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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.

2.6. Transmission electron microscopy

Liver specimens were diced into 1 mm^3, prefixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) solution, followed by three 15-min rinses with 0.1 M phosphate buffer (pH 7.4) and postfixed in cold 1% aqueous osmium tetraoxide for 1 h. After rinsing with phosphate buffer again, the specimens were then dehydrated in a graded ethanol series of 50–100% and then embedded in Epon 812. Ultrathin sections were sliced with glass knives on a LKB-V ultramicrotome (Nova, Sweden), stained with uranyl acetate and lead citrate and then examined under a HITACHI, H-600 electron microscope.

2.7. Respiratory genes

The effect of MC-RR and H–R on the mRNA expression of Myoglobin 1 (Myb1), Myoglobin 2 (Mb2), Cytoglobin (Cyb) and Neuroglobin (Ngb) was evaluated.

2.8. Database and sequence analyses

The Ensembl (http://www.ensembl.org) and NCBI (http://www.ncbi.nlm.nih.gov/) databases were searched for homologous genes and primer premier (Premier Biosoft International) software used to design primers for cloning the respiratory proteins' cDNA based on conserved regions. The primers used for real-time PCR are: ARP; Forward 5′-CCTCTTTCTTCCAGGCTTTG-3′ and Reverse 5′-AGACACTGCCGTTATCATACATC-3′; Myoglobin 1; Forward 5′-ACCACTCACGCCAACAAGCACA-3′ and Reverse 5′-GCCATCACCTTCA CCAGCACCT-3′, Myoglobin 2; Forward 5′-GGAATTGTTATGTT CACGAGGTTAT-3′ and Reverse 5′-GGTGCTTCTTGCCCAGGTT-3′; Neuroglobin; Forward 5′-TTCCTGCTCCCTCTTGGTTT-3′ and Reverse 5′-TTTTCTGCTCCGATGTGGTG-3′ and Cytoglobin Forward 5′-GTCC AGCAGATGGGCAAAGCA-3′ and Reverse 5′-ACCAGCACCTCCAGAAT CACTCG3′.

2.9. RNA extraction

Total RNA was isolated from liver, kidney, heart and brain using Trizol reagent (Invitrogen). Tissues were weighed and homogenized in the right amount of Trizol (100 mg of tissue/1 mL of Trizol). Extracted RNA was treated with RNase and DNase, and the quality and quantity checked photometrically at OD260. Purified total RNA (1 μg) was transcribed with RevertAidTMM-MuLV Reverse Transcriptase and Oligo(dT)18 primer using RevertAidTM first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instruction. The cDNAs of the genes of interest were amplified by PCR using premix Taq version 2.0 (Takara Biotechnology, China).

2.10. Quantitative real-time reverse-transcription PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed using 10-fold diluted cDNA. Levels of mRNA of acidic ribosomal phosphoprotein (ARP), Mb1, Mb2, Ngb and Cyb were evaluated. Acidic ribosomal phosphoprotein Po (ARP) was used as the house keeping gene as it is reported to be unaffected by hypoxia. All primers were tested in advance for specificity by RT-PCR. To avoid amplification of genomic DNA, primer pairs included at least one intron-spanning oligonucleotide. Diluted cDNA equivalent to 10 ng total RNA was applied as template in a reaction volume of 20 μL. Quantitative real-time RT-PCR was performed with the SYBR Green mix according to the manufacturer's instructions, applying 40 amplification cycles (95 °C/15 s, 57 °C/15 s, 72 °C/30 s) and fluorescence measured at the end of each amplification cycle. All assays included a non-template control. First evaluation of qRT-PCR data was done using Option Monitor software 2.03 Version (MJ Research, Cambridge, MA). Efficiency of the reaction was measured by the slope of a standard curve, derived from ten-fold dilution of plasmids.

Table 1
ANOVA (3-way) results of the influence of organ type, MC-RR and varying oxygen level on measured parameters. Underlined values indicate significance ($p < 0.05$).

