Comparative Biochemistry and Physiology, Part C 213 (2018) 47–54

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Effects of calcium and estrogen on the development of the ceratohyal cartilage in zebrafish (*Danio rerio*) larvae upon embryo and maternal cadmium exposure

Su-Mei Wu*, Chong-Kai Su, Li-Hsin Shu

Department of Aquatic Biosciences, National Chiayi University, Chiayi 600, Taiwan

ARTICLE INFO

Keywords:
Maternal Cd
Ceratohyl
Teleosts
Estrogen receptor
Chondrogenesis
Development

ABSTRACT

The present study is to investigate the reason why the ceratohyal cartilage (CH) angle of zebrafish larvae were larger compared to the control group after their female parents were treated with cadmium (F-Cd). However, the CH angle was smaller compared to the control group when embryos were directly exposed to Cd^{2+} for 72 h (D-Cd). Results showed that calcium contents of larvae were lower than the control, but the transporter isoforms *trpv4* and *trpv6* mRNA expressions were significantly increased upon D-Cd treatment. Furthermore, external Ca^{2+} added during D-Cd treatment reveals that the CH angles of larvae did not appear significantly different compared to the control. On the other hand, E2 (17β-estradiol) contents were higher around 1.9 folds in the ovaries of females; CH angle were over 25°, and Cd^{2+} contents were higher around 6 folds than the control group on larvae treated through F-Cd treatment; CH angles and E2 levels on larvae were higher than the control after the larvae were treated with 1.84 μM E2 (D-E2); Estradiol receptor (ER) isoforms ERβ1 and ERα mRNA expressions significantly increased when 0 hpf embryos were either treated with D-E2 or D-Cd. According to the results, we suggested that the CH angle of larvae become larger upon F-Cd treatment due to maternal Cd^{2+} inducing E2 levels. However, the CH angle of larvae appeared to be smaller compared to the control upon D-Cd treatment. We suggested that the CH angle decreased due to the decrease of Ca^{2+} contents upon Cd^{2+} exposure.

1. Introduction

Cadmium (Cd) is dispersed throughout the modern environment mainly as a result of pollution from a variety of sources. Fish larvae are highly sensitive to Cd, and markers of toxicity include premature hatching, decreased growth rates, and induction of developmental abnormalities, such as cyclopia and spinal lordosis (Jones et al., 2001). Cadmium distribution in environments is routinely investigated. The Cd^{2+} concentrations in the Kaohsiung harbor and the clam farms of Chiayi, Taiwan were 17.8 μM (Chen et al., 2007), and 6.4 nM (Liao et al., 2005), respectively; the Cd^{2+} concentration in the protected Liaohe River of China was 10.7 μM (Ke et al., 2017). Our previous study found that 48 h-LC_{50} of Cd^{2+} was 8.9 μM from 24 hpf (hours of post fertilization) zebrafish embryos (Liu et al., 2012). Therefore, the present study conducted uses an exposure concentration of 0–6.1 μM Cd^{2+}.

Potential mechanisms by Cd^{2+} may mimic or inhibit the actions of endogenous estrogens (Henson and Chedrese, 2004). Chouchene et al. (2016) reported that Cd act as a potent anti-estrogen in vivo and in vitro studies. Garcia-Morales et al. (1994) reported that Cd traces also can affect signal transduction pathways, such as stimulating an estrogen response element. In addition, the whole body levels of T4 (thyroxine) and TRs (thyroid hormone receptors) in minnow larvae were significantly decreased upon 2.5 mg/L and 0.5–2.5 mg/L Cd^{2+} exposure, respectively (Li et al., 2014). That proves that Cd^{2+} can induce endocrine disorder at low levels, including HPG axis (Tilton et al., 2003) and HPT axis (Li et al., 2014). Our previous study has found that maternal Cd^{2+} exposure on females can affect its reproduction functions, gamete quality, and their offspring as the angle of ceratohyal cartilage (CH) become abnormal compared to the control larvae (Wu et al., 2013). These studies prove that Cd is an endocrine disruptor, and consequently interested us into investigating whether endocrine disorders are related to the CH angle of larvae.

