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Effect of Commercial Formulation Containing (Fenpropathrin and Pyriproxyfen) on the Development of Chick Embryos

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Abstract

Insecticides are widely used for pest control in agriculture, urban and domestic environments, as well as in veterinary medicine. The current study aimed to determine the possible teratological effects and skeletal malformation induced by the formulation insecticide Sumiprempt containing Fenpropathrin with Pyriproxyfen during the organogenesis period of the developing chick (*Gallus domesticus*) embryo. The eggs were divided into four separate groups; first was considered as control (corn oil) was administered and the other three were experimentally treated groups, *i.e.*, treated Group 1 was given low dose (0.01µg/egg), treated Group 2 was given a medium dose (0.05µg/egg) and the treated Group 3 was given a high dose (0.1µg/egg). The dosage volume was 100µl, and the dose was given on the 7th day of incubation. The morphometric changes, gross morphological malformations and skeletal abnormalities were studied on the 18th day of incubation of chick embryos. Administration of formulation insecticide at various doses resulted in a significant increase in mortality rate, reduction in the weight and length of embryos in a dose-dependent manner. Visible teratological anomalies such as excencephaly, defects in beak, hematoma, anopthalmia, ectopia visceral, delayed retraction of yolk sac and malformed limbs were observed in chick embryos. Additionally, formulation of insecticide led to many skeletal malformations in the skull, limbs, ribs, vertebral column, incomplete ossifications and reduction in the length of bones. The obtained results in chick embryos indicated that exposure to formulation insecticide may be potentially harmful during developmental stages and may lead to undesirable consequences in vertebrates, therefore, its use should be mindful.

Major Findings: The study revealed that exposure to the formulation insecticide Sumiprempt (Fenpropathrin + Pyriproxyfen) during organogenesis in chick embryos resulted in a dose-dependent increase in mortality, reduced body weight and length, and significant teratogenic anomalies such as exencephaly, beak deformities, hematoma, anophthalmia, ectopia viscerale, delayed yolk sac retraction, and limb malformations. Skeletal analysis showed defects in the skull, limbs, ribs, vertebral column, incomplete ossification, and shortened bones, indicating the potential developmental toxicity of the insecticide.

Keywords: Chick Embryos, Fenpropathrin, Insecticide, Organogenesis, Pyriproxyfen, Teratological

1. Introduction

Insecticides are widely employed in agricultural practices to acquire larger crop yields and to manage insects, animals and vector-borne diseases. Previous findings suggested that insecticides might cause detrimental effects because of their possible toxicity to both animals and human beings. However, these insecticides continue to play an important

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part in a wide range of pest control activities across the world. In India, insecticides constitute about 80% of overall pesticides, and farmers use approximately 85,000 tons of insecticides annually. Exposure to these compounds results in malformations and health issues in both invertebrate and vertebrate species, including humans. According to WHO statistics, there are about 3 million human pesticide poisonings each year, with around 250,000 deaths[1].

Insecticides are categorised into many groups depending on their chemical nature, mechanisms of toxic action, and penetration. Insecticides, which are majorly used in agricultural fields are neonicotinoid, methyl carbamates, organophosphorus compounds, chlorinated hydrocarbons and synthetic pyrethroids[2] but over the last decades, the agriculture industry has shown growing interest in a group of systemic insecticides such as synthetic pyrethroids and insect growth regulators and their formulations are currently available in the market. Pyrethroids are insecticides which are synthesised from pyrethrins. Pyrethroids are being employed in various sectors like veterinary, agriculture, horticulture and for household purposes for pest management. Based on their chemical composition, exposure symptoms, target sites, and toxicological consequences, pyrethroids can be divided into Types I and II classes.

Fenpropathrin (α-cyano-3-phenoxybenzyl-2, 2, 3, 3-tetramethylcyclo propane carboxylase) is a Type II synthetic pyrethroid used in agriculture, veterinary medicines and household purposes. It alters the gating kinetics of voltage-sensitive sodium channels, disrupting neuronal function, causing acute neurotoxicity in both insects and non-target species[3].

Insect growth regulators are chemicals used to kill pests and prevent insects from moulting by inhibiting the production of chitin and influencing the growth and development of insects. Pyriproxyfen 2-(1-methyl-2-(4-phenoxyphenoxy)ethoxy) is an efficient pyridine-based, aromatic, non-terpenoidal inhibitor of insect embryogenesis and adult emergence in insects. Pyriproxyfen, being a juvenile hormone analogue, works as an endocrine disruptor, altering the endocrine system and eventually causing harmful health effects in target organisms[4].

Sumiprempt, a formulation insecticide containing a combination of Pyriproxifen 5% EC+Fenpropathrin 15% EC. Sumiprempt insecticide is a low-cost, highly effective insecticide for agricultural use, compared to other insecticide products and has a rapid action. It is widely recommended to control whitefly and bollworms in cotton, whitefly, shoot and fruit borer in brinjal, and whitefly and fruit borer in okra and chilli.

It is well documented that commercial pesticides have negative impacts on birds' growth[5]. The amount of pesticide intoxication on eggs and birds through ingestion, absorption, or inhalation was described in previous research findings[6].

