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In-Ovo Administration of A Commercial Formulation of Chlorpyrifos (50%) and Cypermethrin (5%) Induced Structural Anomalies in Two Generations of Rir Chicks

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Abstract: ***Effects of in-ovo intoxication of an insecticide formulation Nurelle D 505 EC (chlorpyrifos 50% EC + cypermethrin 5% EC) was evaluated in two generations of domestic chick. The investigation covered four groups of fertilized RIR eggs – three for experimental and one for control. Experimental groups of eggs received doses of 0.01, 0.05 and 0.1 µg/egg of combination insecticide (Ci) and the control received corn oil on day zero of incubation. The doses were selected based on our previous study. The eggs were incubated and upon hatching the F1 generation chicks were sorted as per their treatment, tagged and reared on standard diet. Upon sexual maturity the birds from the same groups were allowed pen mating. The fertilized eggs were collected and incubated for the next generation (F2). The eggs were regularly candled every two days till the 18th day of incubation to cull out the unfertile or dead embryos. The dead embryos and hatchlings were given a meticulous visual examination and the rate of morphological malformations was calculated. The study showed that chlorpyrifos and cypermethrin applied in a mixture caused significant increase in the rate of mortalities and decreased hatchability in chick of F1 and F2 generation. The morphological abnormalities caused by the treatment of the Ci were also observed in chicks of F1 & F2 generation. This is the first report of Ci induced structural anomalies in the second generation of chicks and we believe that the test system can evolve as an alternate model for testing two generation developmental toxicity.***

Keywords: Pesticides, Chlorpyrifos, Cypermethrin, structural anomalies, Fertilized RIR Eggs

1. Introduction

Large bodies of evidences explain the vulnerability of a developing embryo to various kinds toxic substances. In the naïve stage of development, the embryo is yet to develop its detoxification mechanisms. The organism builds up in a sequential cascade of events, where there are huge number of sensitive points, which on slightest of a disturbance, might fail to reach their proper endpoints. There has been growing awareness that certain chemicals at levels below those associated with overt toxicity can modulate the developmental mechanisms (Uggini et al., 2012). The period of prenatal development, mainly during the phase of embryogenesis and at the beginning of organogenesis, is delicate. This rapidly differentiating and growing system may be disturbed or changed from normal to anomalous by the introduction of toxic substances (Sahu and Ghatak, 2002, Uggini et al., 2012).

This phenomenon of developmental toxicity was realized after Lenz, (1988) and McBride, (1977) related the usage of sedative-hypnotic drug namely "Thalidomide" by the pregnant mothers to the appearance of an epidemic of limb-reduction malformations in their newborn babies. The extrinsic agents that might cause the development toxicity can be of chemical nature like drugs, lifestyle factors such as alcohol, diet, and environmental toxic chemicals like pesticides or radiations. These factors can lead to structural or functional alteration which intervenes with homeostasis, normal growth, differentiation, development or behavior and prenatal mortality. The structural malformations are not common at the low dose exposure but functional alterations are the most sensitive signs of developmental toxicity (Peterson et al., 1993). The effect of the teratogens can be seen in the prenatal stage, but sometimes the effect comes up with some anomalies in postnatal stages as well. The toxicity of the extrinsic agent shows a relationship between the dose and response. This phenomenon helps us to summarize the status of damage to the organisms due to teratogen dosage level. Under this relationship we can also presume that the organisms have some tolerance for exposure to the low dose.

