INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

www.scienceandnature.org

IMMUNOHISTOCHEMICAL CHANGES IN CEREBRAL CORTEX OF CHICK EMBRYOS AFTER EXPOSURE TO NEONICOTINOID INSECTICIDE IMIDACLOPRID

[1]Vishram Singh, [1]Muktyaz Hussein, [2]Birendra Yadav, [3]Singh, A.K., [4]Hassan, M.A. & [5]Nigar Fatima
[1]Department of Anatomy, Santosh Medical College, Santosh University, Ghaziabad U.P. India.
[2]Department of Physiology, Govt. Medical College Ambedkar Nagar, U.P. India.
[3]Department of Anatomy, MLN Medical College Allahabad, U.P. India.
[4]Department of Com.Medicine, Govt. Medical College Ambedkar Nagar , U.P. India.
[5]Department of Anatomy, Integral Institute of Medical Sciences and Research, Lucknow, U.P. India.

ABSTRACT
Worldwide usage of insecticides enables the increase in agricultural productivity in the twentieth century. Imidacloprid was the first insecticide of the wide group of neonicotinoids introduced to the market. Because of wide application of neonicotinoids in farming, the majority of people in developed countries are chronically exposed to them since they are present in food and in drinking water. Imidacloprid is a widely applied pesticide due to their higher affinity for insect nicotinic acetylcholine receptors, it acts on nervous system. The current study was carried out on 300 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 5µg, 10µg and 20µg in a volume of 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. Dissection of chick head was done for collection of brain. Histological and Immunohistochemical processing was done for cerebrum of chick embryos using Neurofilament Polypeptide (NFP), Glial Fibrillary Acidic Protein (GFAP) Immunohistochemical markers observed under microscope and photographs. The results show that experimental group had comparatively more cases of histological changes, GFAP Immunoreactive in glial cells and NFP Immunoreactive in neuropil and neurons. The result shows patchy clustering and degenerative changes in neurons with focal pyknosis and karyolysis in comparison to controls.

KEYWORDS: Immunohistochemical changes, Imidacloprid, chick embryos and cerebral cortex.

INTRODUCTION

Imidacloprid is a systemic, chloro-nicotinyl insecticide with soil, seed and foliar uses for the control of sucking insects including rice hoppers, aphids, thrips, whiteflies, termites, turf insects, soil insects and some beetles. It is most commonly used on rice, cereal, maize, potatoes, vegetables, sugar beets, fruit, cotton, hops and turf, and is especially systemic when used as a seed or soil treatment. 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 (Kagabu,1997). Through the last decades, the use of neonicotinoids and their intake in the environment has grown rapidly (Jeschke *et al*., 2011), so it is of great importance that we gain knowledge about their impact on environment and on humans. Many epidemiological studies have suggested linkage between chronic exposure to pesticides and neurodegeneration, resulting in lowered cognitive performance and increased prevalence of different types of dementia, such as Alzheimer's, Parkinson's and Huntington's disease, and amyotrophic lateral sclerosis (Baldi *et al.*, 2003; Marambaud *et al*., 2009; Zaganas *et al*., 2013). One of today's commonly used pesticides whose safety has not been sufficiently explored is imidacloprid. Although imidacloprid (belonging to the group of neonicotinoids) is considered safe, it actually has a similar molecular structure as nicotine, which is known to affect nAChRs and adversely influence the development of the mammalian nervous system, especially the developing brain (Kimura-Kuroda J. *et al*., 2012). According to their chemical composition, they can be classified as organochlorines, organo phosphates, carbamates, pyrethroids, neonicotinoids, ryanoids, phenylpyrazoles, and others. Biochemical classification divides pesticides into different groups according to their target organisms: insecticides, herbicides, fungicides, rodenticides, acaricides, algicides, avicides, bactericides, molluscides and nematocides (EPA, 2012). Neonicotinoids are used to protect a lot of different plants, such as leafy and fruiting vegetables, sugar beet, cotton, pome fruit, grains, tobacco, rice, citruses, corn, oilseed rape, potatoes, stone fruit, soy beans, peanuts, artichoke, tea, sunflowers and some others (Jeschke *et al*., 2011). In veterinary medicine, products on the basis of neonicotinoids are used for treatment of external parasites, like fleas and ear mite on cats, dogs, and other domestic animals (Jeschke *et al*., 2011). Imidacloprid acts on nicotinic acetylcholine receptors in central nervous system and causes paralysis and death of insects. Chronic effects