The past studies found that zebrafish embryos treated with E2 or with an aromatase inhibitor had defects in development of craniofacial cartilage (Chen et al., 2014). It is well known that E2 has one specific function on hypercalcemic effect, the concentration of blood-Ca^{2+} can be induced to rise by E2 on rainbow trout, tilapia, and gold fish (Whitehead et al., 1980). Falahatkar et al. (2014) reported that the E2

* Corresponding author at: 300 University Road, Chiayi 600, Taiwan.
E-mail address: sumei@mail.ncyu.edu.tw (S.-M. Wu).

https://doi.org/10.1016/j.cbpc.2018.07.006
Received 1 May 2018; Received in revised form 12 July 2018; Accepted 26 July 2018
Available online 27 July 2018

treatment significantly elevated plasma calcium and phosphors. It has been established that E2 treatment induces vitellogenesis in fishes because vtg is a Ca^{2+}-binding lipoprotein. E2 may be involved in the regulation and elevation of blood plasma Ca^{2+} through an increased uptake from the environmental water or its mobilization from internal sources of Ca^{2+} (Li et al., 2018).

In addition, it has been known for 20 years that Cd^{2+} inhibits Ca^{2+} channels, which causes skeletal deformities and disturbs the overall calcium balance of body (Wicklund Glynn et al., 1994). Our previous study also proved that the Ca^{2+} contents was significantly lower than the control group when the zebrafish embryo (0 hpf) were exposed to 0.2 mM of Cd^{2+} for 72 h (Liu et al., 2012). Ca^{2+} is required during the process of chondrogenesis where committed mescenchymal cells differentiate into chondrocytes. Some of these signaling components are Ca^{2+} sensitive (Matta and Zākāny, 2013), and Ca^{2+} is a signal factor during the skeletogenesis (Webb and Miller, 2000). Therefore, we hypothesized that the changes to embryotic CH angles through the maternal Cd^{2+} exposure compared to wild type embryos might be related to the contents of Ca^{2+} that are directly influenced by the accumulation of Cd^{2+} and indirectly regulated by E2 upon Cd^{2+} treatment.

In fish, Ca^{2+} is taken up mainly by the gills, and mitochondria rich cells are known to be the main cell type involved in the process. These cells contain all molecular components such as the transient receptor potential (TRP) family (including Trpv4, 5 and 6), that are thought to play a role in transepithelial Ca^{2+} uptake (Vanoevelen et al., 2011). Thus, the present study will establish whether Ca^{2+} contents, Ca^{2+} transporter mRNA expressions, E2 levels, and ER gene expressions are related to cartilage development.

In addition, several studies had reported that T4 levels might be connected to chondrogenetic development in the early stage of larvae, as characterized by the decreased T4 content upon Cd exposure in minnow larvae (Li et al., 2014); T4 levels of zebrafish larvae significantly decreased after maternal perchlorate exposure, and caused the length of ceratohyal and Meckel's to become shorter than the control (Mukhi and Patiño, 2007); Liu and Chan (2002) found that 120 hpf zebrafish larvae treated with methimazole and amiodarone lead to shorter jaw length compared to the control, but it could be completely restored by the presence of 10 nM T4. However, it was considered that Ca^{2+} uptake was inhibited by Cd^{2+}; E2 has a specific function on hypercalcemic effect and it can be induced by Cd^{2+}; E2 and Ca^{2+} both are linked with chondrogenetic development in early larvae. Therefore, the present study focuses on the effects of E2 and Ca^{2+} content on the development of the ceratohyal cartilage upon F-Cd, D-Cd or D-E2 treatments.

2. Materials and methods

2.1. Fish and experimental medium

Sexually mature zebrafish (*D. rerio*) of both sexes, obtained from the Institute of Cellular and Organismic Biology, Academia Sinica (Taipei, Taiwan), were kept in an aquarium supplied with de-chlorinated, circulated, aerated local tap water at 28 °C with a 14:10-h light/dark cycle, and were fed Daphnia. Fertilized eggs were either incubated in "zebrafish solution" (E3 solution) at 28 °C, which contained 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 1.25 μM methylene blue (pH 7.2), or exposed to 40 mL of E3 solution mixed with various concentrations of E2 or Cd in petri dishes.

