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Crude extract of cyanobacterium *Radiocystis fernandoi* strain R28 induces anemia and oxidative stress in fish erythrocytes

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ABSTRACT

The cyanobacterium *Radiocystis fernandoi* has been frequently identified in cyanobacterial blooms in Brazil. Recently, *R. fernandoi* strain R28, which produces microcystin (MC)-RR and MC-YR, was isolated from the Furnas reservoir, Minas Gerais, Brazil. The present study evaluated the hematological variables and erythrocyte antioxidant responses, lipid peroxidation (LPO), and genotoxicity in a neotropical fish (*Hoplias malabaricus*) after acute and subchronic exposure to a crude extract (CE) of *R. fernandoi* strain R28. Acute exposure (12 or 96 h) consisted of a single intraperitoneal (i.p.) CE injection, and subchronic exposure consisted of one i.p. CE injection every 72 h for 30 days. After acute exposure, fish exhibited macrocytic anemia (12 h post-injection) followed by normocytic anemia (96 h post-injection). The increased activity of superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, and the glutathione level in the erythrocytes did not prevent oxidative stress, manifested as lipid peroxidation and elevated DNA damage after acute exposure. After subchronic exposure, the hematological variables recovered, and the absence of erythrocyte oxidative stress suggests possible modulation by other biological factors, including a possible decrease in MC uptake by the cells and/or increasing detoxification efficiency that precludes erythrocyte damage.

1. Introduction

Cyanobacterial blooms are common occurrences in freshwater, estuarine, and marine meso- and eutrophic environments (Merel et al., 2013; Paskerovà et al., 2012). Many blooms are natural episodes; however, most are the result of human activities that directly or indirectly elevate the phosphorus and nitrogen levels in the water, changes that create an ideal environment for cyanobacterial growth. Aquatic ecosystem equilibrium and aquatic biota are affected by cyanobacterial blooms due to pH change and reductions in water transparence as well as nighttime dissolved oxygen (Carvalho et al., 2013). The primary concerns in cyanobacterial blooms are related to the production of toxins; these molecules may cause mortality in fish and others organisms due to the toxin presence and changes in the water's physical and chemical characteristics (Chellappa et al., 2008; Chen et al., 2016; Ernst et al., 2001).

In freshwater environments, cyanobacterial blooms are dominated by a single species or composed of a variety of toxic and non-toxic strains, mainly *Microcystis*, *Dolichospermum* (*Anabaena*), and *Cylindrospermopsis* genera and the less common *Radiocystis* Skuja 1948 genus (Carvalho et al., 2013). In the last two decades, the cyanobacterium *Radiocystis fernandoi* Komárek and Komárková-Legnerová 1993, a species that may produce the toxin microcystin (MC), has been frequently identified in Brazilian blooms; it has reached up to 70% of the total cyanobacterial biomass (Anjos et al., 2006; Borges et al., 2008; Fonseca et al., 2011; Jacinavius et al., 2018; Sant'Anna et al., 2008).

MCs, the most widespread cyanobacterial toxins, are potent inhibitors of intracellular protein phosphatases (PP1/PP2A) and are

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hepatotoxic. The chemical structure of MC, [-$_D$-Ala-$_L$-X-erythro-β-methyl-$_D$-isoAsp-$_L$-Y-Adda-$_D$-iso-Glu-N-Methyldehydro-Ala-], represents a heptapeptide with four permanent and two variable amino acids, which are represented by X and Y. The combination of two different amino acids in these positions classify the different MC variants (Carmichael et al., 1988; Wu et al., 2010), for example, the leucine and arginine in MC-LR, the arginine and arginine in MC-RR, and the tyrosine and arginine in MC-YR, respectively, in the X and Y position. More than 100 variants are already identified, and MC-LR, MC-RR, and MC-YR are most common in cyanobacterial blooms (Puddick et al., 2014). The toxicity among variants may differ substantially (6–10 fold; Falconer, 2005).