The chick embryo is a suitable avian model for studying the pharmacology and teratogenicity of various chemicals given during embryonic development. This model is less expensive, easier to monitor and gives quick results. The chronology of chick growth has been illustrated[7,8], which is important for standardising chick development. The effect of formulation insecticide (Sumiprempt) containing Pyriproxyfen with Fenpopathrinon chick developmental studies has not been reported so far.

The present study was aimed at investigating the possible embryotoxic effects of the commercially available formulation insecticide Sumiprmpt (Fenpropathrin+Pyriproxyfen) in the developing chick embryo. Several studies suggest that the inert ingredients in insecticide formulations increase the toxicity of the active ingredients[9]. Hence, a commercial formulation insecticide was chosen for the present study.

2. Materials and Methods

2.1 Animals

Fertilised eggs of *Gallus domesticus* of zero day were selected for the experiment. Eggs of the same size and weight were chosen for the study. The experimental study was performed under the guidelines approved by the Institutional Animal Ethics Committee (IAEC), IIS deemed to be a University (IAEC- Reg No – IAEC/2023/I/3).

2.2 Chemicals

The commercial available insecticide formulation named as Sumiprempt EC (manufactured by Sumitomo Chemical Co, Limited, Chennai, India) contains Fenpropathrin (15%) and Pyriproxyfen (5%) was used in the study. Fenpropathrin (α-cyano-3-phenoxybenzyl-2, 2, 3, 3-tetramethylcyclo propane carboxylase) is a synthetic pyrethroid insecticide which controls a wide range of insects and pests on a variety of crops. Pyriproxyfen2-(1-methyl-2-(4-phenoxyphenoxy)ethoxy) is an insect growth regulator insecticide widely utilised in agriculture and residential pest management all over the world.

2.3 Mode of Administration

Formulation insecticide (Sumiprempt) was diluted in corn oil, and three doses, namely low, medium and high, were made after performing a preliminary test for the selection of doses. A single dose of 0.01, 0.05 and 0.1 µg/ egg (low, medium and high dose, respectively) of formulation insecticide diluted in corn oil was administered into the airspace of fertilised eggs of *Gallus domesticus* on the 7th day of incubation. Eggs in the control group were similarly treated with corn oil. The dosage volume of formulation insecticide and corn oil was maintained at 100µl per egg.

2.4 Experimental Design

For the experimental investigation, 180 fertilised eggs of *Gallus domesticus* (0 day of incubation) were purchased from a hatchery at Ajmer, India. Eggs of the same size were clustered together, and all egg shells were sterilised with 70% alcohol to remove the external contamination. To maintain the appropriate humidity, a one-litre beaker filled with distilled water was placed at the bottom of the incubator. The eggs were placed horizontally on metal racks and rotated twice every day. On the seventh day, eggs were candled, and 20 unfertilised eggs were removed from the experiment as they did not show any development of embryos. The remaining eggs were divided into four groups, with 40 eggs in each group.

The first group was the control, in which (100µl) corn oil was injected, and the remaining three were treated groups. treated Group 1 received (0.01µg/egg) insecticide, treated Group 2 received (0.05µg/egg) insecticide and treated Group 3 received (0.1µg/egg) insecticide. The eggs were injected into the air sac with100µl dose of insecticide on day 7th, the day of incubation, with the help of an insulin syringe.

The 7th day of incubation of the chick embryo was chosen since organogenesis begins with the development of the chorioallantoic membrane, and the vascular network begins to be established next to the shell membrane, facilitating uptake of any toxicants.

Treated and control eggs were sealed after the treatment with paraffin wax, labelled and incubated at 37.5°C with a relative humidity of 70-75% till the 18th day of incubation in a BOD incubator.

2.5 Embryo Collection

The treated and control eggs were opened on the 18th day of incubation, and the embryos were carefully removed and washed with 0.9% saline. Each embryo was individually weighed and examined for morphometric measurement, teratological investigations and was photographed. The embryos were fixed in 95% alcohol for alizarin preparation to study skeletal defects.

2.6 Investigated Parameters

On the 18th day of incubation, the embryos were observed for mortality rate, weight of embryos and gross morphological parameters. Morphological parameters: crown rump length, beak length, neck length, humerus length, radius and ulna length, metacarpus length, femur length. The fibula length, metatarsus length in (cm) were recorded.

Endo-skeletal investigations: Alizarin red S indicator was used for staining the skeleton of chick[10]. Representative samples were photographed with a Nikon D5200 camera. Measurement of the length of the humerus, radius, ulna, femur, tibia and fibula was also recorded.

2.7 Statistical Analysis

Statistical analysis was carried out on the above-mentioned parameters. Values have been expressed as Mean ±Standard Error of Mean (SEM). The collected data of all parameters were statistically examined using normal distribution (Student's T test), and the Mann-Whitney U test was used to compare mortality rates using IBM SPSS Statistics 22 software. The differences in $p \leq 0.05$ are considered to be statistically significant compared to the control.

3. Results

The mortality rate was recorded at the end of experimentation, *i.e.*, 18th day of incubation, by calculating the number of dead embryos that died normally or as a result of formulation administration. The number of both live and dead chick embryos, illustrated in Table 1. The results revealed mortality percentage of embryos in ovo injected by formulation insecticide (fenpropathrin+pyriproxyfen) treated groups at a different doses, *i.e.*, low, medium and high doses (0.01, 0.05 and 0.1µg per egg), respectively was higher as compared to the control group and the mortality rate increased as the concentration of dose increased.

