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Effects of acute and chronic exposures of fluoxetine on the Chinese fish, topmouth gudgeon *Pseudorasbora parva*

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ABSTRACT

Fluoxetine is a selective serotonin reuptake inhibitor used as an antidepressant and has been frequently detected in aquatic environments. However, its effects in fish from Asia remain relatively less studied. In this study, the topmouth gudgeon *Pseudorasbora parva* was exposed to 0, 50, and 200 μg/L of fluoxetine for 4 h and 42 d. The effects of fluoxetine on biometrics were compared to biochemical endpoints indicative of stress in different fish tissues (brain, liver, gills and intestine) following exposures. In fish exposed for 42 d, lipid peroxidation endpoints were enhanced 80% in the liver and gills. Acetylcholinesterase (AChE) activity was increased 40% after exposure to 50 μg/L and 55% at 200 μg/L following 4 h exposure. In contrast AChE was increased 26% (at 50 μg/L) after 42 d of exposures. Enhanced ethoxyresorufin-O-deethylase activity (EROD) was detected only in fish exposed to 50 μg/L of fluoxetine for 4 h. The activity of α-glucosidase (α-Glu) was also induced (at 200 μg/L) after 4 h of exposure. After 4 h of exposure, the activities of proteases in the intestine were generally inhibited at 200 μg/L. Both 4 h and 42 d exposures resulted in an increased hepatosomatic index (HSI) but did not affect the condition factor (CF). Our results demonstrate that fluoxetine significantly altered biochemical endpoints in *P. parva* after acute exposure and the morphological changes in liver size were not observed until 42 d of exposure.

1. Introduction

The production and human usage of pharmaceuticals and personal care products (PPCPs) have been increasing continuously worldwide, with an estimated global production of 13 million tons by the year of 2011 (Brooks, 2014; Brooks et al., 2003; Liu and Wong, 2013; OECD, 2013). Improper disposal of expired and leftover PPCPs, active metabolites and degraded products of PPCPs in the urine and feces from human and livestock are the major sources of these chemicals in the domestic wastewater. In addition, wastewater from medical facilities and pharmaceutical factories are also sources for PPCPs in the environments. Unfortunately, conventional wastewater treatment facilities are rarely capable of removing the PPCPs completely from the wastewater (Huerta-Fontela et al., 2008; Westerhoff et al., 2005) and PPCPs in the aquatic environments can be found at ng/L to μg/L worldwide especially in waters receiving the wastewater effluents (Khetan and Collins, 2007; Kolpin et al., 2002; Metcalfe et al., 2010).

Fluoxetine is a widely prescribed antidepressant for the treatment of human depression and anxiety disorders (Milea et al., 2010). It is a selective serotonin reuptake inhibitor (SSRI) which can increase serotonin concentrations through the block of its reuptake by the serotonin transporter (Stahl, 1998). Fluoxetine has a relatively long half-life from 102 to 385 d due to its recalcitrance to hydrolysis, photolysis and microbial degradation (Kwon and Armbrust, 2006). Its half-life in Japanese medaka *Oryzias latipes* was estimated to be approximately 9 d (Paterson and Metcalfe, 2008). Fluoxetine can be metabolized to norfluoxetine via N-demethylation (Owens et al., 1997; Wong et al., 1995), which can permeate the blood-brain barrier (Hirano et al., 2005; Qu et al., 2009). Both chemicals are detected in treated wastewater effluents and surface waters at concentrations ranging from 0.001 μg/L to 1.3 μg/L in North America (Christensen et al., 2009; Kolpin et al., 2002; Metcalfe et al., 2010; Thibaut and Porte, 2008). Due to their

