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Changes of hematological and biochemical parameters revealed genotoxicity and immunotoxicity of neonicotinoids on Chinese rare minnows (*Gobiocypris rarus*)☆

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ABSTRACT

Adverse impacts of immunity in terrestrial non-target organisms exposed to neonicotinoid insecticides have been reported, but the causal link between insecticide exposure and possible immune alterations in fish remains limited. In the present study, the potential genotoxicity and immunotoxicity of three neonicotinoids (imidacloprid, nitenpyram, and dinotefuran) were assessed in Chinese rare minnows by using a 60-day chronic toxicity test. The hematological and biochemical parameters of juvenile Chinese rare minnows and changes in the transcription of six inflammation-related genes were determined after exposure to neonicotinoids at 0.1, 0.5, or 2.0 mg/L. A clear difference in the frequency of erythrocytes with micronuclei (MN) was observed after treatment with 2.0 mg/L imidacloprid ($p < .05$). Additionally, exposure to 0.5 or 2.0 mg/L imidacloprid significantly increased the binucleated (BN) erythrocytes and those with notched nuclei (NT) ($p < .05$). A serum protein electrophoresis (SPE) assay showed significant alterations in the serum protein in all treatments ($p < .05$), and further analysis indicated decreases in immunoglobulin (Ig) in treatments with 0.5 or 2.0 mg/L imidacloprid or dinotefuran or with 0.1 mg/L nitenpyram ($p < .05$). Moreover, a biochemical assay confirmed that immunoglobulin M (IgM) levels were indeed significantly decreased upon treatment with imidacloprid or dinotefuran at 0.5 or 2.0 mg/L ($p < .05$). In addition, the transcriptional levels of the inflammatory cytokines *IL-6*, *INF-α*, *TNF-α*, and *IL-1β* were markedly down-regulated after all imidacloprid treatments ($p < .05$), whereas the expression levels of only *TNF-α* and *IL-1β* were significantly down-regulated following the 0.5 and 2.0 mg/L dinotefuran treatments ($p < .05$). Taken together, our results clearly demonstrate that imidacloprid, rather than nitenpyram and dinotefuran, can induce genotoxicity. The responsiveness of these immune indicators provides new insight into and evidence of the adverse effects of neonicotinoids on aquatic non-target organisms.

1. Introduction

Neonicotinoid insecticides, a class of chemicals used extensively worldwide for the protection of crop plants, currently account for over 30% of the global market for insecticides (Jeschke et al., 2010; Main et al., 2014). Neonicotinoids (neonics) share a similar mode of action of preferentially binding to insect nicotinic acetylcholine receptors (nAChRs), causing the paralysis and rapid death of insects

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Abbreviations

Neonics	neonicotinoids
nAChRs	nicotinic acetylcholine receptors
CNS	central nervous system
SPE	serum protein electrophoresis
CPF	chlorpyrifos
ALP	alkaline phosphatase
CRP	C-reaction protein

(Tomizawa and Casida, 2003). Therefore, neonics are considered green and safe insecticides for non-target organisms due to their particularly tight binding to the nAChRs of insects (Tomizawa and Casida, 2005). For systemic insecticides, however, only 1.6%–28% of the active ingredients of neonics are taken up by crop plants (Sur and Stork, 2003), and most instead disperse into the wider environment. In addition, due to their relatively persistent accumulation in the environment, they have been detected in soil, wetlands, ground water, and surface water (Prosser et al., 2016). Moreover, neonics applied in crop and soil treatments or spray drift may be transported into bodies of water by surface and sub-surface runoff (Chiovarou and Siewicki, 2007; Jemec et al., 2007). Therefore, the persistent transportation of these pesticides raises concerns about their potential adverse effects on aquatic ecosystems (Gustavsson et al., 2017).

Currently, imidacloprid [1– (6-chloro-3-pyridylmethyl)-*N*-nitroimidazole-lidin-2-ylidene amine], a member of the neonic family, is the most extensively used insecticide in the world (Goulson, 2013; Jeschke et al., 2010). In China, the annual capacity of imidacloprid reached 25,000 tons, with 3000–4000 tons per year for domestic use (Data from China Pesticides Products Reports., 2015). In the California agricultural regions, imidacloprid can be detected in approximately 90% of the surface water (Jeschke et al., 2010). Some studies have reported that the concentration of imidacloprid in several Dutch agricultural surface waters has reached 320 μg/L (Van Dijk et al., 2013). Nitenpyram [(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N*′-methyl-2-nitrovinylidenediamine] and dinotefuran [1-methyl-2-nitro-3-(tetrahydro-3-furylmethyl)guanidine] are two of the strongest water-soluble neonics (Bonmatin et al., 2015). As the application of several classes of neonics (clothianidin, imidacloprid, and thiamethoxam) was gradually banned in some countries, and because there was an increase in the frequency of insect pest resistance to neonics (Shi et al., 2011; Watson et al., 2011), the sales, domestic shipment, and use of these two neonicotinoid insecticides later increased in, for example, Japan (Data from http://www.nies.go.jp/index-e.html, provided by Mizuno, R. in litt., 2012).

