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Tissue bioconcentration and effects of fluoxetine in zebrafish (*Danio rerio*) and red crucian cap (*Carassius auratus*) after short-term and long-term exposure

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HIGHLIGHTS

- Fluoxetine (FLU) caused concentration-dependent effects in fish larvae.
- The effective concentration of FLU on fish in water was as low as 0.1 μg/L.
- FLU levels in fish organs were significantly correlated with its induced effects.
- The accumulation pattern in fish tissue should be used to assess risks of drugs.

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ABSTRACT

Fluoxetion (FLU) is an antidepressant pharmaceutical most commonly detected in the aquatic environment. The present study aims to elucidate the tissue accumulation and effects of FLU using two different fish models. First, the multiple effects and the FLU levels in fish, were examined in zebrafish (*Danio rerio*) embryos exposed to FLU concentrations (0, 0.1, 1, 10, 100, 1000 μg/L) from 4 h post-fertilization (hpf) until 120 hpf. Exposure to FLU accelerated heart rates, postponed hatching time, and increased swimming speed of fish. A dynamic response of acetylcholinesterase (AChE) activity was also displayed in the fish. Second, a 30-day exposure experiment using red crucian carp (*Carassius auratus*) was performed, and it found that the concentration of FLU in fish organs increased with increasing water concentrations, but the highest FLU bioconcentration was present in the lowest FLU exposure group (0.1 μg/L). Finally, 6 days of exposure to 0.1 μg/L of FLU followed by a 6-day clearance experiment was performed with both adult zebrafish and red crucian carp. The FLU levels in different fish organs increased as exposure time increased, but they sharply declined following the 6-day clearance. Correspondingly, the changes in brain AChE activity and in antioxidant parameters in the liver were consistent with the FLU levels in the fish organs. Our study provides fundamental data on the tissue accumulation and concentration-dependent effects in fish exposed to fluoxetine.

1. Introduction

Fluoxetine (FLU), typically marketed under the trade name Prozac, is an antidepressant belonging to the class of selective serotonin reuptake inhibitors (SSRIs). Fluoxetine was developed with specific pharmacological and physiological functions, and it has been used to treat major depression and other psychiatric disorders for over 25 years (Sumpter et al., 2014). The major sources of pharmaceutical chemicals in the environment are discharges from sewage treatment plants (Ferrari et al., 2004). Because of its widespread use and incomplete elimination in most wastewater treatment plants, a wide range of FLU concentrations have been detected in aquatic environments (Brooks et al., 2003; Kolpin et al.,

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2002; Metcalfe et al., 2010), from ng/L levels to low μg/L levels. Although these aquatic concentrations are relatively low, FLU has also been detected in the numerous aquatic species, including fish (Brooks et al., 2005).

Some researchers have suggested that this substance, intended to improve the quality of human health, might also present unrecognized risks to the natural world and even to public health at large (Brooks et al., 2012; Brooks, 2014). Fluoxetine exposure has been shown to affect various physiological processes and behaviors in aquatic organisms. Many studies have reported that long-term exposure to FLU reduces growth and reproductive potential in mussels (*Mytilus californianus*) (Peters and Granek, 2016), affects stickleback (*Gasterosteus aculeatus*) nest quality (Sebire et al., 2015), and also decreases survival, increases abnormal behaviors, and delays predator escape responses in guppies (*Poecilia reticulata*) at environmentally relevant concentrations (Pelli and Connaughton, 2015). Dzieweczynski and Hebert (2012) reported that FLU exposure reduced aggression in male Siamese fighting fish (*Betta splendens*). Although researchers have long recognized the potential effects of environmental FLU exposure on fish, data on its tissue-specific bioconcentration patterns and these pattern-induced effects of FLU in fish are scarce. In particularly, the research on the bioaccumulation pattern in fish embryos/larvae is very limited.

In recent years, the "read-across hypothesis" has made use of clinical and non-clinical data to predict potential effects on fish and other wildlife species (Rand-Weaver et al., 2013; Winter et al., 2010). And, recent studies have also suggested that predicted fish plasma concentrations of SSRIs could be compared to human therapeutic thresholds (H_TPC) to assess potential adverse effects of these drugs in the environment on fish populations (Margiotta-Casaluci et al., 2014; Valenti et al., 2012). Thus, our study exposed fish to a range of FLU concentrations that were predicted to produce plasma concentrations below, equal to, or above those known to induce therapeutic effects in humans (i.e., H_TPC) (Margiotta-Casaluci et al., 2014). We designed short-term and long-term exposure experiments using zebrafish and juvenile red crucian carp in order to: (1) evaluate the potential effects of waterborne FLU on fish embryos/larvae at very early developmental stages; (2) analyze the bioconcentration pattern of FLU in fish tissue after short-term and long-term exposure; (3) investigate the correlation between the potential adverse effects and the concentrations of FLU in fish tissue.