Among the various extrinsic toxic agents, pesticides fall in into a highly considerable category due to their widespread usage and their design to adversely affect the living organisms. Several researches has been performed to report the embryotoxicity and teratogenicity of pesticides on fishes (Datta and Kaviraj, 2003; Köprücü et al., 2006; Sharma and Badre, 2010), amphibians (Bishop, 1992; Carey and Bryant, 1995; Hayes et al., 2006; Krishnamurthy and Smith, 2010, 2011), aves and on mammals. These toxic exposures to the embryos sometime leads to death of the fetus during later phases of development and also various postnatal effects are also reported. Hence, to evaluate the influence of the pesticides on public health and the ecosystem, biological test methods have been developed and applied to remark chemical and physical testing (Fernandez-Alba et al., 2002). Therefore, the present study has been designed to investigate and analyze the possible morphological defects induced by commercially available insecticide Nurelle D 505 EC [chlorpyrifos (50%) and cypermethrin (5%)] in the developing RIR chick embryo for inspecting the mechanisms of teratogenicity as similar arrangement of human teratogenesis can also be suspected from these toxic chemicals.

Earlier studies of the present test substance (combination insecticide Ci), has shown to induce various kinds of axial and skeletal malformation like abnormalities in the development of the skull, vertebral column and the limbs (Uggini et al., 2012). Furthermore, expecting toxic changes in the in-ovo exposed groups (F1 generation) under the influence of the current designed exposure doses of Ci, the toxicity testing was extended to observation of succeeding generation (F2) too. However, the second generation birds were not given any direct exposure of Ci. They were examined to find out if they could bequest any of the toxin induced malformations from the F1 generation. The outcomes of the study would greatly help us in understanding the vulnerability of development against the toxic exposures occurring during early developmental phases.

2. Material and Methods

Test Chemicals
A commercial insecticide formulation of combination insecticide which constituted of chlorpyrifos (50%) and cypermethrin (5%) was used for the study. The details are mentioned in the section material and methods in preceding pages of the thesis.

Fertilized RIR Eggs and Insecticide Injection
Fertilized eggs of RIR breed of Gallus domesticus were collected from Poultry Science Department of Anand Agricultural University, Anand, India). All the eggs were cleaned, marked and injected to receive the respective dosage treatment of the combination insecticide for three experimental groups and corn oil for one control group through air sac method as per (Blankenship et al., 2003). Combination insecticide was diluted in corn oil so as to get doses of 0.01, 0.05 and 0.1 µg/egg based on a prior dose range done by probit analysis (Uggini et al., 2012). Each dose group consisted of 30 RIR eggs.

Incubation
All the treated and control groups were kept in an incubator with capabilities of maintaining and monitoring temperature, humidity and turning the eggs periodically. The temperature in the incubator was maintained at 37°C and relative humidity was kept between 70-80%. On 18th day of incubation viable eggs were transferred to a hatcher till the day of hatch.

Rate of Mortality and Hatchability
The eggs were regularly candled every two days till the 18th day of incubation to cull out the unfertile or dead embryos. The rate of hatchability and mortality were calculated as mentioned previously in the chapter of materials and methods.

Body Weight and Relative Organ Weight
The body weight of the birds on 25th week in F1 generation and on 4th week in F2 generation were recorded. Upon sacrifice, their liver and kidney weight were also recorded and the relative weight of the organ was calculated.

Rate of morphological malformation
The dead embryos and hatchlings were given a meticulous visual examination and the rate of morphological malformations was calculated as mentioned elsewhere in the section materials and methods of the thesis.

Statistical Analysis
All the linier variables are summarized as mean plus or minus standard error. One way analysis of variance followed by Dunnett's multiple comparison was attempted using a Windows-based statistical programme GraphPad Prism to compute the significant difference between the mean values of control and the treatment groups.

3. Results

Rate of mortality and hatchability
The F1 generation showed a highly significant increase in the rate of mortalities in all the Ci exposed groups. The hatchability of the fertile eggs has declined significantly with the increase in the Ci dosage, with the lowest hatchability being found in the highest dose group (Table 1.1).

In the F2 generation too, the pattern of mortalities and hatchability increased and decreased respectively, as in the parent groups. However, when the F1 and F2 generations of a particular group were compared, there observed no significant changes in the mortalities or hatchability. The control birds of F1 and F2 generations showed similar patterns of mortality and hatchability. Similarly the F1generation low dose group and their progeny showed almost similar patterns of mortality and hatchability and the same was true for the other two dose groups and their progeny (Table 1.1).