MCs may also affect other tissues and organs by generating reactive oxygen species (ROS) and inducing oxidative stress (Amado and Monserrat, 2010; Paskerová et al., 2012; Weng et al., 2007). Oxidative stress occurs when ROS production overcomes ROS neutralization by the cellular antioxidant system. The cellular defenses against ROS include biotransformation enzymes from phase I (EROD) and II (glutathione-S-transferase, GST), the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic antioxidants, of which glutathione (GSH) is the most responsive (Livingstone, 2001). Most studies addressed the effects of MC-LR on the liver and other organs (Amado and Monserrat, 2010; Li et al., 2003; Ríos et al., 2014; Sotton et al., 2012); little is known about the effect of other MC variants and/or cyanobacterial extracts on the blood (Cazenave et al., 2006b; Gupta et al., 2003; Prieto et al., 2006). Toxins and other cellular contents from cyanobacterial extracts are transported through the blood to various organs after absorption by the gills or digestive tract; thus, erythrocytes are the first cells that come into contact with MCs. Other cellular compounds may be affected by them. Cellular membrane damage and antioxidant enzyme changes are already reported in human erythrocytes (Sicinská et al., 2006).

Erythrocytes provide O_2 to tissues and remove CO_2 and protons produced during metabolic processes. Unlike mammals, fish erythrocytes are nucleated, contain active cellular organelles even after cell maturation, have functional mitochondria throughout the cell's lifespan, and have a cellular membrane rich in long-chain n-3 polyunsaturated fatty acids that may be oxidized under oxidative stress conditions (Nagasaka et al., 2004). Similar to the liver, fish erythrocytes are considered major sites for ROS production due to their role in O_2 transport, via hemoglobin (Hb), as well as their O_2 utilization and high antioxidant capacity, which helps to avoid Hb denaturation and lipid peroxidation. Erythrocytes represent an excellent model to evaluate toxic effects on fish (Halliwell and Gutteridge, 2015; Ruas et al., 2008).

In this context, the present study evaluated the hematological changes in total blood, the antioxidant and phase II enzyme responses, as well as some oxidative stress biomarkers, including lipid peroxidation and DNA damage, in the erythrocytes of the traira (*Hoplias malabaricus*) after acute and subchronic exposure to *R. fernandoi* strain R28 crude extract (CE). The cyanobacterium *R. fernandoi* strain R28 was isolated from blooms in the Furnas reservoir, Minas Gerais, Brazil; it mainly produces two MC variants, MC-RR and MC-YR, and other minor oligopeptides (Paulino et al., 2017a; Pereira and Giani, 2014; Pereira et al., 2012, 2015). The edible *H. malabaricus* is a neotropical carnivorous top predator species that is widely distributed throughout Brazil and very much appreciated for human consumption. This species is tolerant to numerous adverse environmental conditions (Moron et al., 2003; Rantin et al., 1992; Rios et al., 2005, 2006; Sakuragui et al., 2003), and it is usually found in waters known for cyanobacterial blooms.

2. Material and methods

2.1. Cyanobacterial cultivation, CE preparation, and MC quantification

R. fernandoi strain R28 was cultivated in a 500 mL batch with WC culture medium (Guillard and Lorenzen, 1972) at 25–30 μmol m^{-2} s^{-1} irradiance in a 12 h light:12 h dark photoperiod at 20 °C, according to Pereira et al. (2012), in the Phycology Laboratory of the Botany Department in the Federal University of Minas Gerais. CE was obtained from freeze-dried cyanobacterial biomass using 80% (v:v) HPLC-grade methanol (Panreac AppliChem, Spain) in Milli-Q water (18 MΩ cm^{-1}; Millipore Corporation, UK) and an Ultra Turrax (IKA – T10). The CE was centrifuged at 18,514 × *g* at 18 °C (20 min).

Quantitative confirmation that MC-RR and MC-YR were the major MC variants was performed using reverse-phase high performance liquid chromatography (HPLC-UV, Agilent 1200 Series, Agilent Technologies, Santa Clara, USA) equipped with a G1322A degasser, a G1311A quaternary pump, a G1367B autosampler, a G1316A thermostated column compartment, and a G1316A diode array detector, according to Arandas-Rodrigues et al. (2005). This technique used standard solutions that contained MC-LR, MC-YR, and MC-RR (Sigma, USA). MC concentrations in CE were determined by comparing the peak areas of the test samples with those of the available standards. The MC content in *R. fernandoi* strain R28 was estimated as 2.40 μg MC (1.46 μg of MC-RR and 0.94 μg of MC-YR) per mg of freeze-dried cyanobacterial cells. Considering the final volume of the obtained extract, there was 264.61 μg mL^{-1} of MC-RR and 170.38 μg $mL^{-1}$of MC-YR in the CE (Paulino et al., 2017a). MC-LR was not identified in the CE (Paulino et al., 2017a; Pereira and Giani, 2014; Pereira et al., 2012, 2015).