Dependent Variable	df	F	Sig.
Total antioxidant capacity (TAOC)			
Organ	3	22.59	0.0001
MCRR	2	7.07	0.001
DO	2	13.81	0.0001
Organ*MCRR	6	1.62	0.15
Organ*DO	6	1.52	0.18
MCRR*DO	4	3.26	0.01
Organ*MCRR*DO	12	1.08	0.38
Superoxide dismutase (SOD)			
Organ	3	80.38	0.0001
MCRR	2	56.80	0.0001
DO	2	92.64	0.0001
Organ*MCRR	6	25.00	0.0001
Organ*DO	6	62.37	0.0001
MCRR*DO	4	2.01	0.01
Organ*MCRR*DO	12	3.20	0.0001
Glutathionine peroxidase (GPx)			
Organ	3	91.11	0.0001
MCRR	2	3.11	0.05
DO	2	1.20	0.31
Organ*MCRR	6	15.00	0.0001
Organ*DO	6	10.57	0.0001
MCRR*DO	4	2.08	0.08
Organ*MCRR*DO	12	3.82	0.0001
Catalase			
Organ	3	972.50	0.0001
MCRR	2	0.30	0.73
DO	2	1.67	0.19
Organ*MCRR	6	2.18	0.05
Organ*DO	6	4.07	0.001
MCRR*DO	4	4.25	0.003
Organ*MCRR*DO	12	2.05	0.02
Glucose			
Organ	3	207.92	0.0001
MCRR	2	9.34	0.0001
DO	2	13.81	0.0001
Organ*MCRR	6	0.45	0.85
Organ*DO	6	0.92	0.48
MCRR*DO	4	4.83	0.001
Organ*MCRR*DO	12	0.59	0.84

2.11. Statistical analysis

Three-way analysis of variance (ANOVA) was used to test statistical difference in data between organs (liver, kidney, heart and brain), dissolved oxygen level (normoxia, hypoxia and reoxygenation) and MC-RR (NMG, LMG and HMG) doses while Duncan multiple range test was used for multiple post hoc test. The relationship between the antioxidants under normoxia, hypoxia, reoxygenation and MC-RR was evaluated using Spearman correlation. All statistical analysis was performed using SPSS software (version 15).

3. Results

3.1. Antioxidants and glucose

The level of TAOC in normoxic fish liver was 163%, 98% and 173% lower than in the kidney, heart and brain, respectively. In all organs except the liver, TAOC was lowermost in the group exposed to high MC-RR dose during oxygenation (Fig. 2A). ANOVA showed that organ type, MC-RR dose, oxygen level and interactions between MC-RR and oxygen level significantly affected TAOC level ($p < 0.05$) (Table 1). Superoxide dismutase (SOD) activities in the liver, kidney, heart and brain of control fish were 0.67 ± 0.1,

