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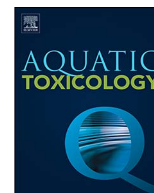

Immunosuppressive effects and associated compensatory responses in zebrafish after full life-cycle exposure to environmentally relevant concentrations of cadmium

Sai-Nan Guo[a], Jia-Lang Zheng[a,*], Shuang-Shuang Yuan[a], Qing-Ling Zhu[b], Chang-Wen Wu[a]

[a] National Engineering Research Center of Marine Facilities Aquaculture, Zhejiang Ocean University, Zhoushan 316022, PR China
[b] Postgraduate Work Department, Zhejiang Ocean University, Zhoushan 316022, PR China

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ABSTRACT

In natural environments, fish survive in polluted water by cadmium (Cd) throughout their whole life cycle. However, little information is available on Cd toxicity considering a life cycle assessment. The present study investigated effects of environmental levels of cadmium (0, 2.5, and 5 μg/L) on immune responses in liver and spleen of zebrafish for 15 weeks, from embryos to sexually maturity. Nitric oxide (NO) levels and iNOS activity declined in liver and spleen of zebrafish exposed to 5 μg/L Cd, suggesting an immunosuppressive effect. The result was further supported by the decreased transcriptional levels of proinflammatory cytokines by Cd, such as interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α) in liver. However, a sharp increase in the mRNA levels of these cytokines was observed in spleen of zebrafish exposed to Cd. The increased mRNA expression of these proinflammatory cytokines may be the secondary effect following immunosuppression and just reflect a compensatory mechanism for coping with the decreased immunity, which may explain an increase in mRNA levels and a decrease in iNOS activity in spleen of zebrafish exposed to Cd. In liver, the down-regulated mRNA levels of iNOS paralleled with the decreased iNOS activity, suggesting a synchronous response from a molecular level to a biochemical level. Positive correlations between mRNA expression levels of nuclear transcription factor κB (NF-κB) and proinflammatory cytokines were also observed, suggesting that NF-κB might be required for the protracted induction of inflammatory genes. The corresponding changes in the mRNA levels of the inhibitor of κBα (IκBαa and IκBαb) may form a feedback loop to restore transcriptional activity of NF-κB. Furthermore, splenic ROS levels were increased by 5 μg/L Cd, possibly activating NF-κB pathway. Taken together, immunosuppressive effects and tissue-dependent compensatory responses were demonstrated in zebrafish after full life-cycle exposure to environmental levels of Cd, indicating a compromise between survival and immunity.

1. Introduction

Cadmium (Cd) pollution of freshwater ecosystems is ubiquitous and has increased dramatically as a result of activities including discharge of municipal effluents, industrial discharges, and mining activities. In industrialized areas in China, Cd concentrations ranged from 1.120–4.474 μg/L in Luan River (Li and Liu, 2009) and even reached 8 μg/L in East Lake (He and Chen, 2007), which are much higher than those in normal freshwater (< 500 ng/L) (Jin et al., 2015). Currently, Cd pollution has become the focus in an area of aquatic toxicology as a consequence of its widespread impacts on vision (Zhang et al., 2015), olfaction (Dew et al., 2015), behavior (Cunningham and Mcgeer, 2015), metal homeostasis (Komjarova and Bury, 2014), redox balance (Onukwufor et al., 2016), DNA modification (Pierron et al., 2014), reproduction (Sellin and Kolok, 2006), and immunity (Giri et al., 2016). In nature environments, fish are continuously exposed to Cd throughout their whole life cycle. Life-cycle examinations possess the possibility to discover low dose exposure influences and can contribute to building the long-term environmentally chemicals' safe concentrations, supplying elaborated message about possible delayed toxicity (Beyger et al., 2012). However, to date, a substantial body of studies have merely elucidated Cd toxicity at different developmental stages of fish, specially focusing on Cd impacts at a sensitive life-stage including embryonic (Hsu et al., 2012; Zhang et al., 2015), larval (Gao et al., 2015; Sassi et al., 2013), or juvenile stages (Sandhu et al., 2014). Life cycle effects by Cd under natural environments are commonly ne-

* Corresponding author.
E-mail address: zhengjialang@aliyun.com (J.-L. Zheng).

