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Organ-dependent response in antioxidants, myoglobin and neuroglobin in goldfish (*Carassius auratus*) exposed to MC-RR under varying oxygen level

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HIGHLIGHTS

- MC-RR and hypoxia–reoxygenation synergistically affect antioxidant defense system.
- Combined MC-RR and hypoxia–reoxygenation increase glucose requirement.
- Extensive hepatocytes damage were caused by MC-RR and hypoxia–reoxygenation.
- Brain Mb1, Mb2 and Ngb mRNAs were induced several folds.

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ABSTRACT

Cyanobacterial bloom, a common phenomenon nowadays often results in the depletion of dissolved oxygen (hypoxia) and releases microcystin-RR (MC-RR) in the water. Information on the combined effects of MC-RR and hypoxia on the goldfish is lacking, therefore, this study is aimed at evaluating the effect of two doses of MC-RR on the antioxidants and globin mRNA of goldfish under normoxia, hypoxia and reoxygenation. The result showed that MC-RR at both doses (50 and 200 μg kg^{-1} body weight) significantly ($p < 0.05$) induced superoxide dismutase activities in the liver and kidney but catalase activities and total antioxidant capacity were low in these organs during hypoxia and reoxygenation compared to normoxia and control. Myoglobin and neuroglobin mRNAs in MC-RR group were significantly induced in the brain only and are believed to protect the brain from oxidative damage. However, other organs were unprotected and extensive damage was observed in the liver cells. Our results clearly demonstrated that MC-RR and hypoxia–reoxygenation transitions were synergistically harmful to the goldfish and could impair its adaptation to hypoxia, especially during reoxygenation.

1. Introduction

Worldwide, the menace of cyanobacteria bloom (cyanobloom) is of serious concern, most especially as the prevalence and intensity of bloom are anticipated to increase in the future under amplified eutrophication and global climatic changes (Okogwu and Ugwumba, 2009; Paerl et al., 2011; Poste et al., 2011). During growth and decay, cyanobacteria deplete dissolved oxygen (hypoxia) and release microcystins (MCs), which create environmental stress (Zhang et al., 2011; Huang et al., 2013). Microcystins (MCs) are produced by cyanobacteria such as *Anabaena flos-aquae* and *Microcystis aeruginosa*, and have the general structure of: cyclo-(D-alanine1-X2-D-MeAsp3-Z4-Adda5-D-glutamate6-Mdha7) (Campos and Vasconcelos, 2010). There are over 80 varieties of MCs, among these, MC-LR (2:Leucine, 4:Arginine), MC-RR (2:Arginine, 4:Arginine) and MC-YR (2:Trysoine, 4:Arginine) are the most toxic and common (Prieto et al., 2006).

The toxicity of MCs is mediated through binding and inhibition of the key cellular enzymes, protein phosphatase 1 and 2A, that leads to hyperphosphorylation of cytosolic and cytoskeletal

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; MC, microcystin; MC-RR, microcystin Arginine Arginine; H–R, hypoxia–reoxygenation; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; TAOC, total antioxidant capacity; Mb, myoglobin, Ngb, neuroglobin; cyb, cytoglobin; NMG, No MC-RR group; LMG, low MC-RR group, HMG, high MC-RR group.

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proteins resulting in the disruption of the hepatocyte cytoskeleton (Ding et al., 2000) and deregulation of cell division, leading to tumor promoting activity (Carmichael, 1994). Several authors, Cazenave et al. (2006), Prieto et al. (2006), Sun et al. (2008), Chen et al. (2009), He et al. (2010), Zhao et al. (2011) and Huang et al. (2013) and references cited have reported the toxicity of MCs to fish, animals and humans.

Hypoxia–reoxygenation (H–R) transitions, which occur due to dissolved oxygen depletion during cyanobloom could cause hypoxic stress in aquatic organisms (Roesner et al., 2006; Taylor and Pouyssegur, 2007; Zhang et al., 2011). Hypoxic stress represents a severe threat to continued cell, tissue, and organism survival through the generation of reactive oxygen. Reactive oxygen species (ROS), such as superoxide anion ($O_2\cdot-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) may directly chemically damage cellular components and tissues particularly targeting macromolecular molecules (proteins, lipids, and nucleic acids) often leading to cellular damage (necrosis) and death (apoptosis), and cumulative organ injury (Lushchak et al., 2001; Prieto et al., 2006). However, ROS is controlled by antioxidant defense systems to avert oxidative stress (Amado and Monserrat, 2010). The antioxidant system consists of superoxide dismutase (SOD), glutathione peroxidase (GP*x*), catalase (CAT), reduced glutathione (GSH) and glutathione reductase among others. Superoxide dismutase dismutase superoxide radicals (HO_2-/O_2-) to the less toxic H_2O_2 while catalase and GP*x* detoxify H_2O_2 to O_2 and H_2O (Lushchak et al., 2001).

