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BEHAVIORAL ALTERATIONS AND NEUROTOXICITY OF IMIDACLOPRID ON FRESHWATER TELEOST *Oreochromis mossambicus*

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Abstract
Agrochemicals are a significant cause of concern for the aquatic environment due to their toxicity, persistence and propensity to build up in the organisms. Imidacloprid (IMI) is a neonicotinoid insecticide widely used across the world. Improper and widespread usage and accidental exposure through agriculture runoff, IMI poses a major threat to non-target aquatic organisms. Although fish being a major source of protein-rich food for the human being, concerns are raised against the health status and vulnerability of fish, leading to the entry of toxicants into the food chain. In this context, the present study is a replicating condition of insecticide exposure in the laboratory, where the toxic potential of IMI with their two sub lethal concentration ($LC_{50/10th}$ and $LC_{50/20th}$) was tested (0.074 ppm and 0.04 ppm). Furthermore, the acute toxicity effect of IMI was validated by the behavioral response, histological alteration and biochemical estimation of Acetylcholine esterase (AchE) in the brain of *Oreochromis mossambicus*. Our result demonstrates that IMI has resulted in neuronal injury in the brain, resulting in severe abnormalities and suggests that alteration in AchE levels can be a good bio- indicator for monitoring neuro-toxicity caused by insecticides.
Keywords: *Imidacloprid, sub-lethal, Oreochromis mossambicus, brain.*

Introduction

The potentially deleterious effect on various natural environment components has elevated a great deal of concern in scientific circles for pesticide management (Subhash *et al.*, 2017; Anand and Taneja,2020). Due to low cost and broad-spectrum toxicity, it is estimated that more than 100,000 tons of pesticides have been applied in India, primarily agricultural pest control (Sindhu *et al.,* 2019). The increase in agricultural production, crop protection, and yield have increased due to the use of chemical pesticides. The abundant use of these chemicals, under the adage, “If little is good, a lot more will be better” has played an essential role in increasing the consumption (Patel *et al*.,2016; Pandya *et al.,* 2018). The annual application of agricultural fertilizers and pesticides is over 140 billion kilograms, a vast source of pollutants through agricultural runoff (Dowd-Uribe*et al.,* 2008;Ficklinand Zhang, 2013;Vymazal and Brezinova, 2014., Patel *et al.,* 2016.). Application of such agrochemicals shows potential health hazards and has become a major concern for aquatic habitat due to their toxicity, persistency and tendency to accumulate in the organisms. (Pandya *et al.*, 2016;Karbalaei*et al*., 2018; Griffin *et al.,* 2019). The application of pesticides has increased several folds in India and is likely to increase in the coming years. The applied insecticides into agriculture fields easily get washed away, enter into the aquatic system in vast quantities and imbalance the ecosystem and induce physiological and biochemical effects on aquatic organisms. Fishes are most essential and highest interacted species of the aquatic ecosystem. They become a bridge between aquatic and terrestrial ecosystems as they are getting used by birds and mammals as a food source. (Sadekarpawar*et al*., 2013; Upadhyay*et al*., 2014; Sadekarpawar*et al*., 2015; Pandya *et al.,* 2020).