for which there is substantial evidence of association with pesticide exposures include cancer, neurodevelopment and behavior effects, other neurological effects including neurodegenerative diseases, birth defects and other adverse birth outcomes, and respiratory diseases. More recently evidence has begun to emerge of associations with obesity, type 2 diabetes and metabolic disease. Some effects last a whole lifetime; some are passed on to future generations. Concern continues to mount about the reality of human exposure to ongoing low doses of mixtures of pesticides, especially those that cause endocrine disruption or damage the developing brain of the unborn foetus.

MATERIALS & METHODS

Study design

The present current study was carried out in the department of Anatomy Govt. Medical College, Ambedkar Nagar and Santosh Medical College Ghaziabad U.P. on 300 fertile eggs of white leghorn chicken weighing between 35 to 55 grams (g) 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 3rd day to discard unfertilized eggs prior to exposure. Eggs were divided into three groups 1, 2 and 3 each group having 50 eggs. Control same as test group, treated with normal same, whereas experimental group 1, 2 and 3 were exposed to Imidacloprid with doses of 5µg, 10µg and 20µg in a volume of 5µl, 10µl and 20µl respectively and control same as experimental group on 3rd 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 crown rump length; size and weight of the embryos and the hardness of the tissue were measured. The dissection of chick embryos was done to observe the gross morphological changes and skeletal anomalies were carefully observed and photographs. Dissection of chick head was done for collection of brain. The brain tissue proceeds for histological processing.

Histological Processing

The size, shape, weight of brains was measured by digital weighing machine and digital vernier caliper. The cerebrum of chick embryos placed in 10% neutral formalin solution for 24-48 hours and thoroughly washed with water before further processing dehydration, clearing, and embedding by automatic tissue processor (Thermo scientific Germany) at histology and research lab of Anatomy department. Serial section of blocks of embedded tissue were cut at 5µm, 7µm and 10µm thickness by Rotary microtome (Thermo scientific Germany) and mounted on glass slides and stained. The cerebrum sections were stain with Haematoxylin and Eosin (H&E) using standard methods to determine histological structures of cerebrum. Histological processing was optimized and neural tissue evaluated for any histological changes in cerebral cortex changes in neuron morphology and cell number examined by light microscope. The specific structural changes to neurons, neuroglial cells were observed and photographs.

Immunohistochemistry Processing

Immunohistochemistry or IHC refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara et al., 2005). IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry).Immunohistochemistry has emerged as a powerful investigative tool that can provide supplemental information to the routine morphological assessment of tissues. The use of Immunohistochemistry to study cellular markers that define specific phenotypes has provided important diagnostic, prognostic, and predictive information relative to disease status and biology. IHC staining was carried out to demonstrate the localization of various specific antigens in the cerebral cortex of experimental and control groups. In our current study we were used two Immunohistochemistry markers namely Glial Fibrillary Acidic Protein (GFAP) and Neurofilament Polypeptide (NFP) for cerebrum of chick embryos. Formalin fixed paraffin embedded tissue sections of cerebrum used for IHC. The cerebrum sections were incubated with primary and secondary antibodies using standard Immunohistochemistry technique (Hsu SM. et. al. 1981).

RESULTS

In the present current study of histological section of cerebrum of chick embryos showing degenerative changes in neurons with arrow in experimental group1 Fig.1 (B), shrinkage of many neurons due to degeneration and patchy clustering with arrow in experimental group 2 Fig.1 (C) and perineuronal edema with degenerative changes in purkinge cells with arrow in experimental group 3 Fig.1 (D) in comparison to control normal Fig.1 (A). In control the cerebral cortex consisting of molecular layer, outer granular layer and outer pyramidal layer. In outer

pyramidal layer nerve cell bodies and pyramidal cells are present and stained with Haematoxylin and Eosin in 400X.

