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The synergistic effects of waterborne microcystin-LR and nitrite on hepatic pathological damage, lipid peroxidation and antioxidant responses of male zebrafish☆

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ABSTRACT

Hazardous materials from decaying cyanobacterial blooms, such as microcystin-LR (MC-LR) and nitrite pose serious challenges to aquatic organisms. To assess combined toxic effects of MC-LR and nitrite on hepatic pathology, lipid peroxidation and antioxidant responses of fish, adult male zebrafish (*Danio rerio*) were exposed to solutions with different combined concentrations of MC-LR (0, 3, 30 μg/L) and nitrite (0, 2, 20 mg/L) for 30 d. The results showed that hepatic pathological lesions progressed in severity and extent with increasing concentration of single factor MC-LR or nitrite and became more severe in co-exposure groups. Concurrently, significant increases in malondialdehyde (MDA) revealed the occurrence of oxidative stress caused by MC-LR, nitrite and both of them, which was indirectly verified by remarkable decreases in the total antioxidant capacity (T-AOC) as well as the transcription and activity of antioxidant enzymes (CAT and GPx). Hepatic mitochondria were damaged as the common action site of MC-LR and nitrite, suggesting that oxidative stress played a significant role in the mechanisms of the hepatotoxicity of MC-LR and nitrite. The depletion of hepatic glutathione (GSH) indicated the importance of GSH/glutathione-S-transferases (GST) system in these two chemicals detoxification. These results clearly illustrated that MC-LR and nitrite have synergistic effects on the histostructure, antioxidant capacity and detoxification capability in the liver of zebrafish. Therefore, the combined pollution of MC-LR and nitrite in eutrophic lakes can reduce the defense mechanism of the fish and accelerate the consumption of GSH, which compromise the survival of the fish during prolonged cyanobacterial blooms episodes.

1. Introduction

In recent years, there are a number of international reports of toxicity episodes that cyanobacterial blooms cause mortality and illness in fish, animals and even humans, which draw great attention in the public (Azevedo et al., 2002; Chen et al., 2009; Wu et al., 2016). Microcystins (MCs), a family of monocyclic polypeptide toxins, are a latent hazardous consequence of algae blooms (Merel et al., 2013). Among more than 100 identified analogous of MCs (Zastepa et al., 2015), microcystin-LR (MC-LR) is considered as the most widely distributed and toxic. It is reported that the concentration of dissolved MCs in Lake Taihu of China reached as high as 35.8 μg/L in the blooming season (Wang et al., 2010). Indeed, nitrite is also a by-product concomitant with the release of MCs during blooms degeneration (Jones and Orr, 1994; Yang et al., 2011). And nitrite concentration varied from 2 to 18 mg/L in shore sites of North American Lakes containing decaying algae and plants (McCoy, 1972; Masser et al., 1999). Environmental concentrations of these two toxins may persist for days and seriously impact waters and aquatic organism (Berg et al., 1987; Lahti et al., 1997; Kopp and

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Heteša, 2000). It thus appears that research on the combined effects of MCs and other water pollutants such as nitrite can provide a more accurate reference for ecological risk assessment of harmful algal blooms.

It is known that the liver is the primary target organ for MCs in fish, with symptoms of intrahepatic hemorrhage, destruction of hepatic structures, cytoplasmic vacuolization, hepatocyte necrosis and degeneration (Li et al., 2005; Li et al., 2007; Li and Xie, 2009; Trinchet et al., 2011; Hou et al., 2017). The hepatotoxicity of MCs is primarily due to the irreversible inhibition of serine/threonine protein phosphatase 1 and 2A (Fischer and Dietrich, 2000; Fischer et al., 2000). Several recent reports showed that oxidative stress is also one of toxic mechanisms of the action of MCs in aquatic organisms (Prieto et al., 2007; Atencio et al., 2008; Hou et al., 2015). Actually, hydrobiontes have developed a physician antioxidant defense system to detoxify the reactive intermediates and repair the resulting damage, which involves enzymatic and non-enzymatic mechanisms. Enzymatic components consist of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferases (GST), while non-enzymatic components include glutathione (GSH), vitamins C and E (Wilhelm, 1996). Many evidences indicated that both cyanobacterial extracts and pure MCs can induce the overproduction of reactive oxygen species (ROS), influence the activities of antioxidant enzymes and lead to an induction in lipid peroxidation (LPO) in diverse aquatic species and organ (Cazenave et al., 2006; Prieto et al., 2007; Atencio et al., 2008; Hou et al., 2015). However, most studies focused on alterations in the antioxidant enzymatic activity after MCs exposure. There is very limited information on the molecular mechanism involved with the antioxidant response of fish during long-term exposure to low MCs concentrations.

