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STUDY ON TOXIC EFFECTS OF NEONICOTINOID INSECTICIDE IMIDACLOPRID EXPOSURE IN CHICK EMBRYOS

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ABSTRACT
The discovery of neonicotinoids as important novel insecticides represents a milestone in insecticide research over the past three decades. The neonicotinoids represent the fastest-growing class of insecticides. Imidacloprid is one of the major representatives of the new generation of neonicotinoid insecticides derived from nicotine isolated from the tobacco plant. Worldwide, it is considered to be one of the insecticides used in the largest volume. The objective of current study is to observe the toxic and teratogenic effects of neonicotinoid insecticide imidacloprid exposure in chick embryos. The current study was carried out on 400 fertile eggs of white leghorn chicken obtained from government poultry farm after taking permission from animal ethical committee. Chicken eggs exposed to Imidacloprid with doses of 2.5µg, 5µg, 10µg and 20µg in a volume of 2.5µl, 5µl, 10µl and 20µl respectively and control same as test group. The embryos were terminated on 21st day, eggs shell broken with a scalpel and embryos removed. The toxic and teratogenic effects observed and recorded. The results show that experimental group had comparatively more cases of teratogenic and embryonic effects; 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. Imidacloprid exposure increases the risks of teratogenic effects with increasing embryonic age. Comparatively higher doses proved more toxic and also caused many embryonic and teratogenic effects.

KEYWORDS: Neonicotinoid insecticide Imidacloprid, chick embryos teratogenic and toxic effects.

INTRODUCTION

The use of pesticides to manage pests in land and water has posed health hazards to live stock and wildlife. Problems and outbreaks have been reported to occur among animals and human from insecticide exposure (Salih and Jaafar, 2013). Prolonged exposure to insecticides is known to cause chronic neurological syndrome, malignant tumors, immunosuppressive action, teratogenic effect, abortion and decreased fertility in experimental animals (Meeker *et al*., 2006). Pesticides are widely used in food production systems and in agriculture sectors of some of the countries because of their increased food demands. Also, a large number of benefits have been derived from the use of pesticides in public health, forestry and domestic sphere. 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. 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 (Pretty J., 2005; Savithri *et al*., 2010) 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. The neonicotinoids, the newest major class of insecticides, have outstanding potency and systemic action for crop protection against piercing-sucking pests. 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 (Felsot A., 2001). 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 (Chen C. *et al*., 2011).

MATERIALS & METHODS

The current study was carried out in the department of Anatomy Govt. Medical College, Ambedkar Nagar and Santosh Medical College Ghaziabad U.P. on 400 fertile eggs of white leghorn chicken weighing between 35 to 55 grams obtained from the government poultry farm after

taking permission from animal ethical committee. 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. 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. The thermostat of the incubator will be set at temperature of 38°C in a humidity inside the chamber will be maintain at 60-80 percent with no additional CO_2 or O_2 and the eggs were tilted three times a day.

Exposure of Neonicotinoid Insecticide Imidacloprid in chick embryos

Eggs will be candled on 2nd day to discard unfertilized eggs prior to exposure. Eggs were divided into four groups A, B, C and D. Each group has 50 eggs each. Control same as test group, treated with same volume of normal saline, whereas test group A, B, C and D were exposed to Imidacloprid with doses of 2.5µg, 5µg, 10µg and 20µg in a volume of 2.5µl, 5µl, 10µl and 20µl respectively and control same as test group on 2nd day of incubation. The solutions were taken in a tuberculin syringe. The broad end of the egg was wiped with a sterile gauze pad moistened with 70 percent 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°C temperature. 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. Parameters namely weight of chick, crown rump length; size and weight of the embryos and the hardness of the tissue were measured. The dissection of chick embryo was done to observe the teratogenic effects, morphological changes and skeletal anomalies were carefully observed and photographs.

RESULTS

In our current study chick embryos were examined on 21st day for toxic and teratogenic effects and we observed Failure of Retraction of Yolk sac (YS) (figure 2, 3 and 6), Growth Retardation (figure 3 and 6), Limbs defects (figure 4), Head Enlargement (figure 5) and Ectopia Viscerale (figure 6) in comparison to controls normal (figure 1) shown in photograph 1 and table 1.

PHOTOGRAPH 1: Showing toxic and teratogenic effects of Neonicotinoid Insecticide Imidacloprid on chick embryos in comparison to controls

In the control groups, either no teratogenic effects, including growth retardation, head enlargement, limb defects, ectopia viscerale and failure of retraction of yolk sac, was seen or if seen, the teratogenic effects were significantly less ($p<0.05$) than the respective study groups. In the study groups, significantly higher number of all teratogenic effects was seen. A chi-squared test for trend revealed a significantly higher ($p<0.05$) number of

teratogenic 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 10µg and 20µg levels. 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 is statistically significant (p<0.001).

