Fish and Shellfish Immunology 82 (2018) 77–83

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Effects of low concentrations copper on antioxidant responses, DNA damage and genotoxicity in thick shell mussel *Mytilus coruscus*

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ARTICLE INFO

Keywords:
Mytilus coruscus
Copper
Antioxidant responses
DNA damage
Genotoxicity

ABSTRACT

For the aim to study potential detrimental effects induced by Cu exposure at low, environmentally relevant concentrations, the in vivo activities at different levels of biological organisations of thick shell mussel *Mytilus coruscus* exposed to two levels of copper were assessed. Cu-induced stresses were evaluated through antioxidant responses, DNA damage and genotoxicity. The results revealed significant higher SOD and CAT activities, and MDA concentration in haemocytes of *M. coruscus* with Cu exposure at 8 μg/L, while only significant accumulation in CAT activity with Cu exposure at 2 μg/L and no significant changes with SOD activity and MDA concentration at this level of Cu exposure. Copper exposure induced DNA damage as induction of OTM value in a time- and concentration-dependent manner. In addition, copper exposure could significantly induced the expressions of MT-10, Hsp70, Hsp90 and C3. The present results deepen the mussels as a suitable model marine invertebrate species to study potential detrimental effects induced by possible toxicants, in combinations at different levels of biological organisations.

1. Introduction

The aquatic environment is often the ultimate recipient of an increasing range of anthropogenic contaminants [1,2], many of which present a range of challenges for the organisms that live in those habitats. The effects of such pollution may sometimes be acute, such as the mass deaths that occur periodically in response to major chemical spills. But while these events capture headlines and attention, it is arguably the day-to-day and comparatively low-level contamination of environments which present the greater long term ecological challenge [3]. In this context, this needs further elaboration to elucidate more realistic exposure scenarios applicable to the environment. Additionally, an integrated approach is also required to assess the biological response at different levels of biological organization [4].

Due to its high electrical and thermal conductibility, copper (Cu) is assumed to be a transition metal and is widespread use in human society [5]. Although there are important natural sources of Cu in the environment, a variety of anthropogenic sources such as agricultural, industrial, and maritime activities have considerably increased Cu releases into aquatic environments worldwide [6,7]. Cu is an essential element for all aerobic organisms due to its role as cofactor of mitochondrial enzymes [8]. Also, in aquatic arthropods and molluscs, Cu is a component of haemocyanin, the oxygen carrier protein [9]. In spite of its necessity, Cu at elevated concentrations in the water acts as a potent toxicant for aquatic animals [10–12]. Copper is known to be redox-active and catalyses the generation of reactive oxygen species (ROS) [13]. In certain circumstances of ROS excess production the protection afforded by antioxidant defence mechanisms might be defeated, thereby leading to oxidative damage to tissue macromolecules including DNA, proteins and lipids [14,15].

Due to their sedentary, ubiquitous, filter-feeders inhabiting in the coastal and estuarine areas, with the addition of the susceptible response to marine or aquatic environment, bivalve molluscs especially the mussels have gained particular concern to perform as the ideal indicators in the assessment of environmental pollution [16–18]. The toxic effects of Cu have been reported for a several marine and

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https://doi.org/10.1016/j.fsi.2018.08.016
Received 6 May 2018; Received in revised form 4 August 2018; Accepted 7 August 2018
Available online 08 August 2018

freshwater mussel species. Regoli and Principato [19] investigated the effects of Cu exposure on the glutathione (GLU), glutathione-dependent and antioxidant enzymes in mussel *Mytilus galloprovincialis* under field and laboratory conditions, and found a significantly lower level of GLU and higher activities of the glyoxalases (GLO) in challenge group compared to control group. de Almeida et al. [20] reported a negative correlations between the enzyme phospholipid hydroperoxide glutathione peroxidase (PHGPx) and malonaldehyde (MDA) levels after Cu exposure, and concluded that PHGPx might play a protective role against lipid peroxidation and thus could be used as a new potential biomarker of toxicity associated with contaminant exposure in mussels. In recent years, Cu was discovered to act as a potent ionoregulatory toxicant in freshwater mussels, and natural dissolved organic matter (DOM) protects against both Cu bioaccumulation and ionoregulatory toxicity [21].

Thick shell mussel, *Mytilus coruscus* inhabiting widely in Yellow Sea, Korean Peninsula, and Hokkaido waters of Japan, has developed to one of the most economically important mussel species in Zhoushan Islands, Zhejiang, China in recent years [22,23]. As a common calcified marine species inhabiting coastal ecosystem, *M. coruscus* attaches to hard substrates in subtidal zones and forms extensive subtidal beds that play an important ecological role and affect the community structure of the associated macrofauna [24]. Therefore, this species may be suitable organisms to study the biological impacts of marine environment factors across China coastal area. In recent years, the information available on the impact of environmental stressors on *M. coruscus* focused mainly on biomarker responses of ocean acidification and thermal stress [25–27], and immune-related activities of biotic and abiotic stresses [23,28–31]. As for the biochemical response of *M. coruscus* to Cu exposure, relevant information was only reported by Li et al. [32] to date, in whose study the effect of acute Cu exposure on *M. coruscus* survival was measured, and the LC_{50} values (the concentration lethal to half of any given species over a certain time) at 48, 72, and 96 h post Cu exposure were corresponding to 0.48, 0.37, and 0.32 mg/L, respectively. Based on this, in the present study we analysed the oxidative stress, DNA damage and genotoxicity of haemocytes to Cu exposure at 8 μg/L and 2 μg/L, corresponding to one in 40 and one in 160 of 96-h LC_{50} value, respectively. The aim of this study was to study potential detrimental effects induced by Cu exposure at low, environmentally relevant concentrations, in combinations at different levels of biological organisations. The results shed a new light on the physiological changes of mussels response to low concentrations Cu exposure, and would be helpful to select suitable biomarkers for marine environmental monitoring, especially for invigilation metal pollution of environmentally relevant concentrations using marine invertebrates, especially mussels.

