Ecotoxicology and Environmental Safety 151 (2018) 199–205

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

The morphological changes and molecular biomarker responses in the liver of fluoride-exposed *Bufo gargarizans* larvae

Xiaoxue Bo, Danyang Mu, Minyao Wu, Hui Xiao, Hongyuan Wang*

College of Life Science, Shaanxi Normal University, Xi'an 710119, China

ARTICLE INFO

Keywords:
Fluoride
Bufo gargarizans
Lipid metabolism
Oxidative stress
Apoptosis

ABSTRACT

The goal of the current study was to evaluate the negative influences of fluoride on liver of *Bufo gargarizans* larvae. *B. gargarizans* larvae were treated with 42.4 mg F^-/L for 0, 24, 48 and 72 h at Gosner stage 37. The morphological changes and responses of molecular biomarkers involved in lipid metabolism, oxidative stress and apoptosis were examined in liver. Disappearance of cell boundaries, degeneration of hepatic parenchyma cells and significant increase in the number of melanomacrophage centres and the quantity of lipid droplets were found in the liver treated with 42.4 mg F^-/L for 72 h. In addition, in the relative expression of acetyl CoA carboxylase 1 (ACC-1), fatty acid elongase 1 (FAE-1), sterol carrier protein 2 (SCP-2), and carnitine palmitoyltransferase-1 (CPT-1), decrease was observed after 24, 48 and 72 h of 42.4 mg F^-/L exposure. Furthermore, the transcript levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were downregulated in tadpoles exposed for 24, 48 and 72 h to 42.4 mg F^-/L, while the transcript level of heat shock protein 90 (HSP90) was upregulated at 42.4 mg F^-/L for 72 h. Also, mRNA expression of Bcl-2-associated transcription factor 1(BCLAF1) and thyroid hormone receptors (TRα and TRβ) was significantly upregulated in tadpoles treated with 42.4 mg F^-/L for 72 h. Therefore, our results suggested that the liver injury induced by fluoride might result from disruption of lipid metabolism, oxidative damage and apoptosis.

1. Introduction

Fluorine is a member of the halogen family (Cao et al., 2013). Fluoride distributes widely on the earth as fluoride compounds as a consequence of its great chemical reactivity (Barbier et al., 2010). Low concentration of fluoride is beneficial for dental health (Kumar and Moss, 2008; Carey, 2014) and bone development (Pereira et al., 2016; Herrera et al., 2017), while excessive fluoride ingestion can cause negative consequences on many animals including amphibians (Zhan et al., 2006; Lu et al., 2010; Iano et al., 2014; Chen et al., 2016). The water quality criteria of fluoride are set only in China (1.0 mg/L), Canada (0.12 mg/L) and German (0.7 mg/L) for aquatic organisms (Cao et al., 2013). However, anthropogenic processes have led to a dramatic increase in the concentration of fluoride compound in water (Camargo, 2003). It was reported that the level of fluoride contamination ranged from 2.3 to 49 mg F^-/L in many aquatic environments (Hardisson et al., 2001; Zheng et al., 2006; Brahman et al., 2013; Rafique et al., 2015). Fluoride content in many aquatic environments is much higher than the water quality criteria of fluoride, leading to the damage of animal health (Barbier et al., 2010).

Numerous studies have explored and found the negative effects of fluoride exposure on amphibians. For example, fluoride could contribute to the teratogenesis of *Xenopus* and *Rana chensinensis* embryos (Goh and Neff, 2003; Chai et al., 2016a). In addition, fluoride exposure increased mortality risk and delayed growth and development in *R. chensinesis* and *Rana nigromaculata* tadpoles (Chen et al., 2016). Moreover, NaF delayed metamorphosis and inhibited skeletal ossification in *Bufo gargarizans* tadpoles (Zhao et al., 2013a; Chai et al., 2017a). Unfortunately, to date, there are few studies about the negative impacts of fluoride on liver of amphibians. The liver serves a key role in the bioconversion processes of xenobiotics in amphibians (Fenoglio et al., 2005). Thus, the livers were used as the target organs to assess the effects of fluoride contaminants on amphibians in the present study.