TABLE 1: Showing toxic and teratogenic effects on 21st day in chick embryos after exposure to Neonicotinoid Insecticide Imidacloprid (IMC) in comparison to controls normal saline (NS)

		Number of embryos in which teratogenic effects is detected											
		Group A			Group B			Group C			Group D		
S. No.	Teratogenic Effects	Study (2.5µL IMC) (n=50)	Control (2.5µL NS) (n=50)	p-value	Study (5.0µL IMC) (n=50)	Control (5.0µL NS) (n=50)	p-value	Study (10.0µL IMC) (n=50)	Control (10.0µL NS) (n=50)	p-value	Study (20.0µL IMC) (n=50)	Control (20.0µL 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 defects	11	0	-	11	0	-	15	2	0.001	15	1	<0.001*
4	Ectopia viscerale	4	0	-	7	0	-	16	0	-	26	0	-
5	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 2: Showing the effect of varying concentrations of Neonicotinoid Insecticide Imidacloprid on decrease 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	5.0µl 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	10µl 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	20µl Normal saline	50	31.1 (30.2-31.2)	3.0	

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

The effects of varying concentrations of Neonicotinoid Insecticide Imidacloprid on decrease weight of chick embryos in comparison to control statistically significant(p<0.001) at 5 µl, 10µl 20µl levels and it was non significant at 2.5 µllevel.

TABLE 3: 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	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	5.0µl 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	10µl 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	20µl Normal saline	50	9.2 (9.0-9.5)	0.85	

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

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, 10µl 20µl levels and it was no significant at 2.5 µl level.

TABLE 4: Mortality in chick embryos after exposed to varying concentrations of Neonicotinoid Insecticide Imidacloprid in comparison to controls.

Groups	Dose	Total number of fertile eggs used	Total number of dead chick embryos (%)	p-value
GROUP A				
Test	2.5 µl Imidacloprid	50	12 (24%)	0.008^{NS}
Control	2.5 µl Normal saline	50	2 (4%)	
GROUP B				
Test	5.0 µl Imidacloprid	50	15 (30%)	<0.001**
Control	5.0 µl Normal saline	50	2 (4%)	
GROUP C				
Test	2.5 µl Imidacloprid	50	19 (38%)	<0.001**
Control	2.5 µl Normal saline	50	5 (10%)	
GROUP D				
Test	5.0 µl Imidacloprid	50	23 (46%)	<0.001**
Control	5.0 µl Normal saline	50	4 (8%)	

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

The mortality induced by exposure of Imidacloprid in chick embryo we found dead embryos in experimental A group 12 (24%), B group 15(30%), C group 19 (38%) and D group 23(46%). In control we observed in A group 2 (4%), B group 2(4%), C group 5(10%) and D group 4(8%) embryos found dead shown in table 5. The mortality rate was 6.5% in control group and 34.5% in experimental group, this difference was statistically significant ($p<0.001$).

DISCUSSION

Imidacloprid was developed in 1985 with the aim of combining compounds with high potency against insects with low mammalian toxicity and favorable persistence. On the basis of animal studies, it is classified as a "moderate toxic" (class II by WHO and toxicity category II EPA)**.** 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 attention of many investigators. Neonicotinoids are widely applied pesticides due to their higher affinity for insect nicotinic acetylcholine receptors. These compounds are extensively applied to control pest insects in different agricultural crops; however they can also affect non target organisms (humans or biota). Still a limited number of studies are referring to neonicotinoids in terms of potential hazard for the additive/cumulative effects on human health and to toxic effects of their transformation products on aquatic nontarget organisms. Imidacloprid is a neurotoxin that is selectively toxic to insects relative to vertebrates and most non-insect invertebrates. Imidacloprid is a neonicotinoid insecticide which produces neurotoxicity through binding or partial binding to specific areas of the nicotinic acetylcholine receptor. Acetylcholine is an important neurotransmitter in both insects and mammals; it is released at the nerve synapse in response to a membrane depolarization which is the hallmark of nerve transmission. 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. In general, toxicity testing is conducted to determine the potential human health and environmental hazards of chemicals (*e.g.* pesticides) and their products. Human toxicology, by definition, is concerned with the effects of chemicals on a single species: man. In this case the research and testing are undertaken with one major objective, protection of the health of the individual. Pesticides are considered as a significant source of diverse pollutants that can cause health implications in humans (Hellweg and Geisler, 2003). It acts as an agonist at the postsynaptic nicotinic acetylcholine receptor (nAChR) in insects (Tomizawa Casida *et al.*, 2005). Exposures to high doses may be associated with degenerative changes in the testes, thymus, bone marrow and pancreas. Cardiovascular and hematological effects have also been observed at higher doses. 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. Akhtar *et al.* (2006) studied on exposure to various environmental chemicals especially pesticides during developmental period is liable to give rise to congenital defects. A specific concern about imidacloprid is that it may cause similar developmental defects as the known teratogen nicotine. For 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). One recent study by Capowiez *et al.* (2006) presents very interesting data. The study was about the effect of neonicotinoids on the behavior of two earthworm species.13 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 (Jacobson & Jacobson, 2002). 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 and Matsuda *et al.*, 2001). It is essential to assess the present environmental load of imidacloprid residues in different food commodities

because imidacloprid is a toxic chemical (Kapoor *et al*., 2010; Tomizawa *et al*., 2003). Toxicity signs of Imidacloprid were evident within 15 minutes following oral administration, might be due to rapid and complete absorption (Bai, *et al*., 1991; Solecki, R. *et al*., 2001) Monitoring Insecticide residues in food for the evaluation of food quality are a priority objective of pesticide research, to avoid possible risk to human health.

CONCLUSION

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. 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, decrease Crown Rump Length and weight of chick embryos as compared to the controls. Comparatively higher doses proved more toxic and also caused many teratogenic effects and developmental defects on chick embryos.

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