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bioavailable properties and persistence in the environment, fluoxetine and norfluoxetine have been detected in aquatic organisms inhabiting streams receiving wastewater effluents. For example, the concentrations of fluoxetine and norfluoxetine in the muscle, liver and brain of three fish species (bluegill *Lepomis macrochirus*, channel catfish *Ictalurus punctatus* and black crappie *Pomoxis nigromaculatus*) in the downstream of wastewater outfalls in north Texas, USA were approximately 0.1 – 1 (for fluoxetine) and 1 – 10 ng/g (for norfluoxetine), respectively (Brooks et al., 2005). Approximately 19 – 70 ng/g of fluoxetine and 33 – 73 ng/g of norfluoxetine were reported in the liver of fish (mixed species) from five effluent-dominated rivers in the USA (Ramirez et al., 2009). It is suggested that trapping of fluoxetine/norfluoxetine in cellular lysosomes may cause retention in biological tissues (Daniel and Wöjcikowski, 1997).

In fish, the LC_{50} values of fluoxetine are generally 2 – 4 orders of magnitude higher than the concentrations reported in the aquatic environments (Brooks et al., 2003; Henry and Black, 2008). For example, the 7 d LC_{50} for western mosquitofish *Gambusia affinis* neonate was determined as 546 μg/L, and 5340 μg/L of fluoxetine caused 100% mortality within 3 d (Henry and Black, 2008). The 48 h LC_{50} of fluoxetine to fathead minnow *Pimephales promelas* was 705 μg/L and the 96 h LC_{50} to sheepshead minnow *Cyprinodon variegatus* was over 2000 μg/L, respectively (Brooks et al., 2003; Winder et al., 2009).

Regarding its biochemical effects, fluoxetine has been shown to alter transcription and activities of antioxidant enzymes as well as the expression of multixenobiotic resistance genes in Mediterranean mussels *Mytilus galloprovincialis* and Asian clam *Corbicula fluminea* (Chen et al., 2015; Franzellitti et al., 2014, 2015). In addition, the reproduction of goldfish *Carassius auratus* was decreased by 54 μg/L of fluoxetine after 14 days of exposure, potentially due to decreased testosterone and milt production coupled with increased circulating vitellogenin and estradiol in males (Mennigen et al., 2010a; Schultz et al., 2011). Isotocin mRNA abundance in the hypothalamus and telencephalon (brain) was also decreased by fluoxetine exposure (Mennigen, 2011). Other neuroendocrine effects in fish include reduced serotonin activity in the brain of hybrid striped bass (*Morone saxatilis* × *M. chrysops*) exposed to 23.2 – 100.9 μg/L of fluoxetine for 6 d (Gaworecki and Klaine, 2008). Similarly, the sexual development of the western mosquitofish was affected by 71 μg/L of fluoxetine after 100 d of exposure. Although most neurological studies of fluoxetine have focused on serotonin and its effects on behavior and reproduction, its effects on AChE, antioxidant physiology, and digestive enzymes in fish remain largely unknown. In addition, growth of aquatic organisms can be markedly reduced by short-term or chronic exposure to fluoxetine (Conners et al., 2009; Stanley et al., 2007). For example, growth was significantly reduced in the fathead minnow *Pimephales promelas* exposed to *S*-fluoxetine at concentrations higher than 51 μg/L for 7 d (Stanley et al., 2007). The reduced growth in the fluoxetine-exposed organisms may potentially be due to lowered feeding rate, decreased food intake, and possible impairment of digestion (Conners et al., 2009; Stanley et al., 2007). Since the digestive enzymes have a close relationship with food digestion rates in fish (Jobling, 1981), the observed reduced growth in the fluoxetine-exposed organisms implies that the digestive enzymes may be a target for this compound.

Although several studies have evaluated the effects of fluoxetine on fish, few have done so with Asian or Chinese species. The topmouth gudgeon *P. parva* is a small freshwater cyprinid originating from Heilong Jiang catchment (China) and neighboring countries (Ma et al., 2017) and has invaded many European countries and other regions of the world (Gozlan et al., 2010). This fish species is ideal for ecotoxicological study due to its small body size, relatively short reproductive cycle (maturity in the first year of life with a life span 3–4 years), and spawning activities, (Gozlan et al., 2010). It was reported that the effects of antidepressants in aquatic organisms can be observed within minutes to hours of exposure (Ford and Fong, 2016). Therefore, the aims of this study were to evaluate the toxicity of fluoxetine in gudgeon following acute (4 h) and chronic exposure.

