Ecotoxicology and Environmental Safety 73 (2010) 1286–1291

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*

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ARTICLE INFO

Article history:
Received 28 May 2009
Received in revised form
27 January 2010
Accepted 26 June 2010
Available online 16 July 2010

Keywords:
Copper toxicity
Synechogobius hasta
Histology of gill and liver
Hepatic metabolism
Fatty liver syndrome

ABSTRACT

The present study was conducted to determine growth, hepatic enzymatic activities and histology in *Synechogobius hasta* exposed to waterborne copper concentrations of 0 (control), 0.15 and 0.3 mg Cu/l, respectively, for 15 days, and explore whether waterborne copper exposure could induce the fatty liver syndrome for the fish species. Growth (WG and SGR) declined, but HSI increased in *S. hasta* with increasing waterborne copper levels ($P<0.05$). Waterborne copper exposure also significantly increased lipid content and reduced protein content in both whole body and liver, and increased copper accumulation in whole body and vertebrae. Copper exposure changed hepatic enzymatic activities (SOD, CAT, SDH, PK, LDH, LPL and HL) and increased hepatic lipid peroxidation level, impaired the histological structure of the gill and liver in *S. hasta*. Thus, our study demonstrated for the first time that waterborne Cu exposure could induce fatty liver syndrome in fish.

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1. Introduction

The increasing industrial and agricultural activities have caused a rise in the presence of contaminants in the environment. In particular, environmental concentrations of heavy metals have been increasing in coastal areas recently. Copper is of particular concern in this aspect because fish are able to bioaccumulate them in the body tissues where they may reach toxic concentrations. Moreover, copper is often used in aquaculture, because it was proven to be an effective way of controlling both toxicoses and infectious diseases in fish ponds. Despite the essential role of copper in a number of enzymatic processes (Solomon and Lowery, 1993), this metal has the potential to exert adverse toxicological effects (Handy, 2003). Therefore, there is a substantial need to understand the toxicity of copper in aquatic organisms. Such studies can provide critical information for the environmental risk assessment of copper in aquatic environments.

The toxicity of copper to fish has been studied extensively by many workers and summarized by Sorensen (1991). While the mechanism of copper uptake and acute toxicity in freshwater fish has been studied in details (Lauren and McDonald, 1987a, b; Wilson and Taylor, 1993a, b), less is known about the mechanism of copper toxicity in marine fish (Wilson and Taylor, 1993a). At present, there are some reports on the short-term and chronic effects of Cu exposure on fish physiology (Beaumont et al., 2000; Antognelli et al., 2003; Handy, 2003; Carvalho and Fernandes, 2008). While acute toxicity of copper in fish is mediated by gill dysfunction, the chronic impact of sublethal copper exposure may include other tissue types, especially liver which has the highest copper concentration (Buckley et al., 1982; Lauren and McDonald, 1987a) and the highest copper accumulation rate during waterborne copper exposure (Grosell et al., 1997, 1998). In fact, the liver has been given particular attention in toxicological investigations of heavy metals in different fish species due to the important role in metabolism (Grosell et al., 1998). Inside the cells, each metabolic pathway is continuously regulated in order to maintain homeostasis and, in general, few key enzymes control the metabolic flux. Although the direct effects of copper on certain glycolytic enzymes have been demonstrated in extracts of mammalian and fish tissues (Isani et al., 1994; Gul et al., 2004), little is known about the effect of copper on glycolytic enzymes after exposing the whole animal to the metal (Beaumont et al., 2000; Antognelli et al., 2003), and on other enzymes related to nutrient metabolism. In fact, the effect of sublethal concentrations of copper on marine fish is not entirely understood.

Synechogobius hasta are widely distributed over the southern coast of Liaoning Province, China. The fish species has been identified as a species destined for diversification of Chinese marine aquaculture (Luo et al., 2008). In recent years, commercial farming of this fish has become of increasing interest in northern China because of its euryhalinity, rapid growth, good taste and high market value (Luo et al., 2008). At present, several studies have been conducted to determine the characteristics of nutrient physiology for the fish species in our laboratory (Luo et al., 2008,

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doi:10.1016/j.ecoenv.2010.06.019

2009a, b). We also found that under natural coasts where the fish lived, *S. hasta* developed fatty liver syndrome, coinciding with excess amounts of waterborne contaminants, such as Cu and Cd. As a series of studies involved in the mechanism of fatty liver occurrence, the present experiment was aimed to investigate the effects of waterborne Cu exposure on growth, hepatic enzyme activities and histology of gill and liver in *S. hasta,* and explore whether sublethal copper exposure in water could induce the fatty liver for the fish species.

