Aquatic Toxicology 166 (2015) 1–9

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

The progestin levonorgestrel affects sex differentiation in zebrafish at environmentally relevant concentrations

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 26 June 2015
Accepted 27 June 2015
Available online 30 June 2015

Keywords:
Progestin
Levonorgestrel
Sex differentiation
Sex ratio
Androgenic activity
Zebrafish

ABSTRACT

Synthetic progestins have become widespread environmental contaminants and may cause adverse effects on fish. In the present study, we investigated the effects of levonorgestrel (LNG) on sex differentiation in zebrafish (*Danio rerio*). Embryos were exposed to LNG at environmentally relevant concentrations (0, 1, 10, 33, and 100 ng/L) and allowed to develop until sexual maturity. Histological examination at 63 days post fertilization (dpf) caused complete sex reversal and 100% males were observed in the 10, 33 and 100 ng/L treatments; gross morphological and histological examination of gonads at 142 dpf further confirmed 100% males at these exposure concentrations. The results indicate androgenic activity of LNG, and masculinization during zebrafish gonadal differentiation. The mRNA expression levels of genes involved in fish sex differentiation and gonadal development were examined at 28 and 42 dpf. Down-regulation of the mRNA expression of aromatase (e.g., *cyp19a1a*, *cyp19a1b*), the forkhead transcription factor gene L2 (*foxl2*) and the Fushi tarazu factor-1d (*nr5a1b*) were observed. In contrast, transcription of the doublesex and mab-3-related transcription factor 1 (*dmrt1*) gene was up-regulated. Androgen receptor (*ar*) mRNA expression was significantly down-regulated at 28 and 42 dpf. Co-exposure to flutamide (an androgen antagonist) and LNG, led to a decrease in the sex inversion potency of LNG. Our study has demonstrated that environmentally relevant concentrations of LNG could alter sex differentiation and gonadal development in zebrafish. Our results also suggest a potentially high ecological risk of LNG to fish populations in LNG-contaminated aquatic environments.

1. Introduction

Progestins (also called progestogens) are synthetic pharmaceutical compounds that are commonly used in contraception and hormone replacement therapies (Zeilinger et al., 2009). Similar to estrogens and androgens, progestins can enter the aquatic environment through wastewater treatment plant effluents, and pastoral agricultural runoff (Chang et al., 2009; Mansell et al., 2011). Although environmental concentrations of progestins are generally only measured at up to tens of ng/L in surface water and hundreds of ng/L in sewage treatment plant effluents (Liu et al., 2011a), the progestins generally display high potency and specificity for biological targets (Fick et al., 2010), many of which are highly conserved between species (Gunnarsson et al., 2008). Recently, there has been a growing concern of the potential adverse impacts that these pharmaceuticals may have on aquatic organisms (DeQuattro et al., 2012; Kumar et al., 2015; Liang et al., 2015a,b; Overturf and Huggett, 2015; Paulos et al., 2010; Petersen et al., 2015; Zhao et al., 2015; Zucchi et al., 2013, 2014).

Levonorgestrel (LNG) is one of the commonly used synthetic progestins in contraceptive pills, emergency contraceptive pills, and contraceptive implants (Besse and Garric, 2009). It has been found in sewage treatment plant effluents and freshwater systems worldwide, including in China (Reviewed by Liu et al., 2011b). The measured concentrations are generally at ng/L levels (e.g., 1–213 ng/L; Al-Odaini et al., 2013; Liu et al., 2011a). LNG has a high potential for bioaccumulation in fish (Fick et al., 2010). In this regard, environmental residues of LNG may pose potential risks to aquatic organisms in the receiving aquatic environment.

Increasing numbers of studies have reported the effects of LNG on aquatic organisms. Most of these studies focused on endocrine disruption and the impairment of reproduction (e.g., inhibition of spawning) in fish (Kroupova et al., 2014; Overturf et al., 2014; Overturf and Huggett, 2015; Runnalls et al., 2013; Svensson et al.,

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http://dx.doi.org/10.1016/j.aquatox.2015.06.013
0166-445X/

2013, 2014; Zeilinger et al., 2009) and amphibians (Hoffmann and Kloas, 2012; Kvarnryd et al., 2011; Lorenz et al., 2011; Säfholm et al., 2011) at low ng/L concentrations. A recent study with three-spined stickleback showed strong androgenic effects on the reproductive cycle in females (Svensson et al., 2013) and disruption of the androgen-dependent reproductive cycle in males (Svensson et al., 2014).

The processes of sexual determination and differentiation in fish are highly plastic and can be modified by many external factors (Devlin and Nagahama, 2002). It is well known that exposure to xenoestrogens or androgens in zebrafish during critical periods of the life cycle can affect sex differentiation, resulting in a change of the sex ratio towards females or males, respectively (Holbech et al., 2006; Örn et al., 2003). As LNG has strong androgenic activity, we hypothesize that LNG may affect sex differentiation during the gonadal differentiation period, which will lead to biased sex ratios of the exposed fish. Therefore, the objective of the present study was to investigate the effects of environmentally relevant concentrations of LNG on sex differentiation in fish.

Zebrafish provide an important vertebrate model for the investigation of chemical effects on sex differentiation, and change to the sex ratio in these fish has been widely regarded as an essential endpoint for the evaluation of sex differentiation (OECD, 2011). In the present study, zebrafish embryos were exposed to environmentally relevant concentrations of LNG until they were sexually mature, and the effects of this on the sex ratio were investigated.

