Imidacloprid and clothianidin insecticides individual and mixture effects on fish *Labeorohita* plasma electrolytes and gill Na^+/K^+ ATPase activities.

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Abstract
Neonicotinoids are a new class of insecticides that are widely used around the world owing to their efficacy and insect selectivity. Imidacloprid and clothianidin are major neonicotinoids which are frequently found in the aquatic environment, especially in streams. The present investigation evaluated the individual and combined toxicity of imidacloprid and clothianidin in the freshwater fish *Labeorohita*. The fish were intoxicated with imidacloprid (66.6 mg/L), clothianidin (30 mg/L) and their combination mixture (IMI: 33.3 and CLO: 15.0 mg/L) for 42 days. The plasma electrolytes such as Na^+, K^+andCl^- andgills Na^+/K^+ ATPase were evaluated. The plasma electrolytes (Na^+, K^+ andCl^-) and gill Na^+/K^+ ATPase activities were significantly inhibited throughout the exposure period. The maximum inhibition was noted on the 28th day of intoxication and is more in the mixture than the individual.

Keywords: Neonicotinoids, Na^+/K^+ ATPase, Electrolytes, Imidacloprid, Clothianidin

Introduction

Neonicotinoid insecticides are a new class of pest controllers extensively applied for crop protection (Jeschke*et al*., 2011; Gomez *et al*., 2020), especially to eradicate piercing and sucking arthropod insect pests (Brandt *et al*., 2016). These pest controllers acquire insect selectivity, a wide range of effects and are safe for application (Simon-Delso*et al*., 2015). In the target insects, neonicotinoids cause noxiousness by conjugating with nicotinic-acetylcholine receptors and cause paralysis, nervous stimulation, and finally death (Goulson, 2013; Hladik*et al*., 2018). Owing to their soluble nature, these are noticed in higer concentrations in the streams (up to 320µg/L), which surpasses the environmentally safe concentrations i.e., up to 1.05µg/L (Sadaria*et al*., 2016; Schmidt *et al*., 2022). Also, the residue concentrations of these insecticides have been extensively found in vegetables, fruits and aquatic animals (Lozowicka*et al*., 2016). The potential toxic effects of neonicotinoids on target and non-aquatic organisms have been reported (Schmidt *et al*., 2022). Still, the communication of neonicotinoids with non-target aquatic animals and their effect on physiology is scanty (Malhotra *et al*., 2021)

Formerly a pollutant that enters and is distributed in the organism tissues should cross the membrane (Tinsley, 1979). Numerous toxic chemical compounds or their active metabolites affect cell damage

by countering mainly by active biological membranes. The substance diffusion via the gill epithelium mainly depends on its lipid/organic solubility (Randall and Brauner, 1996). The organism membrane plays an important role by regulating the movement of contaminants over concentration gradients or charging on the internal space of the membrane boundaries (Goff, 2018). This mechanism is essential for the regular sequence of processes, especially metabolism. Xenobiotic-intoxicated sub-cellular pathology exhibits perturbations of structure and function at the basic molecular level.

The main function of the fish gills is to regulate the acid-base ionic equilibrium and permit selectively essential ions such as Na^+, Cl^- andK^+, and also for protuberance of $HC0_3^-$, H^+ and NH^- ions which are by-products of metabolic mechanisms (Renzis and Michel, 1984; Clausen, 2008). The chloride cells are distinguished by their extreme figures of mitochondria, impenetrable vesicular network in the cytoplasm, elliptical nuclei and excessive transport protein levels, Na^+/K^+ ATPase (Pisam*et al*., 1987). ATPase is responsible for ionic homeostasis in the membrane and plays a vital role in the biological activities of the cell via providing energy in biochemical reactions, therefore ATPase is measured as a good bioindicator in intoxication studies (Galva *et al*., 2012). The Na^+/K^+ ATPase is an extremely conserved membrane-bound enzyme important to maintain cellular ionic homeostasis in the organism (Dang *et al*., 2000). The body fluids electrolytes play an important role to establish buffer zones and different nutrient transport mechanisms to regulate the osmotic equilibrium of body ions (Goff, 2018). Furthermore, they facilitate appropriate ionic equilibrium for regular neuromuscular petulance and cell functions. In the osmoregulation system, Na^+, K^+ and Ca^+ and Cl^- ions play a key role to preserve the hyperosmotic characteristics of freshwater fish (Suvetha*et al*., 2010; Salbego*et al*., 2020). In freshwater fish, the gills chloride cells actively participate in trans epithelial Ca_2^+ and Cl^- absorption is well-recognized, and also active participation in Na^+ uptake (Jurss and Bastrop, 2004; Salbego*et al*., 2020). Na^+/K^+ mediated ATPase is a key enzyme, which actively transports the Na^+ out of the cell and intakes the K^+ into cells via the membrane.