Over the last period, a new class of insecticides, the neonicotinoids, has developed the most important and fastest emerging pesticides on the global market (Jeschke *et al*., 2011; Casida and Durkin 2013; Kakoolaki*et al.,* 2013). When used as plant protection products, neonicotinoids function by becoming distributed systemically throughout the growing plant following seed or soil application. IMI is potential groundwater and surface water contaminant because it can leach and runoff from soil and crops (Desai and Parikh, 2012; PAN Pesticides database, 2016; Tabassum*et al*., 2016). It may also enter water bodies from spray drift or accidental spills, leading to local point-source contamination.There are reports which suggest that the minimum concentration of 10 µg/L IMI in the aquatic environment may have adverse effects on the embryonic and larval stages of common carp. (Islam *et al.,* 2019.) and in *L. variegatus* by decreasing survival, inhibiting behavior, interfering with the growth process, and shortening life span (Sardo and Soares, 2010). Imidacloprid is a neonicotinoid insecticide that causes paralysis of the central nervous system in insects. Moreover, it may also affect the central nervous system of humans and animals (Buszewski*et al*., 2019). Previous work of our lab has also reported physiological, biochemical, and histopathological alteration in *O. mossambicus* in the exposure of IMI in liver, kidney, and gills. (Sadekarpawar*et al*., 2010; Patel *et al.,* 2016) However, there was a lacuna for the evaluation of histoarchitectural alteration and analysis of Acetylcholine esterase activity from the brain; hence the present work is an attempt to assess the neurotoxic potential of IMI regarding Acetylcholine Esterase activity and histological change in the brain of *O. mossambicus* on the exposure of IMI.

Materials and method

Animal maintenance

Mature Tilapia *Oreochromis mossambicus* (15± 2 cm, 25 ± 1.9 g) of similar size in length and weight were acquired from the pure brooders of the Vadodara district and transferred to the laboratory. The acclimation period was 15 days at 27 ± 40°C, pH 7.4 ± 0.5, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L CaCO3 with a 12:12 light: dark photoperiod. Fish were supplied daily with commercial fish food during acclimation. The maintenance of animal and experimental protocols were in accordance with the guideline of APHA-AWWA-WEF (1998).

Experimental protocol

Based on the LC_{50} value (Patel *et al.,* 2016) of IMI sub-lethal study, where the toxic potential of IMI with their two sub-lethal concentrations $LC_{50/10th}$ High Dose (HD) and $LC_{50/20th}$ Low Dose (LD) was tested (0.074 ppm and 0.04 ppm). The experimental regime was maintained in the laboratory for 14 days, with a control group having three replicates in each group. The experiment was performed semi-statically with a group of 10 fishes (5 males and 5 females) in experimental aquaria. All the groups were kept under continuous observation during the experimental period for behavioural study like hyperactivity, restlessness, jerky movement, loss of equilibrium, fin movement and mucus secretion. After the completion of the exposure of 14 days, fishes were caught very gently using a small dip net, one at a time with the least disturbance. They were slowly released in the tough containing 1% clove oil to make it immobile and afterwards allowed to dissect out the brain and was analysed by HE staining for its cellular architectural changes and histological alterations in fish exposed to low dose (LD) and high dose (HD) of IMI as compare to control. After measuring the weight, fresh tissues were fixed in 4% paraformaldehyde for 2 hrs, degraded and embedded in paraffin wax

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and sectioned at 10 -12 um then stained with haematoxylin and eosin and examined microscopically and photographed using a digital camera. After 14 days of exposure, the brains of Tilapia were homogenized in 0.1 M PBS. The homogenate was centrifuged at 10000 g at 4 °C for 15 min to obtain the supernatant. The supernatant was found to determine AChE activity. Triplicate samples from each treatment group were collected and measured the AChE activity by the Elman method 1961.
Statistical analysis- The difference between the mean of control and the exposed fishes was determined by one-way ANOVA using Graph Pad Prism software version 6. If there was any significant difference, a post hoc test was carried out where Dunnett's multiple comparison tests were employed to recognize differences in the alterations that were found between the control and the exposed groups. The significant level of the tests was set at 5% ($p < 0.05$).