Nitrite (NO_2^-) is formed from ammonia by the nitrification process and may be accumulated to very high concentrations in aquatic systems (Jensen, 2003). Elevated ambient nitrite concentrations can generate multifarious physiological interferences in aquatic animals, such as growth suppression, tissue damage, immune function turbulence and endocrine disruption (Woo and chiu, 1994; Chand and Sahoo, 2006; Deane and Woo, 2007; Sun et al., 2014a). The toxicity of nitrite is mainly caused by the active uptake of NO_2^- across the gill epithelium and the subsequent oxidation of hemoglobin to methemoglobin, which could weaken the capacity of binding oxygen and lead to anaerobic condition (Jensen, 2003). Studies have revealed that the imbalance between antioxidant defenses system and ROS is a potential nitrite toxic mechanism (Xian et al., 2012; Sun et al., 2014b; Long et al., 2017). Under nitrite stress, the liver and gill of catfish *Clarias gariepinus* exhibited vacuolization and pyknotic nuclei (Michael et al., 1987). Sun et al. (2014b) reported that prolonged exposure of 15 mg/L nitrite induced ROS overproduction, resulted in oxidative lesions and decreased the activities of CAT, GPx and SOD in the liver of *Megalobrama amblycephala* juveniles. In invertebrates, Wang et al. (2006) found that the activities of SOD, GPx and CAT in the muscle decreased by 82.7%, 80.6% and 67% respectively, when *Macrobrachium nipponense* was exposed to 46 mg/L nitrite. As stated above, MCs and nitrite act differently, but both can stimulate ROS production. Accordingly, the coexist of MCs and nitrite may lead to complicated and considerable changes including antagonistic, synergistic or additive effects. Unfortunately, little is known about the combined toxicity between the two water environmental pollutants, MC-LR and nitrite.

The zebrafish, as a prominent vertebrate model, is widely used for investigating chemical toxicity (MacRae and Peterson, 2003; Hill et al., 2005). In light of the above, a fully factorial toxic experiment was designed in the present study, where adult male zebrafish (*Danio rerio*) were exposed to solutions with different combined concentrations of MC-LR (0, 3, 30 μg/L) and nitrite (0, 2, 20 mg/L) for 30 d. Here, we hypothesized that withstanding of a chronic stress caused by MC-LR can be interfered by another stressor like nitrite. The aims of the present study are, therefore, focused on evaluating the potential combined toxic effects of MC-LR and nitrite on the pathological structure, LPO as well as antioxidant parameters at the protein and transcription levels, and discussing the potential intoxication and detoxification mechanism from the perspective of antioxidant defense system.

2. Materials and methods

2.1. Toxins and chemicals

MC-LR (purity ≥ 95%) was purchased from Express (Express Technology Co. Ltd, TaiWan, China). The toxin was suspended in milliQ water to obtain a stock solution (0.5 mg/ml). Nitrite test solution was prepared by dissolving sodium nitrite ($NaNO_2$, Sinopharm chemical reagent Co. Ltd, Shanghai, China) in 1 L distilled water to acquire a stock solution (10 g/L). All other chemicals utilized in this study were of analytical grade.

2.2. Zebrafish maintenance and experimental design

Adult healthy male zebrafish (AB strain, 3 month old) were obtained from Institute of Hydrobiology, Chinese Academy of Sciences, and then acclimated for 14 days before the experiment. The photoperiod maintained a 14: 10 h (Light: Dark) cycle and water temperature was controlled at 28 ± 0.5 °C. Fish were fed with freshly hatched *Artemia* nauplii to apparent satiation twice daily in order to ensure adequate nutrition.

In view of the environmental concentration and our preliminary experiments (McCoy, 1972; Wang et al., 2010; Hou et al., 2016; Lin et al., 2017), MC-LR and nitrite concentrations ($NaNO_2$) were set at 0, 3, 30 μg/L and 0, 2, 20 mg/L, respectively. Experiments followed a fully factorial design, i.e. there were 9 treatment combinations. After the acclimation period, zebrafish (N = 630) were assigned randomly into 18 glass tanks that contained 15 L water with combined concentrations of MC-LR and nitrite. Each treatment was performed in two replicate tanks (35 fish per tank). The experiment lasted for 30 d and the other conditions were as same as described above for the acclimation period. In order to assure that the combinations of MC-LR and nitrite were constant and comparatively close to the theoretical concentrations, 1/2 water in each tank was replaced every 6 d with water containing relevant concentrations of MC-LR and nitrite. Within the experimental waters the real concentrations of MC-LR were detected every 6 d by the ELISA kit for MC-LR (Beacon Analytical System, Inc., ME, USA), and nitrite concentrations were detected by N-(1-Naphthyl) ethylenediamine dihydrochloride spectrophotometric method according to Tarafder and Rathore (1988) (Table S1, Supporting information). Deviations between the measured real concentrations and the nominal concentrations of MC-LR and nitrite in each treatment group were lower than 20%, which strictly obeyed the proposal of OECD guideline 204 (OECD, 1984).

After 30 d of exposure, zebrafish were anesthetized with a 0.02% buffered MS-222 solution and livers were collected. In each treatment group, each 3 fish's livers were pooled as one replicate and six replicates were used for the analysis of the antioxidant parameters and gene expression respectively. For the pathological study, 6 fish's livers were fixed in 10% neutral buffered formalin and 2.5% glutaraldehyde solution, respectively. All procedures carried out on fish were approved by the guidelines of Institutional Animal and Care and Use Committee (IACUC) of Huazhong Agricultural University, Wuhan of China.