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Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*)

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Abstract

In this study, the impact of 17α-ethynylestradiol (EE_2) on reproduction in zebrafish (*Danio rerio*) was evaluated using vitellogenin (Vtg) induction, mortality rate, growth, sex ratio, gonad histology, fecundity, and sperm parameters as endpoints. Two days post-hatch (2 dph) zebrafish were exposed to solvent control or EE_2 at 0.4, 2, and 10 ng/l for 3 months. At 21 dph, Vtg mRNA expression was detected only in fish exposed to 10 ng/l EE_2. At 90 dph, increased mortality rate and sex ratio (female:male) were observed in fish exposed to 2 and 10 ng/l EE_2. A dose-dependent increase in gonads with underdeveloped gametes was observed in fish exposed to EE_2. At 180 dph, malformation of the sperm duct and reduced number of spermatozoa were found in fish exposed to 2 ng/l and 10 ng/l EE_2. Reduced fecundity and 12 hpf egg viability were found in EE_2-exposed males and females. The number of fish with no expressible milt was elevated dose dependently in EE_2-exposed males, although no difference in sperm density was found. After a 3-month recovery period, growth and sex ratio were partially recovered. Our findings suggest that EE_2 can adversely affect the fecundity, sex differentiation, gametes development, and other reproductive functions of both male and female zebrafish, and some of the toxic effects persist.

Keywords: Zebrafish; 17α-Ethynylestradiol; Gonad development; Reproduction

1. Introduction

In recent years, the effects of environmental endocrine disrupting chemicals (EDCs) on the health of humans and wildlife have been a growing concern among researchers and policy makers (Colborn et al., 1993; Goldman et al., 2000). EDCs have the capacity to modulate the endocrine system and could possibly affect reproductive health of wildlife and even humans. EDCs are widespread, and even some remote areas have been contaminated (Giesy and Kannan, 2001; Martin, 2002; Priddle, 2002; Sandau et al., 2000). The adverse effects of EDCs have been observed in many vertebrate species (Ashby et al., 1997; Tyler et al., 1998). Natural steroidal estrogens modulate sexual differentiation and development in vertebrates. Thus, the hypothesis was made that environmental estrogens (EEs) can disrupt reproductive and developmental functions by mimicking the effects of natural steroidal estrogens. The effluents of sewage treatment works (STWs) represent one of the main sources of EEs (Jobling and Tyler, 2003; Quinn et al., 2004). STW effluents contain complex mixtures of EEs, such as natural estrogens estradiol (E_2), estrone (E_1), synthetic estrogen, 17α-ethynylestradiol (EE_2), phthalates, pesticides, and pharmaceuticals. Higashitani et al. (2003) reported that EEs in STWs contributed to feminization in male carp.

The synthetic estrogen EE_2 is a common component of oral contraceptives (Desbrow et al., 1998). It enters the aquatic environment through domestic STWs. In two independent studies, concentrations of EE_2 in municipal STWs were reported to range from non-detectable to 7.0 ng/l (Desbrow et al., 1998) and non-detectable to 42 ng/l (Ternes et al., 1999). A recent study conducted by the U.S. Geological Survey reported that 5.7% of rivers in the United States had EE_2 concentrations >5 ng/l (Kolpin et al., 2002). Due to the long half-life of EE_2 and its bioconcentration in biota, the concentration of EE_2 in a fish body can be 332-fold higher than that in the environment (Lai et al., 2002), and the potency of EE_2 can be 10–50-fold higher than

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that of E_2 and E_1 in vivo (Thorpe et al., 2003). Numerous studies have shown that fish exposed to EE_2 experienced vitellogenin (Vtg) induction, disrupted sex differentiation, and reproductive failure (Hill and Janz, 2002; Weber et al., 2003; Larsson et al., 1999). Thus, EE_2 could potentially contribute to reproductive dysfunction in wild fish populations, given its high potency and wide distribution.

Previous studies have reported the effects of EE_2 exposure on Vtg induction, sexual differentiation and development and on reproductive function in fish. However, few studies have reported the induction of Vtg expression at early developmental stages after exposure to environmental relevant concentrations of EE_2. Though the deleterious effects of EE_2 on breeding have been reported by determining the total fecundity and fertilization success in many studies (Nash et al., 2004; Schäfers et al., 2007), few have investigated its respective effects in both sexes. In this study, we investigated whether the Vtg mRNA expression could be induced in the first 4 weeks after hatch. By using male/female replacement trials and sperm examination, we also demonstrated the respective impacts of EE_2 on breeding success in both sexes.