Furthermore, the body weight of embryos was recorded at the 18th day of incubation (Table 1).

Administration of formulation insecticide resulted in a dose-dependent significant decrease in the weight of the embryo as compared to control values.

Our results are similar to the results of Szabo *et al.*[11], wherein a higher mortality rate in chick embryos was observed when administered with varying doses of dichlorvos and an atrazine-containing formulation given on the 0th day of incubation. Exposure of flufenoxuron, an insect growth regulator, at a higher dose (20µg/egg) resulted in a 50% mortality rate, and a lower dose (10µg/egg) of flufenoxuron led to a 20% mortality rate in chick embryos. The high rate of embryonic mortality may have resulted from interference in the metabolic process[12].

Petrovova *et al.* studied the effects of bendiocarb on the development of the chick embryo and noted that bendiocarb exposure increased the mortality rates in treated embryos and decreased the rate of hatching[13]. By the results of our study, abamectin, a bio-insecticide, injected with 100 µL of insecticide at different concentrations (360, 540, 900 and 1800 ppm) two days after incubation of the chick embryo, resulted in lower body weight and a significant increase in the mortality rate[14]. Likewise, dose-related body weight reductions were observed in embryos of chicks exposed to malathion at a dose of 5 mg/egg[13,15]. A reduction in body weight of chick embryos that are related to bendiocarb concentrations when administered on embryonic days 5 and 10.

Mobarak and Al-Asmari reported dose associated growth retardation of 6th and 12th day old chick embryo after treatment of insecticide endosulfan and also indicated significant reductions in embryonic wet body weight. The insecticide's effect on growth retardation in developing chick embryos may be attributed to as a result of strong inhibitory effect on metabolism, such as gluconeogenesis and disruption of the retinoid signalling pathway in cells that play an important role in cell proliferation, differentiation and development[16].

Furthermore, Uggini *et al.*[17] discovered that injecting 0.1µg of a combination pesticide formulation containing chlorpyrifos and cypermethrin on the 0th day of incubation of chick hatchlings resulted in a significant decline in body weight. Pinakin *et al.*[18] observed pesticide-induced growth retardation of chick embryos after injecting 100µl of 54 ppm lufenuron, an insect growth regulator on 0th day fertilised eggs of chick embryos.

Moreover Sahu *et al.*[19] found that injecting 25 and 35g of dimecron into unincubated eggs lowered the body weights of 4th, 8th, and 14th day old chick embryos and also shows growth retardation along with significant teratogenic defects during organogenesis[19]. According to Romanoff, low nutritional levels in the middle of the developmental phase may also contribute to loss of weight in embryos[20].

3.1 Morphological and Morphometric Investigations

3.1.1 Morphological Anomalies

The embryos in the control group and the treated groups were studied on the 18th day of incubation. Normal development of head, eyes, beak, feathers and limbs was seen in control groups, as illustrated in Figure 1 (A and B).

Representative figures of various external morphological anomalies recorded in embryos of treated Group 1 with the low dose (0.01µg/egg) of formulation insecticide have been shown in Figure 1 (C, D) in which chick embryo show 18% hemorrhage under the skin, 9% with the short beak, 13% exencephaly, 11% with head enlargement and flexed hindlimb. The most frequent malformation recorded with 23% omphalocele and 21% limb deformities. The other abnormalities were associated with 19% hematoma (Table 2).

Treated Group 2 with medium dose (0.05µg/egg) of formulation insecticide resulted in various morphological

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

Experimental Group	Doses/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 %	38.94±0.08
Treated 1 (low dose)	0.01	40	28	12	30 %	28.82±0.06*
Treated 2 (medium dose)	0.05	40	23	17	42 %	23.42±0.02*
Treated 3 (high dose)	0.1	40	17	23	57 %	19.32±0.04*

Data are represented as Mean±S.E. Statistical difference from the control: * significant at p≤0.05, by the Mann-Whitney U test to compare mortality rates and Student's t test to compare weight of embryos using IBM SPSS statistics 22 software.

anomalies of chick embryos shown in Figure 1 (G, H). Embryos exhibited 32% of omphalocoele, 15% of anopthalmia. 32% of limb deformity, which includes clinodactyly, 35% of hematoma under eye, 18% of oedema in the lower body, 13% of short beak and 35% of haemorrhage under skin, although there was a lower incidence of anomalies with 17% of excencephaly and 13% of sparse body hair (Table 2).

The morphological abnormalities encountered in treated Group 3 with high dose (0.1µg/egg) of formulation insecticide include 15% sparse body hair, 19% of excencephaly and 42% of haemorrhage under skin (Table 2). Representative figures of various morphological anomalies of chick embryos have been shown in Figure 1 (I, K) shows embryo with 30% of anopthalmia, 45% of omphalocoele with growth retardation, 21% edema with short beak, 45% limb deformities and 42% of hematoma under eye. The occurrence of malformations in the high-dose treated group was higher compared to other treated groups.

