

Effects of Barium Chloride Exposure on Hormones and Genes of the Hypothalamic–Pituitary–Gonad Axis, and Reproduction of Zebrafish (*Danio rerio*)

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Abstract Adult zebrafish pairs were exposed to sub-lethal concentrations of BaCl₂ for 21 days, and the effects on reproduction, sex steroid hormones, and transcription of the genes belonging to the hypothalamic–pituitary–gonad (HPG) axis were investigated. The adverse effects on performances of F1 generation were further examined with or without subsequent exposure to BaCl₂. Egg production was significantly decreased, and parental exposure to BaCl₂ resulted in lesser rates of hatching. In males, exposure to BaCl₂ resulted in greater concentrations of E2 along with greater mRNA expression of *cyp19a*. The results demonstrated that BaCl₂ could modulate gene transcriptions and hormone production of the HPG axis in a sex-dependent way, which could cause adverse effects on reproduction and the development of offspring.

Keywords Barium chloride · Endocrine disruption · HPG axis · Reproduction · Zebrafish

Barium is known one of the most abundant elements of the Earth's crust, and has been used in massive amount worldwide. In cosmetics and personal care products,

barium sulfate is allowed to be used in the formulation of eye makeup, lipsticks, and skin products (Cosmetic Ingredient Review 2014). In Korea, face painting is a popular activity among children in various festival, however many of face paints currently on the market contained high level of barium (maximum 4325 ppm) which is greater than regulated safety limit (Korea Consumer Agency 2013). Continuous use of barium compounds in consumer products could led to release into the water environment, hence their potential impact on aquatic organisms is an important concern.

Various studies have reported that barium could cause numerous toxic effects. However, information on toxicity in freshwater organisms is mostly limited to acute lethal effects. For barium chloride (BaCl₂), currently available chronic toxicity information only includes 21 days median effective concentration for immobilization (20.4 mg/L) and reproduction (13.4 mg/L) of *Daphnia magna*, and 30 days median lethal concentration (89.3 mg/L) of *Orconectes limosus* (Hazardous Substances Data Bank (HSDB) 2014).

In fish, reproductive system is primarily controlled by the hypothalamic–pituitary–gonadal axis (HPG) axis which is responsible for regulating gonadotropin and sex hormone dynamics (Nagahama and Yamashita 2008; Sofikitis et al. 2008). Environmental contaminants that disrupt at any point in this axis can adversely affect the function of endocrine system and subsequently affect reproduction. It has been previously shown that several environmental contaminants could disrupt production of steroid hormones or gene transcriptions in HPG axis, subsequently leading to decreased egg production and reduced hatching rates (Ji et al. 2013; Liu et al. 2013; Ma et al. 2012). Until now, the effects of barium compounds on reproduction, hormone production, and gene transcription of the HPG axis are not well understood.

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To fill this knowledge gap, we investigated the endocrine disrupting effects of BaCl₂ on HPG axis and reproduction of zebrafish. The developmental effects on F1 generation were further examined with or without exposure to BaCl₂.

Materials and Methods

Barium chloride (CAS number 10361-37-2) was purchased from Sigma-Aldrich (St. Louise, MO, USA). To measure actual concentrations of the exposure media, water samples were collected from each tank for 3 times during the 21 days exposure period. To determine the content of barium, exposure media were analyzed by ICP-AES (Perkin Elmer Optima 4300 DV, Waltham, MA, USA) with the following conditions: RF power 1300 W, flow rate 1.5 mL/min, plasma gas flow 15 mL/min, auxiliary gas flow 0.2 mL/min, nebulizer gas flow 0.8 mL/min, and wavelength 233.57 nm. The limit of detection (LOD) and limit of quantification (LOQ) were calculated at a signal-to-

noise ratio of 3 and 10, respectively. The LOD for barium was 0.0025 mg/L, and the LOQ was 0.0083 mg/L. The acceptance criteria for the quality control was below 10 % coefficient of variation, and were considered acceptable.