2. Materials and methods

2.1. Chemicals

Fluoxetine HCl (purity > 98%), dimethyl sulfoxide (DMSO), NADPH, 7-ethoxyresorufin, resorufin, DL-dithiothreitol (DTT) and methanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). All glassware and other containers were acid washed, rinsed with deionized water, air-dried before use. All other chemicals of analytical grade were used in this study. All solutions were freshly prepared with double de-ionized water (Milli-Q, Millipore; 18.2 MΩ/cm resistivity).

2.2. Test organisms

P. parva were collected from the Dahuofang Reservoir (a reservoir with relatively pristine watershed which provides drinking water to 23 million residents in Liaoning Province), Liaoning, China (Wu et al., 2017). They were successfully maintained for 3 years (with generation time of approximately 14 months) under the laboratory conditions. The fish were fed freshly hatched brine shrimp (*Artemia franciscana*) nauplii at a rate of 5% (on a wet weight basis) twice daily.

2.3. Determination of 96 h LC_{50}

An acute toxicity test was conducted to determine the 96 h LC_{50} of fluoxetine in *P. parva*. Juvenile fish (~ 60 d old, 130 ± 8 mg, 1.7 ± 0.4 cm) were exposed to 0, 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/L of fluoxetine (in the form of fluoxetine HCl) (nominal concentrations), in glass tanks containing 2 L of moderately hard reconstituted water (MHR) (98.3 mg/L of $NaHCO_3$, 47.6 mg/L of $CaSO_4$, 123.4 mg/L of $MgSO_4$ $7H_2O$, and 4.1 mg/L of KCl). Each concentration had 3 replicates with 5 fish per replicate. The exposure media were renewed every other day and the exposure lasted for 96 h. Food was withheld during the exposure. Water quality parameters were monitored and recorded as follows: pH: 7.8 ± 0.16; DO: 8.9 ± 0.2 mg/L; DOC: 2.1 ± 0.3 mg/L; conductivity: 269 ± 14.6 μS/cm. The photoperiod was 16 h light: 8 h dark, and the temperature was maintained at 23 ± 1 °C.

2.4. Chronic exposure of fluoxetine in P. parva

P. parva with similar size (700.3 ± 45.2 mg, 3.6 ± 0.2 cm) were randomly sampled from the population of over 600 fishes. At this size, the sex of the fish cannot be differentiated. They were acclimated to the experimental conditions for 7 d before exposure. Stock solutions of fluoxetine (1.25 and 5 mg/mL) were prepared with dimethyl sulfoxide (DMSO) and stored at −20 °C. Appropriate amount of the stock solution (280 μL) were added to each glass exposure chamber containing 7 L of exposure media to achieve the specific nominal concentrations of 50 μg/L and 200 μg/L, respectively. Meanwhile, same volume of (i.e., 280 μL) DMSO was added to the control chamber. The final concentration of DMSO in control and exposure aquaria was 4×10^{-5} (v/v). The exposure conditions were similar to that in the 96 h LC_{50} test except that the fish were fed twice daily during the chronic exposure. According to a previous method, a static renewal exposure regime (exposure media renewed every other day) was adopted to maintain the fluoxetine concentration and water quality during the exposure (Silva et al., 2016). The concentrations of fluoxetine were not quantified. A previous study has demonstrated that the fluoxetine concentration (e.g., 534 μg/L) was > 97% of its nominal one (Henry and Black, 2008) and fluoxetine concentration over 5 mg/L was relatively stable in buffer solutions or natural lake water (pH 5 – 9) (Kwon and Armbrust, 2006). It is therefore reasonable to believe that fluoxetine concentrations will remain over 90% of the nominal ones during the exposure in this study.