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Original Article

Effect on chick embryos development after exposure to neonicotinoid insecticide imidacloprid

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ABSTRACT

Introduction: Worldwide, Imidacloprid is one of the most commonly used insecticides belonging to the family of neonicotinoids. The discovery of neonicotinoids as important novel insecticides represents a milestone in insecticide research over the past three decades. Worldwide, it is insecticides. The objective of current study is to observe the effect on development of chick embryos after exposure to varying concentrations of neonicotinoid insecticide imidacloprid.
Methods: The current study was carried out on 400 fertile eggs of white leghorn chicken obtained from government poultry farm In experimental group chicken eggs were exposed to Imidacloprid with doses of 2.5 μg, 5 μg, 10 μg and 20 μg in a volume of 2.5 μl, 5 μl, 10 μl and 20 μl respectively and in control group matching volume of Normal Saline without Imidacloprid were used. The embryos were terminated on 21st day, eggs shell broken with a scalpel and embryos removed. The developmental and teratogenic effects were observed and recorded.
Results: The results show that experimental group had comparatively more cases of developmentaleffects; growth retardation resulting into failure of retraction of yolk sac, limbs defects, head enlargement, ectopia viscerale and decrease weight of chick, crown rump length as compared to controls.
Discussion: Imidacloprid exposure increases the risks of teratogenic effects with increasing embryonic age. Comparatively higher doses proved more toxic to thedevelopment of chick embryos.

1. Introduction

Environmental pollution is a worldwide problem in a modern society. The extensive uses of pesticides are widely used to enhance the crop production and other benefits and have raised concerns about potential adverse effects on the environment, human health and non-target animals.Problems and outbreaks have been reported to occur among animals and human from insecticide exposure.[1] Prolonged exposure to insecticides is known to cause chronic neurological syndrome, malignant tumors,[2] immunosuppressive action, teratogenic effect, abortion and decreased fertility in experimental animals. Pesticide coated seeds are commonly used in agriculture, and may be an important source of food for some birds in times of scarcity, as well as a route of pesticide ingestion. Increased use of chemical pesticides has resulted in contamination of the environment and many associated long-term effects on human health, ranging from short term impacts such as headaches and nausea to chronic impacts such as cancer, reproductive harm, and endocrine disruption. Many other kinds of benefits which are often going unnoticed by general public may be attributed to the use of pesticides. Today, more than 800 products of pesticides are in regular use in India.

The markets of industrialized countries for pesticides are no longer growing as their governments are putting restrictions or limiting the use of pesticides due to their serious health implications to man and his environment.[4,5] Therefore, these companies are looking to developing countries for their increased sales.In the Indian market, the active ingredient (imidacloprid) is embodied in the trade products Gaucho for seed treatment and Confidor for leaf and soil treatment. Its use as a replacement for other insecticides is increasing.

The extensive use of insecticides has been criticized in recent years due to their persistence in the environment and their accumulation in the living tissues of organisms. On the basis of animal studies, it is classified as a “moderate toxic” (class II by WHO and toxicity category II EPA).[6] It is not banned, restricted, canceled, or illegal to import in any country. Therefore, the health risks to humans of this class of insecticides have attracted the

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attention of many investigators. Imidacloprid (IMC) (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) was the first representative of neonicotinoid insecticides to be registered for use and is presently the most important commercial product because of its high efficacy against insects. Besides its agricultural use, it is also used to control houseflies on poultry farms. There is paucity of information available concerning the effects of IMC on animal health, as the insecticide that is likely to be used in future pest control programs.[7]

The neonicotinoids were developed in large part because they show reduced toxicity compared to previously used organophosphate and carbamate insecticides. Most neonicotinoids show much lower toxicity in mammals than insects, but some breakdown products are toxic. Knowledge of the most hazardous substances would enable medical professionals and would-be mothers to minimize foetal exposure to them, helping to achieve the laudable goal of abolishing teratogen-induced malformations. Many insecticides were developed, but many other remaining areas in pest control were filled with neonicotinoids, which have excellent effectiveness and low relative toxicity to the environment and mammals.

