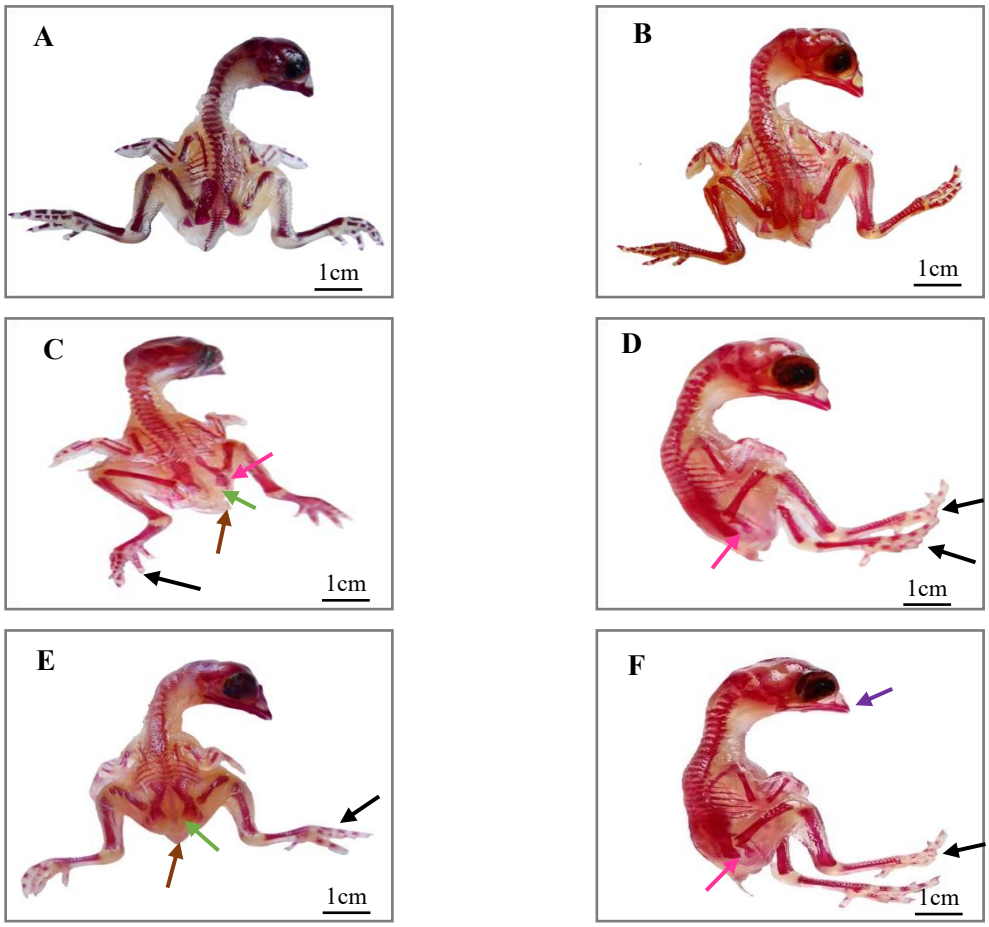
Incomplete ossification of ribs	0%	0%	3.12% (1)
Incomplete ossification of caudal vertebrae	0%	5.8% (2)	9.3% (3)
Incomplete ossification of ilium and ischium	0%	8.8% (3)	12.5% (4)
Incomplete ossification of pygostyle and tail	0%	11.7% (4)	12.5% (4)
Pubis malformation	2.7% (1)	0%	6.2% (2)
Limb malformation and incomplete ossification of digits	5.5% (2)	11.7% (4)	15.6% (5)



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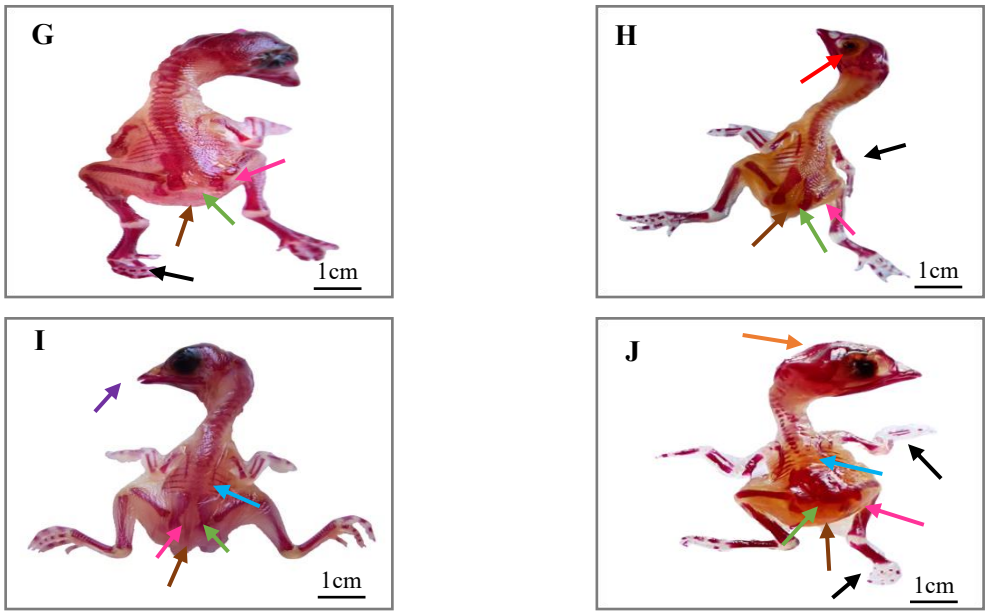

Figure (2). Photograph of endo-skeletal system of 18th day old chick embryos from from the control group (A, B), the low dose (0.0025 µg per egg) pyriproxyfen-treated group (C, D, E, F), and the high-dose (0.005 µg per egg) pyriproxyfen-treated group (G, H, I, J), administered on embryonic day. In the control group (A, B) normal skeletal development and ossification were observed. C displays fusion of ischium and ilium (pink arrow), poor ossification of caudal vertebrae (green arrow), poor ossification of pygostyle (brown arrow) and crooked toes (black arrow). D exhibits abnormal formation of ischium (pink arrow) and flexed toes (black arrow). E shows poor ossification of caudal vertebrae (green arrow), poor ossification of pygostyle (brown arrow) and flexed toes (black arrow). F depicts short beak (purple arrow), abnormal formation of ischium (pink arrow) and flexed toes (black arrow). G reveals fusion of ischium and ilium (pink arrow), absence of caudal vertebrae (green arrow), absence of pygostyle (brown arrow) and crooked toes (black arrow). H shows poorly developed eye (red arrow), absence of ilium (pink arrow), poor ossification of caudal vertebrae (green arrow), poor ossification of pygostyle (brown arrow) and deformed hindlimb (black arrow). I exhibit short beak (purple arrow), poor ossification of thoracic vertebrae (light blue arrow), poor ossification of ilium (pink arrow), poor ossification of caudal vertebrae (green arrow) and poor ossification of pygostyle (brown arrow) and disoriented pelvic girdle (light orange arrow). J displays loss part of skull (orange arrow), poor ossification of thoracic vertebrae (light blue arrow), absence of caudal vertebrae (green arrow), absence of pygostyle (brown arrow) and thinning of radius and ulna, crooked toes (black arrows).