To further understand the potential mechanisms that affect sex differentiation, we have also examined the transcription profiles of a number of genes, including *cyp19*, *dmrt1* (double sex/mab-3 related transcription factor 1), *foxl2* (forkhead transcription factor gene L2) and *nr5a1b* (Fushi tarazu factor -1d, also called *sf1*) in this study. These genes are known to be related to gonadal sex differentiation (von Hofsten and Olsson, 2005). In addition, to better understand whether the effects of LNG occurred via the androgen receptor (AR)-mediated pathway, zebrafish were co-exposed to flutamide and LNG from 20 to 60 days post fertilization (dpf; the critical period of sex differentiation in zebrafish), and the sex ratio was observed. Flutamide is a model mammalian AR antagonist (Ankley et al., 2004). It has been applied in endocrine disrupting chemicals (EDC)-oriented studies with fish, in which it binds to the AR and blocks the AR-mediated responses, as in mammals (Ankley et al., 2004). Our results demonstrate that exposure to environmentally relevant concentrations of LNG caused severe masculinization in zebrafish.

2. Materials and methods

2.1. Chemicals

Levonorgestrel (LNG; CAS 797-63-7; purity ≥ 99%), flutamide (99% ≥ purity), dimethyl sulfoxide (DMSO; CAS 67–68-5; purity ≥ 99.5%) and methanesulfonate (MS-222) were purchased from Sigma–Aldrich (Fluka, Shanghai, China). Deuterated LNG ((−)-Norgestrel-2,2,4,6,6,10-d6 or LNG-d6; CAS 797-63-7; purity ≥ 98%) was obtained from CDN isotopes (Pointe Claire, Quebec, Canada). LNG stock solution (1 mg/mL) and LNG-d6 stock solution (20 mg/mL) were prepared in DMSO and 40% methanol and then stored at 4 °C and −20 °C, respectively.

2.2. Fish husbandry and embryos exposure

Four-month-old zebrafish (*Danio rerio*) of the wild-type (AB strain) were maintained in a semi-static system with charcoal-filtered tap water (pH 7.0–7.4) at 28 ± 0.5 °C with a 14:10 light: dark cycle. The adult fish were fed with newly hatched brine shrimp and dry flake food (Zeigler Brothers, Gardners, PA, USA). Zebrafish embryos were obtained from spawning adults kept together overnight at a sex ratio of 1:1. Embryos were collected after spawning and only those that developed normally were selected for subsequent experiments.

The selected embryos (2 h post fertilization; hpf) were randomly distributed into 1 L glass beakers containing 800 mL of either the LNG exposure solution (1, 10, 33 or 100 ng/L) (or equal to 3.2×10^{-12}, 3.2×10^{-11}, 1.1×10^{-10} or 3.2×10^{-10} M) or the DMSO solvent control. Both the control and treated groups received 0.01% (v/v) DMSO. Three replicates were performed for each exposure concentration and each beaker contained approximately 300 embryos. After 15 days, approximately 250 larvae from each beaker were transferred into 5 L tanks which contained 4 L of the relevant exposure solution. At 30 dpf, approximately 200 larvae were then randomly chosen and transferred into 10 L tanks containing 9 L of the relevant exposure solution. Finally, at 63 dpf, after sampling (see next paragraph), the remaining juvenile fish (20–25 fish from each tank) were maintained in 20 L tanks containing 16 L of the relevant exposure solution, until 142 dpf. All of the exposure solution within each tank was renewed daily. Each tank was checked for dead or malformed fish on a daily basis and any dead fish were removed.

A subset of 15 exposed fish from each treatment replicate was randomly sampled for histological examination at 63 dpf. The fish were anesthetized with 0.01% MS-222 by prolonged immersion, until cessation of opercular movement, then the fish were patted dry with paper towel, and total body length (cm) (from snout to the fork point of caudal fin) and wet weight (g) were measured immediately to calculate the condition factor (*K*-factor = (wet weight (g)/total body length $(cm)^3$) × 100). After measuring, all of the fish were fixed in Bouin's solution for 24 h prior to histological examination.

The fish that were exposed until sexual maturity (142 dpf) were sampled and anesthetized with 0.01% MS-222, then total body length and wet weight were measured and their condition factors calculated. Four fish from each replicate were sampled and the gonads were fixed in Bouin's solution for 24 h prior to histological analysis.

2.3. Histology and sex determination

Gonad morphology and sex determination of the exposed and control fish were performed by histological examination. Following dehydration in a graded series of ethanol solutions, the samples were embedded in paraffin, and 4 μm longitudinal sections (3–4 sections for each fish) were cut through the gonadal region. The sections were stained with hematoxylin-eosin, and examined with an Olympus CX31 light microscope (Olympus, Tokyo, Japan), focusing on sexual differentiation and histological abnormalities of the gonad (e.g., intersex). The gonads were classified as testes or ovaries, based on the presence of spermatogenetic cells or oocytes, respectively, according to Kinnberg et al. (2007). Gonads where a single oocyte at the primary growth stage was observed in otherwise testicular tissue were still classified as testes. Gonads with the presence of both several oocytes and testicular tissue were classified as intersex. Gonads that exhibited no discernible germ cells were classified as undifferentiated. Maturation stages of the ovaries and testes were determined according to Silva et al. (2012). The sex ratios of each group (63 dpf and 142 dpf) and treatment was calculated. All studies were conducted in accordance with the guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China.