2. Materials and methods

The current study was carried out in the department of Anatomy Santosh Medical College Ghaziabad U.P. and Govt. Medical College, Ambedkar Nagar on 400 fertile eggs of white leghorn chicken weighing between 35–55 grams after obtaining permission from the Institutional Animal Ethical Committee at Santosh Medical College, Ghaziabad.

Sample size: 400 fertile eggs used andsample size[8] was estimated using the following formula for difference in proportions in experimental studies.

$$n = \frac{2(\overline{p})(1-\overline{p})\left(Z_{\beta} + Z_{\frac{\alpha}{2}}\right)^2}{(p1-p2)^2}$$

Where n = sample size in each group (assuming equal sized groups)

$$\overline{p} = \frac{(p1+p2)}{2}$$

Z_{β} = Desired power (equals 0.84 for 80% power)

$Z_{\alpha/2}$ = Desired level of statistical significance (typically 1.96 for 95% CI)

P_1 = prevalence of toxic effects in control group

P_2 = prevalence of toxic effects in experimental group.

This study also aims at studying the effects on development of chick embryos with increasing doses of the Imidacloprid. The study plans at three other groups of experiment at 5 μg, 10 μg and 20 μg of Imidacloprid. The sample size calculated for dose 2.5 μg was applied to other groups also. The final sample sizes used in this study are tabulated (Table 1) below.

Table 1
Showing sample size and doses in experimental and control groups.

	Experimental Groups		Control Groups	
	Dose (Imidacloprid)	Sample size	Dose (Normal Saline)	Sample size
Group A	2.5 μg	50	2.5 μg	50
Group B	5 μg	50	5 μg	50
Group C	10 μg	50	10 μg	50
Group D	20 μg	50	20 μg	50

2.1. Eggs Collection and processing

Eggs from stock known to be nutritionally healthy were taken. Eggs were first candled in the order to discard the defective ones and to outline the exact location of the air cell with a pencil and measure the weight of fertile eggs by digital weighing machine. All the eggs were thoroughly washed with soap water solution and incubated immediately in standard electrical digital incubator (Micro Scientific Works Ltd.) with their broad end up where the chorioallantoic membrane is situated and were rotated three times daily along their longitudinal and vertical axis as advised by Olsen and Byerly.[9] The thermostat of the incubator will be set at temperature of 38 °C in a humidity inside the chamber will be maintained at 60–80 percent with no additional CO_2 or O_2 and the eggs were tilted three times a day.

2.2. Exposure of fertile eggs with doses of Imidacloprid Insecticide

Eggs were candled on 2nd day to discard unfertilized eggs prior to exposure. Eggs were randomly divided into four groups A, B, C and D. Each group had 50 eggs each. Control groups same as test were treated with equivalent volumes of normal saline, whereas test groups A, B, C and D were exposed to Imidacloprid with doses of 2.5 μg, 5 μg, 10 μg and 20 μg in volumes of 2.5 μl, 5 μl, 10 μl and 20 μl respectively.

The solutions were taken in a tuberculin syringe. The broad end of the egg was wiped with a sterile gauze pad moistened with 70% alcohol solutions. A hole was drilled in eggshell in the centre of the surface over the air cell with a sterile needle; care was taken not to damage the shell membranes with point of drill. This is to avoid contact of air with the egg membrane. The needle was inserted horizontally into the air cell. The needle was wiped with a sterile gauze pad between each injection and hole of the shell was sealed with Candle melted wax. After injection of drug, eggs were again kept for incubation at 38° Ctemperature.The embryos were terminated and eggs removed from the incubator on 21st day, the egg shell were broken with a scalpel and the embryos were removed. The number of live and dead embryos was noted. Gross abnormalities,teratogenicand developmental effects observed carefully and recorded in all the embryos. Parameters namely weight of chick embryosby digital weighing machine, crown rump length and head circumference of chick embryos by digital vernier calipers were measured. The dissection of chick embryos was done to observe the others internal effects on developmental and morphological changes in chick embryos were carefully observed and photographs.

