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Comparison of waterborne and intraperitoneal exposure to fipronil in the Caspian white fish (*Rutilus frisii)* on acute toxicity and histopathology

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ABSTRACT

Fipronil is an effective insecticide widely used in agriculture with potential ecotoxicological consequences. The median lethal dose (LD_{50}) and concentration (LC_{50}) of fipronil in 16.3 g Caspian white fish, *Rutilus frisii kutum* fingerlings were determined. To determine the LD_{50}, a total of 133 fish were assigned to 19 tanks (7 fish/tank) including one control and 6 treatment groups (300, 450, 550, 650, 750, 850 mg/kg). Fish were injected intraperitoneally and monitored at 96 h. The LD_{50} of fipronil was 632 mg/kg suggesting it was slightly toxic to the Caspian white fish. To determine LC_{50}, 114 fish were assigned to 19 tanks (6 fish/tank) including one control and 6 treatment groups (300, 400, 500, 600, 700, 800 μg/L). The LC_{50} of fipronil was 572 μg/L, which was highly toxic to the fish. The degree of tissue change (DTC) in vital organs from moribund fish exposed via waterborne exposure showed severe damage (DTC: 71 ± 52 for 700 μg/L) in the gill, including aneurisms, extensive fusion and necrosis. The fish exposed through the intraperitoneal route seemed to have severe lesions (DTC: 66 ± 50 for 750 mg/kg) in the kidney, involving hemorrhage, tubular degeneration and necrosis. The liver had no significant differences in DTC values between the two routes and showed pyknosis and sinusoid dilation. Hematoxylin and eosin staining did not show any histological alterations in the brain but nissl staining showed some alterations in distribution of purkinje cells. Generally, this study showed that the route of exposure to fipronil not only affects its acute toxicity but also determines the main target organs of toxicity and histopathological alterations in Caspian white fish.

1. Introduction

Fipronil is a relatively new insecticide with a wide range of uses in agriculture. Fipronil toxicity results from its ability to block gamma-aminobutyric acid-gated chloride channels of neurons in the central nervous system [1]. The increasing use of this pesticide has raised concerns for its harmful effects on human health and the environment [2]. In addition to insects, fipronil has toxic effects on non-target organisms, such as aquatic invertebrates [3], fish [4], some reptiles [5], birds [6] and mammals [7]; and the acute toxicity of fipronil has been determined for these animals.

Median lethal concentration (LC_{50}) and dose (LD_{50}) have been widely used to determine acute toxicity in aquatic and terrestrial animals, respectively. Waterborne administration has advantages such as simulating environmental exposure, involving no anesthesia and less handling of fish and relatively higher absorption rate constant for contaminants. Although waterborne exposure is a common route of toxicant absorption in the aquatic environment, LD_{50} have also been determined in these animals, especially in fish. Compared to waterborne (w.b.) exposure, evaluating intraperitoneal (i.p.) exposure to fipronil in fish has also some advantages. Although both LD_{50} and LC_{50} estimate expressed toxicity, LD_{50} can be a closer estimate of inherent toxicity and is determined based on a whole-body dose (mg/kg) and not water concentration (mg/L) (Hodson, 1988). Moreover, toxicological studies such as detoxification mechanisms in fish, based on LD_{50}, can be more accurately extrapolated to terrestrial mammals. Participants in the Collaborative Workshop on Aquatic Models and 21st Century Toxicology, held at North Carolina State University on May 5–6, 2014, agreed that small fish models can be used as biological model in toxicology and have advantages over mammalian models if standardized protocols are prepared and used [8]. They also recognized the need for extensive studies on fish toxicology and non-water exposure of fish to toxicants. The other reason for determination of the LD_{50} of fipronil in fish is related to its low/moderate water solubility [9] which makes it

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difficult to determine the fipronil dose response relationship. In addition, photolysis can transform fipronil into its metabolites (fipronil-desulfinyl and fipronil-sulfone), which are more toxic than the parent compound for fish [10,11]. Therefore, measurements of fipronil's effects on fish should be considered along with its metabolites. On the other hand, measurement of LC_{50} for larger fish needs larger amounts of fipronil and proper water in a non-static system. Consequently, it is not a good option economically and environmentally. Thus, measurement of LD_{50} of fipronil in fish is necessary to be used for future research, and this study is the first time.

In spite of the advantages and disadvantages cited above, this study was designed to compare the acute toxicities of fipronil through both w.b. and i.p. exposure and to determine the main target of toxicity in Caspian white fish. Previous studies have shown that histopathological studies are a precise and rapid way to show the direct effect of toxicants on target organs [12–14] and similar tests were selected for this study.

Fish are the most important aquatic food and as such can contaminate human populations. In the area south of the Caspian Sea, fipronil is mostly used in rice fields against striped rice stemborer. The streams containing fipronil from the farms enter the Caspian Sea (salinity ≈ 13 ppt) and might affect aquatic life. Caspian white fish (*Rutilus frisii kutum*), belonging to the cyprinidae family, is the most popularly consumed fish in this region and cultured extensively. Thus, both as a model and to provide information concerning the implications of fipronil use, the median lethal dose and concentration of fipronil in fish was studied.