Results

Behavioral Study

Sub-lethal exposure of IMI resulted in an overall increase in behavioral patterns alteration compared to control. Frequency in the alteration of behavior, like restlessness, jerky movement, loss of equilibrium, and hyperactivity, was found to be more increased in HD than in LD treated IMI group and control. A significant change was observed in the tendency of fin movement and mucus secretion also. Based on this study, we can predict the alteration in behavior patterns is dose and exposure time dependent. (Table 1)

Histological Study

A dose dependent alterations in the brain of *O. mossambicus* was observed on the exposure of IMI for a period of 14 days. Severity in histological changes was characterized by vacuolation (VC), necrosis of neurons (NC), with degeneration of neural cells, distended sinusoids (DS), and mild structural damages (SD) were observed in the brain of the fish exposed to a low dose of IMI. Whereas, when exposed to a high dose for a period of 14 days, significant changes were observed in the normal cytoarchitecture of the fish brain compared to control. Severe structural damage, increased necrosis, degenerative changes (DC), mononuclear infiltration (MI), congestion (C), cytoplasmic vacuolization (VC), and severe lesions (L) were also noticed in the brain. A significant haemorrhage (HE) was also noticed in some places. (Figure 1 and Table 2)

Acetylcholine Esterase Activity

A Sublethal exposure of IMI resulted in a significant ($p<0.05$) decline of AChE activity, at high dose only as compared to control. (Figure2).

Exposure time	Day1			Day 5			Day 10			Day 15		
Dose	C	LD	HD	C	LD	HD	C	LD	HD	C	LD	HD
Jerky movement	+	+	+	+	+	+	+	+	++	+	+	++
Hyperactivity	-	-	-	-	+	+	-	+	++	-	+	+++
Restlessness	-	-	-	-	+	+	-	+	++	-	+	+++
Loss of Equilibrium	-	-	-	-	+	+	-	++	++	-	++	+++
Fin Movement	+	+	+	+	++	++	+	++	+++	+	++	+++
Mucus Secretion	-	-	-	-	+	+	-	+	++	-	++	++

Note: (-) →Normal, (+) → mild, (++) → moderate, and (+++) → maximum behaviorpattern

Table 1: Behaviour study in *O. mossambicus* on exposure of sub lethal dose of IMI

