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β-estradiol 17-valerate affects embryonic development and sexual differentiation in Japanese medaka (*Oryzias latipes*)

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ABSTRACT

β-estradiol 17-valerate (EV) is a synthetic estrogen widely used in combination with other steroid hormones in hormone replacement therapy drugs and is detected in natural waters. Although EV is known as an estrogenic chemical, there is still a lack of data on its developmental and reproductive toxicities in fish following exposure to EV during embryo-larval-, juvenile- and adult-life stages in Japanese medaka (*Oryzias latipes*). At the early life stage, the fertilized eggs of medaka were exposed to 1, 10, 100 and 1000 ng/L EV for 15 days, and hatched larval fish were continually exposed to the same concentration range for an additional 15 days. The results showed that exposure to 10 ng/L or above resulted in adverse effects on hatchability and time to hatching, and the number of hatched females was twice that of males at 10 ng/L or above. When the hatched fish were continually exposed to 1, 10 and 100 ng/L of EV for another 40 days, the hepatosomatic index (HSI) was increased in both males and females, and the gonadosomatic index (GSI) was decreased in females, and increased in males. Sex reversal was found in fish exposed to 1 ng/L and above. Quantitative real-time RT-PCR showed that mRNA levels of estrogen receptor α (ER-α) and vitellogenin-I (VTG-I) in the liver of females were significantly down-regulated, while those of vitellogenin-I (VTG-I) in the liver of males were significantly up-regulated at all concentrations. These findings suggest that EV is a reproductive toxicant and estrogenic chemical in both male and female fish.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are of great public and scientific concern, since these chemicals can mimic, block, or interfere with hormones in the body and can subsequently induce detrimental effects on reproductive processes in wildlife and in humans (Saunders, 2005; Caserta et al., 2008; Mélanie et al., 2012). Extremely low doses of estrogenic compounds have shown significantly biological effects both in vivo and in vitro (Caserta et al., 2008; Per et al., 2012). They can delay time to hatching, decrease hatchability, change sexual determination and expression of secondary sex characteristics in a variety of vertebrates (Scholz and Gutzeit, 2000; Saidapur et al., 2001; Zha et al., 2008). Several field studies have shown that these estrogenic responses in wild fish may be due to chemicals present in aquatic environments (Flammarion et al., 2000; Van Aerle et al., 2001) and are related to reproductive failure in fish. Abnormal gonadal development such as intersex (testis-ova) has been increasingly observed in wild fish and has been commonly linked to estrogenic contaminants such 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), based on the fact that these chemicals have the ability to induce intersex when they are tested in the laboratory (Zha et al., 2008). Persistent sex reversal was also found in adult *Xenopus* (*Silurana*) *tropicalis* frogs following larval exposure to the environmental pollutant, EE2 (Pettersson et al., 2006). In toxicological processes, alterations in gene expression are responsible for conventional histological responses. Some genes such as vitellogenin (VTG) and estrogen receptor (ER) genes have been widely used as marker genes to evaluate the effects of estrogenic EDCs (Zhang et al., 2008a; Sellin et al., 2010; Brieno-Enriquez et al., 2012). ERβ gene expression in males and VTG gene expression in females was significantly unregulated when Chinese rare minnows (*Gobiocypris rarus*) were exposed to 0.3 mg/L 2,4-dichlorophenol (2,4-DCP) and above for 21 days (Zhang et al., 2008a). The fact that the hepatic ERα gene in the eelpout (*Zoarces viviparous*) after exposure to E2 and 4-tert-octylphenol for 48 h was significantly transcriptionally up-regulated indicating that the measurement of ER mRNA by the RT-PCR assay is a sensitive method for the detection of estrogenic responses in eelpout

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(Andreassen et al., 2005). In addition, some researchers reported an increase in VTG concentration in aquatic organisms after exposure to estrogenic EDCs such as E2 and EE2 (Irwin et al., 2001; Mitsui et al., 2007).

β-estradiol 17-valerate (EV) is one of the most common sources of free E2 in hormone replacement therapy, in vitro fertilization, and contraceptive drugs, and is used in animal models of reproductive disorders (Seidman et al., 2009; Borgelt and Martell, 2012). In animal farming, estrogens like EV are mainly applied as growth promoters (Andersson and Skakkebæk, 1999), and for developing a single-sex population of fish in aquaculture (Bombardelli and Hayashi, 2005; Senol et al., 2008; Chu et al., 2011). The EV molecule is relatively stable in an aquatic environment and provides slow release of E2 after hydrolization of the ester bond between E2 and short-chain valeric acid. Nonhydrolized EV, however, has the classic structure of a lipid antigen and, as such, may induce an immune response restricted to the lipid antigen presentation pathway (Seidman et al., 2009). It has been reported that low-dose intradermal exposure to 5 μg EV has an inverse effect on the estrus cycle pattern of female rat and can significantly reduce litter size (Seidman et al., 2009). At present, EV has been detected in natural waters in China and its concentration range is approximately 1–10 ng/L (Lei et al., 2009; Jiang et al., 2012). These findings suggest that, under certain circumstances, environmental exposure to EV may act as an endocrine disruptor and adversely affect reproductive outcome. While there is growing field and laboratory evidence which shows the adverse effects of estrogens such as E2 and EE2 on reproduction and development in fish (Zha et al., 2008; Caldwell et al., 2008; Dang, 2010), few studies have focused on the developmental and reproductive toxicity of EV in aquatic species, such as fish. These studies have mainly been concerned with aquaculture application (Bombardelli and Hayashi, 2005; Senol et al., 2008; Chu et al., 2011). However, in aquaculture, only sensitive life stage of fish like sexual differentiation or embryonic stage was exposed to EV to develop single-sex population of fish (Senol et al., 2008). The corresponding single endpoints were sex ratio or sex reversal, and there has been no comprehensive assessment of multiple endpoints such development, growth, reproduction in a full life cycle from fertilization to sexual maturation in fish exposed to EV.