2.3. Statistical Analysis

Dichotomous variables were measured in proportions and continuous variables were measured as Mean± Standard deviation. Calculations were done using Microsoft Excel®. Preparation of graphs and applications of other statistical tests were done using Graph Pad Prism (5.0) software. Chi- squared test or Fisher's exact tests as applicable was used to measure the association between proportions. Chi- squared test for trend was used to measure the association of increasing dose of Imidacloprid over different developmental anomalies. The difference in continuous variables

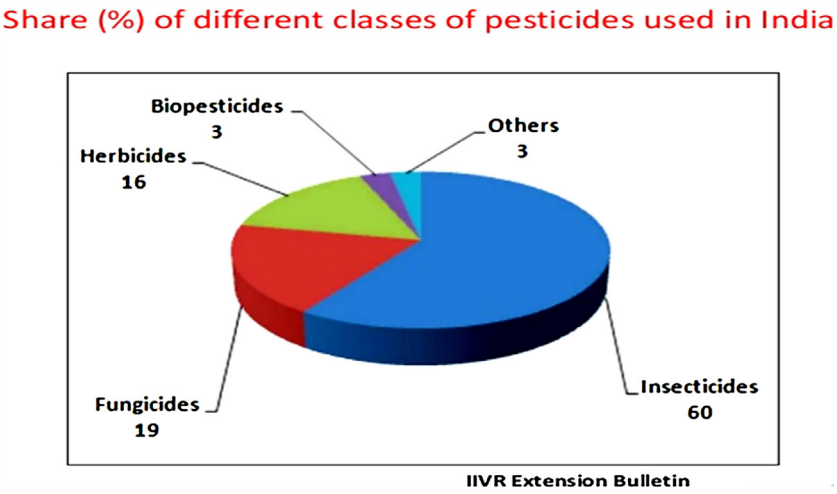

Fig. 1. Showing percentage of different classes of pesticides used in India.

was measured using paired/unpaired *t*-test, as applicable. P-value of less than 0.05 was taken as statistically significant (Fig. 1).

3. Results

In our current study on chick embryos we examined developmental and teratogenic effects namely; Growth Retardation (Fig. 4), Failure of Retraction of Yolk sac (YS) (Fig. 5), Limbs defects (Fig. 2), Ectopia Viscerale (Fig. 3) scanty feathers (Fig. 4) in comparison to controls normal (Fig. 1) shown in Plate 1.

In the control groups, either no developmental effect, including growth retardation, head enlargement, limb defects, ectopia viscerale and failure of retraction of yolk sac, was seen or if seen, the developmental effects were significantly less ($p < 0.05$) than the respective study groups. In the study groups, significantly higher number of all developmental effects was seen. A chi-squared test for trend revealed a significantly higher ($p < 0.05$) number of developmental effects with increasing doses of Imidacloprid for Growth retardation and Ectopia viscerale. Imidacloprid causes teratogenic effects and developmental delays or smaller embryos after exposure to Neonicotinoid insecticide Imidacloprid. The effects of imidacloprid on growth retardation overall statistically significant for embryos at 10 μg and 20 μg levels (Table 2). Imidacloprid had significant adverse effects on embryos failure of retraction of yolk sac although the control group has also shown the failure of retraction of yolk sac but the difference was found to be statistically significant ($p < 0.001$) shown in Table 2.

The effects of varying concentrations of Neonicotinoid Insecticide Imidacloprid on decrease weight of chick embryos in

