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Maternal Bisphenol A exposure impaired endochondral ossification in craniofacial cartilage of rare minnow (*Gobiocypris rarus*) offspring

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ABSTRACT

Bisphenol A (BPA), an endocrine disrupting compound, is present in the aquatic environment. BPA can mimic estrogen and cause adverse effects on development and reproduction in different organisms. As epigenetic modifications due to BPA exposure have been reported, the interest on the effects of this chemical has increased. To assess the potential effects of maternal BPA exposure on offspring bone development, adult *Gobiocypris rarus* (*G. rarus*) females were exposed to 15 μg L^{-1} and 225 μg L^{-1} BPA for 21 days. Eggs were collected after artificial spawning and fertilized with the fresh milt of non-exposed male fish. The offspring were raised in clean water and randomly selected for examination at different development stages. Our results showed that specific effects including poor quality of the embryos, increased malformation (bent spine and tail), and delayed craniofacial cartilage ossification of the larvae. Additionally, the transcripts of ossification related genes were significantly downregulated in offspring, and the lysyloxidase activity decreased. The present study demonstrated the maternal-mediated skeleton toxicity of BPA and its adverse effects on *G. rarus*.

1. Introduction

There is a growing interest on Bisphenol A (BPA) because of its ubiquitous presence and endocrine disruptive effects (Quitmeyer and Roberts, 2007). BPA is widely used in synthetic manufactured products, such as cans coatings, polyvinyl chloride, plastic polycarbonates, and medical equipment (Kalb et al., 2016). It is released into the environment through sewage-treatment effluent, plastics natural degradation, and hydrolysis, causing relatively high concentrations in the environmental media worldwide (Crain et al., 2007). Fast growing trend of BPA use and release, much more BPA contaminations have been detected in wastewater, river water, and seawater, which lead increasing exposure to aquatic organisms (Huang et al., 2012; Yamazaki et al., 2015). Due to its endocrine disrupt toxicity, BPA has detrimental effects on fish immune system (Yang et al., 2015), lipid metabolism (Guan et al., 2016), and reproduction (Chianese et al., 2017; Mileva et al., 2014). The transgenerational studies for BPA toxicity showed it even cause heart disorder in zebrafish offspring (Lombo et al., 2015).

The inherited detrimental effects of BPA on mammal bone development have been well observed. For example in rats, gestational BPA exposure could reduce femur length of the male dams (Lind et al., 2017), and alter femoral geometry in both male and female offspring (Lejonklou et al., 2016). In teleost fish, zebrafish are mostly chosen as animal model to study the effects of endocrine disrupting compounds (EDCs) on craniofacial chondrogenesis. In zebrafish larvae, dioxin suppressed the ceratohyal cartilage growth by reducing both chondrocyte number and size (Burns et al., 2015). Treatment with atrazine, a commonly used herbicide, resulted in craniofacial defects in zebrafish embryos (Walker et al., 2018). Bis-GMA, a BPA polymer used in dentistry, could impair the cartilage ossification of zebrafish embryos (Kramer et al., 2016). In most cases, the fish embryos or juveniles were directly exposed to EDCs. Whether maternal treatment with EDCs affects skeleton development of their offspring is unknown.

Gobiocypris rarus (*G. rarus*), a Chinese freshwater cyprinid that is suitable for aquatic toxicology and has been well used in bioassay past decades (Zhang et al., 2014; Zhong et al., 2005; Zhu et al., 2011). With the hypothesis that maternal BPA exposure could impair cartilage ossification and skeleton morphogenesis on fish subsequent offspring. We measured the quality of embryos, skeleton abnormalities, craniofacial cartilage ossification, and the transcript profiles of skeleton development related genes (*bmp2, bmp4, bmp6*, and so on) as well as enzyme activity of larvae in maternal BPA exposed *G. rarus*. The major findings of this work will be the proof of maternal-mediated skeleton toxicity of BPA on teleost fish.

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2. Materials and methods

2.1. Fish rearing and experimental design

Six months old parent *G. rarus* were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), and raised in glass tanks with dechlorinated tap water (temperature 25 ± 2 °C, pH 8.04 ± 0.22, dissolved O_2 7.18 ± 0.14 mg L^{-1}, ammonia nitrogen 0.44 ± 0.08 mg L^{-1}, total nitrogen 0.55 ± 0.06 mg L^{-1}, total phosphorus 2.20 ± 0.07 mg L^{-1}, and total hardness 188.2 ± 14.2 mg L^{-1}.) with a 14 h: 10 h light-dark cycle. They were fed with *Artemia* nauplii twice daily. After 2 weeks acclimation, the female fish were randomly selected and exposed to BPA (Sigma Chemicals Inc., St. Louis, MO USA) or the solvent control (0.001% dimethyl sulfoxide, DMSO, v/v) for 21 days. The nominal concentrations of BPA were 15 μg L^{-1} and 225 μg L^{-1}, based on environmental levels and our previous reports (Crain et al., 2007; Huang et al., 2012; Yuan et al., 2016; Zhang et al., 2016). The average actual BPA concentrations in water, measured by high performance liquid chromatography (HPLC) (Rezaee et al., 2009), were 13.21 ± 1.32 μg L^{-1} for 15 μg L^{-1} groups and 197.84 ± 24.32 μg L^{-1} for 225 μg L^{-1} groups, no significant difference were found in each treatment after BPA addition for the first time and subsequent each replacement. There were three replicate tanks in each treatment group, with 10 individuals per tank (living space near 1.0 g fish L^{-1}). During the experimental period, half of the exposure water in each tank were replaced daily with fresh solution at the appropriate concentration of BPA.