Body weight and relative organ weight
It was observed that the body weight of the birds after 25 weeks in F1 generation, has significantly reduced in all the three treatment groups and at 4 weeks in F2 generation, the body weight reduction was evident in descendents of mid and high dose groups. The relative weight of liver showed no significant change with the low dose in F1 generation however the relative liver weight increased in the mid dose group while it decreased in the high dose group. The relative weight of the kidney in F1 generation only significantly altered in the high dose group birds when compared to the control birds. In the F2 progeny there was a slight decline of relative kidney weight among the high dose group descendants (Table 1.2).

Morphological Malformations
The morphological abnormalities caused by the treatment of the Ci in general were of following types:

1) Head: Brain exposed through the skull (exencephaly), blood patches (hematomas).
2) Eye: small eye (microphthalmia), missing eyes (anophthalmia), swelling and edema of eye, bulging eyes (exophthalmia).
3) Neck: Wry neck, narrow neck.
4) \Beak: beak deformities and cleft beak.
5) Lower body: Growth retardation, abnormal exposure of internal organs, hematomas.

6) Abdomen: Mushy type.
7) Limbs: Crooked hind limb limit, Spraddle leg, Crippled limbs and Twisted phalanges.

The control chicks of the F1 generation looked healthy and normal (Figures 1.1). The results of the study displayed increase in embryolethality and abnormal survivors with increase in concentrations of Ci treated chick embryos as compared to control embryos. The external malformations displayed by 0.01µg/egg of Ci treated chicks of F1 generation were Narrow neck, crippled and twisted phalanges, spraddle legs, anophthalmia and ectopia viscera (Figure 1.2 -1.5). The birds which received 0.05 µg/egg of Ci showed similar malformations at higher frequency than the former dose, i.e, narrowed necks, mushy body, crippled and twisted phalanges, spraddle legs, exposed viscera and decreased body size (Figure 1.6 – 1.10). The birds of 0.10 µg/egg of Ci group showed malformations at the highest rate (Figure1.11 – 1.17) i.e, hematomas, ectopic viscera, microphthalmia, anophthalmia, exencephaly, limb and phalangeal malformations etc.

The F2 generation control birds displayed normal features (Figure1.18). The F2 progeny of the treated F1 birds however showed various anomalies though they did not receive any kind of direct experimental Ci dosage. The anomalies seen in succeeding generation of 0.01 µg/egg of Ci treated birds were short beak, twisted digits and short limbs, and closed eye (Figure1.19 – 1.21). The progeny of 0.05 µg/egg of Ci treated birds showed malformations like deformed eye, wry neck, narrow neck, mushy abdomen due to unabsorbed yolk, umbilical hernia, exposed internal organs (ectopia viscera/gastroschisis), spraddle legs, crooked legs, crippled legs, twisted phalanges etc (Figure 1.22 – 1.27). The progeny of 0.10 µg/egg of Ci treated birds showed similar malformations though at an increased frequency (Figure1.28 – 1.38).

Table 1.1: The rate of mortality, hatchability and malformation in F1 and F2 generation

Treatment (25µl/egg)		Attribute			
		Dose	% Mortality[@]	% Hatchability[@]	% Malformations[@]
F1 generation	VC	Corn oil	15	85	00
	Ci (µg/egg)	0.01	30**	70*	05*
		0.05	40***	60*	10***
		0.1	53***	47***	25***
F2 generation	VC	Corn oil	20	80	00
	Ci (µg/egg)	0.01 descendents	27*	73*	05*
		0.05 descendents	39**	61**	08**
		0.1 descendents	49***	51***	20***

@ Percentage corrected to nearest whole number; n=30; *p ≤ 0.05, **p ≤ 0.01; ***p ≤ 0.001