2. Materials and methods

2.1. Chemicals

17α-Ethynylestradiol (EE_2) (99.1%) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Stock solutions of EE_2 were prepared in 100% ethanol (analytical grade) at 1 mg/ml and then were serially diluted to stock solutions of 2, 0.4, and 0.08 μg/ml. All additional chemicals used in the experiments were of analytical grade.

2.2. Experimental animals

Wild-type zebrafish (AB* strain) was obtained from the International Zebrafish Center (University of Oregon, OR, USA). Adult zebrafish were maintained in flow-through 2.5-l aquaria and were fed with brine shrimp (*Artemia nauplii*) twice daily. Brine shrimp were hatched in synthetic seawater everyday. At 4 to 18 days post fertilization (dpf), fry were fed with Hatchfry Encapsulon^{TM} (Argent Chemical Laboratories, Redmond, WA, USA). Fish were kept at 28.5 ± 0.5 °C under a photoperiod of 14-h light:10-h dark.

2.3. Water quality

Tap water was filtered with active carbon and a reverse osmosis apparatus (RO; R/O Units HP1200, Aquatic Eco-Systems Inc., Apopka, FL, USA). Analytical grade mineral salt was dissolved in RO water to prepare the standardized synthetic freshwater according to U.S. Environmental Protection Agency guidelines (U.S. EPA, 1986). Synthetic water was aerated and supplied to each aquarium at a flow rate of 9 l/day. Temperature, pH, conductivity, dissolved oxygen, oxidation–reduction potential (ORP), and salinity of aquarium water were monitored continuously (YSI 5200 Recirculating System Monitor, Aquatic Habitats, Apopka, FL, USA). Chlorine, ammonia, nitrate, nitrite, and carbonate hardness were measured once a week.

2.4. F_0 breeding

Adult fish were bred to obtain the eggs used for the EE_2 exposure assays. Fish were transferred to breeding tanks (with a screen of 2 mm^2 porosity) approximately 15 h before spawning (5:00 p.m.). Three males and three females were randomly selected and placed in each tank. At 8:00 a.m. the following day, eggs were collected and examined under a dissecting microscope. Viable eggs were cleaned in freshwater, transferred to sterile petri dishes, and incubated at 28.5 °C until they hatched.

2.5. EE_2 exposure assays

At 2 days post-hatch (dph), viable fry were transferred to 1 l water tanks and exposed to EE_2 (0.4, 2, and 10 ng/l) or solvent control (5 μl/l ethanol). Every tank contained 100 fry and there were four replicates for each treatment. Every day from 2 to 90 dph, a 100% water change was performed and a fresh dose of EE_2 or ethanol was added to the aquaria. From 2 to 16 dph, fry were fed with Hatchfry Encapsulon^{TM} three times per day. At 16 dph, fry were transferred to 2.5 l aerated water tanks and were randomly divided into four replicates for each treatment. Newly hatched brine shrimp were provided twice daily. At 7, 14, 21 and 28 dph, 5–15 fish from each treatment were randomly selected and pooled for Vtg determination (two replicates). Remaining fish were exposed continuously until 90 dph. At 90 dph, 20 fish from each of the four treatments were randomly selected for measurement of weights, lengths, condition factors (*K*-factors) (weight (g)/length $(cm)^3$) × 100) and histological examination of the gonads. The remaining fish were transferred to fresh water in a flow-through system and reared until adulthood (180 dph) for F1 breeding studies.

2.6. Methodologies for measurement of biological endpoints

2.6.1. Vtg determination

Few studies have reported results of Vtg induction after exposure to environmental relevant concentrations of EE_2 in early developmental stages of the zebrafish. In this study Vtg mRNA expression in the first 4 weeks after hatch were determined using RT-PCR. 5 to 15 fish (15 fish at 7 dph, 10 fish at 14 dph, 7 fish at 21 dph and 5 fish at 28 dph) were pooled and homogenized in 5 ml homogenizers. For each treatment, two replicates were used for the Vtg determination. Total RNA was extracted with TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). 2 μg RNA from each of the four treatment groups was used for reverse transcription and three replicates were conducted. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) at 37 °C for 60 min and 94 °C for 5 min. 0.1 μg synthesized cDNA was used for PCR. Primers for Vtg1 and housekeeping gene-elongation factor 1α (Elf-1α) were designed as described in Islinger et al. (2003): Vtg1 upstream primer 5′-GCT GCT GCA TCT GTC