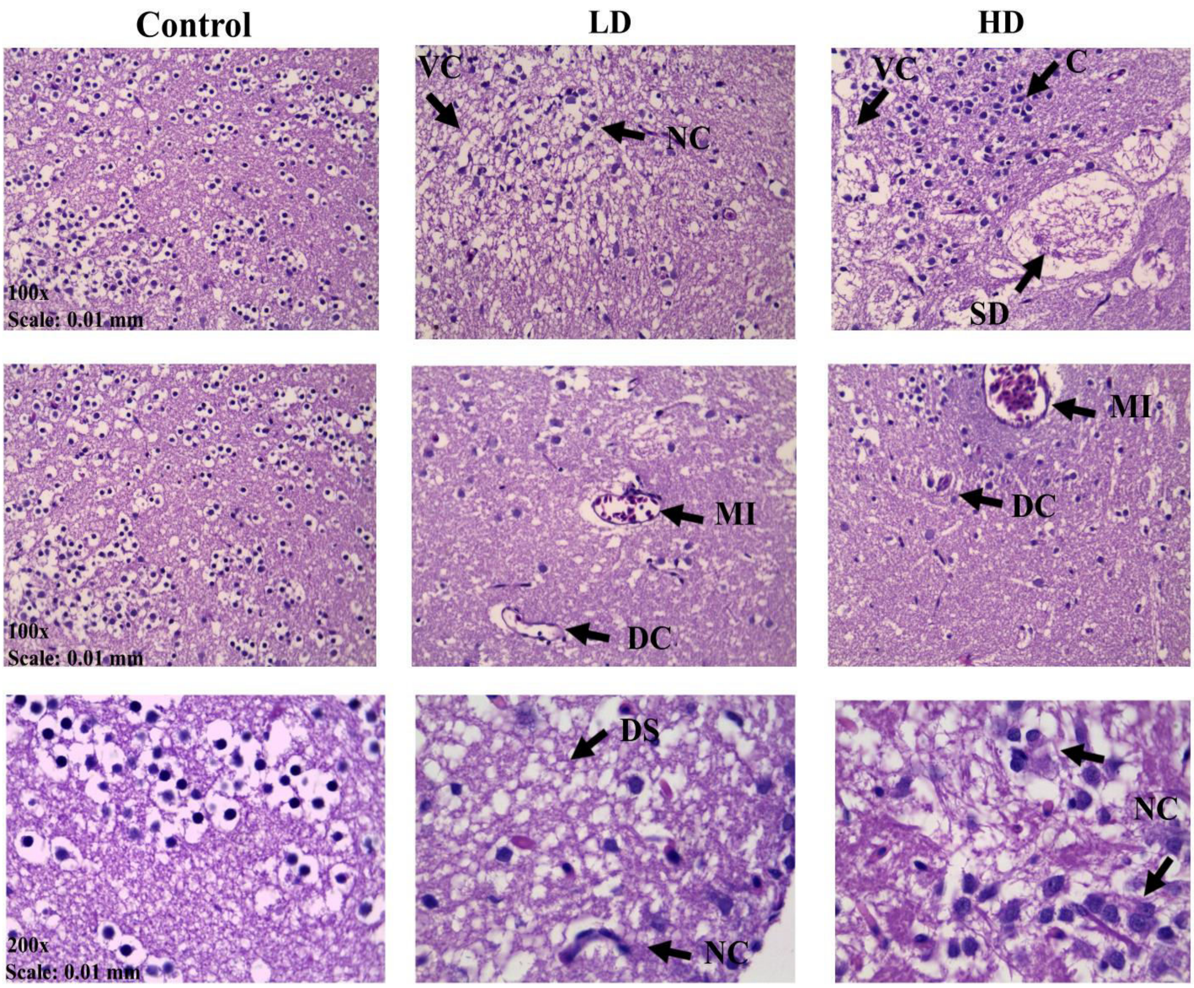

Figure 1: Alteration in histology of brainin *O. mossambicus* on exposure of sub lethal dose of IMI

Characterization	Control	LD	HD
Vacuolation	-	++	+++
Necrosis of neurons	-	++	+++
Distended sinusoids	-	++	+++
Degenerative changes	-	++	+++
Mononuclear infiltration	-	+++	+++
Congestion	-	++	+++
Lesions	-	+	+++
Hemorrhage	-	++	+++

Note: (-)→none, (+) → mild occurance, (++) → moderate occurrence, and (+++) → maximum occurrence

Table 2:Summary of histological changes observed in the brain of *O. mossambicus* subjected to LD and HD of IMI

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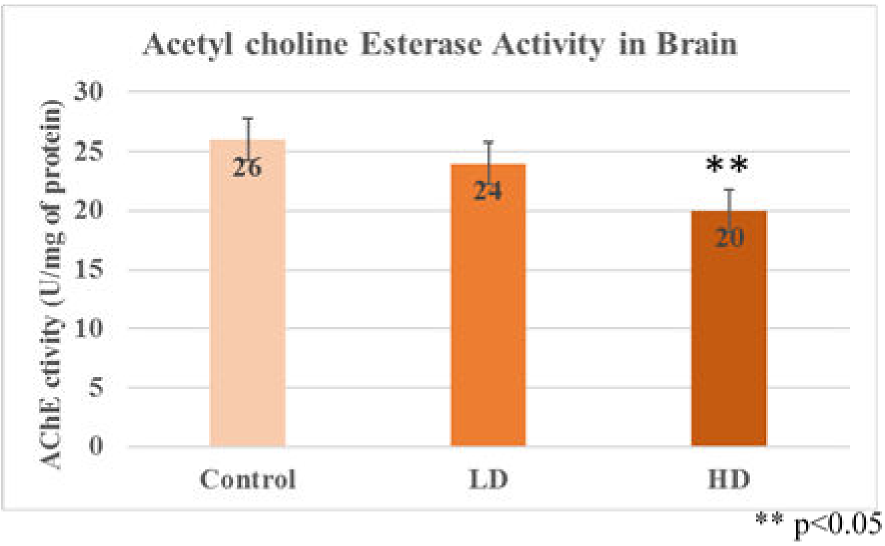