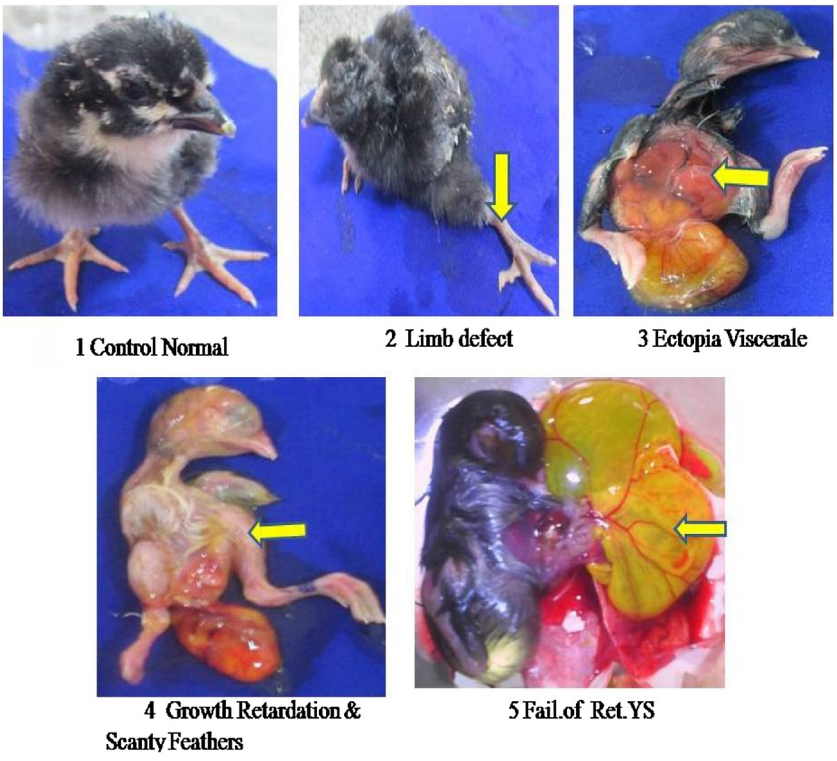

Plate 1. Showing teratogenic effects on chick embryos development after exposure to Neonicotinoid Insecticide Imidacloprid in comparison to controls.

Table 2
Showing the effects on development of chick embryos after exposure to neonicotinoid insecticide imidacloprid (IMC) in comparison to controls normal saline (NS).

S. No.	Developmental effects	Number of chick embryos in which developmental effects detected											
		Group A			Group B			Group C			Group D		
		Study (2.5 μL IMC) (n = 50)	Control (2.5 μL NS) (n = 50)	p-value	Study (5.0 μL IMC) (n = 50)	Control (5.0 μL NS) (n = 50)	p-value	Study (10.0 μL IMC) (n = 50)	Control (10.0 μL NS) (n = 50)	p-value	Study (20.0 μL IMC) (n = 50)	Control (20.0 μL NS) (n = 50)	p-value
1	Growth retardation	18	0	–	24	0	–	25	1	<0.001*	35	2	<0.001*
2	Head enlargement	4	0	–	5	1	<0.001*	5	0	–	10	3	0.071*
3	Limb deformities	11	0	–	11	0	–	15	2	0.001	15	1	<0.001*
4	Beak deformities	7	0	–	6	0	–	10	2	0.027	16	1	<0.001*
5	Ectopia viscerale	4	0	–	7	0	–	16	0	–	26	0	–
6	Scanty feather	8	0	–	12	1	<0.001*	14	0	–	19	1	<0.001*
7	Failure of retraction of yolk sac	39	5	<0.001	33	5	<0.001	34	7	<0.001	40	6	<0.001

* Fisher exact p-value.

Table 3
Showing the effect of varying concentrations of Imidacloprid Insecticide on weight of chick embryos in comparison to controls.

Groups	Dose	Number of chick embryos	Mean weight of chick embryos (in gms) (95% CI)	Standard Deviation	p-value
GROUP A					
Test	2.5 μl Imidacloprid	50	30.8 (29.5–32.2)	4.8	0.047^{NS}
Control	2.5 μl Normal saline	50	32.7 (31.4–34.0)	4.5	
GROUP B					
Test	5.0 μl Imidacloprid	50	29.4 (28.1–30.7)	4.5	<0.001**
Control	5.0 μl Normal saline	50	33.7 (32.5–34.9)	4.2	
GROUP C					
Test	10 μl Imidacloprid	50	27.3 (25.8–28.8)	5.3	<0.001**
Control	10 μl Normal saline	50	31.2 (30.2–32.2)	3.5	
GROUP D					
Test	20 μl Imidacloprid	50	20.5 (18.6–22.5)	6.7	<0.001**
Control	20 μl Normal saline	50	31.1 (30.2–31.2)	3.0	

*Significant **highly significant, ^{NS} Non Significant and 95% Conf. Interval (CI).