2. Materials and methods

2.1. Test chemicals

Fluoxetine hydrochloride (CAS number 56296-78-7, purity ≥ 98.0%) and fluoxetine-d5 hydrochloride (CAS number 1173020-43-3, purity 98.0%) were purchased from CNW Technologies (Shanghai, China). The detail descriptions on chemicals were available in Supporting Information (SI).

2.2. Fish care and maintenance

Adult zebrafish (wild-type, AB strain) were purchased from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) and maintained in flow-through aquarium systems on a 14:10-h light:dark cycle at 28 °C ± 0.5 °C. Juvenile red crucian carp (*Carassius auratus*) were purchased from Lanling bird and flower market in Shanghai, China. Details of the fish were given in the SI.

2.3. Experimental design and sample collection

2.3.1. Short-term exposure of zebrafish embryos

Zebrafish embryos (4 h post-fertilization, 4 hpf) were randomly distributed into 6-well plates with 40 embryos per well, each well containing a different concentration of exposure solution. Exposure solutions contained 0.1, 1, 10, 100, 1000 μg/L FLU; they were prepared using fresh egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.3 mM $MgSO_4$) and had a pH of 6.8–7.2. A blank control group (egg water) was run in parallel. At least three replicate wells for each FLU concentration treatment group and also for the control group constituted biological replicates. In addition, we repeated the exposure experiment several times to obtain enough material for different-endpoint testing. All tested embryos were grown under a 14:10-h light:dark cycle at 28 °C ± 0.5 °C. The test solutions were completely replaced every 24 h. Hatching rate was recorded from 48 hpf to 72 hpf. The heart rate was recorded for 20 s at 48 hpf and 72 hpf under a stereomicroscope, and body length and swimming activity were recorded at 120 hpf. After exposure for 72 hpf and 120 hpf, embryos/larvae from each well were pooled as one sample and stored at −80 °C for the subsequent LC-MS/MS and biological analyses. The analysis of FLU concentrations in exposure solutions were given in the SI.

2.3.2. Long-term exposure of red crucian carp

Red crucian carp were randomly distributed into 10 L glass tanks containing different concentrations of exposure solution, with five fish per tank. FLU exposure solutions were again 0.1, 1, 10, 100, 1000 μg/L; they were prepared using dechlorinated tap water and had a pH of 7.0–7.5, and a control group (dechlorinated tap water only) was run in parallel. Three replicate tanks for each FLU treatment and for the control were used. The test solutions were replaced every 24 h. After 30 days of exposure, the fish were anaesthetized and killed, and individual brain, liver, and muscle samples were separately collected and stored at −80 °C for analysis by LC-MS/MS. The analysis of FLU concentrations in exposure solutions were also given in the SI.

2.3.3. Short-term exposure and purification experiment using adult zebrafish and red crucian carp

Adult zebrafish were randomly distributed into 10 L glass tanks with 15 fish per tank, and red crucian carp were distributed to 10 L glass tanks with six fish per tank. Three replicate tanks were used for each fish species exposure experiment. All fish were exposed to FLU for 6 days at 0.1 μg/L, followed by a 6-day recovery period (clean water), for a total of 12 days. The test solution and water were replaced every 24 h. The adult zebrafish and red crucian carp were anaesthetized and killed on days 0, 3, 6, 9, and 12, and individual brain, liver (viscera), and muscle samples were separately collected and stored at −80 °C for LC-MS/MS and biochemical analysis.

2.4. Extraction of fluoxetine from fish tissue and LC-MS/MS analysis

The FLU in the fish tissue samples was extracted according to methods described previously (Grabicova et al., 2014). And, to analyze the FLU and FLU-d5 in the water and fish tissue samples, we performed LC-MS/MS using an HP Series 1260 high-performance liquid chromatograph and G6460 triple quadrupole mass spectrometer with electrospray ionization. Details of the extraction procedure and the instrumental analysis were given in the SI.

2.5. Biochemical assays

Acetylcholinesterase (AChE) and antioxidant parameters