The total MC-LR equivalents (MC-LReq) in CE was quantified using an enzyme-linked immunosorbent assay kit (ELISA; Beacon Analytical Systems Inc®, USA) in a Molecular Devices SpectraMAX GEMINI X (Molecular Devices, USA) at 450 nm, according to manufacturing instructions. It was calculated as 439.92 μg mL^{-1} and used to obtain the desired concentration for injection into fish.

2.2. Animals and experimental design

H. malabaricus (n = 60; body mass = 236 ± 4.3 g; length = 26.28 ± 0.16 cm) were obtained from the Santa Cândida fish farm (Santa Cruz da Conceição, São Paulo State, Brazil) and acclimated in the laboratory for 30 days in 1000-L tanks at 25 ± 1 °C with dechlorinated water flow, constant aeration, and a 12 h light:12 h dark photoperiod. The fish were fed with live fish every 72 h. Thereafter, the fish were randomly separated into six groups (n = 10 per group) to perform acute and subchronic exposures.

The acute exposure consisted of two control (C) groups and two *R. fernandoi* strain R28 (CE) groups. The C groups received one intraperitoneal (i.p.) injection of 0.5 mL sterile saline solution (0.9% NaCl). The CE groups received one i.p. injection of 0.5 mL CE that contained 120.60 μg MC-RR + MC-YR kg^{-1} fish (or 100 μg MC-LReq kg^{-1} fish) diluted in sterile saline. A blood sample was taken 12 (C12h; CE12h) and 96 h (C96h; CE96h) post-injection. Subchronic exposure consisted of one C and one CE group that received one i.p. injection of 0.5 mL sterile saline (C group) or 0.5 mL CE in sterile saline (CE group), every 72 h, for 30 days (C30d; CE30d); thereafter, blood samples were taken for analyses. This subchronic experimental design simulated fish contamination by feeding, because *H. malabaricus* eats small fish every 3–4 d. The i.p. injection was chosen to specifically examine the dose effect. The MC-LReq dose injected in fish was selected because it does not cause mortality in *H. malabaricus*, but it does cause hepatotoxicity and changes in energy metabolism (Paulino et al., 2017b). At the end of experiments, a blood sample was collected from the caudal vein after fish were anesthetized with benzocaine solution (0.1 g L^{-1}). A whole blood subsample was immediately taken for hematological analyses. The remaining blood was centrifuged, the plasma was removed, and the packed erythrocytes were washed three times in saline and centrifuged at 5620 × *g* (5 min at 4 °C). Thereafter, the erythrocytes were re-suspended in HCl-Tris buffer pH 8.0 (3:1 v/v). The hemolysate was centrifuged (11,235 × *g*, 15 min, 4 °C) to remove broken cells and debris and stored at −80 °C for biochemical analyses.

Table 1
Hematocrit (Hct), red blood cells (RBC), hemoglobin concentration (Hb) and hematological indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of *Hoplias malabaricus* blood from control (C) groups and groups injected with crude extract (CE) of cyanobacteria *Radiocystis fernandoi* strain R28 containing microcytins RR and YR, after acute (12 and 96 h) and subchronic (30 days) exposure. Values are the mean (± SEM). * indicates significant difference from the respective control.

	Acute exposure (one single dose i.p injection)				Subchronic exposure (one dose i.p. injection every 72 h)	
Groups	12 h		96 h		30 days	
	C	CE	C	CE	C	CE
Hct (%)	26.8 ± 0.95	25.9 ± 0.54	27.1 ± 0.89	27.5 ± 0.96	26.5 ± 0.96	23.8 ± 1.30
Hb (g dL^{-1})	4.8 ± 0.34	3.3 ± 0.17*	4.8 ± 0.31	3.4 ± 0.22*	7.3 ± 0.46	7.2 ± 0.22
RBC (E mm^{-3})	202.6 ± 8.98	173.2 ± 9.33*	196.4 ± 7.31	205.9 ± 16.40	203.5 ± 6.53	194.2 ± 12.1
MCV (fL)	129.6 ± 4.42	152.8 ± 8.08*	130.3 ± 3.97	132.2 ± 8.89	132.2 ± 5.33	123.2 ± 2.91
MCH (pg $cell^{-1}$)	26.6 ± 3.47	19.2 ± 0.63*	26.9 ± 3.13	17.1 ± 1.07*	36.2 ± 2.65	36.0 ± 1.34
MCHC (g dL^{-1})	19.9 ± 2.19	12.5 ± 0.66*	19.8 ± 1.96	13.7 ± 0.22*	27.5 ± 2.31	29.3 ± 0.99