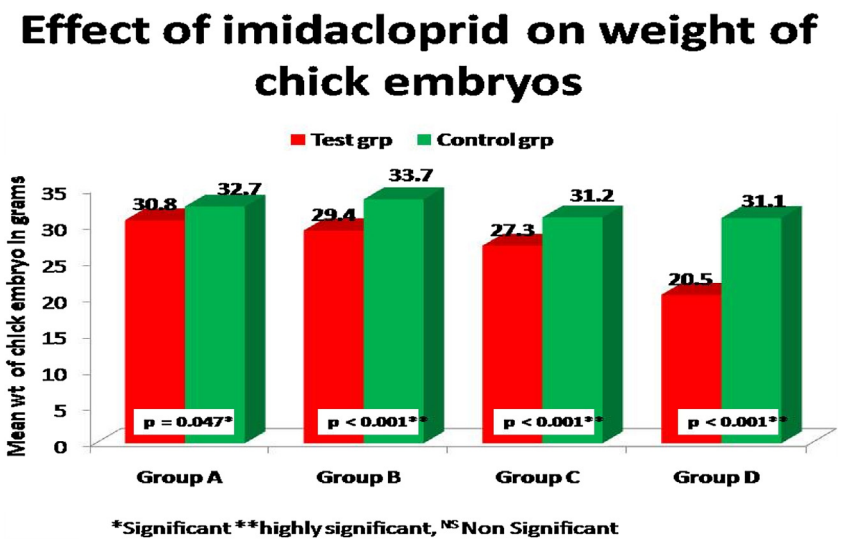

Fig. 2. Showing the effect of varying concentrations of Imidacloprid Insecticide on weight of chick embryos in comparison to controls.

Table 4
Showing the effect of varying concentrations of Neonicotinoid Insecticide Imidacloprid on Crown Rump Length of chick embryos in comparison to controls.

Groups	Dose	Number of chick embryos	Mean CR Length (in cm) (95% CI)	Standard Deviation (in cm)	p-value
GROUP A					
Test	2.5 μl Imidacloprid	50	7.7 (7.4–8.0)	1.01	0.068^{NS}
Control	2.5 μl Normal saline	50	8.0 (7.8–8.3)	0.78	
GROUP B					
Test	5.0 μl Imidacloprid	50	6.8 (6.4–7.3)	1.5	<0.001**
Control	5.0 μl Normal saline	50	8.0 (7.7–8.3)	1.1	
GROUP C					
Test	10 μl Imidacloprid	50	5.4 (5.0–5.8)	1.3	<0.001**
Control	10 μl Normal saline	50	7.6 (7.3–7.9)	1.1	
GROUP D					
Test	20 μl Imidacloprid	50	5.2 (4.7–5.6)	1.5	<0.001**
Control	20 μl Normal saline	50	9.2 (9.0–9.5)	0.85	

*Significant **highly significant and ^{NS} Non Significant. 95% Conf. Interval (CI).