In the current study, exposure of formulation insecticide (fenpropathrin with pyriproxyfen during the organogenesis phase (7th day of incubation) in chick embryos resulted in a number of morphological abnormalities in 18th-day-old chick embryos. The proportionate increase in the number of aberrant embryos was more pronounced when eggs were exposed on the 7th day of incubation, indicating teratogenic susceptibility of the subjected pesticides. Levi[21] and Wells[22] reported that embryonic and fetal phases of embryogenesis are defining characteristics of teratological risks because organs are generally more susceptible to congenital malformations only if subjected during early events of their initiation, or their differentiation and development. In the present study, various types of morphological deformities like exencephaly, anophthalmia, underdeveloped beak, ectopic visceral, limb deformities, oedema, haemorrhage under skin and omphalocoele were observed in treated groups on 18th day old chick embryos.

Uggini *et al.*[17] observed abnormal cell damage in developing embryos while studying the impact of agricultural pesticides on embryonic development and teratogenicity in chick embryos. Anwar investigated that cypermethrin (synthetic pyrethroid) administration at various concentrations (100, 200, and 400) ppm) resulted in morphological deformities in 7th and 11th day incubated chick embryos like reduction in head and eye size, incomplete development and absence of beak in 7th and 11th day incubated chick embryos and the brain, may have been brought on by degenerative alterations in the neurons or might be due to insecticide-induced apoptosis or necrosis[23].

Similar to our results Sardar *et al.*[24] observed that the exposure of lower dose (1.25mg/L) of abamectin, an insecticide in chick embryo resulted in teratological defects like ectopia viscera and limb deformities and a higher

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

Malformations	Groups			
	Control	Treated 1 (Low dose) (0.01µg per egg)	Treated 2 (Medium dose) (0.05µgper egg)	Treated 3 (High dose) (0.1µg per egg)
Anopthalmia	0	0	15	30
Head enlargement	0	11	19	21
Hematoma	0	19	35	42
Short Beak	0	9	13	18
Limb deformities	0	21	32	45
Sparse body hair	0	0	13	15
Ectopic visceral	0	0	15	32
Exencephaly	0	13	17	19
Omphalocoele	0	23	32	45
Haemorrhage under the skin	0	18	35	42
Edema	0	12	18	21

All values were indicated in percentages.