Table 1.2: Body weight and relative organ weight in F1 and F2 generations

Generation	Dose concentration (µg/Egg)	Body weight (gm)	Relative weight	
			Liver(gm/100gm body weight)	Kidney(gm/100gm body weight)
F1 (25 weeks)	Control	1988±8.71	1.67±0.71	0.55±0.03
	0.01	1872±5.21*	2.15±0.13	0.61±0.11
	0.05	1461±11.39**	2.73±0.69*	0.75±0.53*
	0.10	1103 ±9.31***	2.03±0.75*	0.73±0.27*
F2 (4 weeks)	Control	645±11.02	1.52±0.15	0.49±0.01
	0.01	633±7.31	1.77±0.50	0.47±0.17
	0.05	609±18.11*	2.49±0.76	0.45±0.09
	0.10	580±13.90*	1.85±0.26	0.43±0.15*

Values are expressed as mean ± SE; n=30; *p ≤ 0.05, **p ≤ 0.01; ***p ≤ 0.001

Figure 1.1: Control group chicks of F1 generation

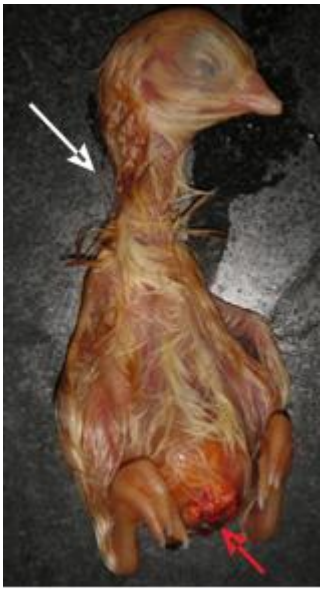
Figure 1.2

Figure 1.3

Figure 1.4

Figure 1.5

Figure 1.2 – 1.5: Chicks of F1 generation receiving 0.01µg Ci per egg (Low Dose)
Narrow neck (white arrow); exposed internal organs (ectopia viscera) (blue arrow); Crippled limbs and twisted phalanges (yellow arrow); Spraddle legs (green arrow); unabsorbed yolk (red arrow)



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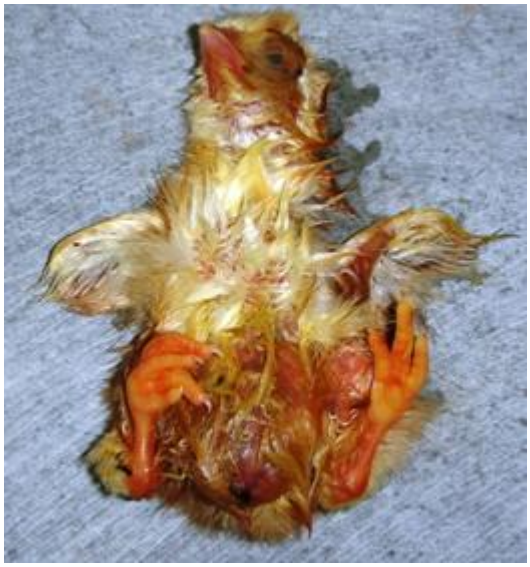

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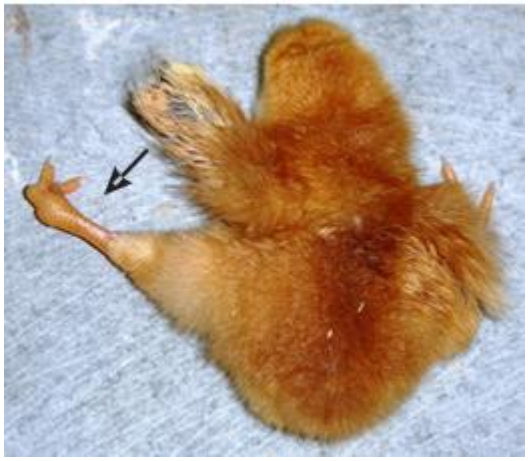