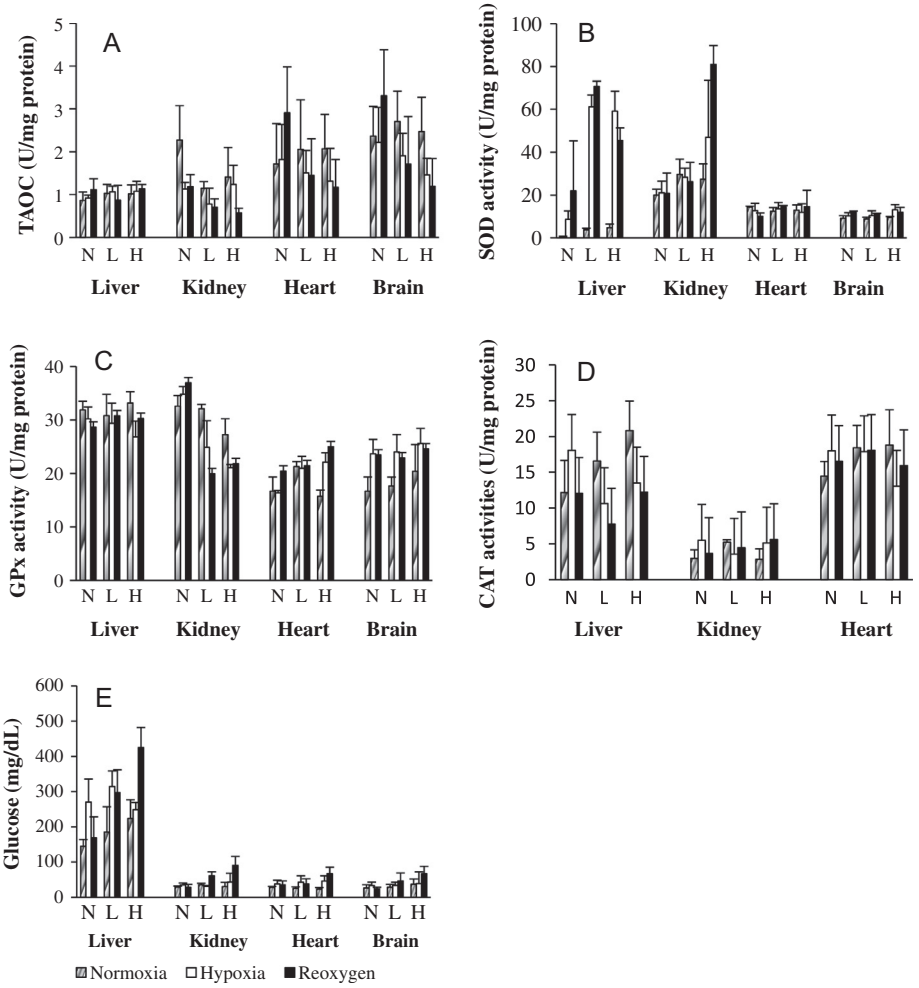

Fig. 1. Variation in (A) total antioxidant capacity, (B) superoxide dismutase, (C) glutathione peroxidase, (D) catalase and (E) glucose in the liver, kidney and heart of goldfish exposed to MC-RR during normoxia, hypoxia and reoxygenation (N = NMG, L = LMG, H = HMG).

17.60 ± 2.6, 14.63 ± 0.4 and 9.72 ± 1.2 U mg^{-1} protein, respectively (Fig. 2B). However, hypoxia caused SOD activities to increase significantly by 92-fold in liver and 2.6-fold in kidney ($p < 0.05$), while reoxygenation increased SOD activities by 106-fold and 4.6-fold in these respective organs in the HMG group. No significant changes were observed in the heart and brain ($p > 0.05$). MC-RR dose, oxygen level and their interaction significantly influenced SOD activities in organ dependent manner ($p < 0.05$).

The activities of GP*x* ranged from 15.75 ± 1.2 to 36.95 ± 1.4 U mg^{-1} protein in liver, kidney, heart and brain (Fig. 1C) and no significant changes were observed between groups in the liver and brain ($p > 0.05$). However, hypoxia and reoxygenation caused 65% and 69% decline in kidney GP*x* activity in the HMG. MC-RR dose but not oxygen level significantly influenced GP*x* activity in an organ dependent manner ($p < 0.05$).

Catalase activities were not significantly varied between groups in all organs except liver ($p > 0.05$). In the liver, hypoxia and reoxygenation significantly decreased catalase activities by 35% and 41%, respectively in the high MC-RR group ($p < 0.05$). Catalase activities were not significantly affected by MC-RR and oxygen level ($p > 0.05$), albeit interactions between both significantly affected catalase in organ dependent pattern ($p < 0.05$). In the liver, hypoxia caused 88% increase in glucose level in the NMG group, which returned to control values after 48 h of reoxygenation but in the HMG group, 48 h-reoxygenation caused 275% increase in glucose. In the kidney, heart and brain, reoxygenation caused 200%, 190% and 82% increase in glucose level, respectively in the high MC-RR group compared to the respective normoxic values. No significant changes were observed in the NMG groups of these organs ($p > 0.05$).

3.2. Protein

The protein values ranged from 1.89 to 4.16 g L^{-1} and differences between groups were not significant in all the organs investigated ($p > 0.05$).

3.3. Correlation

Under MC-RR and H–R transitions, the SOD–CAT–GP*x* association tend to be skewed to the right compared to the control (Fig. 2A–I). This implies that increased SOD activity was not complemented with increased activities of CAT and GP*x*, which means that the H_2O_2 generated by SOD activity is not detoxified swiftly and thus buildup in the cell. Correlation analysis showed that under MC-RR alone, SOD correlated significantly but negatively with CAT ($r = -0.70$, $p < 0.001$), under MC-RR and reoxygenation exposure, SOD correlated significantly and negatively with CAT ($r = -0.72$, $p < 0.001$).

3.4. Transmission electron microscopy

Transmission electron microscopy of liver from control goldfish showed normal cell with nucleus and well arranged rough