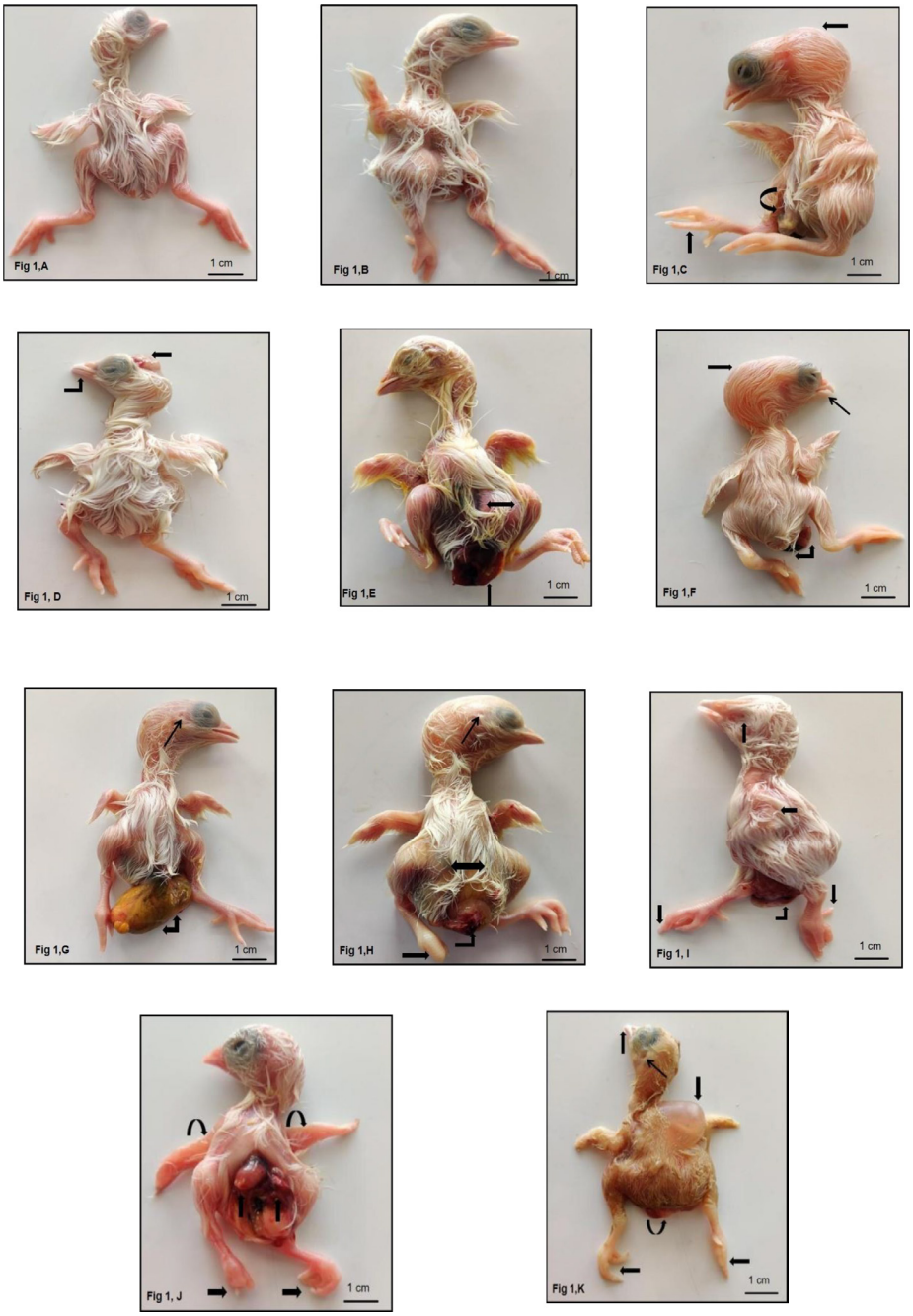


Figure 1. Photograph of 18th day old from control and formulation insecticide treated embryos, control **(A, B)** low dose (0.01 µg/egg) of formulation insecticide treated **(C-E)** medium dose (0.05µg/egg) of formulation treated **(F-H)** and high dose (0.1 µg/egg) of formulation insecticide treated **(I, J, K)**. A and B show normal morphology. **C** showing head enlargement (left arrow), omphalocoele (curved arrow) and flexed limbs (up arrow). **D** showing exencephaly (left arrow) and short beak (bent up arrow). **E.** showing sparse body hair (left-right arrow) and sacral hygroma (up arrow). **F** showing short beak (block arrow), head enlargement (right arrow) and haemorrhage under skin (left up arrow). **G** showing hematoma under eye (block arrow) and omphalocoele (left up arrow). **H** Showing oedema (left right arrow), sacral hygroma (bent up arrow) and clinodactyly (right arrow). **I** show anopthalmia (up arrow), haemorrhage under skin (bent up arrow), absence of forelimbs (left arrow) and flexed hindlimbs (down arrow). **J** showing growth retardation, ectopia visceral (up arrow), scanty feather (curved arrows) and deformed hindlimbs (right arrows). **K** Showing short beak (up arrow), hematoma under eye (block arrow), oedema (down arrow), haemorrhage under skin (curved arrow) and deformed hindlimbs (left arrows).

dose (2.5 mg/kg) of abamectin resulted in failed yolk sac retraction and lowered body weight and embryo length[24]. Furthermore, birth defects such as limb deformities, exencephaly, gastroschisis, and ectopia visceral may be induced by insecticide-mediated vascular perturbation or exogenous collapse of normal development of arteries, veins, and capillaries provided to structures such as the limb, brain, and embryonic intestine[6].

Uggini *et al.*[17] studied the teratogenic effect of the combination insecticide Chloropyrifos (organophosphate) with cypermethrin (synthetic pyrethroid) in chick embryo and found teratological malformations like crooked legs, anopthalmia, beak deformity and twisted phalanges at a dose of 0.05µg/egg injected on the 0th day of incubation[17]. Micropthalmia and defects in the beak might have been induced by suppression of kynurenine formamidase, which inhibits the transformation of tryptophan to essential pyridine nucleotide cofactors in the yolk sac membrane of the embryo. Pesticides interfere with physiochemical processes and lead to morphological alterations[25-27].

3.1.2 Morphometric Measurements

Table 3 shows data on the average crown rump length, beak length and neck length of 18th-day-old chick embryos, injected with both control and formulation insecticide.

The values of average crown rump length, beak length and neck length for an 18th-day-old control chick embryo were 8.91±0.05, 3.45±0.08, and 3.72±0.02, respectively.

Embryos injected with a medium dose (0.05µg/egg) and high dose (0.1µg/egg) showed a significant decrease in the crown-rump length, beak length and neck length as compared to the control values (Table 3).

The harmful impacts of the formulation insecticide dimethoate on the growth of chicken embryos were identified[28]. The findings showed that there was a considerable reduction in the average body mass of embryos. Growth retardation in developing chick could be due to inhibitory effect on metabolism[29]. Moreover, Pushpanjali *et al.*[30] suggested that suppression of gluconeogenesis (main energy source in embryonic life) might be the reason for retardation of growth in crown rump length, beak length and neck length[30]. Growth retardation in the development of the chick embryo may be due to pesticides interference with metabolism, incapability to utilise yolk by growing embryo or disruption of retinoid signaling pathway[16,17].

3.2 Endo-Skeletal Investigations

3.2.1 Skeletal Malformations

Control group: After alizarin staining of 18th day old chick embryos for endo-skeletal examination revealed that the majority of the skull parts had a large degree of ossification. The vertebral column was normally shaped, with no lateral flexion. The sternum was enlarged and entirely cartilaginous. The ischium and ilium were completely ossified and stained red, while the pubis was completely cartilaginous except for the middle part, which was ossified and partially curved. The limb bones, *i.e.*, humerus, radius and ulna, femur and tibiotarsus, carpometacarpus and tarsometatarsus, were entirely ossified except in the epiphyseal regions. The majority of the phalanges were ossified Figure 2A.

