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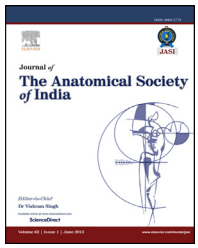

Original Article

# Study on embryonic effects of neonicotinoid insecticide on chick embryos

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ABSTRACT

*Introduction:* Neonicotinoids are a group of insecticides derived from nicotine isolated from the tobacco plant. Imidacloprid is a widely applied pesticide due to their higher affinity for insect nicotinic acetylcholine receptors. Like nicotine, it acts on nervous system. Worldwide, it is considered to be one of the insecticides used in the largest volume. It has a wide diversity of uses in agriculture, on turf, on pets, and for household pests.

*Methods:* Present study was carried out in the Department of Anatomy Government Medical College, Ambedkar Nagar and Santosh Medical College Ghaziabad U.P. on 280 fertile eggs of white leghorn chicken obtained from government poultry farm after taking permission from animal ethical committee. Chicken eggs after having been exposed to Imidacloprid with doses of 5 μg, 12.5 μg, 25 μg, and 50 μg in a volume of 5 μl, 12.5 μl, 25 μl and 50 μl respectively and control same as test group. The embryos were terminated on 18th and 20th days, egg shell broken with a scalpel and embryos removed. Gross abnormalities observed and recorded in all embryos.

*Results:* The results show that experimental group had comparatively more cases of delayed and growth retardation resulting into failure of retraction of yolk sac, limbs defects, neural tube defects as compared to controls. Comparatively higher doses proved more toxic and also caused many developmental defects.

*Discussion:* Neonicotinoid exposure increases the risks of developmental defects with increasing embryonic age. Imidacloprid caused developmental delays and defects on nervous system.



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## 1. Introduction

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 non-target organisms. The usage of high amount of pesticides in environment represents a possible risk for biota and human health due to their potential toxic action. Pesticide substances are biologically active and must be tested to ensure that their use will not give rise to any unacceptable risks to non-target organisms (i.e. humans, animals and plants) or to the environment.

The term 'neonicotinoid pesticides' comprises a group of several different insecticides, but it is usually used to indicate the four that are most widely applied imidacloprid, thiacloprid, clothianidin and thiamethoxam. One of these a representative, imidacloprid (IMI), was the main focus of this research. Imidacloprid represents the new generation of neurotoxic insecticides, which exhibit more selective toxicity for insects relative to mammals. Since being introduced in the insecticide market in 1992, the use of imidacloprid has increased yearly. It ranked as one of the top selling pesticides in the world in 2001–2002. Imidacloprid is a relatively new class of neonicotinoid pesticide with a distinct mode of action.[1] Since it is a systemic chloronicotinyl insecticide that blocks the microtinergic neuronal pathway, it is used for control of sucking insects such as rice hoppers, aphids, ticks, white flies, termites, and turf insects. It is commonly used on rice, soya beans, maize, potatoes, cotton, sugar beets, and kitchen garden vegetables and fruits.[2] 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.[3]

Imidacloprid interacts with the acetylcholine receptor, which is widely conserved across species.[4] In the past few years the agricultural production has been enormously enhanced by the use of many synthetic pesticides. Although, their application is based on selective toxicity for certain organisms yet it has resulted in serious effects on many non-target organisms as well. The use of pesticides has created a type of chemical environment which is proving harmful to the living systems. As a consequence of this, the environmental monitoring and their impact assessment have become the priority areas of research. In Jan. 2013, the European Food Safety stated that neonicotinoids pose an unacceptably high risk to bees, and that the industry-sponsored science upon which regulatory agencies claims of safety have relied may be flawed, concluding that, A high acute risk to honey bees was identified from exposure via dust drift for the seed treatment uses in maize, oilseed ripe and cereals.[5] Pesticides are major contaminants of our environment and many persist in the environment including in various feeds and foodstuffs.

Global pesticide use is increasing, particularly in third world countries. India uses approximately 85,000 tons of pesticides per annum and an 8% increase in pesticide use is expected every year. Imidacloprid was discovered in 1984 at Nihon Bayer Agrochem in Japan by screening novel synthetic compounds for a high affinity to the insect nicotinic AChRs receptors, but with low toxicity to vertebrate species reported by Kagabu.[6] In the Indian market, imidacloprid is included 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. Developmental neurotoxicity study (DNT) revealed decreased body weights, reduced motor activity level and changes in dimensions of brain structures (reduction in the thickness of corpus callosum and a decreased width of caudate putamen). 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. For obvious reasons no studies of teratogenicity are conducted during embryogenesis of humans.

## 2. Materials and methods

The present study was carried out in the department of Anatomy Govt. Medical College, Ambedkar Nagar and Santosh Medical College Ghaziabad U.P. on 280 fertile eggs of white leghorn chicken (Cochran WG. Sampling Techniques)[7] obtained from the government poultry farm after taking permission from animal ethical committee. Eggs from stock known to be nutritionally healthy as well as free from genetic defects 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 place immediately in standard electrical digital incubator (Macro Scientific Pvt. 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.[8] 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 $O_2$.