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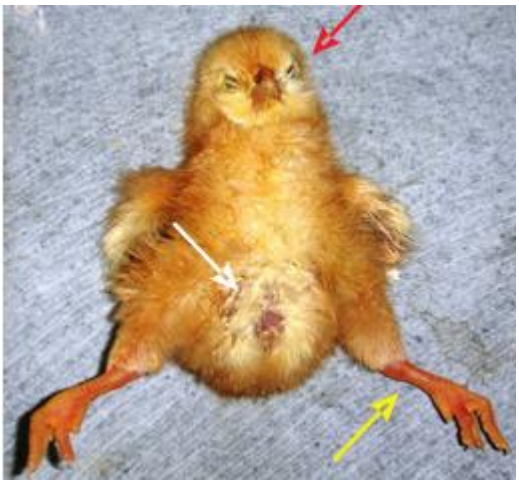

Figure 1.9

Figure 1.10

Figure 1.6 – 1.10: Chicks of F1 generation receiving 0.05µg Ci per egg (Mid Dose)
Narrow neck (white arrow); Umbilical hernia (red arrow); Mushy chick (Fig. 1.7); Crippled limbs and twisted phallanges (black arrow); closed eyes (red arrow); Spraddle legs (yellow arrow); unabsorbed yolk (white arrow) and small sized body (Fig. 1.10)

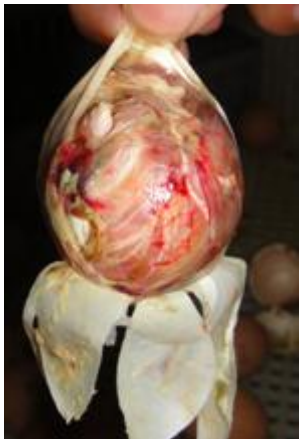

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Figure 1.13

Figure 1.14

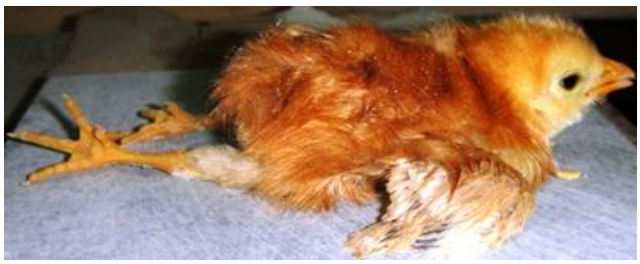


Figure 1.15

Figure 1.16

Figure 1.17

Figure 1.11– 1.17: Chicks of F1 generation receiving 0.1µg Ci per egg (High Dose)
Unhatched chick and hematomas (Fig. 1.11); Narrow neck (white arrow); exposed internal organs (ectopia viscera) (red arrow); deformed eye (yellow arrow); Exencephaly (green arrow); abnormal phalanges (blue arrow); umbilical hernia (black arrow); Crippled limbs (Fig. 1.15); closed eyes (maroon arrow); unabsorbed yolk (dark blue arrow), wry neck (purple arrow); sticky and small sized body (Fig. 1.17)

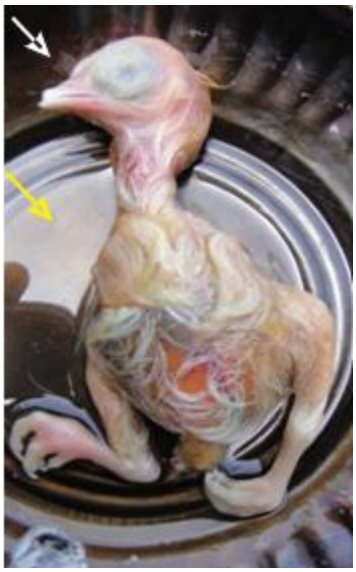
Figure 1.18: Control group chicks of F2 generation

Figure 1.19

Figure 1.20

Figure 1.21

Figure 1.19– 1.21: Chicks of F2 generation receiving 0.01µg Ci per egg (Low Dose)
Twisted digits (yellow arrow); short limbs (white arrow); Twisted phalanges (red arrow), anophthalmia (black arrow)



