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Chronic toxicity of copper on embryo development in Chinese toad, *Bufo gargarizans*

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ABSTRACT

This study examined the effects of copper exposure on embryonic development of Chinese toad, *Bufo gargarizans*. Firstly, the LC_{50} values from 24 to 96 h of exposure were 3.61×10^{-6} M, by means of a 4 d toxicity test with *B. gargarizans* embryos. Secondly, Chinese toad embryos were exposed to 10^{-9}–10^{-6} M copper from mid gastrula stage to operculum completion stage. Measurements included mortality, tadpole weight, tadpole total length, growth retardation, duration of different embryo stages and malformation. Embryonic survival was not affected by copper. Relative to control tadpoles, significantly decreased weight and total length were found at 10^{-9}–10^{-6} M reduced percentage of the embryos in right operculum stage after 10 d exposure to copper and reduced percentage of embryos in operculum completion stage after 12 d exposure to copper were also observed. Moreover, the duration of embryonic development increased at neural, circulation and operculum development stage in copper-treated groups. For the scanning microscope and histological observation, the abnormalities were malformation of wavy dorsal fin, flexural tail, curvature body axis, yolk sac oedema and reduced pigmentation in the yolk sac. Histopathological changes in olfactory, retinal epithelium and skin were also observed. DNA strand breaks exposed to the copper were analyzed by DNA ladder. In conclusion, copper induced toxic effects on *B. gargarizans* embryos. The present study indicated chronic toxicity tests may provide more accurate way in formulating the "safe levels" of heavy metals to amphibian.

1. Introduction

Copper is a common trace element in the environment. In recent years, concentrations of copper in the environment have increased dramatically due to widespread use such as electrical generator, radio and television sets, heating system water pipes and wiring (Johnson et al., 2007). Excessive copper could lead to water contamination and poor water quality. Therefore, copper has received much attention due to its toxicity, especially to aquatic organisms (Mount, 1968; Li et al., 1998).

There have been many studies documenting toxicity of copper exposure in different species. In mammals, some studies reported that chronic Cu^{2+} poisoning may occur with a relatively low hepatic Cu^{2+} level, and it is characterized by hepatic and renal cell degeneration, hemoglobinuria, jaundice and early death (Todd and Thompson, 1965; Knobeloch et al., 1994; Sugawara et al., 1995). Regarding humans, there also exists evidence that involves people of varying ages with different lengths of a low-level of exposure to Cu^{2+} where symptoms exerted by intoxication with metal, such as vomiting, nausea, abdominal cramps, dizziness, and diarrhea in a relative short period after exposure (Knobeloch et al., 1994). Moreover, waterborne copper exposure can exert a variety of physiological effects on fish, including the disruption of sensory system function, which has wide-reaching implications for fish behavior. In developing fish larvae, copper is known to affect key parameters, such as survival and growth and more recently has been shown to interfere with the octavolateral system (Johnson et al., 2007).

Amphibian embryos have a close connection with water due to their unique life habitat. The high-sensitivity of amphibian embryos to environmental pollutants (Cooke, 1981; Bantle et al., 1989; Herkovits and Pérez Coll, 1993; Blaustein et al., 1994; Carey and Bryant, 1995; Herkovits et al., 1996) renders them an indicator for assessing the water quality and pollution (Boyer and Grue, 1995; Ugarte et al., 2005). The latest paper reported that *Xenopus laevis* appeared to increase malformations and possibly reduce growth during organogenesis using the Frog Embryo Teratogenesis Assay Xenopus Assay (FETAX) by aquatic toxicity of copper oxide nanomaterial (Nations et al., 2011). Copper hazard was evaluated by means of Toxicity Profiles curves from 24 to 168 h (7 d) of exposure on *Bufo arenarum* embryos. Copper toxicity depends on the concentration and time of exposure (Herkovits and Helguero, 1998).

Bufo gargarizans belongs to *Bufonidae Bufo*, and is common in China. Tadpoles mainly live on algae, detritus and higher plants. But to our knowledge, no reports have been made regarding toxic effect of copper on *B. gargarizans* embryo development. In this study, we examined mortality, growth retardation, development arrest and malformation of embryos collectively. The present study

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aimed to use a multidisciplinary approach to assess the complex effects of copper on *B. gargarizans* embryonic development.

2. Materials and methods

2.1. Animals

Three Chinese toads (*B. gargarizans*) mating pairs were collected in February 2011 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, embryos were raised in our laboratory for experiment.

2.2. Test materials

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 1×10^{-3} M. And then, these storing solutions were diluted with tap water to experiment concentration needed in this research. Dissolved oxygen and ammonia 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 conductance and salinity.