### 2.1. *Method for injection of neonicotinoid (Imidacloprid) in chick embryos on 3rd day*

Eggs will be candled on 3rd day to discard unfertilized eggs prior to injection. Eggs were divided into four groups A, B, C & D. Each group has 35 eggs each.[9] Control same as test group, treated with same volume of normal saline, whereas test group A, B, C & D were exposed to Imidacloprid with doses of 5 μg, 12.5 μg, 25 μg and 50 μg in a volume of 5 μl, 12.5 μl, 25 μl and 50 μl respectively on 3rd day of incubation shown in Table 1.

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 egg shell 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

**Table 1 – Shows doses of neonicotinoid (Imidacloprid) in chick embryos in different groups.**

| Doses | Groups | | | |
|---|---|---|---|---|
| | A (5 µg) | B (12.5 µg) | C (25 µg) | D (50 µg) |
| Normal saline (Control) | 5 µl | 12.5 µl | 25 µl | 50 µl |
| Imidacloprid (Test) | 5 µl | 12.5 µl | 25 µl | 50 µl |

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.

### 2.2. *Methodology*

After injection of drug, eggs were again kept for incubated at 38 °C temperature and before hatching eggs were broken to collect embryos for examination on 20th day of incubation. The embryos were terminated and eggs removed from the incubator on 18th and 20th days, the egg shell were broken with a scalpel and the embryos were removed. The number of live and dead embryos was noted. Gross skeletal malformation were carefully observed and recorded in all the embryos. The embryos decapitated and the heads were fixed in 10% formaldehyde for a period of 1 day through 14 days. The dissection of chick embryo head was done and brains removed from the skull. Parameters namely crown rump length, size of the head and the hardness of the tissue was measured. The embryonic effects of imidacloprid on chick embryos were observed and photographs.

## 3. Results

The chick embryo were examined for gross abnormalities and we observed in test groups (T) Failure of Retraction of Yolk sac (Figs. 3, 5 and 6), Growth Retardation (Figs. 1–4 and 6), Limbs

**Fig. 1 – Control normal.**


**Fig. 2 – Test failure of retraction of yolk sac.**

**Fig. 3 – Failure of retraction of yolk sac and growth retardation.**

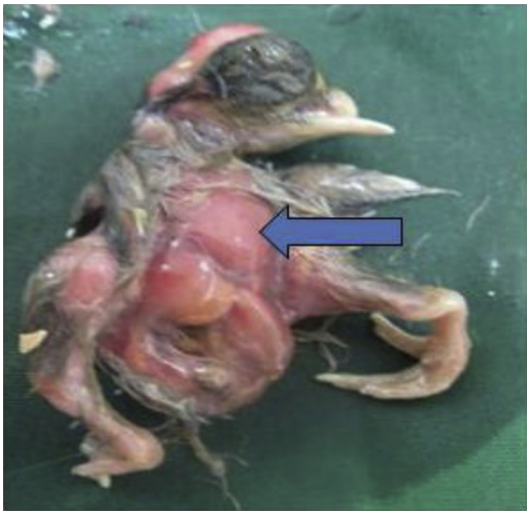

**Fig. 4 – Test growth retardation and scanty feathers.**

**Fig. 5 – Test head enlargement.**

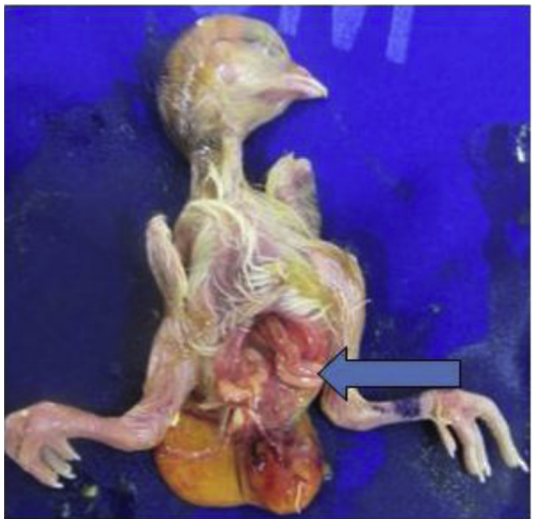

**Fig. 6 – Test ectopia viscerale.**

deformity (Fig. 4), Head Enlargement (Fig. 5), Beak deformity and Scanty feathers (Fig. 4), Ectopia Viscerale (Fig. 6) and control (C) normal shown in Table 2. Imidacloprid caused developmental delays or smaller embryos. The effects of imidacloprid on growth retardation overall statistically significant for embryos at 25 μg and 50 μg levels. Imidacloprid had a significant adverse effects on embryo 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 ($p > 0.001$).

