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Chronic effects of mercury on *Bufo gargarizans* larvae: Thyroid disruption, liver damage, oxidative stress and lipid metabolism disorder

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ABSTRACT

Mercury is severely detrimental to organisms and is ubiquitous in both terrestrial and aquatic ecosystems. In the present study, we examined the effects of chronic mercury (Hg) exposure on metamorphosis, body size, thyroid microstructures, liver microstructural and ultrastructural features, and transcript levels of genes associated with lipid metabolism, oxidative stress and thyroid hormones signaling pathways of Chinese toad (*Bufo gargarizans*) tadpoles. Tadpoles were exposed to mercury concentrations at 0, 6, 12, 18, 24 and 30 μg/L from Gosner stage 26–42 of metamorphic climax. The present results showed that high dose mercury (24 and 30 μg/L) decelerated metamorphosis rate and inhibited body size of *B. gargarizans* larvae. Histological examinations have clearly exhibited that high mercury concentrations caused thyroid gland and liver damages. Moreover, degeneration and disintegration of hepatocytes, mitochondrial vacuolation, and endoplasmic reticulum breakdown were visible in the ultrastructure of liver after high dose mercury treatment. Furthermore, the larvae exposed to high dose mercury demonstrated a significant decrease in type II iodothyronine deiodinase (Dio2) and thyroid hormone receptor α and β (TRα and TRβ) mRNA levels. Transcript level of superoxide dismutase (SOD) and heat shock protein (HSP) were significantly up regulated in larvae exposed to high dose mercury, while transcript level of phospholipid hydroperoxide glutathione peroxidase (PHGPx) was significantly down regulated. Moreover, exposure to high dose mercury significantly down regulated mRNA expression of carnitine palmitoyltransferase (CPT), sterol carrier protein (SCP), acyl-CoA oxidase (ACOX) and peroxisome proliferator-activated receptor α (PPAPα), but significantly up regulated mRNA expression of fatty acid elongase (FAE), fatty acid synthetase (FAS) and Acetyl CoA Carboxylase (ACC). Therefore, we conclude that high dose mercury induced thyroid function disruption, liver oxidative stress and lipid metabolism disorder by damaging thyroid and liver cell structures and altering the expression levels of relevant genes.

1. Introduction

As a highly hazardous metal pollutant worldwide, mercury (Hg) is ubiquitous in the environment from both natural and anthropogenic sources. The natural sources of mercury include crustal movements, volcanic eruptions and weathering of rock minerals and soils (Dan and Strode, 2008; Tan et al., 2009). Anthropogenic sources include the release of mercury from alkali metal treatment, coal incineration, medical waste and gold and mercury mining activities (Pirrone et al., 2010; US Geological Survey, 2000). Mercury pollution from anthropogenic activities and industrialization had resulted in several catastrophes of mercury poisoning in Japan and Iraq (Bakir et al., 1973; Hylander, 2001; Kudo et al., 1998), and recently many high-risk sites have been identified in other countries (Bernhoft, 2012; Driscoll et al., 2013; Li et al., 2009). Therefore, in recent years, more and more investigations have been done on mercury toxic effects, and mercury has become the focus of global environmental and biological health (Amos et al., 2013; Bernhoft, 2012; Driscoll et al., 2013; Krabbenhoft and Sunderland, 2013).

Most of the studies about adverse effects of mercury on amphibians are concerned about larvae mortality, morphological deformation and developmental inhibition. For example, mercury can cause death and malformation of *Xenopus laevis* embryos and it can also increase mortality and size, and reduce metamorphosis in *Silurana tropicalis* tadpoles

Abbreviations: Dio2, type II iodothyronine deiodinase; Dio3, type III iodothyronine deiodinase; TRα and TRβ, thyroid hormone receptor α and β; SOD, superoxide dismutase; HSP, heat shock protein; PHGPx, phospholipid hydroperoxide glutathione peroxidase; CPT, carnitine palmitoyltransferase; SCP, sterol carrier protein; ACOX, acyl-CoA oxidase; PPAPα, peroxisome proliferator-activated receptor α; FAE, fatty acid elongase; FAS, fatty acid synthetase; ACC, Acetyl CoA Carboxylase