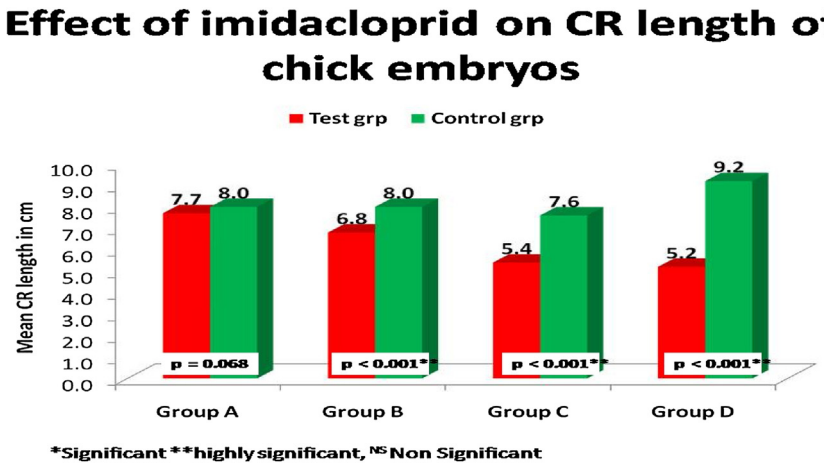

Fig. 3. Showing the effect of varying concentrations of Neonicotinoid Insecticide Imidacloprid on Crown Rump Length of chick embryos in comparison to controls.

comparison to control statistically significant ($p < 0.001$) at 5 μl, 10 μl, 20 μl levels and it was statistically non-significant ($p \geq 0.05$) at 2.5 μl level (Table 3 and Fig. 2).

In present study showing effects of varying concentrations of Neonicotinoid Insecticide Imidacloprid on decrease Crown Rump of chick embryos in comparison to control statistically significant ($p < 0.001$) at 5 μl, 10 μl 20 μl levels and it was no significant at 2.5 μl level (Table 4 and Fig. 3).

The effects of varying concentrations of Neonicotinoid Insecticide Imidacloprid on Head Circumference of chick embryos in comparison to control statistically significant($p < 0.001$) at 5 μl, 10 μl 20 μl levels and it was no significant at 2.5 μl level (Table 5 and Fig. 4).

4. Discussion

The use of pesticides has created a type of chemical environment which is proving harmful to the living systems. Neonicotinoids are widely applied pesticides due to their higher affinity for insect nicotinic acetylcholine receptors.

India being agrarian state needs to take specific steps to educate farmers about pesticides ill effects and their judicious use so to limits its hazardous effect to the non-target species that are exposed to it directly or indirectly as taken along with food etc being residue in the agricultural products. Imidacloprid is a neurotoxin that is selectively toxic to insects relative to vertebrates and most non-insect invertebrates. In mammals, the primary effects following acute high-dose oral exposure to imidacloprid are mortality, transient cholinergic effects (dizziness, apathy, locomotor effects, labored breathing) and transient growth retardation. Pesticides are considered as a significant source of diverse pollutants that can cause health implications in humans.[10]

Akhtar et al. (2006) studied on exposure to various environmental chemicals especially pesticides during developmental period is liable to give rise to congenital defects.[11] A specific concern about imidacloprid is that it may cause similar developmental defects as the known teratogen nicotine. For

Table 5
Effect of varying concentrations of Neonicotinoid Insecticide Imidacloprid on Head Circumference of chick embryos in comparison to controls.

Groups	Dose	Number of chick embryos	Mean HC (in cm) (95% CI)	Standard Deviation (in cm)	p-value
GROUP A					
Test	2.5 μl Imidacloprid	50	5.9 (5.6–6.1)	1.0	0.067^{NS}
Control	2.5 μl Normal saline	50	6.2 (6.0–6.3)	0.55	
GROUP B					
Test	5.0 μl Imidacloprid	50	5.5 (5.2–5.8)	1.0	0.053*
Control	5.0 μl Normal saline	50	5.8 (5.6-6.0)	0.74	
GROUP C					
Test	10 μl Imidacloprid	50	4.4 (4.0–4.8)	1.3	<0.001**
Control	10 μl Normal saline	50	5.9 (5.8–6.1)	0.63	
GROUP D					
Test	20 μl Imidacloprid	50	4.6 (4.1–5.0)	1.5	<0.001**
Control	20 μl Normal saline	50	6.4 (6.1–6.6)	0.97	

*Significant **highly significant and ^{NS} Non Significant. 95% Conf. Interval (CI).

developmental studies, chicken embryos are a model organism because they are inexpensive, easy to control with dosing and sensitive to toxins and are vertebrates reported by Ejaz and Woong (2006).[12]

