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The synthetic progestin megestrol acetate adversely affects zebrafish reproduction

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ABSTRACT

Synthetic progestins contaminate the aquatic ecosystem, and may cause adverse health effects on aquatic organisms. Megestrol acetate (MTA) is present in the aquatic environment, but its possible effects on fish reproduction are unknown. In the present study, we investigated the endocrine disruption and impact of MTA on fish reproduction. After a pre-exposure period of 14 days, reproductively mature zebrafish (*Danio rerio*) (F0) were exposed to MTA at environmental concentrations (33, 100, 333, and 666 ng/L) for 21 days. Egg production was decreased in F0 fish exposed to MTA, with a significant decrease at 666 ng/L. The exposure significantly decreased the circulating concentrations of estradiol (E2) and testosterone (T) in female fish or 11-keto testosterone (11-KT) in male fish. MTA exposure significantly downregulated the transcription of certain genes along the hypothalamic-pituitary-gonadal (HPG) axis. MTA did not affect early embryonic development or hatching success in the F1 generation. The present study showed that MTA is a potent endocrine disruptor in fish, and short-term exposure to MTA could significantly affect reproduction in fish and negatively impact the fish population.

1. Introduction

Many environmental contaminants present in aquatic environments can interfere with the normal endocrine functions of aquatic organisms. Pharmaceuticals, including natural and synthetic steroid hormones, are among the most active compounds in this environment (Christen et al., 2010; Runnalls et al., 2010). The adverse effects of natural and synthetic steroidal estrogens such as 17α-ethinylestradiol (EE2) on aquatic organisms have been extensively studied by researchers worldwide (Caldwell et al., 2008; Runnalls et al., 2013); however, surprisingly little attention has been paid to the environmental health effects of progestins.

Progestins are widely used in oral contraceptives either alone or in combination with estrogens (e.g., EE2) (Erkkola and Landgren, 2005). They are also applied in veterinary medicine and other hormonal therapies (Lonergan, 2011). These compounds can be released into the aquatic environment from sewage treatment plants, pharmaceutical industries, and agricultural areas (e.g., farm animal waste) and are now environmental contaminants worldwide (Kolodziej et al., 2003). Progestins have been detected in surface water (Chang et al., 2009; Vulliet et al., 2011; Chang et al., 2011) and waste waters (Chang et al., 2009, 2011; Vulliet et al., 2007; Pu et al., 2008; Liu et al., 2011) in many countries. Of these, the synthetic progestin megestrol acetate (MTA) has been widely used as an active pharmaceutical ingredient for clinical application (Argiles et al., 2013). A previous study in China reported that MTA was one of the major synthetic progestins detected in urban rivers (at concentrations of up to 34 ng/L) (Chang et al., 2009). Another study found that MTA was the predominant synthetic progestin (41 ± 25 ng/L) in the influent wastewaters obtained from seven wastewater treatment plants (WWTPs) in Beijing, China (Chang et al., 2011). In China, synthetic progestins, which originate from excretion by humans and livestock, could contribute to the major environmental contamination of the aquatic ecosystem.

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Recently, several studies have reported that even at low concentrations, natural progesterone (P4) or synthetic progestins (e.g., levonorgestrel, norethindrone, desogestrel, and gestodene) may cause endocrine disruption and inhibit reproduction in fish (Atteke et al., 2003; Zeilinger et al., 2009; Paulos et al., 2010; Murack et al., 2011; DeQuattro et al., 2012; Runnalls et al., 2013; Svensson et al., 2013, 2014) and frogs (Kvarnryd et al., 2011; Lorenz et al., 2011; Safholm et al., 2012; Hoffmann and Kloas, 2012). However, their underlying mechanisms are not yet well-known. Despite the widespread usage of synthetic progestins and their high prevalence in environmental water bodies, surprisingly they have received very little attention, and the hazards and environmental risks they pose to aquatic organisms are not well-known. Currently, it is not known whether MTA in the environment could have the potential to adversely affect reproduction in fish.

Therefore, we investigated the effect of MTA on the reproduction of zebrafish exposed to environmental levels of MTA (33, 100, 333, and 666 ng/L). Many endocrine disruptors are known to modulate sex steroid signaling and the hypothalamic-pituitary-gonadal (HPG) axis (Villeneuve et al., 2007; Ankley et al., 2009; Ji et al., 2013) by interfering with endocrine functions and affecting both development and reproduction. Thus, we also measured the concentrations of sex hormones in zebrafish and examined the effect of MTA on the transcription of key genes along the HPG axis, with the intention of identifying potential target genes and the potential mode of action of MTA. The present study showed that MTA adversely affects reproduction in zebrafish in a dose-dependent manner after short-term exposure.