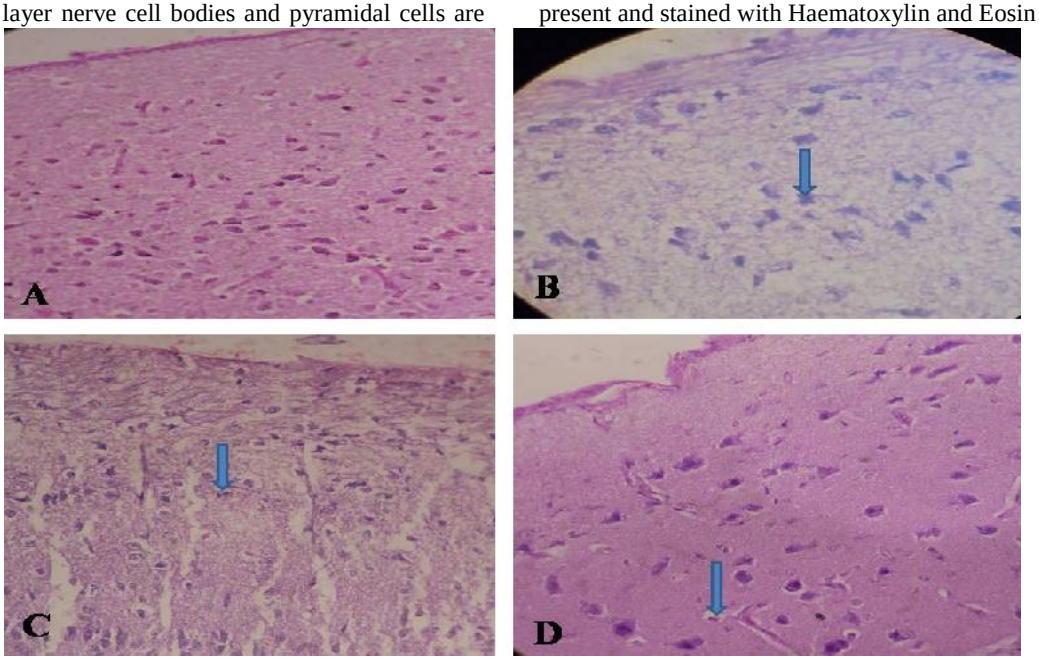


FIGURE 1- Photomicrographs of histological Haematoxylin and Eosin stained section of cerebral cortex of the chick embryos in experimental group1 (B), experimental group 2 (C), experimental group 3 (D) and comparison with control group (A) in H&E 400x.

In our present study we found expression of Glial Fibrillary Acidic Protein (GFAP) Immunohistochemistry marker in cerebral cortex was Immunoreactive in glial cells. The experimental group1 showing patchy clustering and degenerative changes in neurons with (arrow) Fig.2 (B). In experimental group 2 and 3 showing reactive and neoplastic astrocytes, degenerative changes in neurons with karyolysis and focal pyknosis with (arrow) Fig.2 (C) and (D) respectively. The expression of GFAP in comparison with experimental group to control group was normal Fig.2 (A). We did not found any evidence of inflammation or granuloma or malignancy in our present current study. Glial Fibrillary Acidic Protein (GFAP): It is one of the 5 major types of cytoplasmic filaments. It is present in normal, reactive and neoplastic astrocytes, developing reactive and neoplastic ependymal cells, developing and neoplastic oligodendrocytes. GFAP is also expressed in peripheral nerve sheath tumors and in mixed tumors of salivary and sweet glands.