2. Materials and Methods

2.1. Procurement of animals:

The Labeorohita (25 ± 5 gr) was obtained from a local supplier and was given a prophylactic dip in 2% salt solution for 1 min, followed by oxytetracycline treatment (15 mg/L) for the first three days. The fish were allowed to acclimatise to laboratory conditions for 2 weeks prior to the experiment and they were fed commercially available feed.

2.2. The physicochemical parameters of water:

The regular mean standards of water conditions throughout the 42 days mentioned below: Temperature 28 ± 2°C, pH 7.4 ± 0.6, dissolved oxygen levels 8.45 ± 0.24 mg L^{-1}, total hardness of the water 415 ± 1.8 mg L^{-1} as calcium carbonate concentrations ($CaCO_3$), alkalinity 344 ± 1.8 mg L^{-1} as calcium carbonate concentrations and total chlorides 245.24 ± 1.8 mg L^{-1}.

2.3. Pesticides and chemicals:

Neonicotinoid insecticides imidacloprid and clothianidin were selected for the

present study. The analytical grade Imidacloprid (CAS No: 38261-41-3) and Clothianidin (CAS No: 210880-92-5) insecticides and chemicals procured from Sigma-Aldrich (PESTANAL®).

2.4. Experiment design:

The *Labeorohita*was exposed to one-third of LC_{50} concentration and the experiment was driven into 4 groups: the control group (acetone only), the IMI group (66.6mg/L), the CLO group (30mg/L) and the Mix group (IMI: 33.3 + CLO: 15.0 mg/L). Forty-two (42) fish were exposed to each group and the concentrations were added directly to the water daily. The experiment was evaluated at 7, 14, 21, 28, 35 and 42 days, four fishes from each group were anaesthetized and dissected, isolated the tissue carefully and kept in a deep freezer (-20°C) until further estimations.

2.5. Blood plasma electrolytes ($Na^+/K^+/Cl^-$) analysis:

Blood was collected from the caudal vein by a disposable syringe which was treated with heparin and immediately transferred into heparinised vials. The samples were centrifuged at 10,000gx for 15 min, and the plasma was separated for electrolytes estimation described by the technic of Maruna (1958) and chlorides were estimated by the slight modification technic of Tietz (1990).

2.6. The activity of Na^+/K^{+-} ATPase (EC 3.6.1.3):

The fish gills were dissected and the tissue was washed gently and homogenated by using an ice-cold sucrose solution. The gills homogenate was centrifuged at 3000gx for 15 min, the obtained supernatant was centrifuged again at 12000gx for 30 min. The supernatant was once again centrifuged at 3,5000gx for 30 min. The supernatant was estimated for Na^+/K^{+-} ATPase enzyme activity described by the method of Shiosaka*et al*. (1971). The specific enzyme activity was expressed as micro-moles of inorganic phosphorus liberated/min/mg protein.

2.7. Statistical analysis:

The obtained data analysis by SPSS and Duncan's multiple range tests were used to estimate the variances between exposure and control means and the values are Mean ± SE (n=6), with significant variances from *p*>5%.