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(Davidson et al., 2011; Prati et al., 2002). Moreover, growth inhibition were found in *Dendrosophus bogerti* larvae and *Rana sphenocephala* larvae when exposed to mercury (Eliana et al., 2010; Unrine et al., 2004). In addition, there are still many studies which focused on mercury exposure and bioaccumulation in amphibian larvae in field environments (Bank et al., 2007; Bergeron et al., 2010; Loftin et al., 2012; Rowe and Heyes, 2017; Unrine et al., 2005). Although the adverse effects of mercury on amphibians are well studied, the studies on liver pathology, oxidative stress and lipid metabolism of amphibians are very scarce.

It is well known the liver is the principal organ being responsible for the balance of lipogenesis (fatty acids) and lipolysis (fat catabolism via β-oxidation) (Cave et al., 2016; Dentin et al., 2005). Many enzymes and proteins are involved in lipid metabolism, which may be affected by Hg. Examples include acyl-CoA oxidase (ACOX), carnitine palmitoyl transferase (CPT) and sterol carrier protein (SCP), which are involved in the decomposition of fatty acids (Gilde et al., 2003; Schroeder et al., 2007; Zeng and Li, 2004), while acetyl CoA carboxylase (ACC) and fatty acid elongase (FAE) and fatty acid synthetase (FAS) are related to fatty acid synthesis (Asturias et al., 2005; Jakobsson et al., 2006; Salie and Thelen, 2016). Moreover, peroxisome proliferator activated receptor α (PPAPα), a kind of transcriptional factor, is involved in lipid metabolism via regulation of the expression of genes related to the catabolism of fatty acid (Pawlak et al., 2015). Thyroid hormones are also believed to be important in regulating the growth and development of tadpoles, especially in regulating amphibian metamorphosis (Brown, 2005; Darras and Van Herck, 2012), and recent studies have shown that thyroid hormones also play a substantial role in hepatic lipid metabolism (Cioffi et al., 2013; Sinha et al., 2014). To this date, there are few studies on the effects of mercury on thyroid hormones and lipid metabolism in aquatic organisms.

Amphibians are considered to have antioxidant defense systems, mainly composed of enzymes and non-enzyme antioxidants, which scavenge excess reactive oxygen species (ROS) (cells produce a series of ROS during metabolism) and avoid oxidative damage to cells (Lushchak, 2011). However, long-term stress on animals will increase excessive ROS generation and then weaken the ability of anti-oxidation and the ability to resist external environment disturbance, which will threaten animal lives to a certain extent (Lushchak, 2011; Novo and Parola, 2012). Superoxide dismutase (SOD) and phospholipid glutathione peroxidase (PHGPx) are two highly conserved antioxidant enzymes against oxidative stress in antioxidant defense systems (Margis et al., 2008), and heat shock protein (HSP) is also one of the defense systems to study cell antioxidant damage (Gupta et al., 2010; Lee et al., 2013). Previous studies have shown that SOD, HSP and PHGPx are important molecular biomarkers of oxidative stress in aquatic organisms (Jiang et al., 2012; Mathieu-Denoncourt et al., 2014; Valavanidis et al., 2006; Woolfson and Heikkila, 2009; Yang et al., 2010). Therefore, the expression of SOD, PHGPx and HSP genes in liver was measured to examine the oxidative stress of tadpoles by mercury exposure.

Bufo gargarizans, endemic to eastern Asia, is widely distributed in artificial ponds and drains, and is sensitive to environmental changes. It is an excellent indicator organism for studying the impacts of environmental pollutants on physiological and biochemical indices in our laboratory. The aim of the present study was to determine the direct effects of chronic exposure to mercury at concentrations of 0, 6, 12, 18, 24 and 30 μg/L using *B. gargarizans* larvae. We predicted chronic exposure to mercury would negatively influence amphibian larvae survival, body length and mass at metamorphosis climax and time to metamorphic climax. Meanwhile, we expected mercury chronic exposure would cause adverse effects on thyroid gland and liver health. Especially, we would evaluate mercury chronic toxic effects by assessing relative expressions of the genes associated with hepatic lipid metabolism (CPT, SCP, ACOX, PPARα, FAE, FAS and ACC), liver oxidative stress (SOD, PHGPx and HSP) and thyroid functions (Dio2, Dio3, TRα and TRβ).