The goldfish, *Carassius auratus,* which routinely experiences hypoxia–reoxygenation (H–R) episodes in its natural habitat have evolved a complex behavioral, physiological and biochemical strategies to cope with this condition. Hypoxia tolerance is achieved by increased buffering capacities, metabolic suppression and through a well coordinated antioxidant system (Lushchak et al., 2001). Recently, studies have shown that globin proteins (haemoglobin, myoglobin, neuroglobin, cytoglobin and globin X) also play a vital role in the adaptation of goldfish to H–R transitions (Fraser et al. 2006; Roesner et al., 2008). Myoglobin, cytoglobin and neuroglobin mRNA levels and proteins in various organs are increased under hypoxia and are thus believed to play significant roles in the adaptation of hypoxia-tolerant fishes to H–R transitions (Roesner et al., 2008).

Although MCs and H–R transitions co-occur in freshwaters and have been extensively studied, independently, there is however paucity of information on the combined effects of these environmental stressors on hypoxia-adapted fish. To the best of our knowledge, only Martins et al. (2011) studied the effect of MC-LR on the cardio-respiratory responses of *Oreochromis niloticus* to hypoxia. This study therefore, aims at elucidating the response of hypoxia-tolerant goldfish to H–R transitions under MC-RR intoxication with a view to providing an insight into the effects of MC-RR on the adaption of this fish to H–R. We hypothesized that MC-RR and hypoxia–reoxygenation transitions will synergistically affect the antioxidant system and the globin genes and consequently reduce the fitness of the goldfish to survive during hypoxia and re-introduction of oxygen.

2. Materials and methods

2.1. Toxin extraction

The cyanobacterial material used in this experiment was collected from surface blooms of Lake Dianchi, Yunnan province in China. MC-RR was extracted from freeze-dried crude algae three times with 75% methanol using well established protocol in our laboratory as detailed in Li et al. (2005). The identity, purity and quantity of MC-RR was checked using a reverse-phase high-performance liquid chromatography (LC-20A, Shimadzu Corporation, Kyoto, Japan) and the concentrations determined by comparing the peak areas of the test samples with those of the standards available (MC-RR, Wako Pure Chemical Industries, Japan). The purified MC-RR (>98% pure) was suspended in normal saline (0.9% NaCl) prior to use.

2.2. Fish care and experimental protocol

Adult goldfish weighing 253.8 ± 29.3 g of both sexes purchased from a fish farm in Wuhan were acclimated to water temperature (25 ± 2 °C) and dissolved oxygen (6–8 mgL^{-1}), and fed commercial diet for two weeks prior to the experiment. After acclimation, the fish were randomly divided into three groups namely; group1-No MC-RR group (NMG), group 2- Low MC-RR group (LMG) and group 3- High MC-RR group (HMG). Each group was further subdivided into two sub-groups, normoxic and hypoxic sub-groups. Groups 2 and 3 were intraperitoneally injected with 50 $\mu g\ kg^{-1}$ BW ($MC\text{-}RR_{50}$) and 200 $\mu g\ kg^{-1}$ BW ($MC\text{-}RR_{200}$) of MC-RR, respectively, which corresponds to 2% and 8% of the lethal dose (LD_{50}) of carp, respectively (Gupta et al., 2003). No MC-RR was administered to Group 1 and it served as the control group.

Oxygen in the hypoxia aquaria was displaced by nitrogen sparging of dilution water and dissolved oxygen maintained at 0.8 ± 0.2 mgL^{-1} for 48 h and monitored continuously, while the normoxia sub-groups were maintained at dissolved oxygen level of 6–8 mgL^{-1}. After 48 h, the hypoxia tanks were re-oxygenated and monitored for additional 48 h. Water quality parameters (pH, dissolved oxygen and temperature) were measured twice daily from all aquaria, temperature in all aquaria was controlled at 25 ± 2 °C. All experiment was in triplicate.

At 6 h, 12 h, 24 h and 48 h intervals during hypoxia and reoxygention treatments, nine (9) fish were sampled from each normoxic, hypoxic and reoxygenated tanks of the NMG, LMG and HMG groups. The sampled fish were euthanized by cervical transection in ice and the brain, heart, liver and kidney removed in that order, divided into different parts, shock frozen in liquid nitrogen and stored at −80 °C until needed.

2.3. Tissue homogenate preparation

Tissue homogenate (10%) was prepared by weighing the tissue and homogenizing tissue in normal saline according to the weight-volume ratio 1:9. Homogenates were centrifuge at 1000–3000 rpm for 10 min and the supernatant was then divided into aliquots and stored at −80 °C for biochemical analysis. All operations were performed at 0–4 °C.

2.4. Measurement of glucose and antioxidant enzyme activities

Total antioxidant capacity (TAOC), glucose and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GP*x*) were measured in the liver, kidney, heart and brain. All biochemical analyses were performed using assay kits from (Nanjing Jiancheng Bioengineering Institute, China) and following the manufacturer's guide. Spectrophotometric analysis for SOD and glutathione were done using microplate reader (Thermo Electron Corporation), while all other biochemical analyses were carried out using spectrophotometer (Shimadzu UV-2550).

2.5. Protein analysis

Protein concentration was measured in all the organs by the Bradford method with Coomassie Brilliant Blue G-250 (Bradford, 1976) using bovine serum albumin as a standard.