2. Materials and methods

2.1. Fish

Two hundred and fifty Caspian white fish fingerlings (mean body weight: 16 ± 3 g) were obtained from the Shahid Rajai Fish Proliferation and Culture Center (Sari, Mazandaran Province, Iran). The fish were randomly divided into groups without determination of the male: female ratio. Fish were acclimated for 2 wk prior to the test, and fed commercial fish food until the day before fipronil exposure.

2.2. Determination of 96 h LD_{50} value for fipronil

2.2.1. Fish environment and handling

Nineteen plastic tanks (1000 L capacity) including a negative control tank (no replicate) and treatment tanks with water-shower aeration and a semi-static system were used. Non-chlorinated well water with characteristics showed in Table 1 was used. The photoperiod was 13 h light and 11 h dark. The volume of water used in each tank was about 200 L.

2.2.2. Preparation and injection of fipronil solution

Fipronil (98% purity, 50:50 racemic mixture) was purchased from the Moshkfam Fars Chemical Company (Shiraz, Iran). Stock solutions of fipronil were prepared in 6 amber glass vials containing 5cc sunflower oil and 108, 162, 198, 234, 270, 306 mg fipronil and one glass vial containing only 5cc sunflower oil. To dissolve fipronil in the oil, the stocks were vortexed for 30 min. Before the injection, the fish were anesthetized using phenoxyethanol, and weighed. For each treatment, 0.25 ± 0.05cc of the standard solution was i.p. injected into the fish using an insulin syringe based on the weight of each fish.

Table 1
Characteristics of groundwater used for the experiment.

Parameter	Value
pH	6.9 ± 0.3
Dissolved oxygen (mg/L)	7.8 ± 0.5
Temperature (° C)	18.1 ± 0.8
Total hardness (mg/L)	394 ± 7
Total dissolved solid (mg/L)	440 ± 20
EC (μs/cm)	860 ± 20
Nitrate (mg/L)	0.9 ± 0.3
Nitrite (mg/L)	0.009 ± 0.003
Bicarbonate (mg/L)	410 ± 10

2.2.3. Experimental design for LD_{50}

There were 6 treatment groups with three replicates and 7 fish in each group. After some experimental tests for estimation of lethal dose range, fipronil was i.p. injected into the fish at 300, 450, 550, 650, 750, 850 mg/kg of fish weight. The fish were monitored for 96 h (4 d) for any mortality and then sacrificed for histopathological tests.

2.3. Determination of 96 h LC_{50} value for fipronil

2.3.1. Experimental design

There were 6 treatment groups with three replicates and 6 fish for each group. Nineteen plastic tanks (20 L capacity) including a negative control tank (no replicate) and treatment tanks with air pump aeration and static system were used for determining the LC_{50}. Oxygen dissolved concentration and pH were maintained around 8 mg/L and 7.5, respectively. After acclimation, 6 fish were randomly transferred into each tank containing 15 L of non-chlorinated well water and 4.5, 6, 7.5, 9, 10.5 and 12 mg fipronil (without solvent) for 96 h and the number of dead fish were recorded daily. Moreover, to record any changes in behavior, fish were observed for about 1 h once daily.

2.4. Histopathological tests

After 96 h of exposure, three moribund fish from the 450, 550, 650 and750 mg/kg, and 400, 500, 600 and 700 μg/L (the treatment groups which had enough moribund fish) fipronil exposed tanks and three fish from the control tank were sacrificed by decapitation, dissected, and the gills, livers, kidneys and brains were fixed in Bouin's solution for 48 h. The tissue were rinsed in a graded series of ethanol to be dehydrated, cleared in xylene, embedded in paraffin, sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H & E). Nissl staining was also done for the brain tissue according to Parent et al. [15]. Three random sections per fish tissue were observed under the light microscope (Olympus Co, Tokyo, Japan) and photographed using a Microscope Camera Eyepiece (Dino-Lit Premier AM7023; AnMo Electronics Corporation, Taiwan). The histological alterations for each organ studied were assessed semi-quantitatively for the degree of tissue change (DTC), according to the procedures of Poleksic and Mitrovic-Tutundzic [16]. The alterations were classified into three stages, including stage I (without alteration, i.e., normal functioning of the tissue), stage II (some to severe damage), and stage III (very severe and irreparable damage). DTC was calculated using the following formula: DTC = (1 X SI) + (10 X SII) + (100 X SIII) where SI, SII and SIII is equal to the summation of alterations in each stage. Then, $0 \le$ DTC ≤ 10 indicates normal functioning of the organ; $11 \le$ DTC ≤ 20 indicates slight damage to the organ; $21 \le$ DTC ≤ 50 indicates moderate damage to the organ; $50 \le$ DTC ≤ 100 indicates severe lesions and $100 <$ DTC indicates irreversible damage to the organ.

2.5. Statistical methods

Data analysis was done using MedCalc (ver. 16.8.4) statistical software (Microsoft Partner, Korea). The acute toxic effect of fipronil on the Caspian white fish was determined by the use of Finney's probit analysis. A 95% confidence interval was calculated for the analysis. Sigma Plot ver. 11 software (Systat Software, Inc., CA, USA) was used for statistical analysis. The Mann–Whitney test was used for comparison of DTC results. The significance level was set at $P < 0.05$.