2.3. Copper exposure experiment

2.2.1. Experiment 1: acute toxicity for the determination of 96 h LC_{50}

The acute toxicity assays were carried out in static fashion for 96 h. Embryos were exposed to six copper concentrations (and a control) range from 1×10^{-6} to 4×10^{-5} M(1×10^{-6}, 2×10^{-6}, 4×10^{-6}, 6×10^{-6}, 8×10^{-6}, 1×10^{-5}, 2×10^{-5} and 4×10^{-5} M of copper, respectively). Three replicates for each concentration were used and each replicate consisted of a glass beaker containing 500 mL of respective treatment solutions and 30 toad embryos at tail fin circulation stage (stage 20) (Gosner, 1960). Water temperature was maintained at 17 ± 1 °C. During the exposures, mortality was monitored at 0, 4, 8 and 12 h and then at each 12 h interval in tap water till the end of the test (96 h of exposure), and used to calculate the 96 h LC_{50}. Dead embryos were removed and discarded after each observation.

2.2.2. Experimental 2: chronic toxicity experiment

All exposures took place with tap water, which was insolated for 3 d at room temperature (17–18 °C). Toad embryos were raised in this research without any feeding. According to the first experiment, LC_{50} of copper for *B. gargarizans* after 96 h exposure was 3.61×10^{-6} M. In this experiment, besides the control group, concentrations of copper were defined to be 1×10^{-9}, 1×10^{-8}, 1×10^{-7} and 1×10^{-6} M. Embryos of the control group were raised in insolated tap water only. Each stochastic 100 embryos at late cleavage stage (stage 11; approx. 30 h post-fertilization) were maintained in 4 glass aquaria evenly (60 cm × 30 cm × 20 cm) with 4 L test solution at 17 ± 1 °C with a 12 h light: 12 h dark photoperiod. Each copper concentration was tested in four replications. In order to keep the copper concentration, the test solutions were completely renewed every day with constant copper dose.

During the copper exposure periods, developing embryo was observed every 2 or 4 h from late cleavage stage (stage 11) until operculum completion stage (stage 25) to determine which stage they were in. Developmental stages were determined using the staging criteria of Gosner (Gosner, 1960). In order to record the accurate time of embryonic development, the time of embryonic development of selected exposure periods are divided into four parts: gastrula development (stage 11–12), neural development (stage 13–16), circulation development (stage 17–22), and operculum development (stage 23–25). Furthermore, dead embryos, if any, were removed and counted every 12 h in exposure process. Until hatching of tadpoles or till the end of experiment, each individual was weighted once on an analytical balance having readability of nearest 0.001 g. Total lengths of the tadpoles were measured to the nearest 0.01 mm by Tesa-Cal Dura-Cal Digital electronic calipers. Both normal and abnormal development of the embryos was photographed by a Zeiss Discovery V12 stereoscope equipped with Cannon 7D digital camera.

2.4. Scanning electron microscopy

Scanning electron microscopy (SEM) was conducted using a Hitachi S-570. SEM was utilized to verify malformation as described below. SEM was also employed to visualize variations in *B. gargarizans* embryo skin morphology during organogenesis with exposure to copper.

2.5. Histological analyses

For histopathological analysis, control and exposed larvae were fixed in 4% paraformaldehyde for 24 h. After fixation, larvae were rinsed in tap and distilled water, dehydrated in an ascending ethanol series and cleared in xylene before embedding in paraffin wax. Parasagittal serial sections of 5 μm thickness were cut on a microtome and stained with Hematoxylin–Eosin. The slides were mounted in Canada balsam and left to dry on a hot plate for 2 hours before observation under the microscope.

2.6. Analysis of DNA fragmentation and laddering

Apoptosis of cells was measured by DNA ladder. After exposure, DNA has been isolated from the larvae of *B. gargarizans* exposed to different copper concentration. Fifty milligram tissue was homogenized in 2 mL lysis buffer (0.1 mL protease K, 4.9 mL SNET) at centrifuge tube. Tubes were incubated at 55 °C water bath for 12 h. Then, 2 mL mixture of hydroxybenzene, chloroform and iso-amyl alcohol in the ratio of 25:24:1 in volume, were added standing for 30 min, and then centrifuge at 1800 rpm min^{-1} for 5 min. Transfer 0.5 mL supernatant into a new tube, and mixed with 0.5 mL isopropanol throughly. Centrifuge at 8000 rpm min^{-1} at 4 °C for 15 min. The precipitate of DNA was exsiccated by 1 mL 70% alcohol, and was solubilized in the double distilled water (Sambrook and Russell, 2001). Analysis of DNA ladder was based on the technique of DNA electrophoresis in a 1% agarose gel.

2.7. Data analysis

All the statistics were performed with the software SPSS 16.0. Differences between the different concentrations copper treatment and control embryos were compared using One-Way analysis of variance (One-Way ANOVA) and Chi-square test. The LC_{50} was analyzed by General Linear Model (GLM).

3. Results

3.1. The 96 h LC_{50} of embryos

Differences between concentrations were tested by linear regressions of the mortality rate as dependent variable, the concentration of copper as independent variable in 24, 48, 72 and 96 h, respectively. There is a significant linear dependence between mortality rate and copper concentration. Mortality increased significantly with increasing copper concentration and exposure time. However, no embryos