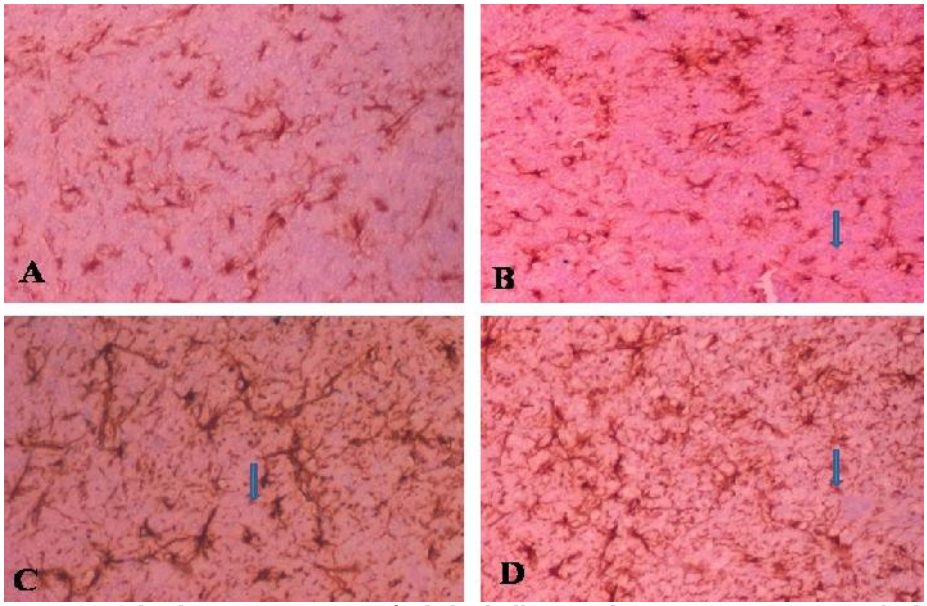


FIGURE 2- Photomicrographs showing expression of Glial Fibrillary Acidic Protein (GFAP) in cerebral cortex of chick embryos in Experimental group 1 Fig.2 (B), Experimental group 2 Fig.2 (C) and Experimental group 3 Fig. 2 (D) in comparison to control Fig.2 (A) in 400 X.

Neurofilament Polypeptide (NFP): Neurofilaments represents intermediate filaments of neurons and their processes. They are expressed in tumors of neuronal origin of differentiation like neuroblastoma, Medulloblastoma and Retinoblastoma. Positivity is also seen in merkel cell tumor of skin, Endocrine tumors of pancreas, Carcinoid tumors, Parathyroid tumors and neoplasms of endocrine nature. In our present study we observe expression of Neurofilament Polypeptide Immunohistochemistry marker of cerebral cortex was Immunoreactive in neuropil and neurons. The experimental group 1 showing patchy clustering aggregation in neurons with arrow Fig.3 (B). In experimental group 2 and 3 showing degenerative changes in neurons with focal markers pyknosis and karyolysis with arrow Fig.3 (C and D) respectively. The expression of Neurofilament Polypeptide in comparison to control group was normal Fig.3 (A).