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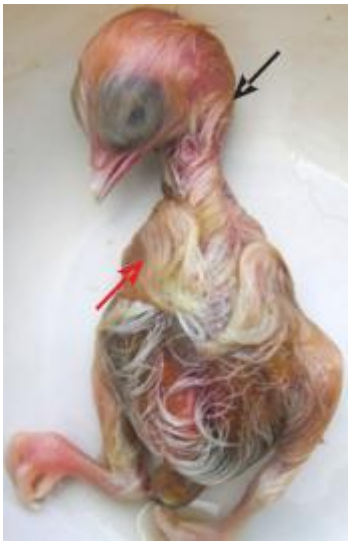

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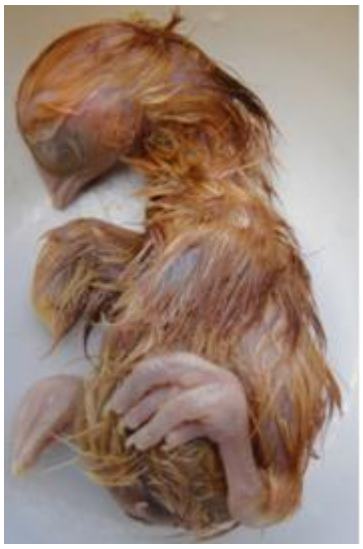

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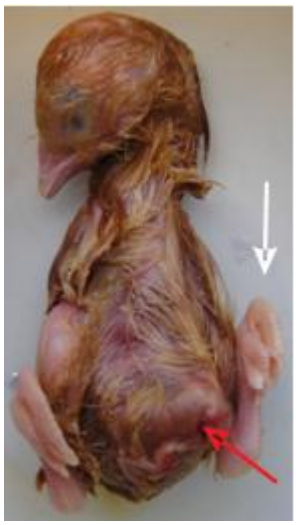

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Figure 1.26

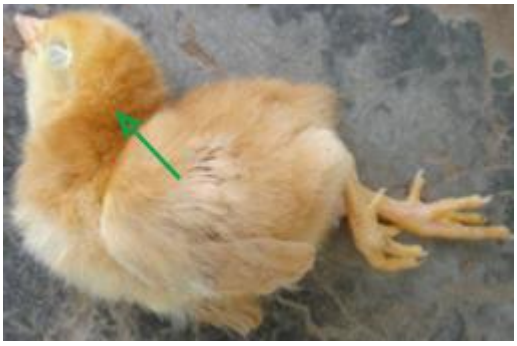
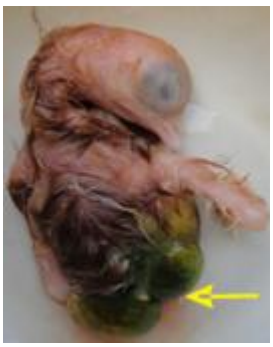


Figure 1.27

Figure 1.22– 1.27: Chicks of F2 generation receiving 0.05 µg Ci per egg (Mid Dose) Abnormal beak (white arrow), ectopia viscera (red arrow); swelling and edema of eye (blue arrow); Narrow neck (black arrow); mushy abdomen (unabsorbed yolk) (Fig. 1.24) Crippled legs (white arrow); umbilical hernia (red arrow); Spraddle legs (yellow arrow); closed eyes (blue arrow); wry neck (green arrow)

Figure 1.28



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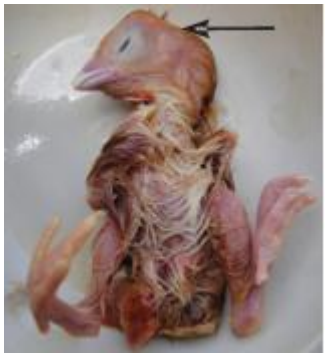