3. Results

The gill ATPase enzyme activity was inhibited and continued up to 42 days, the highest inhibition was noticed on day 28 in all groups (IMI: 35%, CLO: 47% and Mix: 75% respectively) Fig. 1.

The plasma Na^+ levels were decreased through the exposure period up to day 42, and the maximum reduction was observed on 28 days of exposure (IMI: 37%, CLO: 41% and Mix: 55%) Fig. 2. The plasma K^+ levels were reduced up to 42 days of the exposure period, the maximum decrease was noticed on the day 28 intoxication period (IMI: 23%, CLO: 26% and Mix: 36%) Fig. 3. Plasma Cl^- levels were inhibited throughout the exposure period in all groups, the highest decrease was observed in the day 28 exposure period (IMI: 22%, CLO:26%, and Mix: 40%) Fig. 4. The binary mixture of IMI and CLO (Mix group) in the experiment had more effects than the individual insecticides.

4. Discussion

Fish gills are the main constituents of gas exchange organs, which have an extremely

complex vasculature architecture and are enclosed by an extreme surface extent epithelium that facilitates a narrow barrier between the fish blood and the surrounding environment (Hsia *et al*., 2013). The total cardiac output diffuses into the gill vasculature before inflowing the dorsal artery and the circulation. In addition, the branchial epithelial tissue is the transportation system's main location that counteracts the consequences of ionic and osmotic gradients, and the primary location of body fluid pH equilibrium and metabolic end-product excretion (Evans *et al*., 2005). Therefore, the gills epithelium of fish is a versatile organ that plays a vital role in a group of physiological consequences of the surrounding and internal environment variations

There is clear evidence involving the chloride cells as the prime location of ionic exchange across the gill epithelium (Maetz and Bomancin, 1975; Perry, 1997; Pisam*et al*., 1987). The ATPase activity considers an important indicator of intra-cellular activity during the intoxication of pesticides (Kalivaradan and Ramudu, 2005). Significant continuous decreases in gills Na^+/K^+ ATPase activity were noticed in the fish *Labeorohita* on exposure to IMI, CLO and a Mix of neonicotinoid insecticides for 42 days. Environmental xenobiotics can directly interact with the membrane-bound enzymes or amend the Na^+/K^+ ATPase activity owing to the interruption of the energy-constructing metabolic mechanism and plasma electrolytes (Watson and Beamish, 1980; Salbego*et al*., 2020; Dogan *et al*., 2022; Veedu*et al*., 2022).

The integrity of the membrane is crucial to continue cation homeostasis. The Na^+/K^{+-} ATPase is an essential membrane-bounded protein and important for specific lipids activity. Therefore, a significant inhibition in branchial Na^+/K^{+-} ATPase activity might have been caused by neonicotinoid insecticides-induced damage to the gill tissue membrane/plasma proteins and lipids (Malhotra *et al*., 2021; Veedu*et al*., 2022). The present results suggest insecticides' inhibition of Na^+/K^+ ATPase activity affected ions' transportation through the gill epithelial membrane. Different kinds of environmental contaminate/xenobiotics predominantly consumed from the surrounding water are through the gills (Randall and Brauner, 1996). Fish gill tissue is the major location of exchanges of water by diffusing mechanism (Motais*et al*, 1969). Significantly inhibited values for Na^+/K^+ ATPase enzyme activity in gill tissue were noticed after 42 days of intoxication to neonicotinoids. To continue ionic homeostasis through exposure to environmental pollutants, fish activate/adjust several physiological and biochemical mechanisms to detoxify (Roesijadi, 1996) and reinstate the gill ionic uptake process (McDonald and Wood, 1996). In the crayfish *Astacusleptodactylus*, which is intoxicated to the sub-lethal concentrations of neonicotinoid insecticide thiamethoxam, has been noticed to inhibition of Na^+/K^+ ATPase activity in gills, the decreased trend is increased with the concentration (Salbego*et al*., 2020; Uçkun*et al*., 2021). Individual and combination neonicotinoid insecticides thiamethoxam and acetamiprid intoxication in fish *Catlacatla* reduced the gills' Na^+/K^+ ATPase activity in effects to individual and more in binary combination (Veedu*et al*., 2022).