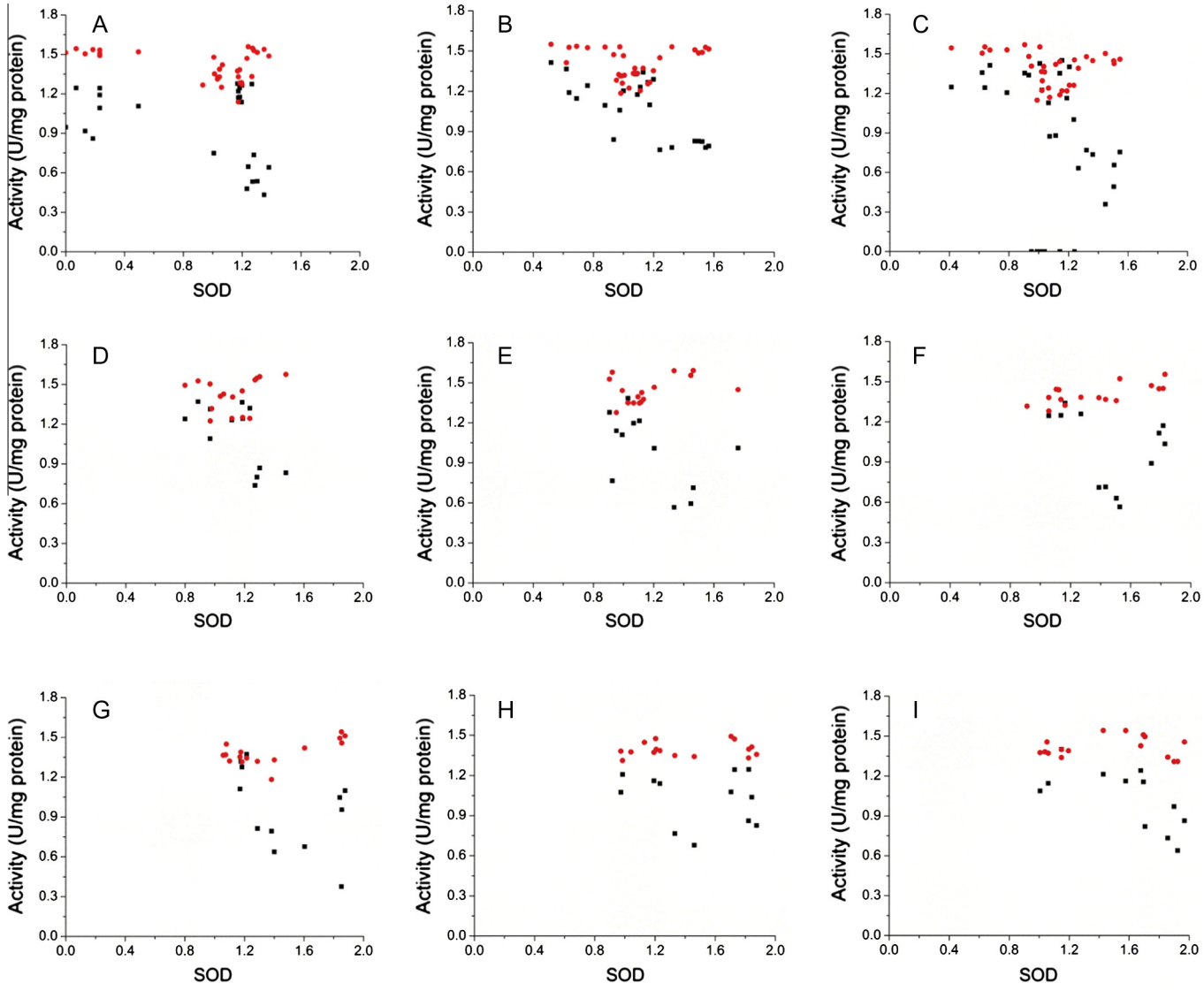

Fig. 2. The effect of MC-RR and varying oxygen level (hypoxia–reoxygenation) on SOD–CAT–GP*x* association in goldfish (black dots = CAT, red dots = GP*x*, *x*-axis = SOD). A = control (normoxia), B = low MC-RR, C = high MC-RR, D = hypoxia, E = reoxygenation, F = low MC-RR and hypoxia, G = low MC-RR and reoxygenation, H = high MC-RR and hypoxia, I = high MC-RR and reoxygenation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

endoplasmic reticulum and mitochondria (Fig 3A). After 48 h exposure to MC-RR, swollen and vacuolized mitochondria, dilated endoplasmic reticulum and proliferation of lysosome (Fig. 3B) were observed. After 48 h exposure to MC-RR and hypoxia, proliferation of ribosome, distortion of hepatocytes architecture, vacuolization of cytoplasmic, increased presence of lipid droplet and distorted plasma membrane were noticed (Fig. 3C and D). After 48 h of reoxygenation, further vacuolization and loss of cristae and matrix of mitochondria, swollen endoplasmic reticulum and vacuolization of cytoplasm was evident (Fig. 3E).

