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Endocrine disruption in Chinese rare minnow (*Gobiocypris rarus*) after long-term exposure to low environmental concentrations of progestin megestrol acetate

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ABSTRACT

Synthetic progestins are widely used pharmaceutical agents that have become common contaminants in the aquatic environment. The potential adverse effects of long-term exposure on aquatic wildlife, however, are not fully understood. The aim of this study was to investigate the endocrine disruption in Chinese rare minnow *(Gobiocypris rarus)* in response to megestrol acetate (MTA) exposure. Newly-hatched Chinese rare minnow larvae were exposed to MTA at a nominal concentration of either 1 ng/L (detected concentrations ranged from 0.18 to 0.93 ng/L) or 10 ng/L (detected concentrations ranged from 4.27 to 9.64 ng/L) for 6 months and the effects on growth, sex steroid hormones, gonadal histology, and steroidogenic genes expression were determined. After 6 months of exposure to a nominal concentration of 10 ng/L MTA, the body weight and condition factors were significantly increased in fish of both sexes. Exposure to a nominal concentration of 10 ng/L MTA significantly reduced plasma concentrations of estradiol and 11-ketotestosterone in female fish while also reducing testosterone and 11-ketotestosterone in male fish. Gonad histology revealed significantly reduced proportions of vitellogenic oocytes in female fish exposed to a nominal concentration of 10 ng/L MTA and induction of atretic follicles in female fish exposed to both nominal concentrations of MTA. The expression of *cyp19a1a* and *cyp17a1* in the gonads was up-regulated in the ovaries while down-regulated in the testes. Our results indicate that MTA can induce endocrine disruption in Chinese rare minnow at the low concentrations found in contaminated environments. This indicates a potentially high ecological risk from MTA to fish populations in MTA-contaminated aquatic environments in China and may also in other regions.

1. Introduction

The presence of steroid hormones with high biological activity contaminating aquatic environments is becoming an increasing cause for concern (Fent et al., 2006). Progesterone and other synthetic progestins are a group of steroid hormones widely used in human and veterinary medicine with a variety of therapeutic applications, some of which are being used in agriculture as growth promoters (Fent, 2015). These steroids can enter aquatic ecosystems through waste-water and agricultural run-off (Fent, 2015). They have been detected worldwide in surface waters, waste water, flush water and waste from livestock farms (Chang et al., 2009, 2011; Liu et al., 2011a, 2014; Zhang et al., 2017). Their concentration in surface waters ranges from the low ng/L range to over 100 ng/L (Fent, 2015). Megestrol acetate (MTA) is a synthetic progestin most commonly used in medicine for such as contraception, cancer treatment, and improving patients' appetite (Argilés et al., 2013; Besse and Garric, 2009; Gómez-Canela et al., 2014). It is also used in livestock as growth promoter in some countries (Fent, 2015; Peng et al., 2008). Though there is no available data about the consumption figures of MTA yet, the measured concentrations in the aquatic environment may indicate its high consumptions. MTA has been detected in wastewater treatment plant (WWTP) influent (0.14–150 ng/L) and effluent (0.1–20 ng/L), as well as in surface waters (0.02–34 ng/L) in many countries including China (Chang et al., 2009, 2011; Fan et al., 2011; Zhang et al., 2011), Japan (Chang et al., 2008), Switzerland (Zhang et al., 2017), and Spain (Gómez-Canela et al., 2014). Of note, the concentrations of MTA in surface waters have been found to be higher in China (up to 34 ng/L) than in other countries

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tested (Chang et al., 2008, 2009; Zhang et al., 2017); these concentrations may have the potential to result in adverse effects on aquatic organisms (Fent, 2015).

An increasing numbers of studies have reported adverse effects of progesterone and synthetic progestins (levonorgestrel, norethindrone, gestodene, drospirenone, desogestrel, dydrogesterone norgestrel and chlormadinone acetate) on reproduction (fecundity, reproductive behavior, and reproductive cycle), endocrine disruption (hormone levels and gonadal histology) and sex differentiation in fish, mainly in zebrafish and fathead minnow (Cardoso et al., 2017; Fent, 2015; Siegenthaler et al., 2017). MTA can induce alterations in sex hormone concentrations and gonadal histology at concentrations equal or greater than 33 ng/L and significantly inhibit egg production at a concentration of 666 ng/L in adult zebrafish (Han et al., 2014; Hua et al., 2016). However, the majority of research into the toxicological effects of progestins has been based on short-term exposure in adult fish and few studies have investigated the effects of long-term exposure at the low concentrations found in the environment.

The Chinese rare minnow (*Gobiocypris rarus*) is an endemic fish species inhabiting the upstream waters of the Yangtze River in China. It makes an appropriate native model fish for the investigation of endocrine-disrupting effects of chemicals in China due to its favorable characteristics including its small size, ease of culture, short life cycle, prolific year-round egg production, and sensitivity to chemical contamination (Wang and Cao, 2017; Zhong et al., 2005). Due to species differences, the existing research on the toxicological effects of progestins conducted in zebrafish and fathead minnow may not represent the potential ecological risks to native wild fish populations inhabiting contaminated waters in China. As an endemic fish species and representative of cyprinid fish (the most important component of the fish fauna in China), the response of Chinese rare minnow to environmental contamination would shed light upon the general condition of most of the Chinese freshwater fish (Wang and Cao, 2017).

