Aquatic Toxicology 170 (2016) 24–30

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Effects of copper on growth, metamorphosis and endocrine disruption of *Bufo gargarizans* larvae

Chao Wang[a], Gang Liang[a], Lihong Chai[b,c], Hongyuan Wang[a,*]

[a] College of Life Science, Shaanxi Normal University, Xi'an 710119, China
[b] School of Environmental Science and Engineering, Chang'an University, Xi'an 710054, China
[c] Key Laboratory of Subsurface Hydrology and Ecological Effect in Arid Region of Ministry of Education, Xi'an 710062, China

ARTICLE INFO

Article history:
Received 21 August 2015
Received in revised form 30 October 2015
Accepted 31 October 2015
Available online 5 November 2015

Keywords:
Copper
Growth
Metamorphosis
Endocrine disruption
Bufo gargarizans

ABSTRACT

Chinese toad (*Bufo gargarizans*) tadpoles were exposed to copper (1, 6.4, 32 and 64 μg L^{-1} copper) from the beginning of larval period through completion of metamorphosis. We examined the effects of chronic copper exposure on mortality, growth, time to metamorphosis, tail resorption time, body size at the metamorphic climax (Gs 42) and completion of metamorphosis (Gs 46) and thyroid gland histology. In addition, type 2 and 3 iodothyronine deiodinase (*Dio2* and *Dio3*), thyroid hormone receptors (TRα and TRβ) mRNA levels were also measured to assess disruption of TH synthesis. Our result showed that 6.4–64 μg L^{-1} copper concentration increased the mortality and inhibited the growth of *B. gargarizans* tadpoles. In addition, significant reduction in size at Gs 42 and a time delay to Gs 42 were observed at 6.4–64 μg L^{-1} copper treatments. Moreover, histological examinations have clearly revealed that 64 μg L^{-1} copper caused follicular cell hyperplasia in thyroid gland. According to real-time PCR results, exposure to 32 and 64 μg L^{-1} copper significantly up-regulated mRNA expression of *Dio3*, but down-regulated mRNA expression of TRα and TRβ mRNA level. We concluded that copper delayed amphibian metamorphosis through changing mRNA expression of *Dio3*, TRα and TRβ, which suggests that copper might have the endocrine-disrupting effect.

1. Introduction

The global decline of amphibian populations has been well documented, and exposure to environmental toxicants, such as heavy metals, is among many proposed causes (Sparling, 2003; Alford, 2010). Heavy metals are not degraded in the environment, and thus are likely to act as a chronic stressor that may affect multiple generations (Lance et al., 2013). Over the past decade, contamination of aquatic environment by heavy metals has gained increasing attention. Because aquatic ecosystem is the ultimate recipient of various pollutants originating from natural and anthropogenic sources, their accumulation and persistence constitutes a serious threat to aquatic organisms (Wei and Yang, 2015). Copper is a kind of metal that may be of particular significance to amphibian populations, and it is often present in aquatic habitats used by amphibian species (Linder and Grillitsch, 2000; Bridges and Semlitsch, 2005). In recent years, concentrations of copper in aquatic systems have increased due to numerous anthropogenic activities including industrial discharges, urban or agricultural runoff, and mining operations; aquatic organisms can experience chronic exposure (Lance et al., 2013). In fact, copper concentrations ranging from 50 mg L^{-1} to >560 mg L^{-1} have been reported in polluted freshwater areas all over the world (USEPA, 2007) This concentration is far more higher than Freshwater-quality chronic copper criterion of the U.S. Environmental Protection Agency (U.S. EPA) (9–13 μg L^{-1}) (USEPA, 2002). Copper can be toxic at concentrations slightly above the normal physiological range (Herkovits and Helguero, 1998). Therefore, copper has received much attention due to its toxicity, especially to aquatic organisms (Mount, 1968; Li et al., 1998).