Medaka is recognized as an appropriate model for the evaluation of EDC effects (Arcand-Hoy and Benson, 1998; Ishibashi et al., 2004; Sun et al., 2007). In particular, its large eggs with clear chorions facilitate the observation of embryos. The early life stages of medaka are also considered to be relatively sensitive to estrogenic chemicals (Chikae et al., 2004).

Therefore, the present study aimed to evaluate the developmental and reproductive toxicities in fish following exposure to low concentrations of EV during embryo-larval-, juvenile- and adult-life stages in Japanese medaka (*Oryzias latipes*). The entire exposure time was 70 days and the corresponding endpoints included time to hatching, hatchability, sex ratio, gross abnormalities, HSI and GSI. The underlined estrogenic mode of action (MoA) of EV was evaluated by transcriptional expression of both VTG and ER genes at various concentration levels.

2. Materials and methods

2.1. Test compounds and test fish

β-estradiol-17-valerate (EV) was purchased from Sigma–Aldrich, USA. The different concentrations of EV stock solutions were prepared with HPLC-grade DMSO. All the other chemicals were analytical or HPLC grade.

In our laboratory, Japanese medaka (d-rR strain) fish were kindly provided by Y. Wakamatsu (Laboratory of Freshwater Fish at the bioscience Center of Nagoya University, Japan). The brood stock has been maintained for more than 4 years. Medaka were kept in charcoal-dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg CaCO3/L) at a constant temperature (25 ± 1 °C) with a photoperiod of 16:8 h (light:dark). The brood stock was fed three times daily, once with newly hatched brine shrimp (*Artemia nauplii*) and twice with commercial food (TetraMin®, Germany).

2.2. Exposure and experimental design

At the early life stage, embryos less than 4 h post-fertilization were used in the exposure experiments.

Paired stock fish are maintained in the laboratory. Spontaneously spawned eggs were carefully collected from the ventral side of stock females (about 40 females) within a few hours of natural fertilization. Eggs were obtained from clutches by gently rolling them with a finger. Eggs were disinfected by placing them in a 0.9% solution of hydrogen peroxide for 10 min (Marking et al., 1994; Sun et al., 2007), and then checked for fertilization using a dissecting microscope. Based on the results of an initial range-finding study (data not shown), embryos were exposed to nominal EV concentrations of 1, 10, 100 and 1000 ng/L in dilution water (charcoal-dechlorinated tap water) for 15 days. In addition, dilution water controls (DWC) and solvent controls (SC) were included in the experimental design. The SC and all EV exposure groups contained 0.1 ml/L DMSO and 1% methylene blue whereas the DWC groups contained 1% methylene blue only. Treated and control embryos were randomly assigned to different treatments in glass dishes containing 100 mL each test solution (30 embryos/dish). Three replicates were used for each concentration and control. Embryos were incubated in a 16:8 h light:dark photoperiod cycle at 25 ± 1 °C. Eighty percent of each test solution was renewed every 24 h. Embryos were observed twice daily at which time dead embryos (identified by the incorporation of methylene blue) were removed. Hatchability, time to hatching and gross abnormalities were recorded.

The hatched larval fish were continuously maintained at the concentration for an additional 15 days. The fish were observed daily. All dead fish were removed and recorded. After the additional 15 days of exposure, the genetic sex ratio was determined simply by the color of the fish. In the d-rR strain of medaka, sex-linked genes determine orange-red coloration in males and white color in females (Edmunds et al., 2000; Scholz and Gutzeit, 2000). The medaka (*Oryzias latipes*) has an XY-XX genetic sex determination system. In the d-rR strain the genetic sex, i.e. XX or XY, is phenotypically apparent since only the Y-chromosome carries an allele for the formation of orange-red chromatophores (Scholz and Gutzeit, 2000). In this study, the color on the tails of the fish was the main criterion for determining males or females. For Japanese medaka, the male or female fish in about 15 days post-hatch can be distinguished by color (Sun et al., 2007).

At the later developmental stage, ten fish including five females and five males were randomly assigned to a 5 L glass aquarium and duplicate aquaria were used at each exposure level. Fish were continuously exposed to EV concentrations of 1, 10 and 100 ng/L. In addition, DWC and SC were included in the experimental design. The SC aquaria and all EV exposure aquaria received 0.1 ml/L DMSO whereas the DWC received dechlorinated tap water only. In view of the relative stability of the EV molecule in an aquatic environment, the exposure and control solution was renewed once every 48 h. Fish were kept under semi-static conditions at 25 ± 1 °C with a photoperiod of 16:8 h (light:dark). Treated and control fish were continually exposed for 40 days.

The entire test duration was 70 days. The medaka fish were fed three times daily. Feeding consisted of newly hatched brine shrimp (*Artemia nauplii*) for days 0–7, and a commercial granule