2. Materials and methods

2.1. Experimental species and animal care

The tadpoles were originated from sexually mature male and female toads of *B. gargarizans,* which were collected in February 2017 from Qinling Mountains, Shaanxi Province, China (109°06′52″E, 34°00′56″N). Each couple was held in approximately 50 mm water in large plastic tank with screened lids. After spawning naturally, embryos were reared in another aquarium with shallow water (50 mm) under the light cycle of 12 h light and 12 h dark photoperiod. Animal care and treatments were agreed by Animal Bioethical Committee of Shaanxi Normal University.

2.2. Water quality

All the water used in the experiment is tap water with aeration for more than three days at room temperature (18–19 ℃). A waterproof portable meter (PC300, Clean) was used to measure water conductivity in dechlorinated tap water. Total organic carbon was monitored by a TOC analyzer (TOC-5000A; Shimadzu), and water dissolved oxygen and pH were measured by a multi-parameter water quality analyzer (GDYS-201M; Little Swan). All the measurements were taken before the exposure and each aquarium was measured twice weekly. The water conditions were as follows: specific conductivity is 234 ± 44 ms/cm, TOC is 3.68 ± 0.78 mg/L, dissolved oxygen is 6.8 ± 0.5 mg/L, and pH is 7.4 ± 0.3.

2.3. Chronic toxicity experiment

A stock solution of 1000 μg/L of mercury was obtained by dissolving $HgCl_2$ powder into distilled water ($HgCl_2$, 99.99% purity; Sigma-Aldrich). Five mercury concentrations of 6, 12, 18, 24 and 30 μg/L were made by diluting the stock solution with dechlorinated tap water and control was achieved with dechlorinated tap water.

6 and 12 μg/L was set as low concentrations from mercury LD 50 experiment to *B. gargarizans* and 24 and 30 μg/L was set as high concentrations from China integrated wastewater discharge standard (Ma et al., 2015). The mercury concentrations in test water were analyzed before exposure experiments by using atomic absorption spectrometer (ZEEnit 700P, Analytik Jena). The limit of detection was 0.001 mg/L and relative standard deviation was less than 5%. Test solutions were entirely renewed every 48 h to keep the appropriate concentration of mercury and water quality.

Tadpole developmental stages are identified based on Gosner (1960). The healthy tadpoles reaching Gosner stage (Gs) 26 were randomly selected and transferred to aquaria with 60 tadpoles per aquarium. In addition, five experimental groups exposed to 4 L treatment solutions with 6, 12, 18, 24 and 30 μg/L mercury, respectively, and control group exposed to 4 L dechlorinated water only. All aquaria were kept at about 18 ± 1 ℃ on a 12 h light and 12 h dark regimen throughout the exposure. There were 3 replicates per treatment. During exposure to mercury, tadpoles were fed on lettuce leaves boiled in 100 ℃ water for 3 min in each aquarium 2–3times each day.

During mercury exposure experiment, the growth of tadpoles was monitored every day and dead tadpoles were removed and recorded at any time. The exposure experiment didn't end until half of all tadpoles (90 individuals) in control group reached metamorphic climax of Gs 42 (Sun et al., 2018). By that time, metamorphosis percentage were calculated by recording individuals of larvae reaching Gs 42 in each replicate aquarium and by using 60 as total number to assess the mortality rate of each treatment and control. Total length, body mass, tail length, and hind-limb length were measured for each tadpole reaching Gs 42. Each individual was measured to the nearest 0.02 mm by Tesa-Cal Dura-Cal Digital electronic calipers. Moreover, each individual at Gs 42 was weighed on an analytical balance having readability to the