Adult zebrafish (*Danio rerio*; AB strain) were obtained from a commercial fishery (Green Fish, Seoul, Korea). The fish were acclimated in tanks containing dechlorinated tap water for 3 weeks at 28 ± 1°C under a photoperiod 16:8 h light/dark in Molecular and Environmental Toxicology Laboratory at Yongin University (Yongin, Korea). The fish were fed with commercially available mosquito larvae (Green Fish, Seoul, Korea) twice daily *ad libitum*, and the remaining food and feces were removed 30 min after the feeding.

In the F₀ exposure study, 10 mating pairs per treatment group were exposed to control, 0.1, 1, or 10 mg/L BaCl₂ for 21 days, following OECD test guideline 229 (OECD 2009). Definitive test concentrations for each compound were determined via preliminary range-finding tests. Two replicates with 5 mating pairs were used for each control or treatment, and fish were placed in a spawning aquarium (30 L tank with 25 L exposure medium). Exposure

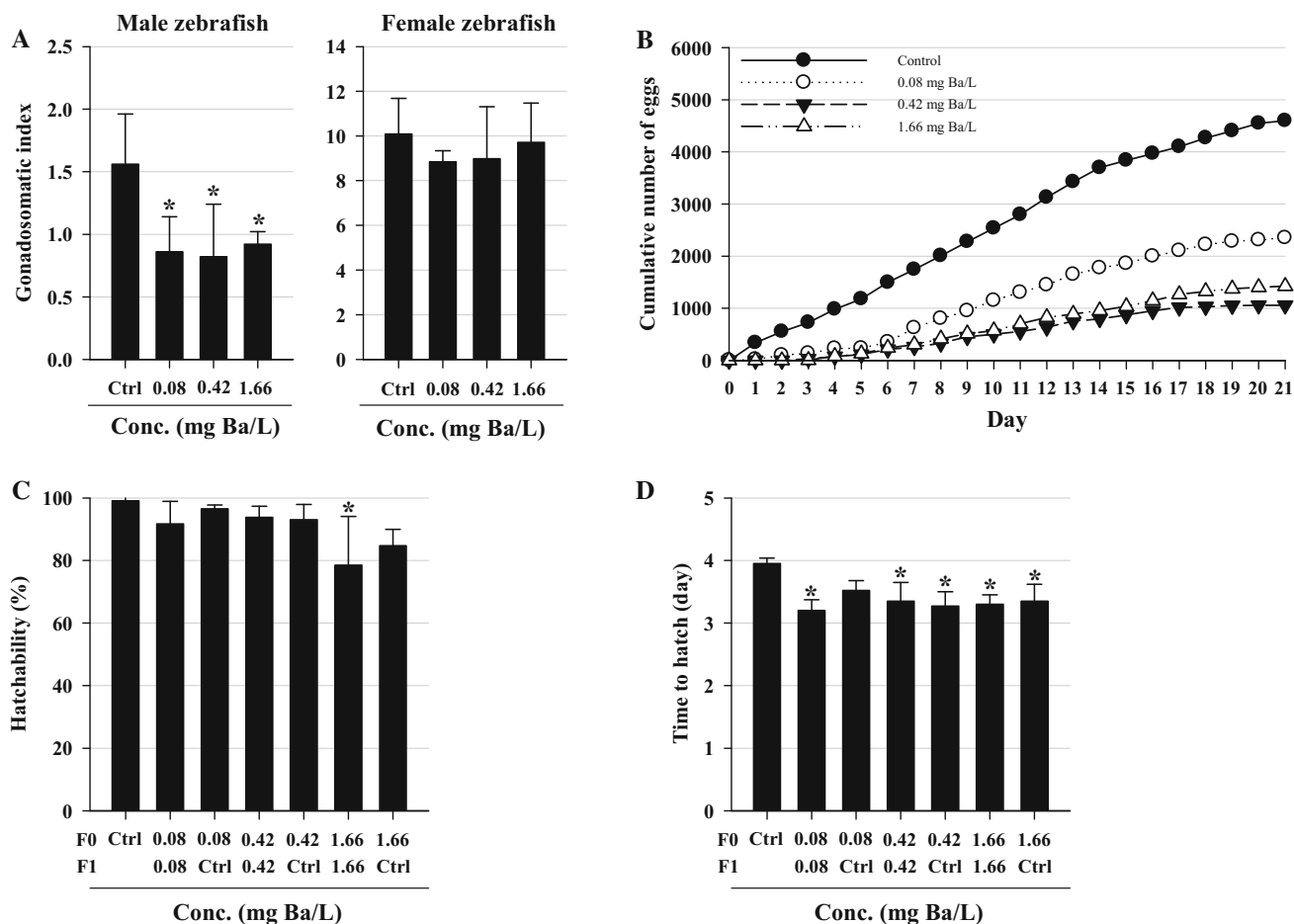


Fig. 1 Effects on **a** gonadosomatic index of F₀ fish, **b** cumulative number of eggs, **c** hatchability in offspring, and **d** time to hatch in offspring after 21 days exposure of F₀ fish to BaCl₂. The values are mean ± SD. Asterisk (*) indicates significant difference from control ($p < 0.05$)

medium was renewed daily, by carefully decanting 50 % of old medium and adding freshly prepared test medium. Fish were fed with mosquito larvae twice daily. Dead fish were removed as soon as detected. The number of eggs spawned was counted and recorded daily. Exposure medium was routinely monitored for pH, conductivity, temperature, and dissolved oxygen. On conclusion of the exposure, all surviving fish were euthanized using 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA). Gonad and total weight were measured for each fish, and gonadosomatic index (GSI; $100 \times$ gonad weight/body weight) were calculated. For measurement of sex steroid hormones and gene transcription, 4 male and 4 female fish were randomly sampled from each treatment.