3.5. Respiratory gene expression

Evaluation of real-time RT-PCR results showed that acidic ribosomal phosphoprotein Po (ARP) used as the house keeping gene was 1.2-fold up-regulated in the kidney and 1.2-fold down-regulated in the heart. Consequently, all mRNA expressions were normalized using total mRNA content.

Brain Mb1 mRNA expression was significantly up-regulated by 27% and 115% above the control (normoxia) during hypoxia and reoxygenation, respectively in the HMG, Mb2 was induced by 22% (hypoxia) and 88% (reoxygenation) while Ngb mRNA expression was significantly ($p < 0.05$) up-regulated by 35% and 91%, respectively during the same period (Fig. 4A). A time series analysis of the dynamics of the expression of these genes in the brain showed that Mb1, Mb2 and Ngb transcripts increased by 74%, 63% and 71%, respectively, 6 h after exposure to MC-RR and hypoxia, but plummeted to near control level thereafter (Fig. 4B). However, 18 h later, the expression level of these genes was up-regulated by 165%, 160% and 175%, respectively (>twice the hypoxia values) and remained significantly ($p < 0.05$) high 24 h after reoxygenation, albeit declined to control values 24 h later. In the liver, kidney and heart, Mb1 and Cyb were non-significantly induced ($p > 0.05$). ANOVA showed that MC-RR and varying oxygen level synergistically influenced Mb1, Mb2 and Ngb levels in the brain only ($p < 0.05$).

4. Discussion

Several studies have shown that exposure to environmental stressors such as microcystin-RR (MC-RR) and hypoxia–reoxygenation (H–R) transitions pose serious challenges to