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glected.

Cd exerts its toxicity by the production of reactive oxygen species (ROS) (Cuypers et al., 2010). ROS are products of normal cellular metabolism in aerobic organisms (Valko et al., 2007) and play an indispensable role in anti-microbial defenses (Leto et al., 2009). However, high levels of ROS can become important mediators of damage to immunity (Zheng et al., 2016a). Mounting evidence indicates that ROS function as a signal initiation molecule to induce inducible nitric oxide synthase (iNOS) and subsequent increase in nitric oxide (NO) generation by activating multiple signal pathways including nuclear factor kappa B (NF-κB) (Eguchi et al., 2011; Kim et al., 2013; Liu et al., 2012; Wang et al., 2015a). NO is thought to the best known of effector molecules, whose diverse effects in inflammatory response are linked to NO reactivity (Bogdan, 2001). The generation of NO is mainly induced by iNOS which is early response gene in the inflammatory process and is commonly used as inflammatory markers in response to a variety of stimuli (Ramyaa et al., 2013). Proinflammatory cytokines act as modulators of immune responses, including interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1β (IL-1 β), and tumor necrosis factor-α (TNF-α). These cytokines are functionally active in inflammation of teleosts (Bo et al., 2015; Grayfer et al., 2008; Karan et al., 2016; Zhang et al., 2016), transcriptionally controlled by NF-κB (Zante et al., 2015). Normally, NF-κB are sequestered in the cytoplasm bound to its inhibitor of κBα. Once stimulated by some stimuli, NF-κB separate from IκB and translocate into the nucleus where both transcription factors can regulate the transcription of inflammatory genes. The complexity of inflammatory responses reflects a likewise complex mechanism of regulation, which is dependent on distinct signal pathways regulating either pre-existing enzyme activity or new molecule synthesis. However, in fish models, the immunotoxicological responses of Cd have been slightly evaluated and caused controversial results in fish. In fish exposed to Cd, a simulated-inflammatory process was reported by several researchers (Jin et al., 2015; Zhao et al., 2015), while an immunocompromised effect was suggested in other studies (Giri et al., 2016; Guardiola et al., 2013). Even in our recent studies, opposite inflammatory responses have been also demonstrated in zebrafish exposed to Cd (Yuan et al., 2017; Zheng et al., 2016a, 2017), depending on sampling and exposure time. Further studies are still needed to ascertain that whether Cd-induced immunotoxicology is tissue-dependent and what associated molecular mechanisms are.

In the current research, to mimic fish life cycle exposure to Cd in nature environments, zebrafish embryos (24 h post-fertilization) were subjected to environmentally relevant levels of Cd^{2+} (0, 2.5, and 5 μg/L) for 15 weeks until they were sexually mature. The objective of current research was to (1) uncover the molecular mechanisms of inflammatory responses by determining survival rate and levels of ROS, NO, and lipid peroxidation (LPO), specifically focusing on changes in the activity levels of iNOS, and the gene transcriptional levels of iNOS, IL-6, IL-10, IL-1 β, TNF-α, NF-κB, to (2) emphasize a potential tissue-dependent inflammation in liver and spleen.