After exposure for 21 days, the tail of each zebrafish was transected. Blood per each fish was collected from caudal vein in a glass capillary tube, and centrifuged at $2000 \times g$ for 5 min. Plasma sample (1 μ L) was dissolved in 120 μ L ELISA buffer, and used to quantify E2 (Cat no. 582251) and T (Cat no. 582701) by use of ELISA kit (Cayman Chemical Company), following the manufacturer's instructions.

Brain and gonad were collected from each fish after exposure for 21 days and reserved in 250 μ L RNeasy lysis reagent (QIAGEN, Korea Ltd., Seoul, Korea) at -20°C until analysis. Effects on steroidogenic transcription were evaluated as described elsewhere (Ji et al. 2013) with minor modification. Briefly, Transcriptions of 22 genes as well as two housekeeping gene including β -actin and *18s ribosomal RNA* (*18S rRNA*) were measured. Total RNA was isolated from the sample by use of the RNeasy mini kit (QIAGEN). One micrograms of total RNA for each sample were used for reverse transcription by use of the iScriptTM cDNA Synthesis Kit (BIORAD, Hercules, CA, USA). The LightCycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany) was used to perform quantitative real time polymerase chain reaction (PCR). For

quantification of PCR results, the threshold cycle (C_t) values for each gene of interest were normalized to that of reference genes using the delta delta C_t method. Normalized values were used to calculate the degree of up-regulation or down-regulation expressed as a fold difference compared to normalized control values.

On days 7, 8, and 13 of fish exposure, fertilized eggs were collected from each tank. Among them, forty-eight eggs were randomly selected per each tank, i.e., 96 eggs per each treatment group, and separately placed in 96-well plate (Corning Life Sciences, CA, USA) containing 250 μ L exposure water (the same concentration) or clean water (control) for 6 days under static conditions. Hatching rate and time to hatch were also determined.