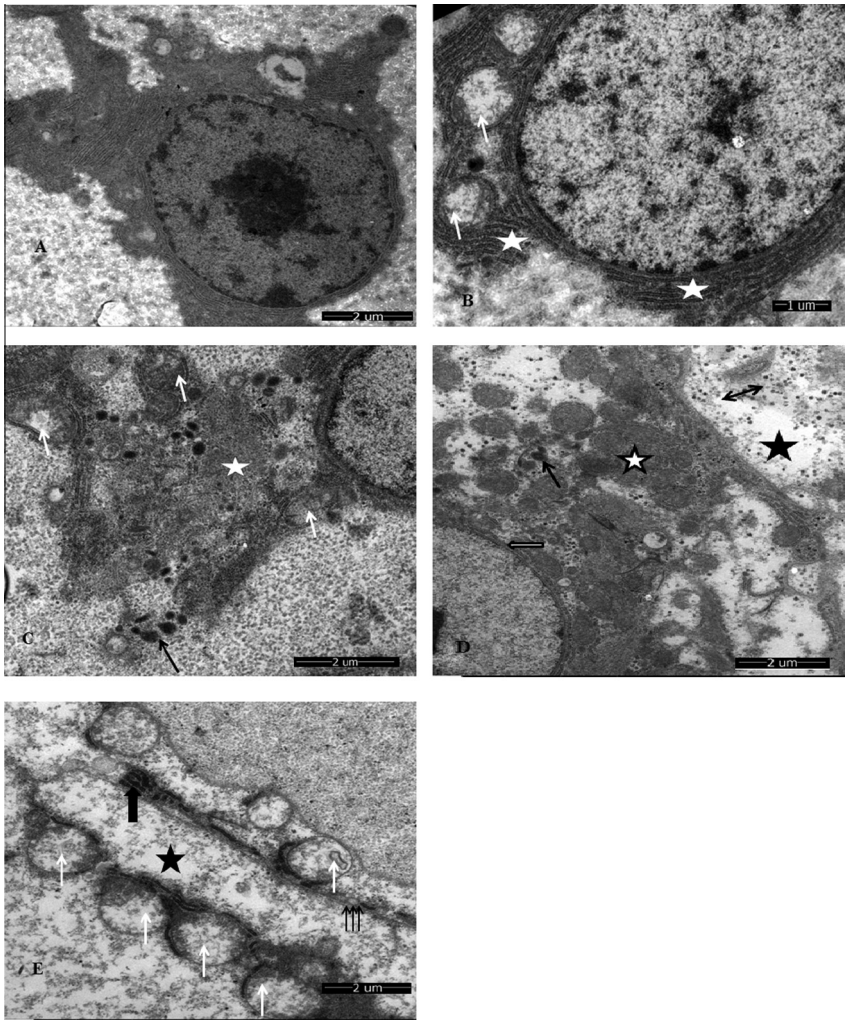

Fig. 3. Ultrastructure of the liver showing the control hepatocyte (A). Effect of MC-RR and varying oxygen on goldfish hepatocytes at 48 h normoxia (B), 48 h hypoxia (C and D) and 48 h after reoxygenation (E). (B) swollen and vacuolized mitochondria (white arrow) vacuolization and swollen, dilated endoplasmic reticulum (white star) and (C) lysosome proliferation (single black arrow) at 48 h and (D) ribosome proliferation (double-headed arrow), distortion of hepatocytes architecture (black star with insert white star), cytoplasmic vacuolization, lipid droplet (black arrow) and distorted plasma membrane (E) mitochondrial vacuolization, cytoplasmic vacuolation (black star), swollen endoplasmic reticulum (thick black arrow) and loss of cristae and matrix (triple arrow).

aquatic organisms (Lushchak et al., 2001; Amado and Monserrat 2010; Campos and Vasconcelos, 2010; Martins et al., 2011). However, many fish species have evolved strategies to cope with these stressful conditions, especially hypoxia. The goldfish, *C. auratus* is hypoxia tolerant and have evolved several mechanisms to adapt to hypoxia (Fraser et al., 2006). These mechanisms include metabolic suppression, stimulation of anaerobic glycolysis, coordinated antioxidant system and globin molecules (Roesner et al. 2008,). However, information is sparse on response of goldfish to the combined assault of MC-RR and H–R transitions. This study for the first time show combined effect of these stressors on the antioxidant system and some globin mRNA in *C. auratus*.

Previous studies show that biochemical toxicity of both MC-RR and H–R is mediated by oxidative stress through overproduction of radical oxygen species (Campos and Vasconcelos, 2010; Amado and Monserrat, 2010). Li et al. (2005) and Jiang et al. (2011) reported increased production of ROS in the hepatocytes and liver of *Cyprinus capio* exposed to MC-LR. ROS promotes lipid peroxidation and protein carbonylation, which devastates cell integrity (Bagnyukova et al., 2006; Jiang et al., 2011). Antioxidants (SOD, CAT, GP*x*, GST, GSH and GR) are deployed by fish and mammals to remove ROS and avert cellular damage (Lushchak et al., 2001; Li et al., 2011).

In the present study, we observed significant several fold increase in SOD activities in the liver and kidney of the goldfish dosed with MC-RR (LMG and LMG) during hypoxia and reoxygenation but CAT and GP*x* activities in the liver and kidney declined. There are several plausible explanations to the deactivation of CAT and GP*x* during the exposure of goldfish to MC-RR and H–R. Firstly, these enzymes could have been inactivated by high levels of ROS, especially superoxide ions (HO_2^-/O_2^-), generated by combined MC-RR and H–R assault (Lushchak et al., 2001). Superoxide ions (HO_2^-/O_2^-), when allowed to accumulate have been reported to deactivate CAT and GP*x* (Jiang et al. 2011; Bagnyukova et al., 2006) and as a consequence may lead to sluggish removal and consequent buildup of H_2O_2. CAT and GP*x* are first choice for the detoxification of H_2O_2 (Halliwell and Gutteridge, 1999) and when deactivated allow accumulation of H_2O_2. Secondly, the products of lipid peroxidation and protein carbonylation such as lipid peroxides could also deactivate CAT and GP*x* as suggested by Bagnyukova et al. (2006). Thirdly, it is possible that MC-RR and H–R could have acted at the gene level to down-regulate CAT and GP*x* transcripts as reported by Olsvik et al. (2006) and Sun et al. (2008). Zhao et al. (2008) reported down-regulation of GP*x* transcript in *C. auratus* exposed to microcystins, which affected the availability of this antioxidant. We strongly feel that these