The use of molecular biomarkers is an important tool to assess the biological impacts of contaminants (Handy et al., 2003). To detect the toxicity of fluoride on *B. gargarizans*, we selected some biomarker genes for analysis. It is known that excessive fluoride can cause oxidative stress (Shanthakumari et al., 2004; Agalakova and Gusev, 2012), impair lipid metabolism (Gao et al., 2008; Ranjan et al., 2009) and induce apoptosis (Miao et al., 2013). Oxidative stress, the recognized mechanism of F toxicity, is induced by the imbalance between generation and removal of free radicals (Jhala et al., 2008; Vasant and

* Corresponding author.
E-mail address: hongyuanwang@snnu.edu.cn (H. Wang).

https://doi.org/10.1016/j.ecoenv.2018.01.027
Received 15 November 2017; Received in revised form 9 January 2018; Accepted 11 January 2018

Narasimhacharya, 2011; Zhou et al., 2015). Superoxide dismutase (SOD), glutathione peroxidase (GPx) and heat shock proteins (HSPs) are major players that reduce oxidative stress through counteracting free radicals (Menon and Rozman, 2007; Simoncelli et al., 2015). In addition, oxidative damage has been shown to be implicated in apoptosis (Kannan and Jain, 1996; Chandra et al., 2000). Apoptosis, programmed cell death, is a crucial self-regulatory mechanism to maintain homeostasis for multicellular organisms (Lin et al., 2016). BCLAF1, a pro-apoptotic transcriptional factor, has an important function in perturbing apoptotic pathways (Lee et al., 2012). Furthermore, lipid peroxidation is also induced under conditions of excessive ROS production (Barbier et al., 2010). Balance of lipid metabolism is pivotal for the function of cells, tissues, and organisms (Luo et al., 2017). Acetyl CoA carboxylase 1 (ACC-1) and fatty acid elongase 1 (FAE-1) have been found to play a large role in lipogenesis, while acyl-CoA oxidase 1 (ACOX-1), sterol carrier protein 2 (SCP-2), carnitine palmitoyltransferase-1 (CPT-1) and peroxisome proliferators-activated receptor α (PPARα) have been shown to be associated with fatty acid β-oxidation (Wu et al., 2017; Chai et al., 2017b). Thus, there are compelling reasons to use these genes described above as molecular biomarkers to evaluate oxidative stress, apoptosis and lipid metabolism in liver of amphibians in the present study.

B. gargarizans belongs to Amphibia Bufonidae Bufo and distributes in most regions of China. Amphibians are sensitive to aquatic contaminants because of unshelled eggs, highly permeable skin, and living in aquatic environments at embryos and larvae life periods (Alford et al., 2001; Rowe et al., 2003). These features lead amphibians to study of the negative effects of pollutants on aquatic organisms (Patar et al., 2015; Chai et al., 2016b). In our laboratory, *B. gargarizans* has been proved to be an excellent test animal to evaluate negative consequences of contaminants (Wang et al., 2015; Wu et al., 2017). This experiment was focused on detecting the morphological changes and molecular biomarker responses in the liver of fluoride-exposed *B. gargarizans* larvae. Firstly, changes of histological features, including the number of melanomacrophage centres and the quantity of lipid droplets were analyzed in liver. Besides, we detected responses of molecular biomarkers related to hepatic oxidative stress, apoptosis and lipid metabolism using RT-PCR to explore the toxic effects of fluoride exposure at the genetic level.

2. Materials and methods

2.1. Animal husbandry

In February 2017, five pairs of adult male and female *B.gargarizans* were obtained from Qinling Mountains, Shaanxi Province, China (109°06′52″E, 34°00′56″N). Each couple was placed in one aquarium with shallow water (50 mm). When oviposition occurred, eggs were maintained in our laboratory for research. Eggs developed in dechlorinated tap water (water temperature, 18 ± 1 °C; light period, 12 h light: 12 h dark).