Statistical analyses were performed using SPSS[®] version 18.0 K, for Windows[®] (SPSS, Chicago, IL, USA). The value p less than 0.05 were considered statistically significant. Differences between control and exposure groups were investigated using Kruskal–Wallis test or one-way analysis of variance (ANOVA) with Dunnett's test. Before conducting ANOVA, normality of distributions and homogeneity of variances were confirmed. To evaluate dose–response relationship, the linear regression analysis was conducted.

Results and Discussion

The present study demonstrates that BaCl_2 has the potential to induce endocrine disruption in reproductive system by changing sex hormone homeostasis and accompanying genes along the HPG axis. The average measured concentrations for the nominal concentrations of 0.1 mg/L BaCl_2 (0.06 mg Ba/L), 1 mg/L BaCl_2 (0.66 mg Ba/L), and 10 mg/L BaCl_2 (6.60 mg Ba/L) were 0.08, 0.42, and 1.66 mg Ba/L, respectively. Measured levels at the lowest concentration were within 20 % of difference from the

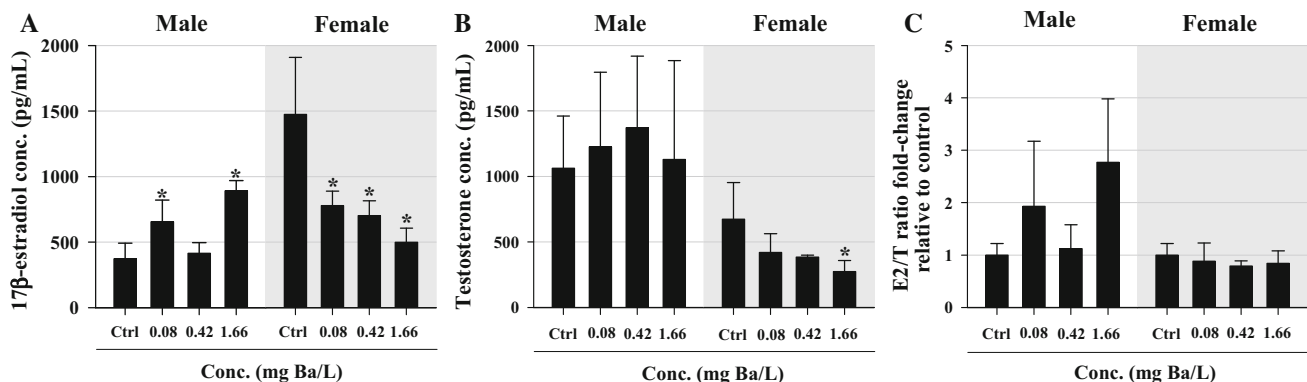
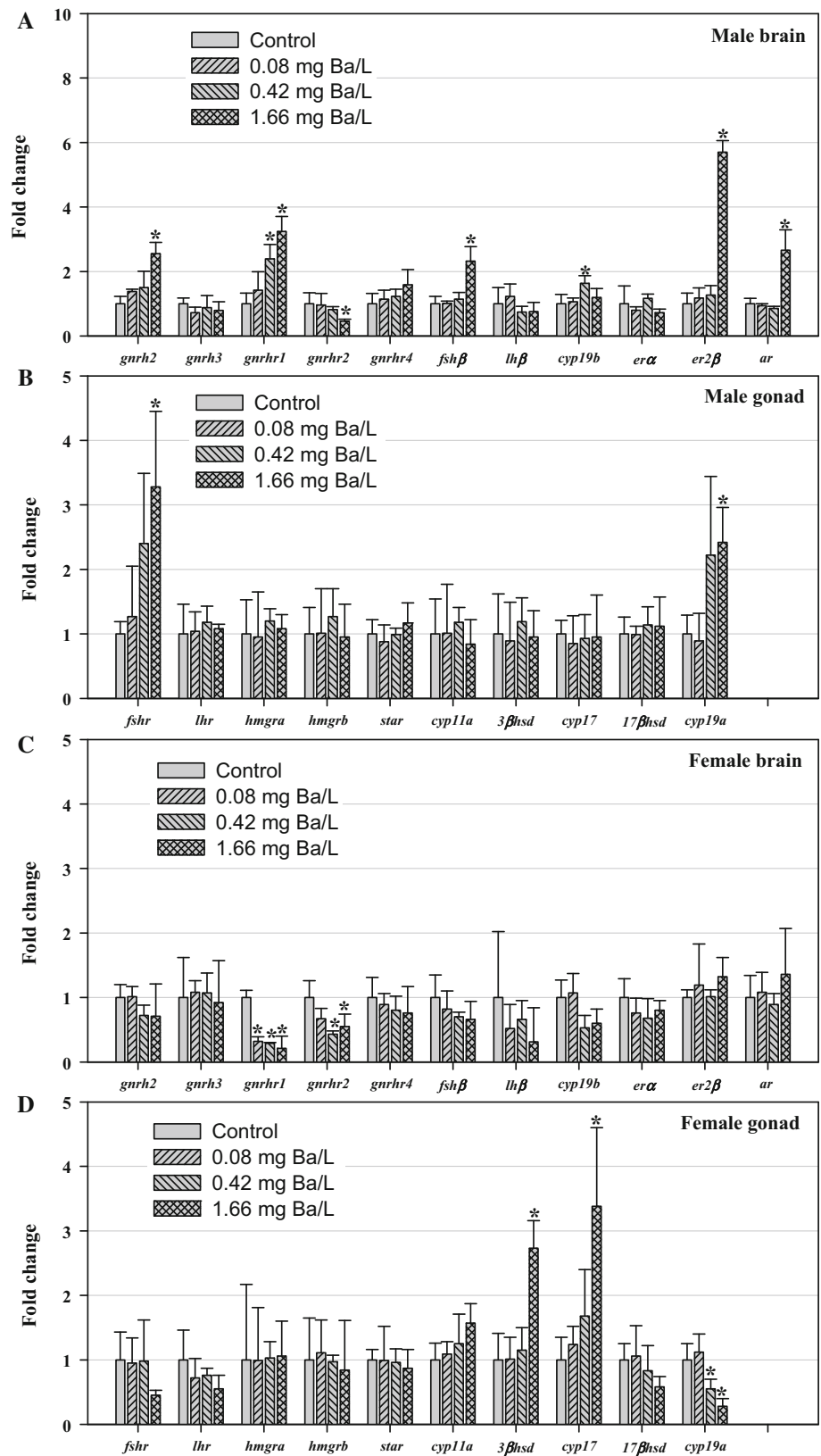