The lethal effects and malformations induced by Imidacloprid in chick embryo we found dead embryos in test A group 7 (20%), B group 9 (25.7%), C group 12 (34.2%) and D group 16 (45.7%) Table 2. In control we observed in A group all chick embryo were live, B group 2 (5.7%), C group 3 (8.5%) and D group 3 (8.5%) embryos found dead shown in Table 3. The mortality rate was 5.7% in control group and 31.4% in test group, this difference was statistically significant ($p > 0.05$).

**Table 2 – Shows embryonic effects on 20th day in chick embryos after injection of imidacloprid.**

| S.no | Abnormalties | Groups | | | | |
|---|---|---|---|---|---|---|
| | | Control | A | B | C | D |
| 1. | Growth retardation | 0 | 5 | 7 | 13 | 17 |
| 2. | Head enlargement | 0 | 0 | 0 | 2 | 3 |
| 3. | Limbs deformities | 0 | 0 | 1 | 3 | 5 |
| 4. | Short beak | 0 | 0 | 0 | 1 | 2 |
| 5. | Ectopia viscerale | 0 | 0 | 0 | 2 | 3 |
| 6. | Failure of retraction of yolk sac | 3 | 09 | 12 | 15 | 18 |

## 4. Discussion

Pesticides can be classified according to their target, their mode or period of action, or their chemistry. They may be chemical substances, biological agents (such as viruses or bacteria), antimicrobials, disinfectants or devices used against any pest studied by Saravi and Shokrzadeh.[10] Newer classes of highly selective, systemic and single mode activity pesticides were introduced in the early 1990s and promised to address pest more specifically. Over the past twenty years, a class of systemic insecticides called neonicotinoids gained increasing interest in the agricultural sector.

The study of congenital anomalies is known as teratology. If an organ or organism clearly oversteps the reasonable limits in any range of variation, then the condition is known as abnormality, anomaly or malformation. 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 studied on exposure to various environmental chemicals especially pesticides during developmental period is liable to give rise to congenital defects.[11] Administration of pregnant rats with a single extraperitoneal injection of imidacloprid at the rate of 337 mg/kg b. wt produced neurobehavioral deficits, increased expression of glial fibrillary acidic protein in the motor cortex and hippocampus in offspring rats reported by Abou-Donia et al.[12] In 90 days oral toxicity study with imidacloprid in female rats at the concentration of 20 mg/kg/day evidenced decreased activity of acetylcholinesterase (AchE) in brain, spontaneous locomotor activity, histopathologically

**Table 3 – Shows lethal and embryonic effects induced by Imidacloprid in chick embryos.**

| Doses | No. of fertile eggs used | No. of dead embryos | No. of lire embryos |
|---|---|---|---|
| Control (N.S.) (A) 5 μl | 35 | 0 | 35 |
| (B) 12.5 μl | 35 | 2 | 33 |
| (C) 25 μl | 35 | 3 | 32 |
| (D) 50 μl | 35 | 3 | 32 |
| Imidacloprid (A) 5 μl | 35 | 7 | 28 |
| (B) 12.5 μl | 35 | 9 | 26 |
| (C) 25 μ1 | 35 | 12 | 23 |
| (D) 50 μl | 35 | 16 | 19 |

cerebellum of brain showed degenerative changes in purkinji cells and loss of granules in granular layer studied by Bhardwaj et al.[13]

Administration of imidacloprid at the rate of 80 mg/kg b wt/day through oral gavage for 28 days resulted in neurotoxicity which was evident from histopathological changes in brain like marked congestion in cerebellum, degeneration of purkinje cells with loss of dendrites, vacuolation around neurons, shrunken neurons, chromatolysis and ultra structural alterations like vacuolar mitochondria, apoptotic nuclei with disrupted and margination of chromatin material reported by Soujanyaet al.[14] One recent study by Capowiez et al presents very interesting data. The study was about the effect of neonicotinoids on the behaviour of two earthworm specie.[15] P.E.Natekar et al observed malformations in Methotrexate treated group of chick embryo were stunted growth, break deformities, limb deformities, scanty feathers, short wings and ectopia viscerale.[9] Recent findings suggest that thiamethoxam binds, compared to the other neonicotinoids sales products, in a different way, possibly to a different site of the receptor in aphids studied by H. Kayser et al.[16]

Imidacloprid residues, which are of toxicological significance in plant and animal commodities, include imidacloprid and its metabolites containing the 6-chloropyridinyl moiety. This moiety is common for several insecticidal neonicotinoids, which exert neurotoxicity via blockage of the nAChRs.[17] 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.

## 5. Conclusion

The Imidacloprid exposure increases the risk of developmental defects specially on nervous system. In the light of present study, it can be concluded that the imidacloprid is a potential teratogenic compound and therefore its use should be limited. Results show that experimental group had comparatively more cases of growth retardation resulting into failure of retraction of yolk sac, head enlargement, limbs defects and ectopia viscerale as compared to the controls. Comparatively higher doses proved more toxic and also caused many developmental defects on chick embryo.

## Conflicts of interest

All authors have none to declare.

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