2. Materials and methods

The research was conducted in Panjin Guanghe Fisheries Co. Ltd. Two experiments with waterborne copper exposure were conducted. The first experiment was involved in the acute copper toxicity for *S. hasta*, determining the 96 h median lethal concentration (LC_{50}). The second experiment evaluated chronic effects of waterborne copper exposure on growth, hepatic enzymatic activities, gill and hepatic histology of *S. hasta*. For the two experiments, copper was added as cupric sulfate in distilled water for stock concentrations. Individual test solutions in the first and second experiment were obtained by adding the appropriate volume of the primary stock to the dilution water. The copper concentration in the test tanks was measured using inductivity coupled plasma mass spectrometry (ICP-MS, IRIS Advantage (HR), Thermo Jarrel Ash Corporation, Boston, MA, USA). We assure that the experiments performed on animals, animal care and all protocols followed the ethical guidelines of Huazhong Agricultural University for the care and use of laboratory animals.

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

S. hasta were taken from the local ponds and transferred to indoor fiberglass tanks (300-l in water volume) for 10-day acclimatization. The tests consisted of six concentration groups (zero control, 0.30, 0.46, 0.71, 1.10 and 2.60 mg Cu/l, respectively), three replicates per group, with 10 fish in each replicate. During the 96-h experiment, the water was aerated continuously. Each test solution was renewed daily. Water temperature was maintained at 26 °C, dissolved oxygen was 6.28 ± 0.2 mg/l, pH 8.0 and salinity 28.

During the exposures, mortality was monitored at 0, 3, 6, and 9 h and then at each 12-h interval to the end of the test, and used to calculate the 96 h LC_{50}. The criteria for death were no gill movement and no reaction to gentle prodding. Dead fish were removed and discarded after each observation. The results of the LC_{50} at 96 h were computed using the probit analysis computer program (Finney, 1971) and was 0.77 mg/l.

2.2. Experimental 2: sublethal experiment

According to the first experiment, LC_{50} of copper for *S. hasta* after 96 h exposure was 0.77 mg/l. In the second experiment, *S. hasta* were exposed to three copper treatments at the concentrations of zero control, 0.15 and 0.30 mg Cu/l (corresponding to 0%, 20% and 40% of 96 h LC_{50}), respectively. All of the treatments were carried out in 3 replicates, with 20 fish per replicate. Prior to the experiment, the fish from a local pond were kept in 300-l circular fiberglass tanks for 10-day acclimatization. At the beginning of the experiment, uniform-sized fish (mean initial body weight: 13.73 ± 0.67 g) were stocked in nine fiberglass tanks with 20 fish each. The experiment was carried out in static aquarium system, and continuously aerated under constant temperature (25 ± 1 °C) and salinity (28), respectively), normal photoperiod (14 h/10 h light/dark) and daily feeding.

During the acclimation and experimental period, fish were fed trash fish twice a day. Trash fish was fed at 10% (based on the wet weight of the trash fish) of the biomass. The Cu content of trash fish was not determined directly. However, since the organisms of all treatments were fed the same food and the same daily ration, any histological alternation in comparison to the control group was taken as indicative of the effect of waterborne Cu used in the experiment. To ensure that water quality and maintain copper levels, water was renewed 100% every 12 h, and copper exposure was carried out by the dilution in distilled water of cupric sulfate and Cu concentrations were monitored during the experimental period by the methods of ICP-MS. Dead juveniles and uneaten feed were removed every day in the morning (11:00 h) and afternoon (14:00–16:00 h) when the water was renewed. The experiment continued for 15 days.