Fig. 2 Effects of BaCl_2 on **a** 17 β -estradiol (E2) concentration, **b** testosterone (T) concentration, and **c** E2/T ratio in zebrafish. The results are shown as mean \pm SD. Asterisk indicates significant difference from control ($p < 0.05$)

Fig. 3 Profiles of gene expression in zebrafish exposed to BaCl₂. Observations in **a** male zebrafish brain, **b** male zebrafish gonad, **c** female zebrafish brain, and **d** female zebrafish gonad. The results are shown as mean \pm SD, gene expression is expressed as fold change relative to control. Asterisk (*) indicates significant difference between exposure groups and the control group ($p < 0.05$)



nominal concentration. At higher concentrations, however, the measured concentrations were relatively lower than the nominal concentrations. The average measured concentrations were mainly used for presentation of the results throughout the present study.

Dramatic decrease in egg production along with significant decrease in GSI of male zebrafish observed at ≥ 0.08 mg Ba/L (i.e., ≥ 0.1 mg/L BaCl₂ for nominal concentration) (Fig. 1a, b) suggest that BaCl₂ has the potential to inhibit the normal growth of gonad and further affect fertility. Gonadosomatic index, an index of gonad size relative to fish size, has been used as a biomarker to examine possible adverse effects of endocrine disrupting chemicals (Hutchinson et al. 2006). Alterations of GSI value have been reported in several fish species exposed to xenoestrogens such as E2 (Bjerselius et al. 2001) and 4-nonylphenol (Kang et al. 2003). Decreases in GSI may indicate alteration in the numbers and sizes of Sertoli cells and germ cells (Miles-Richardson et al. 1999; Kinnberg et al. 2000).