Many studies have demonstrated adverse effects of copper exposure to amphibians. For example, copper may result in deformities in *Xenopus laevis* and *Bufo gargarizans* embryos (Haywood et al., 2004; Xia et al., 2012). In addition, exposure to 100 μg L^{-1} copper decreased swimming performance in northern leopard frogs larvae (*Rana pipiens*) (Chen et al., 2007), and copper also reduced body size in gray treefrog (*Hyla chrysoscelis*) and Arabian toad larvae (*Bufo arabicus*) (Parris and Baud, 2004; Barry, 2011). Moreover, recent studies have demonstrated that copper exposures can delay metamorphosis, and reduce metamorphic success rate in southern leopard frogs (*Lithobates sphenocephalus*) and *B. gargarizans* (Lance et al., 2012; Chai et al., 2014), and also altered thyroid gland size, diameter and number of follicle in *B. gargarizans* larvae (Chai et al., 2014).

* Corresponding author. Fax: +86 029 85310546.
E-mail address: hongyuanwang@snnu.edu.cn (H. Wang).

http://dx.doi.org/10.1016/j.aquatox.2015.10.023
0166-445X/

Amphibian metamorphosis is dependent on thyroid hormone (TH), which induces the suite of molecular and cellular changes that cause a tadpole to transform into a frog (Bonett et al., 2010). TH includes thyroxine (T4) and 3,5,3′-triiodothyronine (T3). The main bioactive form of TH is T3, which facilitates metamorphosis by regulating a cascade of genes controlling morphogenesis and development (Brown and Cai, 2007; Croteau et al., 2009). T3 concentration is determined by relative activities of type 2 and 3 iodothyronine deiodinases (*Dio2* and *Dio3*). Majority of T3 is locally derived from the localized metabolism of T4 to T3 catalyzed by *Dio2* in tissues. The expression of *Dio3* allows tissue-specific regulation of intracellular T3 and T4 through the conversion of T4 to inactive rT3 (reverse T3) and active T3 to inactive T2 (Kuiper et al., 2006). Thyroid hormone actions are mediated by TH receptors (TRα and TRβ). TRα mediates early events of metamorphosis that are mainly growth programs. When the TH concentration is high enough it induces TRβ, which then contributes to the late changes that involve cell death and organ remodeling (Brown and Cai, 2007). Thus, both *Dios* and TRs play important roles in the control of amphibian metamorphosis. The molecular characterization of TH signaling programs that are subjected to disruption by copper is an important, yet relatively poorly studied area of developmental and toxicological research.

B. gargarizans is a wide distributed amphibian species throughout the agricultural landscapes in China. *B. gargarizans* has been used as a model animal for detecting chemicals effects in our laboratory (Xia et al., 2012; Zhao et al., 2013; Chai et al., 2014; Wang et al., 2015). In our previous study, we examined the effects of chronic copper exposure on *B. gargarizans*. However, expressions of genes involved in metamorphosis were not detected. For these reasons, in this study we examined the effects of chronic copper exposure on mortality, growth and developmental rate of *B. gargarizans* tadpoles with various copper concentrations at 0, 1, 6.4, 32, and 64 $\mu g\,L^{-1}$ from Gs 26 to Gs 46 (Gosner, 1960). Morphological examinations were performed on thyroid gland. In addition, we assessed genes involved in thyroid hormone pathways, including deiodinases (*Dio2* and *Dio3*) and thyroid hormone receptors (TRα and TRβ) as a way to assess toxicity and possible endocrine disruption by copper.

2. Materials and methods

2.1. Test species and animal husbandry

B. gargarizans lives in most area of China. This species reproduces in lakes, ponds, swamps, old riverbeds with semi-flowing water. The larvae mainly live on algae and detritus.

Five mating pairs were collected in February 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). After spawning naturally, embryos were raised in another aquarium with shallow water (50 mm). All aquaria were kept at $18 \pm 1\,^{\circ}C$ under a 12 h light and 12 h dark photoperiod.