2.2. Reagents and solution preparation

Test solution of 100 mg/L NaF was prepared by adding 400 mg sodium fluoride (NaF, 99% pure, Sigma-Aldrich, St. Louis, MO) to 4 L dechlorinated tap water, which is equivalent to 45.24 mg F^-/L (nominal concentrations). The measured concentrations of F^- for control (dechlorinated tap water) and treatment solution were obtained through a fluoride combination electrode (STISE22, Ohaus Instrument Co. Ltd., Shanghai, China) connected with a pH/mV meter (Starter 3100). The standard curve was plotted by serial dilution of the F^- 1000 ppm standard. Total ionic strength adjustment buffer (TISAB) was added into each solution before measuring. The solution was stirred at a constant speed by the magnetic stirrer, the electrode tip was subsequently put into the solution. Record the mV value after the reading was stable. Draw the mV value (vertical axis) against F^- concentration (horizontal axis) to establish the standard curve. The actual F^- concentrations of control (dechlorinated tap water) and test solution were determined on the basis of standard curve. The uniform water quality conditions were maintained as follows: TOC, 2.81–4.57 mg/L; conductivity, 189–281 mS/cm; total chlorine, 0.22–0.38 mg/L; dissolved oxygen, 6.4–7.4 mg/L; pH, 7.0 −7.4; total hardness, 160–180 mg $CaCO_3$ L^{-1}.

2.3. Experimental design

Embryos at Gosner stage 2 (Gosner, 1960) were cultured in tank (50 cm × 20 cm × 20 cm) containing 4 L dechlorinated tap water. The tap water used in the experiment was fully aerated for 3 d at room temperature (17–18 °C). There were 120 embryos in each tank. Three parallel groups were established in the experiment. Aquaria was kept as follows: temperature, 18 ± 1 °C; light period, 12 h light: 12 h dark. Embryos were not offered food. Larvae fed on fresh boiled vegetables. Tap water in the glass aquaria was changed every alternate day.

At Gs 37, 80 larvae with uniform size were selected randomly and starved for 48 h. Then larvae were assigned to tank (50 cm × 20 cm × 20 cm) with 4 L treatment solution of 42.4 mg F^-/L. For RT-qPCR studies, livers of three larvae were dissected out at 0, 24, 48, 72 h after fluoride exposure, respectively. For liver histological analysis, nine larvae were sampled at 0, 24, 48, 72 h during the exposure, respectively. Three larvae with abdominal incision were fixed by immersion in Bouin's solution for 48 h, and preserved in solution of 70% alcohol. Livers of six larvae were processed for optimal cutting temperature (OCT) compound embedding. Larvae treated with fluoride for 0 h were used as controls.

2.4. Histopathological studies

Livers of three larvae were dissected out from control and treated larvae, respectively. Following this, livers were dehydrated by passage through graded serial of alcohol and embedded in paraffin wax, sections were cut at 7 μm and stained by hematoxylin and eosin (H&E). In addition, non-consecutive cryosections of 14 μm thickness were cut and stained with oil red O for lipid droplets assessment. The staining method was as follows: sections were placed into oil red O in dextrin for 20 min, then sections were washed in running tap water briefly. Subsequently, slides were counterstained using hematoxylin for 15 min and rinsed with water until blue. The stained slides were observed under light microscope and photomicrographs were obtained.

2.5. Qualitative analysis of melanomacrophage centres and lipid droplets

To quantify the number of melanomacrophage centres and evaluate the quantity of lipid droplets per unit area, images of five non-consecutive sections were captured for each larva (n = 3 larvae per group), respectively. Subsequently, fifteen square areas (100 μm × 100 μm) were selected randomly for each treatment group and analyzed using the Image J software (NIH, Windows version). The number of melanomacrophage centres and the quantity of the lipid droplets were analyzed according to Chai et al. (2017b) and Deutsch et al. (2014), respectively. Then, mean values of the number of melanomacrophage centres and the quantity of lipid droplets per unit area were calculated. Results were presented as mean ± SD.

2.6. RNA isolation and cDNA synthesis

Total RNA was extracted from liver of *B. gargarizans* at various exposure time of fluoride using Trizol reagent (Invitrogen). RNA purity and concentration were tested using the Nanodrop. Following this, RNA was converted into cDNA using TIANScript RT kit (Tiangen biotech).