2.3. Hematological analyses

Hematocrit (Hct, %) was determined via microcentrifugation in heparinized glass capillary tubes. The hemoglobin concentration (Hb, g dL^{-1}) was determined using the cyanmethemoglobin method with Drabkin's solution (Collier, 1944), and a spectrophotometer (BEL Photonics SP 1105, Tecnal, Brazil) was used to measure the absorbance at 540 nm. The red blood cell count (RBC, n mm^{-3}) was determined using a Neubauer chamber after 1:200 blood dilutions into a formaldehyde-citrate solution. The mean cell volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg $cell^{-1}$), and mean cell hemoglobin concentration (MCHC, g dL^{-1}) were calculated using Hct, Hb, and RBC measurements.

2.4. Biochemical analyses

The activity of SOD (U mg^{-1} Hb) was determined according to McCord and Fridovich (1969) at 550 nm, based on the cytochrome C inhibition rate by superoxide radicals. One SOD unit corresponds to the amount of enzyme necessary to produce a 50% inhibition in the rate of cytochrome C reduction. CAT activity (μmol mg^{-1} Hb) was determined using H_2O_2 concentration decay at 240 nm (Beutler, 1975), and the activity of GPx (nmol mg^{-1} Hb) was determined via NADPH oxidation in the presence of tert-Butyl hydroperoxide (t-BOOH) at 340 nm (Beutler, 1984). The GST activity (nmol mg^{-1} Hb) was measured at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB), according to Habig and Jakoby (1981). The GSH level (nmol mg^{-1} Hb) was determined as described by White et al. (2003), based on the reaction of naphthalene-2 3-dicarboxaldehyde (NDA) with GSH to form a fluorescent compound (excitation at 472 nm and emission at 528 nm). Lipid peroxidation was assessed by determining lipid hydroperoxide (LPO, nmol mg^{-1} Hb), following the method described by Jiang et al. (1992). This measurement occurs after Fe^{2+} oxidation to Fe^{3+} in the presence of orange xylenol reagent (Ferrous Oxidation-Xylenol Orange -FOX), which forms Fe^{3+}-xylenol with absorbance at 560 nm. All assays were performed in a SpectraMAX M5 microplate reader (Molecular Devices, USA). The Hb concentration (mg Hb) was used to calculate enzyme activity and the GSH and LPO concentrations.

2.5. Genotoxic analyses

The comet assay with peripheral blood (erythrocytes) was performed according to Speit and Hartmann (1999). Comets were scored using a Zeiss epifluorescence microscope. For each liver slide, 100 cells were analyzed according to the method of Collins et al. (1997). They were scored as belonging to one of five classes, from undamaged (0) to maximally damaged (4), predefined with reference to the tail intensity. The comet score for a group could range from 0 (completely undamaged = 100 cells x 0) to 400 (maximum damage = 100 cells x 4).

2.6. Statistical analyses

Data are presented as the mean ± standard error of the mean (S.E.M.). The normality of the acute assay data was verified using the D'Agostino-Pearson test, and a one-way analysis of variance (ANOVA) was applied to compare control and experimental groups, followed by Bonferroni's post hoc test. To compare data in the subchronic assay, Student's *t*-test (two-tailed) and F test were used to compare variances. The comet assay data were analyzed using the Mann–Whitney *U* test. All tests were performed using GraphPad Prism 6.0, and data significance was designated at $p < 0.05$.

3. Results

No fish died during either acute or subchronic exposures to *R. fernandoi* CE. This extract decreased the Hb concentration, RBC and MCHC count, and increased MCV at 12 h post-injection ($p < 0.05$). After 96 h, the Hb concentration and MCHC were lower than those of the controls, and the MCH was also significantly decreased ($p < 0.05$; Table 1). Immature erythrocytes, which have a lower Hb content than mature erythrocytes, were very low in the controls, and there was a non-significant increase in the *H. malabaricus* peripheral blood after acute exposure. There were no changes in the hematological variables after subchronic exposure (Table 1).