After the 21-days exposure period, the female fish were induced artificial spawning and the eggs were collected for fertilization according to the established artificial insemination protocol (Zhu et al., 2011). Briefly, females were injected of 1 IU human chorionic gonadotrophin (Chorulon; Intervet Co. The Netherlands) to superovulate, and their abdomen mildly compressed to harvest mature oocytes after 12 h convalescence. Then the eggs were transferred to glass petri dishes and fertilized with some fresh milt produced by non-exposed males immediately. The fertilized eggs were placed in glass petri dishes containing clean water and kept in incubator sustained at 25 ± 2 °C with a 14 h: 10 h light-dark cycle. The egg diameter, fertility rate, and malformation rate were calculated from 4 h post-fertilization (hpf) to hatching. Besides, the spontaneous coiling rate and the embryonic heart rate were recorded from 24 to 48 hpf according to Wang et al. (2014) and Ramlan et al. (2017). The hatching rate and body length were detected post hatching. To evaluate the morphological development, larvae were anesthetized with MS-222 (0.04%, Sigma Chemicals Inc., St. Louis, MO USA), and observed by optical microscope (Nikon 80i, Japan). All experimental procedures were approved by the Animal Ethics Committee of Northwest A&F University.

2.2. Observation of offspring craniofacial cartilage and bone

Larvae were fixed at 12, 24, 36, 48 days post-fertilization (dpf), and stained with alcian blue (Sigma Chemicals Inc., St. Louis, MO USA) and alizarin red (Sigma Chemicals Inc., St. Louis, MO USA) to observe the craniofacial cartilage ossification (Burns et al., 2015). In detail, larvae were anesthetized with MS-222 (0.04%, Sigma Chemicals Inc., St. Louis, MO USA), and fixed overnight in 10% formaldehyde, then dehydrated with graduated concentrations of ethanol. The cartilages were stained with 0.1% alcian blue and 0.1% alizarin red in 80% ethanol with 20% glacial acetic acid solution at 37 °C overnight. After rehydrated, the stained larvae were preserved in glycerinum at 4 °C and observed by compound microscope (Nikon 80i, Japan).

2.3. Quantitative real-time PCR (qRT-PCR) assay of the larvae

Total RNAs were extracted from the whole body of 30 larvae using TRIZOL reagent (Takara, Dalian, China), and treated with RNase-free DNase I (Fermantas, Canada) to remove genomic DNA contamination following manufacturer's instructions. RNA integrity was checked by analyzing 28S ribosomal RNA (rRNA) and 18S rRNA ratios with 1% agarose gel electrophoresis. The quality of RNA was ascertained by spectrophotometric method with a nanodrop spectrophotometer (Thermo Electron Corporation, USA). The cDNAs were synthesized from 3 μg total RNA with M-MLV reverse transcriptase (Invitrogen, Waltham, MA, USA) and oligo $(dT)_{18}$ primer in a 20 μL final reaction volume, and also ascertained by the spectrophotometric method and agarose gel electrophoresis.

The qRT-PCR was performed using SYBR Green ExTaq II kit (Takara, Dalian, China) and CFX96 Real-Time PCR system (Bio-Rad, California, USA). The PCR reaction was carried out in a final volume of 25 μL, with 12.5 μL SYBR Green Premix ExTaq™, 0.4 μM of each forward and reverse primers, and 2.5 μL RT reaction solutions. Each sample was analyzed in triplicate using the following protocol: initial denaturation 95 °C/30 s, denaturation 40 cycles of 95 °C/5 s, and annealing 62 °C/30 s. CFX Manager Software (Bio-Rad, California, USA) was used to analyze the density of SYBR green I and to determine the threshold cycle (Ct) value. Results of gene expression were analyzed using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

Ossification-related mRNA expression profiles of *bmp2, bmp4, bmp6, runx2, sox9a, col2a1,* and *loxl1* were evaluated in the larvae. The primers were designed by the corresponding genes consensus regions and listed in Table S1. Both *β-actin* and *ef1α* were used as reference genes according to our previous publications (Yuan et al., 2014). The geometric mean of two reference genes was used to normalize these target genes (Vandesompele et al., 2002).

2.4. Examination of lysyloxidase (LOX) activity

Lysyloxidase activity was measured using Amplite Fluorimetric Lysyloxidase Assay Kit (AAT Bioquest Inc., USA). In brief, the whole body homogenate samples were mixed with horseradish peroxidase reaction solution, and incubated at 37 °C for 30 min in the dark. The fluorescence intensity was quantified by a multi-detection microplate reader (Synergy HT, Bio Tek, USA), at the excitation wavelength of 540 nm and the absorption wavelength of 590 nm. Each corresponding sample fluorescence intensity normalized to protein concentration, which has been assayed using BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), was regarded as a relative fluorescence unit (RFU). Each treatment was detected in triplicate (n = 30).

2.5. Statistical analysis

All data are expressed as the mean ± standard error (SEM). Homogeneity of variances was analyzed by Levene's test. Normality of the data was tested using the Kolmogorov–Smirnov test. When necessary, data were transformed by arc-sin square root transformation prior to statistical analysis. Data were analyzed by a general mixed model (with repeated measures), followed by one-way analysis of variance (ANOVA) with Duncan's test by using SPSS 20.0 software (SPSS, Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

3. Result

3.1. Reproduction toxicity on the embryos upon maternal BPA exposure

In the present study, we totally collected 3100 eggs, 3187 eggs, and 3129 eggs in the CON, BPA15, and BPA225 groups respectively. The egg diameter, body length, and the rate of fertility, spontaneous coiling, embryonic heart, hatching, and malformation are shown in Fig. 1. Maternal BPA exposure significantly decreased the egg diameter and spontaneous coiling rate, while increased the embryonic heart rate and