Important indicators for zebrafish embryogenesis, i.e., hatchability and time to hatch, were affected by parental exposure to BaCl₂. Significant decrease in hatchability in eggs exposed to 1.66 mg Ba/L (10 mg/L BaCl₂ for nominal concentration) continuously throughout the hatching period (Fig. 1c). In addition, parental exposure to BaCl₂ fastened hatching period even when the eggs were transferred to clean water (Fig. 1d). The present observations clearly show that parental exposure to barium compounds could influence the development of the next generation embryo without the exposure.

Exposure to BaCl₂ significantly modulated concentrations of sex hormones and transcripts of genes involved in HPG axis, and these effects were sex dependent. Sex steroid hormones (E2 and T) and their balance play important roles in sex differentiation, determination and maturation of teleost (Devlin and Nagahama 2002; Fenske and Segner 2004). Therefore, altered sex hormone levels observed in the present study may explain reproduction failure. In male fish exposed to BaCl₂, significant increase of E2 at 1.66 mg Ba/L was accompanied by up-regulation of *cyp19a* gene, while no significant difference was observed in concentrations of T (Figs. 2a, b, 3b). These results indicate that changes in E2 hormones were regulated by *cyp19* (aromatase) enzyme, which catalyzes the conversion of androgen to estrogen. In female fish, significant decrease of E2 was accompanied by down-regulation of *cyp19a*, which was the opposite direction to those observed in male fish (Figs. 2a, 3d). In addition, concentrations of T were significantly decreased following exposure to 1.66 mg Ba/L (Fig. 2b). This result was accompanied by the down-regulation of *17βhsd* gene (Fig. 3d), which has an important role for basal biosynthesis of T. The reason for the sex-dependent alterations of the steroid hormones and steroidogenic gene transcriptions was

not determined in the present study, and warrants further investigations.

The transcriptions of the genes involved in steroidogenic pathways were affected by exposure to BaCl₂ (Fig. 3). In males, expressions of *gnrh2*, *gnrhr1*, *fshβ*, *cyp19b*, *er2β*, and *ar* mRNAs in brain, and *fshr* and *cyp19a* mRNAs in testis were significantly up-regulated by the exposure to BaCl₂ (Fig. 3a, b). Expression of *gnrh2* mRNA in male zebrafish exposed to 1.66 mg Ba/L was significantly lesser compared to those in the control male fish (Fig. 3a). In females, expressions of *gnrhr1* and *gnrh2* mRNAs in brain and *cyp19a* mRNA in ovary were significantly down-regulated (Fig. 3c, d). Expressions of *3βhsd* and *cyp17* mRNAs in ovary from female fish exposed to BaCl₂ were significantly up-regulated in a dose–response manner (Fig. 3d). Gonadotropin hormones play important roles in regulation of spermatogenesis in male fish, by binding to receptors such as follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone receptor (*lhr*). Up-regulation of *gnrh2*, *gnrhr1*, and *fshβ* genes in the male brain observed in the present study suggests possible change in spermatogenesis and subsequent reproductive dysfunction. The greater abundances of transcription level of *fshr* gene in the gonads from male fish might be a response to greater *fshβ* gene transcription of the pituitary. These results correspond well with the fact that exposure to BaCl₂ could indirectly affect gonadotropin hormones.

Our results clearly show that chronic exposure to BaCl₂ could affect reproduction, sex hormone production and the transcription of genes in HPG axis in fish, although the detailed mechanisms of sex dependent responses remain unknown. Further study on the crossover interactions with other endocrine systems at environmentally relevant concentration will provide more holistic understanding of the observed endocrine disruption by barium exposure.

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