The aim of this study was to assess the endocrine-disrupting effects of long-term environmental exposure to low concentrations of MTA in *Gobiocypris rarus*, which could predict the population-level effects in the contaminated aquatic environment. With long-term exposure encompassing the sensitive early developmental stages, we hypothesis that MTA would affect the fish health at concentrations lower than that (e.g., 33 ng/L) used in the short-term exposure of adult fish. We investigated the effects of MTA on toxicological endpoints including growth parameters, somatic indices, pathological gonadal changes, sex hormone concentration, and steroidogenic gene expression.

2. Materials and methods

2.1. Chemicals

Megestrol acetate (MTA, CAS 595-33-5, purity > 99.7%), dimethyl sulfoxide (DMSO, CAS 67-68-5, purity ≥ 99.5%) and methanesulfonate (MS-222) were purchased from Sigma-Aldrich (Fluka, Shanghai, China). Progesterone-d9 (P-d9, CAS 15775-74-3, purity > 98%) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Other reagents used in this study were of analytical grade. MTA stock solution (1 mg/mL) was prepared in DMSO and stored at 4 °C. P-d9 stock solution (20 mg/mL) was prepared in 100% methanol, and stored at − 20 °C.

2.2. Experimental animals

Adult Chinese rare minnows were maintained according to previously described protocols (Zha et al., 2008). Briefly, the brood stock were raised in dechlorinated tap water at a constant temperature of 25 ± 2 °C, with a photoperiod of 16:8 h (light:dark). The fish were fed twice daily with commercial food pellets (Trea, Germany) and newly-hatched brine shrimp (*Artemia nauplii*). Embryos were obtained from spawning adults paired at a sex ratio of 1:1. Embryos were collected after spawning and newly-hatched and normally-developed larvae were selected for subsequent experiments after approximately 72 h post fertilization (hpf).

The newly-hatched larvae at 0 days post hatch (dph) were randomly distributed into 1 L glass beakers containing 800 mL of exposure solution containing either 1 or 10 ng/L (equal to 2.6×10^{-12} or 2.6×10^{-11} M, respectively) MTA, or the DMSO solvent control. Both the control and treatment groups received 0.01% (v/v) DMSO. Four replicates were performed for each exposure concentration and each beaker contained approximately 100 larvae. After 15 days, the larvae from each beaker were transferred into 5 L tanks containing 4 L of the relevant exposure solution and subsequently transferred into 10 L tanks containing 9 L of the relevant exposure solution at 30 dph. At 70 dph, 30–40 fish from each tank were randomly transferred to a 30 L tank containing 20 L exposure solution and maintained there until the end of exposure. In cases where the number of individuals in each tank differed, additional fish were removed to avoid density-dependent effects. The entire exposure period lasted for 6 months with half of the water in each tank renewed daily, with fresh water containing corresponding nominal concentrations of MTA. The fish were maintained in accordance with the guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China (http://www.nicpbp.org.cn/sydw/CL0238/2741.html).

2.3. Tissue and blood sampling

Following 6 months of either MTA exposure or control conditions, fish were fasted for 24 h and then euthanized using an overdose of MS-222 (300 mg/L) by prolonged immersion until opercular movement ceased. The total body length (cm) from snout to the fork point of the caudal fin and wet body weight (g) were measured immediately to calculate the condition factor (K = (wet body weight (g)/total body length $(cm)^3$) × 100). The gonads were excised, and weighed to determine the gonadal somatic index (GSI), and then immediately frozen in liquid nitrogen and stored at − 80 °C. The sex of the fish was examined to determine the sex ratio. Blood was collected from the caudal vein, centrifuged at 7000 × *g* for 5 min at 4 °C to separate the plasma, and the supernatant was collected and stored at − 80 °C. Gonads of 8 fish per treatment (2 fish from each replicate tank) were randomly sampled and fixed in Bouin's solution for 24 h and then stored in 70% ethanol for further histological processing.

2.4. Sex hormone assay

Plasma samples from three individual fish of the same sex were pooled and analysis of sex hormone concentrations were performed as previously described (Drevnick and Sandheinrich, 2003). Briefly, 10 μL of plasma 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 stream of nitrogen gas. The residues were dissolved using the buffer provided in the detection kits. Estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) concentrations were measured using enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). The detection limits were 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.

2.5. Gonadal histology

The fixed gonad samples were dehydrated, embedded in paraffin, sectioned, and then stained as previously described (Hua et al., 2016). Ovarian staging identified primary oocytes (PO), corticalalveolar oocyte (CAO), vitellogenic oocytes (VO), mature oocytes/preovulatory oocytes (MO/PreO), and atretic follicles (AF), while testicular staging