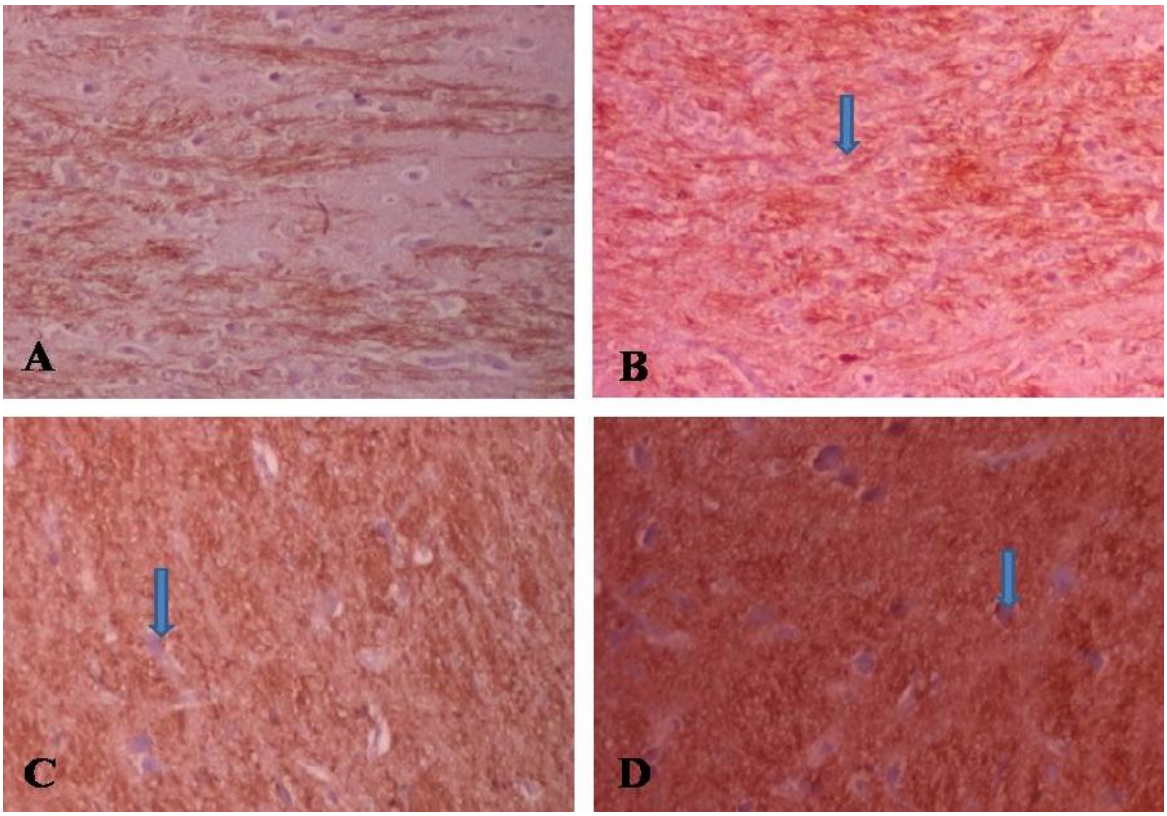


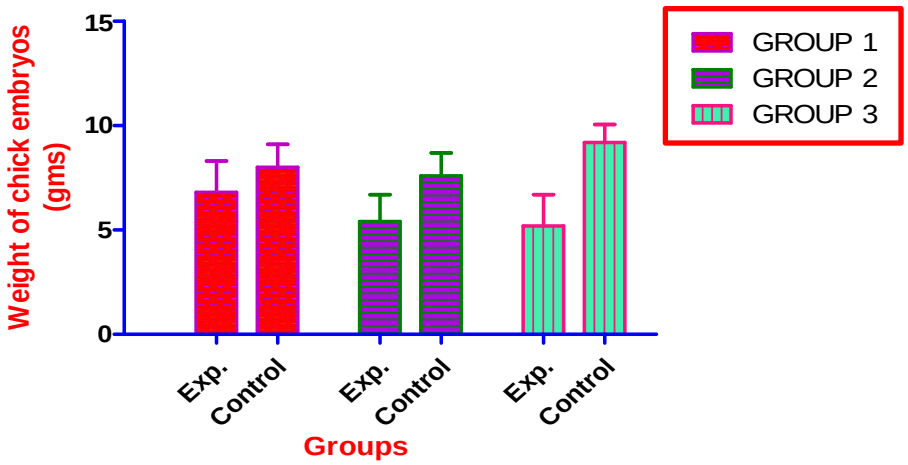
FIGURE 3- Photomicrographs showing expression of Neurofilament Polypeptide (NF) in cerebral cortex of chick embryos Experimental group1 Fig.3 (B), Experimental group 2 Fig. 3 (C) and Experimental group 3 Fig.3 (D) in comparison to Control normal Fig.3 (A) in 400X.

TABLE 1: Showing of the effects of Neonicotinoid Insecticide Imidacloprid on weight of chick embryos in comparison to controls group.

Groups	Dose	Number of chick embryos	Mean weight of chick embryos (in gms) (95% CI)	Standard Deviation	p-value
GROUP 1					
Experimental	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 2					
Experimental	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 3					
Experimental	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 showing decrease weight of chick embryos (grams) in comparison to control and statistically significant($p<0.001$) at 5 µl, 10µl 20µl levels in table 1 and Graph 1.



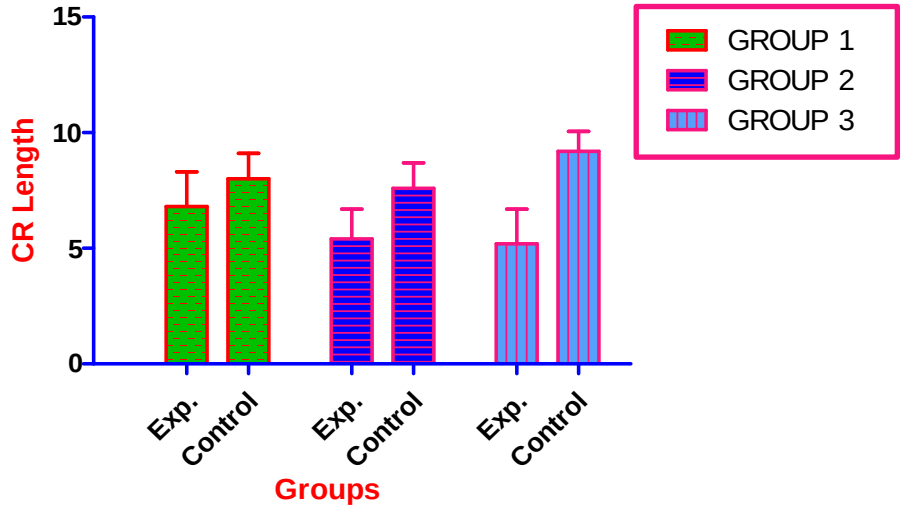
GRAPH 1: Effect of Neonicotinoid Insecticide Imidacloprid showing decrease weight of chick embryos Experimental (Exp.) groups in comparison to controls group.