3.2.2 Formulation of İnsecticide Treated Groups

The chick embryos treated with a low dose of formulation insecticide exhibit skull malformation (11%), like short upper and lower jaws, ossification in frontal and parietal bones (Figures 2 (C, D), cervical scoliosis (11%). Ossification of cervical, thoracic and caudal

Table 3. Morphometric measurements of 18 ED chick embryos after treatment with formulation insecticide on the 7th day of incubation

Fetal growth parameters	Groups			
	Control	Treated 1 Low dose (0.01µg per egg)	Treated 2 Medium dose (0.05µg per egg)	Treated 3 High dose (0.1µg per egg)
Crown rump length (cm)	8.91±0.05	8.89±0.04	7.26±0.06*	6.29±0.02*
Beak length (cm)	3.45±0.08	3.41±0.05	2.77±0.03*	2.21±0.01*
Neck length (cm)	3.72±0.02	3.71±0.03	2.21±0.01*	1.56±0.08*

Data are represented as Mean±S.E. Statistical difference from the control: * significant at p≤0.05, by Student's t test. Using IBM SPSS Statistics 22 software.

vertebrae (10%, 11% and 19%, respectively). About 13% of embryos showed incomplete ossification of ribs, 17% of embryos showed ossification of pygostyle, and 28% of embryos showed incomplete ossification of the tail. Limb malformation constitutes about 23% (Table 4).

The chick embryos that received medium dose of formulation insecticide showed malformations in skull (21%) reduced pygostle (31%), ossification in cervical, thoracic and caudal vertebrae (13%, 17% and 28%, respectively), fusion of ilium and femur (Figure 2 (E, F)), scoliosis (15%), incomplete formation of ribs (17%). About 33% of embryos had malformation in the limbs (Table 4).

Chick embryos treated with a high dose of formulation insecticide showed various skeletal anomalies (Table 4). Defects of the skull constitute about 36%. Skull malformation showed loss of part of its bones, like ossification in the frontal and parietal and short lower and upper jaws.

Embryos that suffered from malformations in the vertebral column showed incomplete ossification in cervical, thoracic and caudal vertebrae (19%, 31% and 42%, respectively). About 23% of embryos had incomplete ossification in ribs, 21% of embryos exhibited scoliosis, and 37% of embryos showed ossification of the pygostyle. Moreover, malformations in limbs, including flexed toes with a percentage of 46% (Figures 2 (G, H)) and a higher number of skeletal malformations, were found in high-dose-treated embryos.

The skeletal system is one of the possible targets of pesticide toxicity, and pyrethroids have been reported to cause reduced bone formation[22]. Thus, treatment of embryos until day 7 of incubation impacts skeletal development, as shown in the study by Osdoby and Caplan, where the mineralisation of the long bones in the chick embryo hind limbs occurs by the 8th day of development[31].

Skeletal anomalies such as poor ossification of bone in fertilised embryos of the chick may result from inhibition with a synthetic pyrethroid deltamethrin insecticide in endochondral and intramembranous pathways, which are primary ossification pathways of skeletal development in living vertebrates[32]. Similarly, Hoffman and Eastin also observed skeletal deformities in mallard embryos exposed to malathion, diazinon, and parathion, such as cranioranchis, scoliosis and fusion of cervical vertebrae and concluded that skeletal deformities are caused by cholinergic system disruption[33].

According to Uggini *et al.*[17] pyrethroids are known to affect neurotransmission, and this interference with neural activity and acetylcholinesterase activity can result in a variety of vertebral anomalies in chick embryos[17]. B*hask*ar *et al.*[32] reported skeletal defects in Gallus domesticus chick embryos exposed to deltamethrin (synthetic pyrethroid) containing formulation insecticide, such as scoliosis, poor ossification of bone, caudal regression, deformed ribs, poor ossification of frontals and parietal in skull and thinning of upper and lower limb bones[32]. Additionally, skeletal malformations were reported in chick embryos treated with a formulation of insecticide quinalphos, such as ossification of thoracic vertebrae and caudal vertebrae, shortness of humerus, radius, ulna and curled toes[34].

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

Endo-skeletal malformations	Groups			
	Control	Treated 1 (Low dose) (0.01µg per egg)	Treated 2 (Medium dose) (0.05µg per egg)	Treated 3 (High dose) (0.01µg per egg)
Skull	0	11	21	36
Incomplete ossification of cervical vertebrae	0	10	13	19
Incomplete ossification of thoracic vertebrae	0	11	17	31
Incomplete ossification of caudal vertebrae	0	19	28	42
Incomplete ossification of ribs	0	13	17	23
Scoliosis	0	11	15	21
Incomplete ossification of the pygostyle	0	17	31	37
Limb malformation	0	23	33	46
Incomplete ossification of the tail	0%	28%	36	42

All values were indicated in percentages.