2.2. Cd and E2 exposure medium

The concentration of 0, 0.033, 0.33, 4.45 and 6.1 μM Cd^{2+} were used to expose 0 hpf embryos of zebrafish. The Cd^{2+} medium was prepared using completely dried $CdCl_2$ dissolved in 1 mL concentrated HCl; double-deionized water was used to prepare the 89.3 mM Cd^{2+} stock solution, which was then diluted by 2500–10,000 folds of zebrafish solution before being used in exposure experiments (pH ≒ 7.2). The exposure concentration of Cd^{2+} is confirmed by atomic absorption spectrophotometry (Z-5000, Hitachi, Japan). During all experiments, the test containers were cleaned with HNO_3 and thoroughly rinsed with double-deionized water before use. The E2 (17β-estradiol) medium was dissolved in 30 mg of 30 mL of Dimethyl sulfoxide (DMSO). The final concentration was 3.6 M for E2 of stock solution. It was stored at −20 °C until testing, which was diluted with the exposure doses. All chemicals ($CdCl_2$, DMSO, 17β-estradiol) were purchased from Sigma (St. Louis, MO).

2.3. Isolation of total RNA for quantitative measurement of ERα, ERβ1, ERβ2, Trpv4, Trpv6 and β actin by real-time PCR

Total RNA was extracted using an RNA Trizol B kit (Teltest, Paisley, Scotland). Forty-five larvae were homogenized in 4.5 mL RNA Trizol B reagent. The homogenate was mixed with 200 μL of chloroform, shaken gently for 15 s, and kept on ice for 5 min before centrifugation at 11,630 × *g* for 10 min at 4 °C. An aliquot of 600 μL of supernatant was mixed well with 600 μL of isopropanol and 3 M of acetic acid, and stored at 15 °C for 10 min before further centrifugation at 11,630 × *g* for 10 min at 4 °C. The supernatant was removed, and the precipitate was washed with 75% ethanol. The precipitate was allowed to dry for 30 min and was dissolved again in an adequate amount of diethylpyrocarbonate (DEPC, Sigma) to obtain total RNA. The RNA extract was dried at 37 °C for 5–10 min before measuring its total RNA content in a spectrophotometer (Hitachi U-2000) at an OD 260/280 nm.

The total amount of RNA was subsequently extracted with a QuickPrep Micro mRNA purification Kit (Amersham Pharmacia, Piscataway, NJ). Finally, the mRNA pellets were precipitated with 0.1 mg glycogen, 1/10 volume of 3 mM NaOAc, and 95% ethanol; it was all then stored at −20 °C until complementary DNA synthesis (cDNA) was done. mRNA (0.36 μg) was reverse-transcribed in a final volume of 20 μL containing 0.5 mM dNTPs, 2.5 μM oligo $(dT)_{18}$, 5 mM dithiothreitol, and 200 units of PowerScript reverse transcriptase (Clontech, Palo Alto, CA) for 1.5 h at 42 °C, followed by a 15-min incubation period at 70 °C. The cDNA samples were then stored at −20 °C.

Quantitative real-time PCR (qPCR) was carried out using a SYBR Green dye (Qiagen, Hilden, Germany)-based assay with an ABI Prism 7000 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Wellesley, MA) according to the manufacturer's instructions. Primers targeting the *ERα*, *ERβ1*, *ERβ2*, *Trpv4*, *Trpv6* and endogenous control gene, β-actin, were designed using the Primer Express 2.0 software (Applied Biosystems). In each assay, 25 ng of cDNA was amplified in a 20-μL reaction containing 2× SYBR Green Master mix, 300 nM of the forward and reverse primers, and nuclease-free water. The primers designed for the consensus of the selected genes, and β-actin is in the following list.

Primer	Forward (5′-3′)	Reverse (5′-3′)
ERα	CTG TCT GCT CAC GAC AG	AGC CAC AGT TGC TAA GAG
ERβ1	CGC TCG GCA TGG ACA AC	CCC ATG CGG TGG AGA GTA AT
ERβ2	CAG ACC TCT GTC TCA GCA G	CAG CAG ACA CAG CAG CTT G
Trpv-4	GCT TAT GAT ACC ACA GAA GAC	ACC AAC ACA GAG TAG ATG AA
Trpv-6	TCC TTT CCC ATC ACC CTC	CAC TGT GGC AAC TTT CGT
β-actin	ATT GCT GAC AGG ATG CAG AAG	GAT GGT CCA GAC TCA TCG TAC TC