TABLE 2: Showing the effects 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 1					
Experimental	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 2					
Experimental	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 3					
Experimental	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 the effects of varying concentrations of Neonicotinoid Insecticide Imidacloprid on Crown Rump of chick embryos decrease Experimental groups in comparison to control was statistically significant($p<0.001$) at 5 µl, 10µl 20µl levels in table 2 and graph 2.



GRAPH 2: Effect of Neonicotinoid Insecticide Imidacloprid showing decrease Crown Rump Length of chick embryos experimental (Exp.) groups in comparison to controls group.

DISCUSSION

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 (Gervais J. A. et al., 2010). Imidacloprid is highly toxic to beneficial insects, such as bees and other pollinators, and to other terrestrial and aquatic invertebrates. In invertebrates, imidacloprid has a cumulative and irreversible mode of action. By affecting the populations of the organisms on the bottom of the food chain, neonicotinoids have the potential to indirectly affect other organisms, such as birds, fish, reptiles, etc.

Taking everything into account there are many indices pointing out that imidacloprid could cause neurodegenerative processes in mammals and in humans at long-term exposure to low concentrations. It might also be involved into the pathogenesis of neurodegenerative diseases such as Parkinson and Alzheimer. Histopathology, the microscopic study of diseased tissue, is an important tool of anatomical pathology. In terrestrial and aquatic animals, insecticides produce toxic effects on different tissues. For understanding the pathological conditions of the animal, histological studies pave a way, to have a clear understanding as to how these insecticides cause injury to the tissues. It is essential to have an insight in to the histological analysis of the tissues. Histopathological changes with different pesticides have been reported earlier. (Frick et al., 1971, Newmaun and Maclean, 1974; Couch, 1977; Peguiguot et al., 1978; Kumar and Pant, 1985 and Usha Rani, 1986). But the literature on histopathology of imidacloprid on rat is scanty. Hence an attention has been made in the present investigation. There are detailed studies of developmental immunotoxicity of imidacloprid in Wister rats (Lalita Gawade et al., 2013). Imidacloprid induced histological and biochemical alterations in liver also found in female albino rats. Chronic exposure to imidacloprid also induces inflammation and oxidative stress in the liver and central nervous system of rats (V.Duzguner, S. Erdogan, 2012). Toor et al, 2013), Japanese quail exposed to imidacloprid in layer chickens (A.M. Kammon et al, 2010).

CONCLUSION

In the light of current study it is clearly indicated that imidacloprid induced marked histopathological and Immunohistochemical changes in cerebral cortex of chick embryos. The results of present study concluded that Neonicotinoid Insecticide imidacloprid is a potential teratogenic compound and the use of imidacloprid should be restricted to the minimal possible quantities. In the present study various histopathological changes observed in cerebral cortex of experimental groups showing degenerative changes in neurons with patchy pyknosis and focal ependymal lining seen. The expression of Neurofilament Polypeptide in cerebral cortex was Immunoreactive in neuropil with scattered neurons and expression of GFAP was Immunoreactive in glial cells. The effects of Imidacloprid showing decrease weight and Crown Rump Length of chick embryos experimental groups in comparison to controls group.

ACKNOWLEDGMENTS

I express my gratitude to Dr. Arvind Kumar Singh, Assistant Professor, Department of Com.Medicine, Rana BP Singh, Lecturer (Statistics), Department of Com.Medicine, Dr P.K.Singh, Professor & Principal and Dr Rajesh Gautam, Lecturer, Department of Pathology. Dr Ajay Kumar Singh, Lecturer, Department of biochemistry, Govt. Medical College Ambedkar Nagar U.P. India., for suggesting the research problem, statistical analysis, constant encouragement and valuable guidance. I also thankful to my wife Mrs. Nigar Hussain for the strength and help.