2.2. Water chemistry

Copper(II) sulfate pentahydrate with a purity of approximate 99% was obtained from Sigma Chemical Company, St. Louis, MO, USA. The $CuSO_4{\cdot}5H_2O$ was solubilized in distilled water into a final concentration of 64 $mg\,L^{-1}$. And then, these storing solutions were diluted with tap water to experiment concentration needed in this research. Dissolved oxygen, ammonia and total hardness of tap water were measured using GDYS-201 M multi parameter water quality analyzer (Little Swan, China). PC300 waterproof portable meter (Clean, USA) was used to monitor water conductivity and salinity. Total organic carbon (TOC) was measured by a TOC analyser (TOC-5000A, Shimadzu, Japan). The experimental copper (ions) concentration was measured using STARTER 3100 (OHAUS, USA) with #Cu0150X (Van London-pHoenix, USA). Dissolved oxygen was about 6.0–7.5 $mg\,L^{-1}$. The pH ranged from 6.84 to 7.25. Conductivity, total chlorine and TOC was 196–268 $\mu S\,cm^{-1}$, 0.1–0.4 $mg\,L^{-1}$ and 2.86–4.75 $mg\,L^{-1}$, respectively. The total hardness ($CaCO_3$) was 123.6–132.7 $mg\,L^{-1}$. The mean experimental copper (ions) concentrations $0.94 \pm 0.04\,\mu g\,L^{-1}$, $6.21 \pm 0.04\,\mu g\,L^{-1}$, $30.41 \pm 0.47\,\mu g\,L^{-1}$ and $61.68 \pm 0.32\,\mu g\,L^{-1}$.

2.3. Chronic toxicity experiment

Each stochastic 30 larvae at Gs 26 determined using generally accepted staging criteria from Gosner (Gosner, 1960) were maintained in glass aquaria evenly (50 cm × 20 cm × 10 cm) containing 5 L test solution. Each treatment was conducted in quadruplicate. Tadpoles were fed on boiled vegetables when being exposed to copper. Any unconsumed food or feces was siphoned from the tanks daily to provide optimal conditions for tadpoles.

Stock solutions of 64 $mg\,L^{-1}$ Cu^{2+} were prepared weekly by adding 0.25 g $CuSO_4{\cdot}5H_2O$ to 1 L distilled water. The nominal concentrations of copper used were 1, 6.4, 32 and 64 $\mu g\,L^{-1}$ (1.6×10^{-8}, 1.0×10^{-7}, 5.0×10^{-7} and 1.0×10^{-6} $mol\,L^{-1}$) respectively. In one replicate five tadpoles were randomly selected for thyroid histological examinations at Gs 42. For other triple replicate, all of tadpoles were prepared for monitoring metamorphosis process. A control group exposed to clean insolated tap water was included in each experiment. In order to maintain the appropriate concentration of copper and water quality, fifty percent of test solutions volume was renewed daily and entirely replaced every 48 h. Dead larvae were removed, and survival were evaluated when renewing the solutions.

During the exposure period, tadpoles were inspected daily for survivorship. The random sampling was conducted on day 16, day 32 and day 48 respectively after copper exposure, five tadpoles from each aquarium (total of 20 per treatment) were collected randomly and euthanized by formaldehyde. The samples were rinsed in distilled water and then weighed, measured for total length. The developmental stages of *B. gargarizans* larvae were determined using generally accepted staging criteria from Gosner (Gosner, 1960).

At the metamorphic climax (initiation of forelimb emergence) and completion of metamorphosis, total length and body weight were recorded once for each Gs 42 and Gs 46 tadpole until 30 tadpoles in control group completed metamorphosis. Each individual was weighted once on an analytical balance having readability of nearest 0.001 g. Lengths of the larvae were measured to the nearest 0.01 mm by Tesa-Cal Dura-Cal Digital electronic calipers. In addition, the time of larvae forelimb initiation (from Gs 26 to Gs 42) was recorded daily for each replicate aquarium. Tail resorption time (from Gs 42 to Gs 46) was also recorded. The experiment was terminated on day 63 after copper exposure.

2.4. Histological analyses

For histopathological analysis, at Gs 42, five tadpoles were randomly collected from both controls and copper treatments for thyroid histological examinations. Tadpoles were fixed in 4% paraformaldehyde, dehydrated through an ascending ethanol series and xylene and embedded in paraffin. Serial longitudinal sections at 6 μm thickness were cut, and stained with hematoxylin-eosin. The sections were observed and photographed under Nikon ECLIPSE 80 iMicroscope equipped with a computer and imaging system.