Figure 2: Acetylcholine Esterase Activityof the brainin *O. mossambicus* on exposure of sub lethal dose of IMI

Discussion-

In this study, the acute toxicity effect of IMI was studied in terms of alterations in the behavioral pattern, histopathological changes, and AChE activity in the brain of tilapia (*O. mossambicus*). The major aim was to validate the neurotoxic potential of IMI; the work was focused on alteration in behavior, AChE levels, and correlate it with the histoarchitectural changes in the brain.

Behavior provides a unique perspective on the physiology and ecology of an organism and its environment, operating through the central and peripheral nervous system (Halappa and David, 2009). Since behavior is not a random process, instead of a highly structured and anticipated sequence that ensures fitness and survival of the species, behavioral endpoints serve as a valuable tool to distinguish and evaluate the effects of exposure to environmental stress. Alteration in the fish behavior provides relevant indices for ecosystem assessment, any change in their behavior indicates the deterioration of water quality, so they are considered a biological indicator(Renick*et al.*, 2016). In the present scenario, the control fish were active and alert. They had a well-synchronized movement, whereas, in the case of IMI, exposure exhibited a dose-dependent severity in hyperactivity, loss of equilibrium, restlessness, and jerky movement. These symptoms may be due to inhibition of AChE activity leading to the accumulation of Ach in cholinergic synapses ensuing hyperstimulation. Since inhibition of AchE activity is a typical characteristic of neonicotinoids (Lionetto*et al.*,2003;Tomizawa, and Casida,2011);our findings corroborate with observations made by Rossi *et al.*, 2020 in sentinel freshwater fish following sublethal exposure of neonicotinoids. The observed behavioral changes also suggest that the brain was affected by IMI exposure. To confirm these histological studies were conducted.Histology is used as a biomonitoring tool in toxicity studies as it provides early warning signs of disease. Histopathological observations have been commonly used to determine the health of the entire ecosystem population for toxicity testing of the effects of xenobiotic compounds at the sub-organismal or organismal level.(Joseph *et al.*, 2010; David and Rao, 2015). The damage and injury in different organs are usually dependent on dose, duration, exposure, and type of pesticides. The utility of histological lesions is sensitive and reliable indicators of fish health as reported in early studies. (Desai and Parikh 2012;Sadekarpawar*et al.*, 2015 b). Tissues obtained from the control group showed regular features with no apparent lesions. However, dose-dependent alterations in the brain were observed, such as Hyperplasia, edema, necrosis, vacuolation, and overall increase in brain cells. Vacuolation and increased cell size may have been due to glycolysis leading to microsomal and mitochondrial dysfunctions. (Bose *et al*., 2013;Rajini*et al*., 2015;Xavier and Kripasana,2020).The recent results of the present study show that the IMI causes major neurotoxicity in the brain and impairs its behavioral pattern. The problem can be more severe in the fish farms as the ponds are habitually located in or near the agricultural land, which is loaded with pesticides of all kinds. To this, if the groundwater used for domestic purposes can exacerbate the problem with simulated laboratory experimental conditions. Therefore, a scientific detoxification approach is necessary to improve the health of these economically valuable fish and reduce the losses caused by anthropogenic stress.

Conclusion

The study suggests that sub-lethal concentration of IMI leads to neurotoxicity, and fishes were found to be highly stressed, therefore it is necessary to carry out field studies and check the fish health status and IMI residues in the environment. Furthermore, monitoring AChE levels can be used as a well-supported marker for IMI neurotoxicity in fishes in general and tilapia in particular.

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Compliance with ethical standards The research carried out is compliance with ethical standards.

Ethical approval- All the procedures were in accordance with the guidelines of APHA-AWWA-WEF (1998) for which the care and use of animals were followed.

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