Fig. 1 shows the changes in the antioxidant enzyme activities and GSH and LPO levels in *H. malabaricus* after acute contamination (12 or 96 h) with *R. fernandoi* strain R28 CE. Twelve h post-injection, the activity of SOD, GPx, and GST increased ($p < 0.05$), CAT activity and the GSH level did not change, and the LPO concentration increased ($p < 0.05$). After 96 h, the activities of SOD, CAT, and GPx were similar to those of the control fish (Fig. 1A–C), but the GST activity and the levels of GSH and LPO were significantly increased (Fig. 1D–F).

After subchronic *R. fernandoi* strain R28 CE injection every 72 h for 30 days, the antioxidant enzyme activities differed from those observed during acute contamination. SOD activity decreased (Fig. 2A), CAT and GST activities and GSH content did not change (Fig. 2B, D, and E), GPx activity increased (Fig. 2C), and the LPO level decreased (Fig. 2F).

The DNA damage score in *H. malabaricus* erythrocytes showed greater variability in the control fish than in those exposed to *R. fernandoi* CE during acute exposure; there were significant increases in fish exposed to CE for 12 h ($p < 0.05$) but not 96 h (Fig. 3A). After subchronic exposure, there was no change in the DNA damage score between the control and exposed fish (Fig. 3B).

4. Discussion

H. malabaricus exhibited great tolerance to the compounds present in the *R. fernandoi* strain R28 CE, which primarily contains MC-RR and MC-YR (Pereira et al., 2012, 2015), as was confirmed by HPLC–MS

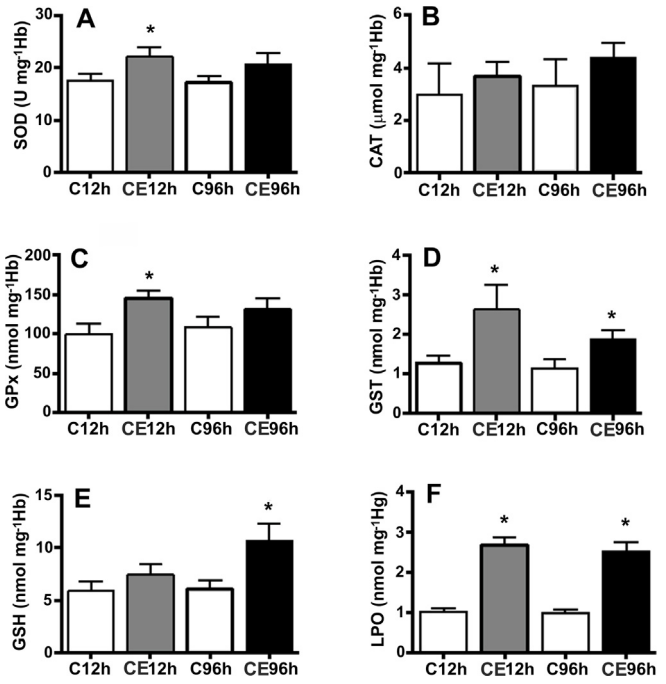

Fig. 1. Enzyme activities of superoxide dismutase (SOD, **A**), catalase (CAT, **B**), glutathione peroxidase (GPx, **C**) and glutathione-S-transferase (GST, **D**); levels of glutathione (GSH, **E**) and lipid hydroperoxide (LPO, **F**) in erythrocytes of *H. malabaricus* from control groups (C) and groups exposed to crude extract (CE) of cyanobacterium *Radiocystis fernandoi* strain R28 containing microcystins RR and YR, after acute (12 and 96 h) exposure. Values are mean (± SEM). *indicates significant difference from the respective control (C).