2.3. Sampling and samples analysis

At the end of the 15-day sublethal experiment, 24 h after the last feeding, all fish were counted and weighed to determine survival, weight gain (WG), specific growth rate (SGR). After obtaining the final total weight of fish in each tank, four fish per tank were randomly selected, weighed and measured for body lengths, then frozen at −70 °C for subsequent determination of whole body composition. Remaining fish from each aquarium were randomly selected, dissected in ice to obtain gill, liver, white muscle and vertebrae samples, and condition factor (CF), and viscerosomatic index (VSI) and hepatosomatic index (HSI) were calculated. They were then stored at −70 °C for subsequent analysis. For enzymatic analysis, liver was removed using sterile forceps, placed in sterile 15 ml glass tissue grinders and stored at −70 °C (not longer than 2 weeks) for subsequent analysis. For histological observation, the left lobe of liver (sliced into 3 mm thick slabs) and the second gill arch of the right side of each fish were collected, fixed in 10% neutral buffered formalin for 24 h and prepared for histological analysis.

Proximate analysis consisted of determining moisture, ash, crude protein, lipid (AOAC (Association of Official Analytical Chemists), 1995) and copper contents of various tissues. Crude protein ($N \times 6.25$) was determined by the Kjeldahl method after an acid digestion using an auto Kjeldahl System (2300-Auto-analyzer, Sweden). Crude lipid was determined by the ether-extraction. Moisture was determined by freeze drying at −55 °C. Cu content of whole body, white muscle and vertebrae were analyzed using ICP-MS, and the sample response compared against that generated for a standard calibration curve. Quality assurance/quality control (QA/QC) procedures included analysis of three method blanks (purified water), three certified biological reference tissues (NRC DORM-2, NRC LUTS-1 and NRC DOLT-2, National Research Council) and two randomly selected duplicate samples per 20 samples. Recovery of Cu from certified biological reference tissues mentioned above ranged from 94% to 104%. The Cu detection limits are 0.04 ng/ml (water) and 0.8 ng/g dry weight (tissue). These analyses were conducted in duplicate.

For enzymatic analysis, liver samples were homogenized in 0.1 M Tris–HCl buffer at 4 °C, pH 7.4, to make a 10% (W/V) homogenate. The homogenates were centrifuged at 16,000*g* for 5 min at 4 °C and then the supernatants were collected for enzyme analysis. The assays were run in triplicate. The following enzyme activities were measured: superoxide dismutase (SOD, EC1.15.1.1) as described by Misra and Fridovich (1972), catalase (CAT, EC 1.11.1.6) according to Aebi (1984), succinate dehydrogenase (SDH, EC 1.3.99.1) as described by Slater and Bonner (1952). Pyruvate kinase (PK, EC 1.11.1.7) was determined following Carbonell et al. (1973), lactate dehydrogenase (LDH, EC1.1.1.27) according to Jones and Sidell (1982), malic dehydrogenase (MDH, EC 1.1.1.40) according to Ochoa (1955), hepatic lipase (HL, EC 3.1.1.3) following Ehnholm et al. (1975) and modified by Burgaya et al. (1989). Lipoprotein lipase (LPL, EC 3.1.1.34) activity was measured using labeled triolein-^{3}H as a substrate, according to the modified methods by Ballart et al. (2003). All enzyme activities were expressed as U (units) per mg of soluble protein. Soluble protein content of liver homogenates was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Lipid peroxidation was also determined in liver samples according to the method described by Livingstone et al. (1990) in terms of malondialdehyde (MDA) equivalents using the thiobarbituric acid (TBA) reaction.

2.3.1. Histology of gill and liver

Samples of liver and gills were fixed for 24 h in 10% neutral buffered formalin. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Sagittal sections of 6 μm thickness were stained with hematoxylin and eosin (Woods and Ellis, 1994), and then prepared for light microscopy.

2.4. Statistical analysis

Results are presented as mean ± SD. Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogornov–Smirnov test. The homogeneity of variances among the different treatments was tested using the Barlett's test. Then they were subjected to one-way ANOVA and Duncan's multiple range test. Difference was considered significant at $P < 0.05$. All statistical analyses were performed using the SPSS 10.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA).

3. Results

3.1. Growth performance and body composition

Growth (WG and SGR) and survival significantly declined with increasing waterborne Cu levels ($P < 0.05$) (Table 1). CF and VSI showed no significant differences among the treatments ($P > 0.05$) (Table 2). The HSI increased with the waterborne copper concentration ($P < 0.05$). Increased lipid content and reduced protein content were observed in both whole body and liver when waterborne copper levels increased from 0 to 0.30 mg/l ($P < 0.05$). Cu concentrations in whole body and

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