The toxic materials may cause impairment to the gill structure, thus plummeting the O_2 ingestion and upsetting

the osmoregulatory mechanism. The Na^+, K^+, Ca_2^+ and Cl^- ions in different tissues were significantly decreased when intoxicated with different insecticides, indicating they might have changed the membrane's penetrable characteristics and deranged the ionic pump due to tissue damage (Reddy and Philip, 1994; Salbego*et al*., 2020). The plasma $Na^+/K^+/Cl^-$ levels were decreased in *Cyprinus carpio* intoxicated with a sub-lethal dose of synthetic pyrethroid cypermethrin (Suvetha*et al*., 2010). In the present study, Na^+, K^+ and Cl^- ionic concentration was continuously inhibited by the neonicotinoid insecticides individually and combined. Similar results were reported in the fishes exposed to different classes of insecticides individually and combined (Narra, 2016; Salbego*et al*., 2020; Veedu*et al*., 2022).

The decreased effect on Na^+/K^+ ATPase is commonly attributed to functional and structural impairment to the gill tissue as a consequence of the toxicant long-term exposure caused to bioaccumulate in tissues (Pelgrom*et al*, 1995; Veedu*et al*., 2022). These mechanisms are clearly evidenced in the histopathological alterations such as the gills epithelial architecture modifications and cell apoptosis supporting the present inhibition activity of branchial Na^+/K^+ ATPase (Pivovarov*et al*., 2018). Damage to aerobic respiration as detected by augmented LDH and reduced SDH activities or unbalanced integrity of the membrane can interrupt the active gradient sodium pump, subsequently in modified intra-cellular content of the ions and the surrounding environment (Clausen, 2008). The neonicotinoids alter the activity of ATPase may be suggesting that the gill ionic equilibrium and transportation were impaired may be due to triggers of the other physiological, biochemical, and neurological effects that might compromise numerous body mechanisms (Dogan *et al*., 2022; Veedu*et al*., 2022). In fish exposed to neonicotinoid insecticides, time-dependent histopathological changes such as lamellar epithelial congestions and epithelial hyperplasia were observed (Günal*et al*., 2020; Salbego*et al*., 2020; El-Garawani*et al*., 1022).

Conclusion

In conclusion, the ATPase enzyme is not only intently participating in the ionic active transport system and protecting the ionic balance of the membrane but is also involved in other biological activities. In the present study, the neonicotinoid insecticides such as imidacloprid and clothianidin individually or combinedly alter the fish osmoregulatory system, the impairment is more in binary mixture. The Na^+/K^+ ATPase enzyme regulates the branchial ionic transport (Na^+, K^+, Ca^+ and Cl^-) and maintains the ionic and osmotic equilibrium in freshwater fishes, and the decrease of the enzyme activity indicates that the insecticides especially the neonicotinoid mixture might have disturbed the ionic and osmotic balance. The investigation of the ATPase enzyme is very important to assess environmental pollution, especially pesticide-induced damage to the fish gill osmoregulatory and body fluid electrolyte regulatory system.

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Figures Legends

Figure 1: Gills Na^+/K^+ ATPase enzyme activity of *Labeorohita* intoxicated to IMI, CLO and a Mix for 42 days. Each value represents Mean ± SE and * indicates insignificant (n=6).

Figure 2: Plasma/serum Na^+ levels of *Labeorohita* intoxicated to IMI, CLO and a Mix for 42 days. Each value represents Mean ± SE and * indicates insignificant (n=6).

Figure 3: Plasma/serum K^+ levels of *Labeorohita* intoxicated to IMI, CLO and a Mix for 42 days. Each value represents Mean ± SE and * indicates insignificant (n=6).

Figure 4: Plasma/serum Cl^- levels of *Labeorohita* intoxicated to IMI, CLO and a Mix for 42 days. Each value represents Mean ± SE and * indicates insignificant (n=6).