REFERENCES

A.M. Kammon, R.S.Brar, H.S.Banga, S.Sodhi (2010). Patho-biochemical studies on hepatotoxicity and nephrotoxicity on exposure to chloropyrifos and imidacloprid in layer chickens, Veterinarski Arhiv., 80:663-672.

A. Usha Rani (1986). Effect of cadmium on some aspects of physiology and histology in the edible fresh water Teleost, Tilapia mossambica (Peters). Ph.D Thesis, Sri Venkateswara University, Tirupati, India.

Baldi I., Lebailly P., Mohammed-Brahim B., Letenneur L., Dartigues J.-F., Brochard P. (2003). Neurodegenerative diseases and exposure to pesticides in the elderly. American Journal of Epidemiology, 157: 409-414.

D.F.Frick, H.F. Kraybih and J.M. Dimitraff (1971). Toxic effects of cadmiumareview.Environ.Res.,4:71-85.

EPA. (2012). Types of Pesticides. U.S Environmental Protection Agency. http://www.epa.gov/pesticides/about/types.htm (3.6.2014).

Gervais, J. A., Luukinen, B., Buhl, K., Stone, D. (2010). Imidacloprid technical fact sheet. National Pesticide Information Center, Oregon State University Extension Services http://npic.orst.edu/factsheets/imidacloprid.pdf. (26.3.2014).

Hsu SM, Raine L, Fanger H. (1981) use of biotin-avidin peroxidase complex (ABP) in Immuno peroxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem., 29:577-580.

J.A.Couch (1977). Ultrastructural study of lesions in gills of a marine shrimp exposed to cadnium. J. Invertebrata Pathology., 29:267-286.

Jeschke P, Nauen R. (2005). Neonicotinoid insecticides. In: Comprehensive Molecular Insect Science. Oxford, UK, Elsevier: vol. 5: 53-105

Jeschke P., Nauen R., Schindler M., Elbert A. (2011). Overview of the status and global strategy for neonicotinoids. Journal of Agricultural and Food Chemistry, 59: 2897-2908.

J. Peguiguot, M. Gire and Pand A Moga (1978). Eur. J. Toxicol., 8:165-168.

Kagabu S. (1997) Chloronicotinyl insecticides discovery, application and future prospective. Rev Toxicol., 1:75-129.

Kimura-Kuroda J., Komuta Y., Kuroda Y., Hayashi M., Kawano H. (2012). Nicotine-like effects of neonicotinoid insecticides acetamipride and imidacloprid on cerebellar neurons from neonatal rats. PLoS ONE, 7, 2: 1-11.

L.Gawade, S.S. Dadarkar, R.Husain, M.Gante (2013). A detailed study of developmental immunotoxicity of imidacloprid in wistar rats. Food and Chemical Toxicology, Volume 51:61-70.

Marambaud P., Dreses-Werringloer U., Vingtdeux V. (2009). Calcium signaling in neurodegeneration. Molecular Neurodegeneration, 4: 20.

M.W. Newmaun and S.A. Maclean (1974). Physiological response of the cannew, Tautogoloabrus adspersus to cadmium, VI Histopathology, NOAA Tech. Rep. NMFS.SSRF-681:27-33.

Ramos-Vara (2005). JA Technical Aspects of Immunohistochemistry, Vet Pathol 42 (4): 405-426. Doi.,10:1354/vp.42-4-405.

S. Kumar and S.C. Pant (1985). Renal pathology in fish (Puntius conchonius, Ham) following exposure to acutely lethal and sublethal concentratons of monocrotophos. B & CT., 35(2):228-33.

V. Duzguner, S. Erdogan (2010). Acute oxidant and inflammatory effects of imidacloprid on the mammalian central nervous system. Pestic. Biochem Physiol., 97:13–18.

Zaganas I., Kapetanaki S., Mastorodemos V., Kanavouras K., Colosio C., Wilks M.F., Tsatsakis A. (2013). Linking pesticide exposure and dementia: What is the evidence? Toxicology, 307: 3-11.