Figure 1.30

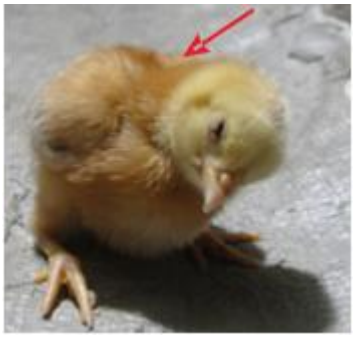
Figure 1.31

Figure 1.32

Figure 1.33

Figure 1.28– 1.33: Chicks of F2 generation receiving 0.1µg Ci per egg (High Dose)
Abnormal beak (white arrow); abnormal legs (green arrow); exposed internal organs (ectopia Viscera) (yellow arrow); Exencephaly (red arrow); deformed eye (blue arrow); Narrow neck (black arrow); umbilical hernia (pink arrow); unabsorbed yolk (orange arrow)

Figure 1.34



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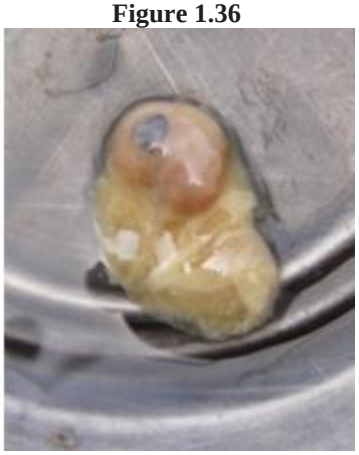

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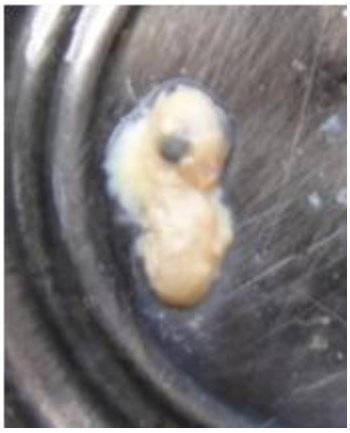

Figure 1.38

Figure 1.34– 1.38: Chicks of F2 generation receiving 0.1µg Ci per egg (High Dose).
Abnormal beak (white arrow); abnormal legs (yellow arrow); Wry Neck (red arrow); Small sized body (Fig. 1.36); Undeveloped chick embryos (Fig. 1.37 and 1.38)

4. Discussion

The present study represents an attempt to investigate the abnormality in the chick embryos due to the in-ovo Ci exposure. The in-ovo Ci exposure in different doses has lead to the decline in the general health and body growth of the F1 as well as the F2 generation birds, as evident from the decline in the body weight and alterations in the relative weights of the liver and kidney. In this study malformed growth was observed and it is been researched that for the proper growth and development, uninterrupted oxidative phosphorylation (as a source of every), cell proliferation and differentiation is required. In the current study, the Ci resulted growth retardation possibly because of the deranged metabolic machinery involved in the ATP production. Embryonic period is characterized by heightened cellular activities which require continuous supply of energy. In a subsequent biochemical analysis we have observed that Ci treatment resulted in deviant carbohydrate metabolism. Similar pinion was made by several workers while explaining the pesticide induced hampered development Garg et al., 2004; Pushpanjali et al., 2005. Moreover, interruptions to retinoid signalling pathway during embryonic development was also cited as a possible cause of altered development of vital organs of the body and hence, stunted growth (Lemaire et al., 2005).

The occurrence of external malformations observed in present study such as exencephaly, anophthalmia, narrow or twisted neck, deformed beak, hematomas, ectopic viscera, wry neck, muscle twitching, twisted digits, growth retardation are quite similar to earlier investigations reported in chick embryo and abnormal survivors exposed to various kinds of pesticides (Friedberg and Gartner, 1990; Khalil and El-Sayed, 2000; Sahu and Ghatak; 2002, Petrovova et al., 2009; Mobarak and Al-Asmari, 2011; Pinakin et al., 2011; Nitu et al., 2012; Uggini et al., 2012). It is known that structural abnormalities are amongst the most serious of the possible side effects of any insecticide and may happen before or shortly after birth.