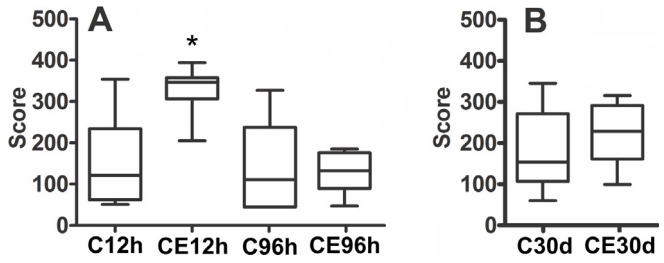

Fig. 3. DNA damage scoring in the erythrocytes of *H. malabaricus* from control groups (C) and groups exposed to crude extract (CE) of cyanobacteria *Radiocystis fernandoi* strain R28 containing microcystins RR and YR after acute (12 and 96 h, one dose) and subchronic (30 days) exposure. Values are mean (± SEM). *indicates significant difference from the respective control (C).

analysis. Although, no fish died during acute or subchronic exposure, the CE compounds, including MC-RR and MC-YR, induced several changes in the hematological variables and caused oxidative stress in the peripheral blood erythrocytes after acute exposure. The absence of significant hematological changes after subchronic exposure suggests a shock phase response during the first 96-h CE exposure and possible biochemical adjustments in the erythrocytes to continuous exposure (one dose injection every 72 h for 30 days).

The changes in the hematological variables indicate the occurrence of macrocytic anemia 12 h post-CE injection that was characterized by unchanged Hct but significant reductions in RBC, Hb concentration, MCH, and MCHC, and increased MCV. The Hct value depends on RBC and MVC changes; the absence of change in RBC, and the elevated MCV indicated the erythrocyte physiological response to maintain its O_2 carrier capacity transport during the first 12 h of exposure. Macrocytic anemia following 12-h CE exposure was followed by normocytic anemia 96 h post-injection. This state was characterized by RBC and MCV recovery to control levels, but the Hb concentration remained lower than the control. MCs contained in the CE are known to reduce Hct, Hb, and RBCs, all of which cause normocytic anemia in fish. This state was reported by Zhang et al. (2007) in goldfish (*Carassius auratus*) i.p. injected with 50 and 200 µg MC-LReq kg^{-1} fish (primarily MC-RR and MC-LR). Similar results were reported in silver carp (*Hypophthalmichthys molitrix*) i.p. injected with 250 µg MC-LR kg^{-1} fish (pure MC-LR; Vajcová et al., 1998) and in human hemodialysis patients in Caruaru (Pernambuco State, Brazil; Pouria et al., 1998). Normocytic anemia generally shows reduced hematopoiesis and Hb production (Köprücü et al., 2006), changes that suggest possible disruptive action of MC or other CE compounds in the fish erythropoietic tissue that promotes hemoglobin reduction in mature erythrocytes, as observed in the present study after 96-h exposure. MC is known to induce methemoglobin (met-Hb) formation (Sedan et al., 2013; Sicinska et al., 2006), and thus the Hb reduction in the erythrocytes, and the possible increase of met-Hb concentration in them, may affect O_2 uptake by the fish gills. Consequently, this change reduces the O_2 transport to the tissues, which contributes to decrease the aerobic metabolism and energy production in the tissues. However, after subchronic CE exposure, the recovery of the blood variable levels to near-control values demonstrated the high capacity of *H. malabaricus* to restore blood hematological properties.

The *H. malabaricus* erythrocyte antioxidant enzyme responses clearly showed that the *R. fernandoi* strain R28 CE generated ROS-induced changes in SOD, GPx, and GST activity during the first 96 h of exposure. The $O_2^{-}\cdot$ is usually produced during Hb auto-oxidation (Misra and Fridovik, 1972), which probably increased 12 h after CE injection and induced a significant increase in SOD activity. SOD