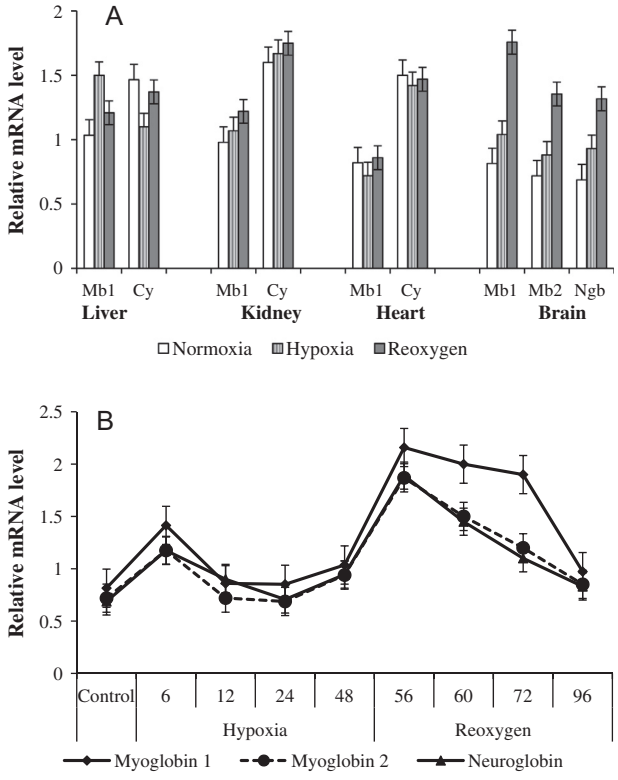

Fig. 4. (A) The expression of globin genes in the liver, kidney, heart and brain of goldfish exposed to MC-RR under normoxia, hypoxia and reoxygenation and (B) changes in myoglobin1, myoglobin2 and neuroglobin transcripts with time in the brain of goldfish exposed to MC-RR under varying oxygen level.

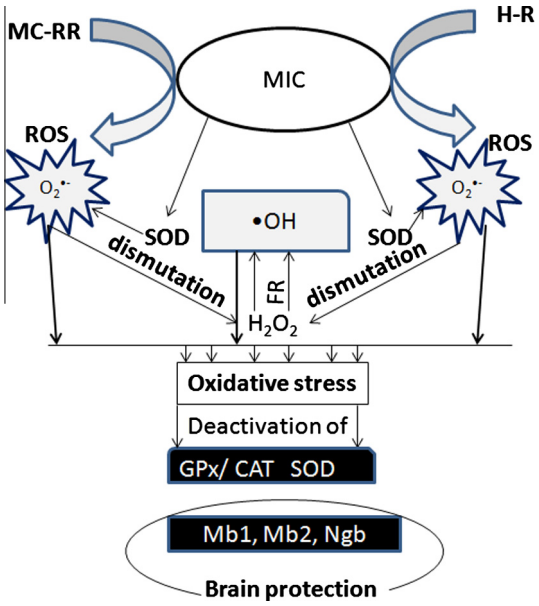

Fig. 5. Proposed pathway of reactive oxygen species (ROS) generation and pathogenesis during exposure to microcystin-RR (MC-RR) and hypoxia–reoxygenation (H–R) processes and the role of myoglobin1 (Mb1), myoglobin2 (Mb2) and neuroglobin (Ngb) in protecting the brain of *C. auratus* (O_2 = oxygen, FR = Fenton reaction, MIC = mitochondria, $O_2^{\bullet-}$ = superoxide ion, ·OH = hydroxyl ion, H_2O_2 = hydrogen peroxide, SOD = superoxide dismutase, GP*x* = glutathione peroxidase, CAT = catalase).

three processes in concomitant were instrumental to the deactivation of CAT and GP*x*. Prieto et al. (2006) and Jiang et al. (2011) also observed inhibition of CAT activities in tilapia and common carp exposed to MC-LR, respectively. Accumulation of superoxide ions, deactivation of CAT and GP*x*, and consequent H_2O_2 accretion portend grave dangers to living cells.

Superoxide ions and hydrogen peroxide (H_2O_2) are highly toxic and could also reduce metal ions (Cu^+ and Fe^{+2}) in Fenton reaction to produce the potent toxin hydroxyl radicals (·OH) (Halliwell and Gutteridge, 1999; Lushchak et al., 2001). Ding et al. (2000), Lushchak et al. (2001), Prieto et al. (2006), Jiang et al. (2011) and Zhao et al. (2011) have also shown that the accumulation of these ROS lead to lipid peroxidation, protein carboxylation, DNA damage, loss of cell architecture, impaired mitochondrial function, apoptosis and necrosis.