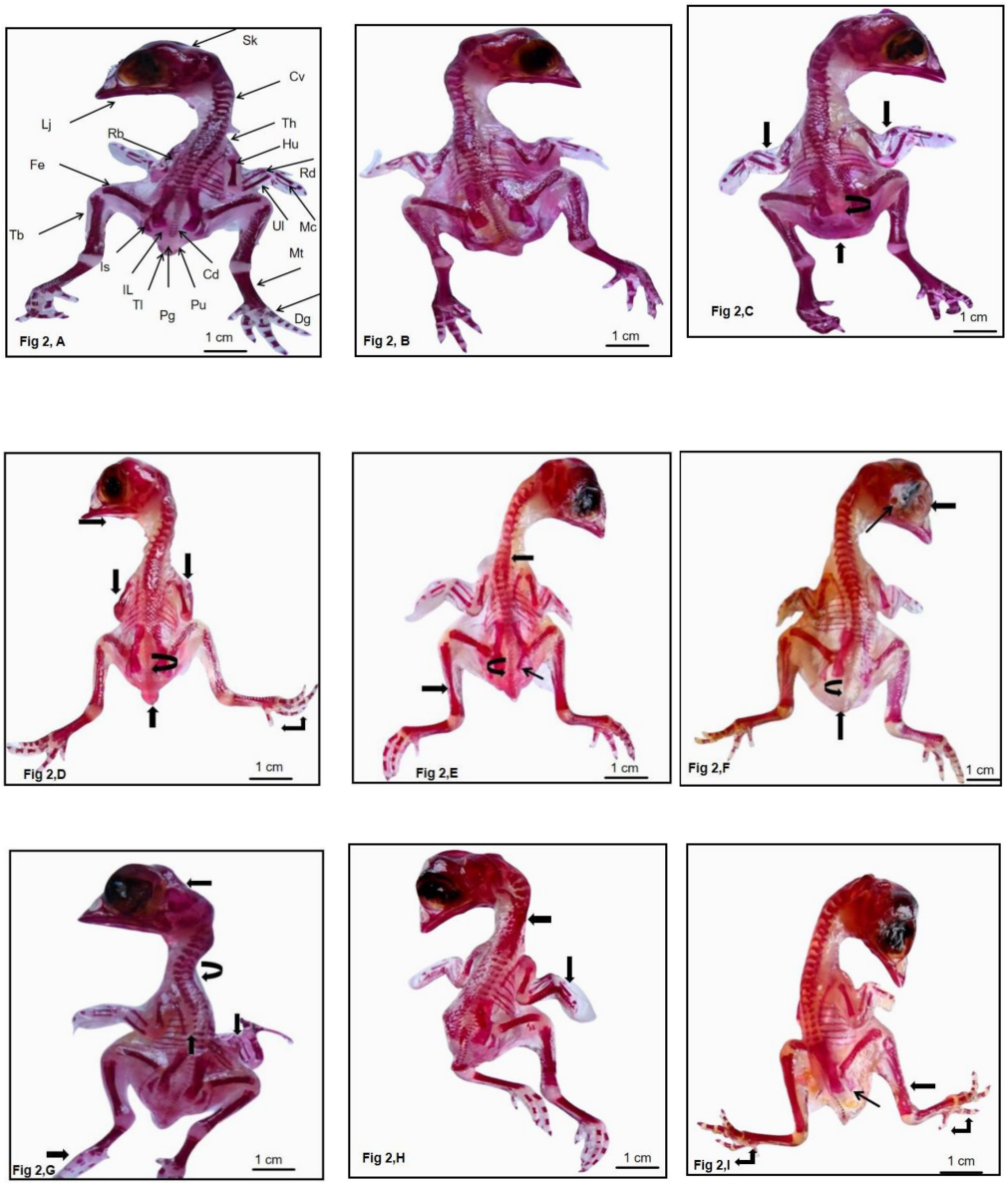


Figure 2. Photograph of the endoskeletal system of 18th-day-old chick embryos from control and formulation insecticide-treated groups. (A, B) Control, (C, D) low dose (0.01 µg/egg) of formulation insecticide treated, (E, F) medium dose (0.05 µg/egg) of formulation treated and (G, H) high dose (0.1 µg/egg) of formulation insecticide treated. **A, B** showed a well-formed structure of endoskeleton with normal ossification. (Sk: skull, Lj: lower jaw, Cv: caudal vertebrae, Th: thoracic vertebrae, Hu: humerus, Rd: radius, Ul: ulna, Mc: metacarpals, Rb: ribs, Fe: femur, Tb: tibiotarsus, Mt: metatarsus, Dg: digits, Cd: caudal vertebrae, Is: ischium, IL: ilium, Pg: pygostyle, Pu: pubis, Tl: tail). **C** shows thinning of radius and ulna (down arrow), ossification of caudal vertebrae (curved arrow), and incomplete ossification of pygostyle (up arrow). **D** shows short jaws (right arrow), poorly developed upper limb bones (down arrow), incomplete ossification of caudal vertebrae (curved arrow), flexed toes (left up arrow) and kinked tail (up arrow). **E** poor ossification of thoracic vertebrae (left arrow) and caudal vertebrae (curved arrow), fusion of ilium and femur (block arrow) and thinning of tibiotarsus (right arrow). **F** shows a poorly developed eye (left arrow), hematoma under the eye (block arrow), and incomplete ossification of caudal vertebrae curved arrow) and incomplete ossification of pygostyle and tail (up arrow). **G** shows loss of part of skull (left arrow), poor ossification of frontal and parietal, scoliosis (curved arrow), poorly developed vertebral column (up arrow), absence of right upper limb bones (down arrow) and poorly developed digits (right arrow). **H** shows poor ossification of cervical vertebrae (left arrow) and poor ossification of radius and ulna (down arrow). It shows abnormal formation of tibiotarsus bone (left arrow), poor ossification of ischium (block arrow) and flexed limbs (left up arrow).