2. Materials and methods

2.1. Fish maintenance and treatment protocol

Adult zebrafih (AB strain) were cultured in charcoal-filtered and aerated tap water at 27.5 ± 0.5 °C under a light/dark cycle of 12 h:12 h. Embryos were obtained from healthy adult fish with the sex ratio of male to female 2:1. Spawning was induced in the next morning when the light was turned on. Fertilized and normal embryos were collected within 0.5 h of spawning and rinsed in charcoal-filtered and aerated water twice. Embryos of 24 h post fertilization were exposed to Cd at concentrations of 0 (control), 2.5, and 5 μg/L for 15 weeks, three tanks per level, with 70 fertilized eggs in each tanks. The concentrations of Cd exposure referred to environmental relevance, as suggested by Faucher et al. (2008) and Sandhu et al. (2014). The fish were fed commercial flake food (Tetra, Germany) and freshly hatched *Artemia nauplii* three times daily at a rate of 5.0% of body weight (Su et al., 2016). Water temperature was maintained at 27.5 ± 0.5 °C with a 12L:12D photoperiod. Dissolved oxygen and pH were 7.27 ± 0.45 mg L^{-1} and 7.43 ± 0.26, respectively. Cd concentrations for the control, 2.5, and 5 μg/L groups were 0, 2.44 ± 0.07, and 4.81 ± 0.27 μg/L, respectively. Water was renewed 100% at 7 a.m. every morning, stock solution of Cd was added at the time of the water change. The Cd concentrations were measured using flame atomic absorption spectroscopy (FAAS).

At the end of the 15-week period, 24 h after the last feeding, all fish were euthanized with a 0.02% tricaine methanesulfonate solution (MS-222). Liver and spleen samples was immediately frozen in liquid nitrogen, and stored at −80 °C until biochemical determinations and RNA extraction. RNA was extracted from liver from 3 fish and spleen from 10 fish. Biochemical analysis was produced in liver from 10 fish and in spleen from 30 fish. We assured that all experiments, animal care, and protocols followed the ethical guidelines of the Zhejiang Ocean University for the care and use of laboratory animals.

2.2. Biochemical analysis

Tissues were homogenized in an ice-cold 0.1 M phosphate buffered solution (PBS, pH 7.4) containing 1 mM of ethylene diamine tetraacetic acid (EDTA), 0.5 M of saccharose, 0.15 M of KCl, and 1 mM of dithiothreitol (DTT). The homogenates were centrifuged at 500 × *g* for 15 min at 4 °C to precipitate large particles and centrifuged again at 12,000 × *g* for 30 min at 4 °C. The supernatants were maintained at 4 °C until being measured for biochemical analysis.

ROS content was determined using DCFH-DA (LeBel et al., 1992). Briefly DCFH-DA solution was co-incubated at 37 °C for 20 min. DCF fluorescence intensity was measured. LPO levels were determined by the thiobarbituric reactive species assay, which measured the production of malondialdehyde, according to the method described by Livingstone et al. (1990). The production of NO was determined spectrophotometrically at 540 nm following Griess reaction as described by Sessa et al. (1994), by measuring a stable oxidative end-product of NO, nitrite. iNOS activity was determined using iNOS activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Soluble protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. Four biological replicates and two technical replicates were used in the analysis.

2.3. Gene expression analysis

10–30 mg liver tissue was homogenized in 1 mL ice-cold RNAiso reagent (TaKaRa, Dalian, China) to isolate total RNA, based on the phenol–chloroform extraction method. The integrity and quality of total RNA were evaluated by electrophoresis on 1.5% formaldehyde-agarose gels and measurements of absorption at 260 and 280 nm, respectively. When 18S and 28S rRNA bands were evident and the 260/280 absorption ratio was 1.8–2.0, 0.5 μg of total RNA was used for first-strand cDNA synthesis using the PrimeScript® RT reagent Kit (TaKaRa, Dalian, China). Samples of cDNA were double diluted to determine amplification efficiency and ensured that PCR efficiency was between 95% and 105%.

The specific primers were designed using the Primer 5.0 (Table 1). Amplified PCR products were purified by Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 (TaKaRa, Dalian, China), subcloned using the pGEM-T Easy Vector System (Promega, Nepean, Canada). The plasmid was used for transformation of JM109 Competent Cells (Promega, Nepean, Canada) and clones with inserts were sequenced (Sangon, Shanghai, China). The sequences obtained were verified by NCBI Blast.

Q-PCR reactions (20 μL) were performed in 96-well plates in an Applied Biosystems Prism 7500 Sequence Detection System (Applied

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