Epidemiological studies have shown neurobehavioral and cognitive deficits and increased susceptibility to disease in offspring at various developmental stages, all associated with maternal exposure to neurotoxic chemicals during pregnancy.[13]

It is essential to assess the present environmental load of imidacloprid residues in different food commodities because imidacloprid is a toxic chemical.[14,15] Imidacloprid toxicity is high in case of lung exposure, when it is inhaled in form of aerosols, but very low when inhaling dust. After the rats' absorption of imidacloprid, it was distributed throughout the whole body except the fatty tissues, the central nervous system (CNS), and the mineral portion of bones.[16] There are detailed studies of developmental immunotoxicity of imidacloprid in Wister rats reported by Lalita Gawade et al., 2013.[17]

Chronic exposure to imidacloprid also induces inflammation and oxidative stress in the liver and central nervous system of rats (V.Duzguner, S. Erdogan, 2012),[18] Japanese quail exposed to imidacloprid in layer chickens (A.M. Kammon et al., 2010).[19] Animal studies are important because, in some instances, they have shed light on mechanisms of teratogenicity and because when such an agent causes similar patterns of anomalies in several species, human teratogens should also be suspected.

Recently imidacloprid has raised concern because of reports of egg shell thinning; reduced egg production and hatching time which are considered as signs of possible endocrine disruption Berny et al. (1999)[20] and Matsuda et al. (2001).[21] A specific concern about imidacloprid is that it may cause similar developmental defects as the known teratogen nicotine. For developmental

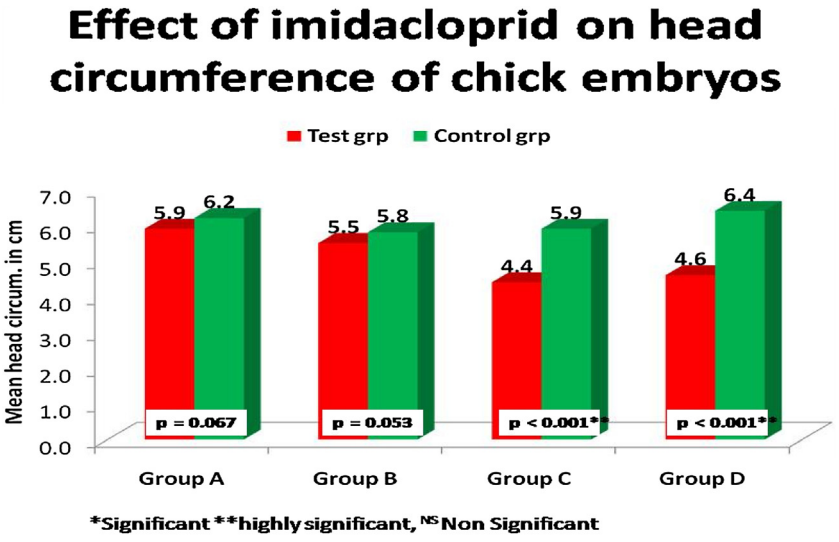

Fig. 4. Effect of varying concentrations of Neonicotinoid Insecticide Imidacloprid on Head Circumference of chick embryos in comparison to controls.

studies, chicken embryos are a model organism because they are inexpensive, easy to control with dosing, sensitive to toxins, and are vertebrates reported by Ejaz and Woong (2006).[22]

5. Conclusion

In our current study, it can be concluded that the Neonicotinoid Insecticide imidacloprid is a potential teratogenic compound and therefore its use should be limited. Results shows that experimental group had comparatively more cases of growth retardation resulting into failure of retraction of yolk sac, head enlargement, limbs defects, ectopia viscerale, scanty feathers, beak defects, decrease Crown Rump Length, decrease weight and head circumference of chick embryos as compared to the controls.

Comparatively higher doses proved more toxic and also caused many teratogenic and developmental defects on chick embryos. It can be recommended that usage of neonicotinoid insecticide imidacloprid should be prevented or limited in the environment where pregnant animal or woman live.

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Further reading

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