2. Materials and methods

2.1. Mussel rearing, copper exposure, and sampling

Healthy thick shell mussel *M. coursucs* adults (weight 65.0 ± 8.5 g, length 9.2 ± 0.8 cm), were obtained from the Dongji Island aquaculture base (Zhoushan, Zhejiang Province, China). After removing the algae and shell residue on the surface, these mussels were acclimated in a plastic tank filled with air-pumped natural filtered sea water (salinity 28 ppt) under the laboratory conditions for 6 days. Following, artificial seawater (salinity 28 ppt) prepared with sea salts dissolved in deionized (double distilled) water (ddH2O) was used to gradually (20% per day) replace the natural seawater, until the acclimation media consisted of only artificial seawater. Mussels were maintained under this condition for 6 days. The cultural conditions are as 24 ± 1 °C, and 16: 8 h (light: dark) photoperiod regime, and spirulina powder was fed daily.

Stock solution of copper was freshly prepared by dissolving the copper (II) chloridedihydrate ($CuCl_2$) in ddH_2O (nominal 0.85 g Cu/L). Fresh stock solution was prepared daily. Exposure solutions were prepared by adding a copper stock solution to artificial seawater which had been previously aerated for 24 h to achieve CO_2 equilibrium and stabilization. Exposure solutions were kept at 24 ± 1 °C to equilibrate for 24 h prior to adding mussels. Glass aquariums were used to keep mussels during the challenge assay, and they were pre-washed with 1% HNO_3 and thoroughly rinsed with ddH_2O before use. The challenge experiment was set up using two levels of exposure solutions (nominal 8 μg/L and 2 μg/L of Cu, corresponding to the 1/40 and 1/160 of 96-h LC_{50} [32], respectively) for a total of two treatments. In the control group, nothing was added to the artificial seawater. The experiment lasted for 18 days in triplicate with 40 mussels per replicate in each group. Five individuals were sacrificed for haemocytes collection according to Huang et al. [33] at 0, 3, 6, 12 and 18 d post treatment, following these haemocytes were pooled to reduce individual variation and to provide sufficient cells for total RNA extraction and haemocyte lysate supernatant preparation.

2.2. Assays of enzyme activities

The antioxidant enzymes activities were measured as we described previously [23]. Briefly, the activity of superoxide dismutase (SOD) in haemocytes was measured based on the aerobic reduction of nitroblue tetrazolium (NBT) at 535 nm by superoxide radicals [34]. One unit (U) of SOD activity was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%. Haemocyte catalase (CAT) activity was assayed by monitoring the decomposition of H_2O_2 at 240 nm for 1 min [35]. One U of enzyme activity was defined as the degradation of 1 mmol H_2O_2 per second per mg tissue protein. The protein levels of these enzymes were assayed using corresponding detection kits (Jiancheng, China) according to the manufacturer's instructions. The soluble protein content was determined based on the Bradford assay using bovine serum albumin (BSA) for the standard [36].

2.3. Malondialdehyde (MDA) levels

The level of lipid peroxidation was determined by measuring the malondialdehyde (MDA) level. The MDA was assessed by the thiobarbituric reactive species (TBARS) assay according to the method described by Ohkawa et al. [37].

2.4. Comet assay

The single cell gel electrophoresis (comet assay) was employed to measure the DNA damage. The assay was performed according to Singh et al. [38] with little modification. Firstly, microscope slides were submerged in boiled 1.0% normal melting point agarose (NMP) solution, following removed the agarose slides and allow to dry at room temperature. A 100 μL mixed suspension containing 25 μL of haemocyte suspension and 75 μL of 0.7% low melting point agarose (LMP) was added on the slides and spread evenly by coverslips at about 37 °C, and the slides were allowed to solidify at 4 °C for 10 min. Secondly, the slides were immersed in ice-colded cell lysing buffer in dark at 4 °C for 2 h and electrophoresed for 20 min at 25 V. Thirdly, the slides were neutralized with cold neutralization buffer (0.4 M Trise-HCl, pH 7.5) for 10 min, dehydrated in ethanol for 15 min and stored in the dark at 4 °C. Finally, the slides were stained with SYBR Green and analysed at 400 × magnification under an fluorescence microscope (Leica Germany). Fifty randomly chosen cells on each slide were examined and the CASP (comet assay software project) image analysis system was used to measure the comet tail moment [39]. The comet tail moment is widely considered as a fragment product of DNA, in which the tail and tail length is highly associated with the extent of DNA breakage, and olive tail moment (OTM) value is the mean distance of DNA migration in tail, which could be automatically calculated by CASP image analysis system for each cell [40]. The assay was performed in triplicate.