2. Materials and methods

2.1. Chemicals

Megestrol acetate (MTA, CAS 595-33-5, purity >99.7%) and dimethyl sulfoxide (DMSO, CAS 67–68-5, purity ≥99.5%) were purchased from Sigma–Aldrich (Fluka, Shanghai, China). Progesterone-d9 (P-d9, Cat. No. P755902, purity >98%) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All the other reagents used in this study were of analytical grade. MTA stock solution (1 mg/mL) was dissolved in DMSO and stored at 4 °C.

2.2. Fish maintenance and experimental design

Four-month-old wild-type zebrafish (strain AB) 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 fish were fed newly hatched brine shrimp and pellet food (Zeigler Brothers, Gardners, PA, USA). The fish were housed in 30-L glass tanks with 16 L of water in each tank. Three replicate tanks containing six males and six females each were used. The experiment consisted of a 14-d pre-exposure period in order to establish the reproductive capacity of unexposed fish and to provide tank-specific baseline data for potential statistical comparison after initiation of chemical exposure (Ankley et al., 2001; Kunz et al., 2006). After the pre-exposure period and successful spawning has been established, chemical treatment was then started. The zebrafish were exposed to nominal concentrations of MTA (33, 100, 333, and 666 ng/L) (or equal to 0.086, 0.260, 0.866 and 1.732 nM) for 21 days, and the eggs were collected daily. The experiments included a water control and a solvent control (SC). Both the SC and exposure groups received 0.001% (v/v) DMSO. The water of the tanks exposed to MTA was renewed daily. After 21 days of exposure to MTA, the embryos were collected and divided into two groups: one group received continued treatment with the same MTA concentrations as did their parents, and the other group received no further MTA treatment. The hatching, malformation rates, and survival during the early developmental stage were also recorded. The zebrafish were maintained in accordance with guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China. All animals were treated humanely and with the aim of alleviating any suffering.

2.3. Tissue sampling

After F0 individuals were treated for 21 days, the fish were anesthetized in MS-222 (0.03%; Sigma–Aldrich, Shanghai, China). The body length (cm) and weight (g) were recorded to determine the condition factor (K = (body weight/body length^3) × 100). Blood samples were collected from the caudal vein of the fish with a glass capillary and transferred into heparin sodium-rinsed tubes. The brain and gonad were excised, and gonads were weighed for gonadosomatic index (GSI) calculation, then immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.4. Sex hormone assay

Plasma was obtained by centrifugation (7000 × g for 5 min at 4 °C) of the blood sample. The plasma samples from three individual fish of the same sex were pooled as one replicate. Plasma extraction and the determination of sex hormone concentrations were performed with a modified method as described previously (Drevnick and Sandheinrich, 2003). Briefly, 10 μL of plasma from each biological replicate was diluted to 400 μL using ultrapure water (Milli-Q, Millipore, Billerica, MA) and extracted twice with 2 mL of ethyl ether. The ether phase was collected and evaporated under a gentle stream of nitrogen gas. The residues were redissolved with the buffer provided in the detection kits, and estradiol (E2), testosterone (T), and 11-keto testosterone (11-KT) concentrations were measured using enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA; detection limits: 20 pg/mL for E2, 6 pg/mL for T, and 1.3 pg/mL for 11-KT). The intra- and inter-assay coefficients of variance (CV) were <20%, <15% and <15%, for the E2, T, and 11-KT assays, respectively.

2.5. Quantitative real-time polymerase chain reaction (PCR) analysis

Extraction, purification, and quantification of total RNA, first-strand cDNA synthesis, and qRT-PCR reactions were performed as described previously (Chen et al., 2012). Quantitative real-time PCR was analyzed on ABI 7300 System (PerkinElmer Applied Biosystems, Foster City, CA, USA) using the SYBR® Green PCR kit (Toyobo, Tokyo, Japan). The primer sequences of the selected genes were obtained by using the online Primer 3 program (http://frodo.wi.mit.edu/). Table 1 lists the gene names, their abbreviations, and the primer sequences of the selected genes. Prior to the transcriptional assay, we assessed the amplification efficiencies of primers and the transcriptional stability of five candidate genes (*rpl8, 18s, β-actin, gapdh, ef1α*) commonly used as reference genes for each organ from fish of both genders. Then, *β-actin* was selected as the reference gene for transcriptional assay in female brains, male brains, and testes, and the *gapdh* gene was selected as the reference gene for female ovaries. The relative transcriptional abundance of each gene to its corresponding SC group was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).