In our present findings, endo-skeletal anomalies such as beak deformities, curled toes and flexed digits were also observed. The beak deformity in our findings could be due to chondroitin sulphate deficiency caused by interference with an NAD-dependent mechanism. The curled toes and folded digits can be due to muscular strain in the feet during the toe bone ossification. This could be as a result of the embryo not receiving certain esterase-metabolised substances from the yolk sac[26].

Our present observation of the induction of skeletal deformities, such as reduced ossification of vertebrae and deformed ribs and beak defects due to different doses of formulation insecticide (0.01, 0.05µg and 0.1µg per egg), treated eggs was consistent with those reported in growing chicks by treatment with RPR-V, an organophosphate[35]. Absence of caudal vertebrae was also detected in the present research. Similar findings were seen in embryos of eggs collected after maternal enrofloxacin treatment[36].

The vertebral abnormalities are linked with the disruption of the cholinergic system and acetylcholinesterase inhibition. Acetylcholine inhibition impacts migration of target cells, proliferation and differentiation of cells, and any interference with the functioning of acetylcholinesterase (AChE) during early development would cause deleterious effects[5].

In the present study, the formulation insecticide teratogenic tendency likely involves altered or interrupted cell signalling, inappropriate apoptosis, and/or defective neural tube closure, in addition to being a cholinesterase inhibitor. As a result, a wide range of defects were identified in chick embryo such as growth retardation, teratological defects and skeletal malformations. Hence, the potential of fenpropathrin with pyriproxyfen formulation insecticide may cause inadequate utilisation of yolk content, disruption in signalling pathways, chondroitin sulphate deficiency and inhibition of the cholinesterase.

3.2.3 Length of Ossified Centres

Measurement of the arrangement of ossification centres in long bones revealed differences in length between groups (Table 5). The average length of humerus, radius, ulna, carpo-metacarpus, femur, tibiotarsus and tarso-metatarsus of 18th day old control chick embryo were 1.72±0.08, 0.69±0.05, 0.52±0.07, 0.45±0.03, 1.62±0.08, 2.35±0.05 and 1.53±0.08, respectively.

Insignificant change was seen in the length of humerus, radius, ulna and femur, carpo-metacarpus, tibiotarsus and tarso-metatarsus in the low-dose treated group.

The treatment with medium dose (0.05µg/egg) of formulation insecticide resulted in a significant decrease in the length of the humerus, radius, ulna and femur. The chick embryos treated with a high dose (0.1µg/egg) of formulation insecticide resulted in a significant decrease in the length of humerus, radius, ulna, femur, carpo-metacarpus, tibiotarsus and tarso-metatarsus as compared to the control.

Changes in body weight, morphometric parameters, and incidence of gross morphological malformations, alterations were found in the current study during the development of chick embryos due to administration of a commercially available formulation insecticide (Sumiprempt) appears to have contributed and suggested insecticide-mediated teratological effect.

Table 5. The effect of formulation insecticide injection on the length (in cm) of ossified centres of long bones in 18th day old chick embryos in different groups

Average length of bones (cm)	Groups			
	Control	Treated 1 (Low dose) (0.01µg per egg)	Treated 2 (Medium dose) (0.05µg per egg)	Treated 3 (High dose) (0.1µg per egg)
Humerus	1.72±0.08	1.52±0.09	1.35±0.05*	1.25±0.04*
Radius	0.69±0.05	0.60±0.04	0.36±0.08*	0.22±0.03*
Ulna	0.52±0.07	0.48±0.03	0.21±0.02*	0.18±0.05*
Carpo-metacarpus	0.45±0.03	0.42±0.06	0.29±0.05	0.15±0.03*
Femur	1.62±0.08	1.50±0.03	1.26±0.03*	1.21±0.02*
Tibiotarsus	2.35±0.05	2.28±0.04	2.25±0.05	2.19±0.04*
Tarso- metatarsus	1.53±0.08	1.49±0.03	1.32±0.02	1.27±0.02*

Data are represented as Mean±S.E. Statistical difference from the control: * significant at p≤0.05, by Student's t test using IBM SPSS statistics 22 software.

4. Conclusion

The current study concludes that *in-ovo* administration of Fenpropathrin with Pyriproxyfen formulation insecticide in the form of Sumiprempt insecticide has greatly increased the teratogenic effect on embryological development of *Gallus domesticus*. The administration of formulation insecticide with respect to a crucial period of embryogenesis affects the morphometric parameters of chick embryo and causes various gross morphological abnormalities with marked reduction in average body weight in 18th-day-old chick embryos. It is also evident from the results that the given low dose (0.01µg/egg), medium dose (0.05µg/egg), and high dose (0.1µg/egg) in the study were embryotoxic and teratogenic to a non-targeted organism, the chick. So it is suggested that the formulation used in the present study should be utilised with attention, and farmers and workers should also be made aware of the possible risks of insecticides on human health so that they can use them judiciously and wear protective equipment while working with these insecticides.

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