In China, the presence of imidacloprid has been detected in more than 90% of urine samples from rural and urban Chinese subjects (Wang et al., 2015). To date, limited evidence has elucidated a correlation between human health and exposure to neonics (Cimino et al., 2017). However, adverse effects of neonics have been reported in other terrestrial non-target organisms (Belzunces et al., 2015; Goulson, 2013). In non-human vertebrates, research from Liu et al. (2016) indicated that exposure to imidacloprid can interfere with neurogenesis and may increase the risk of neural dysplasia during chick embryo development. Additionally, significant induction of inflammation and oxidative stress in the liver tissue and central nervous system (CNS) of female Wistar rats was detected following exposure to 1 mg/kg/BW/day imidacloprid (Vesile and Erdogan, 2012). In aquatic invertebrates, studies have focused on the acute and chronic toxicity of neonics to mollusks (*Lymnaea stagnalis*) (Vehovszky et al., 2015) and lampmussels (*Lampsilis fasciola*) (Belzunces et al., 2015). Recent studies in aquatic vertebrates have reported on the genotoxicity and neurotoxicity of neonics (Ge et al., 2015; Topal et al., 2017; Yan et al., 2015). In addition, clothianidin is involved in the reduction of the immune defenses to deformed wing virus in honey bees (Di et al., 2013). In rats, induction of pro-inflammatory cytokines (i.e., TNF-α, IL-1b, IL-6, IL-12 and IFN-γ) was observed during chronic imidacloprid exposure (Vesile and Erdogan, 2012). However, to our knowledge, studies on the immunotoxicity of neonics after the exposure of aquatic species are scarce.

Fish are widely distributed and cover practically all aquatic ecosystems (Forné et al., 2010). Since fish are a sufficiently sensitive bio-indicator to changes in the aquatic environment, they provide an excellent model for chemical safety assessment and environmental toxicological studies in aquatic systems (Liang and Zha, 2016). Chinese rare minnows (*Gobiocypris rarus*) have been recommended for toxicity testing in China due to their small size, ease of culture, short life cycle, prolific egg production, and high fertilization and hatching rates. Therefore, the first aim of this study was to determine the genotoxicity of neonics (imidacloprid, nitenpyram and dinotefuran) in Chinese rare minnows. Second, we examined the immunotoxicity and formed conclusions about the potential mechanisms of action of neonics in aquatic organisms.

2. Materials and methods

2.1. Chemicals

The neonics imidacloprid (CAS No. 138261-41-3, purity ≥96%), nitenpyram (CAS No. 150824-47-8, purity ≥95%), and dinotefuran (CAS No. 165252-70-0, purity ≥97%) were purchased from J&K Chemical Ltd. (Hebei, China). Stock solutions of imidacloprid, nitenpyram, and dinotefuran were made in acetone and stored in the dark at 4 °C. The final acetone concentration was less than 0.01%.

2.2. Animals

A brood stock of Chinese rare minnows was raised in a flow-through system with dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg $CaCO_3$/L; temperature 25.0 ± 0.4 °C, expressed as the mean ± standard error of the mean (S.E.M.) and a photoperiod of 16:8 h (light:dark) that has been used for testing chemicals in our laboratory (Hong et al., 2016; Zha et al., 2017). Fish were fed twice daily with newly hatched brine shrimp (*Artemia nauplii*) and with a commercial pellet food (Trea, Germany) at a total rate of 0.1% body weight per day. Throughout the study, all fish were cared for in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals for the Science and Technology Bureau of China. For sacrifice, all fish in the current experiment were anesthetized with Tricaine (MS-222) before sectioning.

2.3. Experimental design

Two-month-old healthy juvenile Chinese rare minnow offspring from the same pair of brood stock were used in this experiment. The body weights and lengths were 0.32 ± 0.11 g and 25.82 ± 0.58 mm, respectively. All experiments were performed with a constant temperature of 25 ± 1 °C, under illumination with a 16:8 h 1ight:dark period. Exposure was initiated after 14 days of acclimatization. Fish were exposed to various concentrations of imidacloprid, nitenpyram and dinotefuran (0, 0.1, 0.5, and 2.0 mg/L; nominal concentrations), and acetone served as the solvent control