Our results also showed a significant inverse relationship between SOD and CAT in the group exposed to both MC-RR and reoxygenation, which indicates that SOD activities may directly or circuitously inhibit CAT activity. Perhaps accumulation of H_2O_2 and its reaction products such as ·OH may be the cause of the widespread damage observed in hepatocytes of the goldfish exposed to MC-RR and H–R. Swollen and vacuolized mitochondria, vacuolized, swollen and dilated endoplasmic reticulum, lysosome and ribosome proliferation, cytoplasmic vacuolization, lipid droplet and distorted plasma membrane were among the observed abnormally in the liver of goldfish exposed to MC-RR and H–R. Such abnormally are attributable to oxidative damage (Zhao et al., 2011). All these strongly indicate that MC-RR and H–R synergistically compromised the survival of goldfish under the latter conditions. Declined TAOC in the kidney, heart and brain of the goldfish dosed with MC-RR during hypoxia and reoxygenation congruously support this opinion. Furthermore, exposure of goldfish to MC-RR and H–R appears to blur the coordination between the antioxidants, thus allowing the slow removal/accumulation of ROS. This is typified by the skewing of the graph of GP*x* and CAT/SOD (Fig. 2A–I) to the right in the HMG group compared to MC-RR alone, hypoxia alone or control.

Superoxide dismutase and GP*x* activities in the brain were relatively low even during reoxygenation. It could be that the ROS level in the brain was insufficient to elicit response from the antioxidants or that these antioxidants were deactivated. However, the mRNA of myglobin 1 (Mb1), myoglobin 2 (Mb2) and neuroglobin (Ngb) were significantly upregulated in the brain of fish exposed to both MC-RR and H–R. Mb1 and Mb2 have been previously reported to show hypoxia induction at the mRNA level in the goldfish (Roesner et al., 2008). These globins have been shown to play a substantial role in the survival of goldfish exposed to oxidative stress. Although there are several studies on the expression and regulation of Ngb (Burmester and Hankeln, 2004; Roesner et al., 2008), its role in the adaptation of the goldfish to hypoxia is still poorly understood. Some studies (Bentmann et al., 2005) suggest that Ngb may facilitate O_2 delivery just like Mb whereas others (Fraser et al., 2006; Riggs and Gorr, 2006) believe that it may play a role in the detoxification of reactive nitrogen (RNS) and reactive oxygen species (ROS) and hypoxia-related signaling (Fago et al., 2006). Our results lend support to the latter proposition and we believe that Ngb and Mb may complement/supplement the antioxidants in the detoxification of RNS and ROS. In this study, although Mb1, Mb2 and Ngb were significantly up-regulated by 27%, 22% and 35% during hypoxia, the expression of these genes was even more enhanced during re-oxygenation by 115%, 88% and 91%, respectively. Our argument is, if the main function of these globins (Mb1, Mb2, Ngb) was enhanced-oxygen delivery, then the expression levels should be higher during hypoxia than reoxygenation. Rather, the reverse was the case as Mb1, Mb2 and Ngb transcripts were induced by over twice the hypoxia value (when O_2 supply was short) during reoxygenation (when the goldfish was gravely endangered by ROS and RNS). Taylor and Pouyssegur (2007) showed that cells/organism exposed to hypoxia face even more

danger from ROS during reoxygenation. Therefore, we postulate that these globins may serve neuroprotective roles against oxidative stress. Neuroglobin along Mb1 and Mb2 apparently protect the goldfish brain during high oxidative stress and perhaps when the primary antioxidants are inactivated by ROS overproduced during exposure to highly stressful conditions as exemplified by combined MC-RR and H–R assault as shown in Fig. 5. The mechanism by which this is achieved is probably unclear at this point but necessitates further studies.

Compared to the control group, goldfish dosed with MC-RR had significantly ($p < 0.05$) high glucose level in all organs during reoxygenation. High glucose level suggests enhanced glycogenolysis and mobilization of glucose for glycolysis. By implication the MC-RR group needs a lot of energy to survive post-hypoxia period. However, oxygen-independent glycolysis that occur during hypoxia yields 15-times less ATP than oxygen-dependent glycolysis (Richards, 2011) and more fermentable substrates are required to supply energy. Therefore glucose and glycogen reserves may be nearly depleted during prolonged hypoxia. Thus, goldfish intoxicated with MC-RR and subjected to prolonged hypoxia may lack the necessary energy to survive reoxygenation-induced stress.

5. Conclusion

Intraperitoneal injection of MC-RR followed by exposure to hypoxia and reoxygenation decreased some antioxidant activities and total antioxidant capacity in most organs and caused extensive damage to hepatocytes. Although neuroglobin and myoglobin mRNAs were significantly induced in the brain and are believed to protect the brain from ROS, other organs (liver, kidney and heart) were unprotected. Thus, exposure of goldfish to MC-RR and H–R may reduce the chances of *C. auratus* to survive hypoxia and reoxygenation episodes.

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