The application of pesticide might create a number of such processes which are not natural and interferes with natural developmental process of an organism. And the major interfering mechanism reported is oxidative stress which leads to malformed development of an organism (Hodgson and Levi, 1987 and Paskova et al., 2011). The other reasons behind these abnormalities could be induction of mutation in genes due to these teratogens, and the mutation might inhibit the proliferation and differentiation of the cells and thus hampers the development of the organism. The embryotoxicants are also known also induce DNA fragmentation (Giri et al., 2003; Uggini et al., 2013). The other assumptions might be that the teratogens could injure the roof plate of the neural tube which alters the formation and development of the eye (Sahu and Ghatak 2002). It was proposed that hematomas could be a result of malformed craniofacial cleft. The effects of Ci on the development of wings, feathers, beak and legs might be due to binding of the insecticide with calcium binding protein calmodulin which results in decrease in intracellular Ca^{2+} as suggested by Rashatwar and Matsumura, (1985) and thereby hindering the proper intracellular communication resulting in deviant cellular activities and abnormal development.

In this study the fertilized RIR eggs are injected with toxins on the day one of their incubation. The absence of a functional detoxifying machinery in the liver probably has lead to the potentiation of effects of the toxin. The development and functioning of liver happens after 4th-5th day in the chick embryo, so the xenobiotic substance gets accumulated during the initial days of the development which causes an augmented teratogenicity. Similar observations made by Romanoff, (1960) consolidate the above notion. Thus, it might be possible that the insecticide residues as stated by many alter and hinder the process of cellular activities during development which results in deformity in the limbs in late developmental stage and thus leads to the sculpture of malformed legs.

The other malformations like hematomas and edema formation under the influence of Ci treatment might be due to widespread vascular damage and insufficient utilization of the yolk. This observation is in agreement with reference to the pericardial and peritoneal edema researched in fish eating birds, exposed to organochlorine insecticides (Gilbertson et al., 1991).

Hence it is quite evident that application of the combination insecticide on the RIR fertilized eggs will give malformed embryos, where the insecticide disturbs the normal developmental process required for proper growth of embryos and their skeleton formation. Therefore looking at the observations made during this study, it is recommended that combination insecticide should be applied with caution as it can be dangerous for both humans and domestic animals.

The present investigation reveals that an in-ovo exposure of the Ci at various doses can lead to hampered growth and development of an organism. Which might also causes highly deleterious effects witnessed as an array of morphological malformations. Moreover it was amply evident that the toxic mechanisms of derailed development

have been carried on to the succeeding generation. Through this we could analyze the potential hazard that the extraneous chemicals might impose on the development of an organism. It is likely that the teratogenic inclination of the Ci might involve more than one kind of cellular or molecular injury, which may include an altered or interrupted cell proliferation and developmental mechanics, or inappropriate or defective closure of neural tube. Thus it has to be made mandatory to regulate the usage of these environmental toxicants so that they do not enter into the food chains. Moreover, a guideline should be given to the farmers to make a logical application of the pesticide to save the animals and human beings as well.

5. Conclusion

The study shows that environmental toxic substances are sensitive not only to adult animals, but also to descendants. During their inovo growth, the toxicants greatly influenced the health of the parent generation and also hindered the embryonic development of the next generation. The study of morphology, mortality and hatchability showed that the animal of F2 generation had weakened structural anomalies in contrast with the animals directly receiving the pesticide dose. This result illustrates the need for more than one generation of screening of the recognized developmental toxicants to obtain the much-needed evidence behind many unexplained human diseases about which the aetiology is still at large.

6. Acknowledgment

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Disclosures Conflict of interest: I, Asiya Khan declare that I have no conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by the author.

Animal studies: All institutional and national guidelines for the care and use of laboratory animals were followed.

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