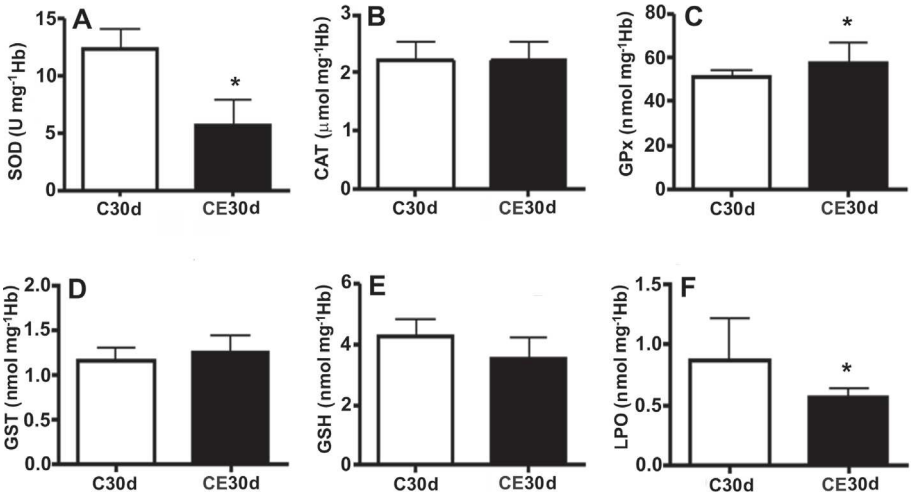

Fig. 2. Enzyme activities of superoxide dismutase (SOD, **A**), catalase (CAT, **B**), glutathione peroxidase (GPx, **C**) and glutathione-S-transferase (GST, **D**); levels of glutathione (GSH, **E**) and lipid hydroperoxide (LPO, **F**) in erythrocytes of *H. malabaricus* from control groups (C) and groups exposed to crude extract (CE) of cyanobacterium *Radiocystis fernandoi* strain R28 containing microcystins RR and YR, after subchronic (30 days) exposure. Values are mean (± SEM). *indicates significant difference from the respective control (C).

catalyzes the $O_2^{-}\cdot$ dismutation into H_2O_2. The CAT-GPx system is the primary mechanism against H_2O_2; CAT degrades H_2O_2 without consuming cellular reducing equivalents and is activated at high H_2O_2 levels (Halliwell and Gutteridge, 2015). Additionally, GPx uses GSH as a substrate, and thereby represents an efficient process for removing H_2O_2 and other organic peroxides; it is activated at low H_2O_2 levels (Pandey et al., 2011). Thus, the increased GPx activity in the *H. malabaricus* erythrocytes may indicate low H_2O_2 production. In human erythrocytes, which are destitute of a nucleus and cytoplasmic organelles such as mitochondria, lysosome, and Golgi apparatus, the disturbance of SOD and CAT activity by MC-LR results in high levels of H_2O_2 and cellular damage (Sicinska et al., 2006).

The $O_2^{-}\cdot$ participates in hydroxyl radical (OH·) formation, which has great potential to induce cellular damage (Christian, 2000). This ROS is not eliminated by enzymatic reaction, but it can be scavenged by GSH and other antioxidants. GSH, a tripeptide composed of glutamic acid, cysteine, and glycine, is synthetized in fish erythrocytes and serves as an effective cellular protector that is capable of reducing ROS (Lima et al., 2006; Sk and Bhattacharya, 2006). During exposure to oxidative agents, GSH is oxidized more rapidly than Hb and other cellular constituents, thereby protecting them (Jacob and Jandl, 1966; Yonezawa et al., 2005). GSH also plays an important role in MC detoxification to form an MC-glutathione conjugate via GST. This process represents the first step of MC detoxification; it is followed by degradation to a MC-cysteine conjugate compound in various animal organs, a reaction that enhances MC water solubility, reduces its toxicity, and promotes its excretion (Cazenave et al., 2006a, 2006b; Li et al., 2014; Pflugmacher et al., 1998, 2001). MC-LR and MC-YR conjugated with GSH and cysteine are 3-to10-fold less toxic than MCs alone (Metcalf et al., 2000). GST activation in *H. malabaricus* erythrocytes suggests the conjugation of MCs present in the CE to GSH, as occurs in other tissue cells with MC-LR (Amado and Montserrat, 2010; Jiang et al., 2011; Sedan et al., 2013). However, the absence of change in GSH levels 12 h after the i.p. injection may indicate a limiting factor for MC elimination from erythrocytes, a deficiency that would cause cellular toxicity denoted by elevated LPO levels and DNA damage. MC incorporation into the cell by organic anion-transporting polypeptides (OATP) via GSH exchange reduces the detoxification capability of the cell (Amado and Montserrat, 2010). Thus, it is possible that MCs present in *R. fernandoi* CE, together with other cellular compounds, reduce ROS scavenging and thus increase DNA damage 12 h post-i.p. injection. However, 96 h post-CE injection, DNA damage did not differ from the controls, data that indicate transient erythrocyte DNA damage. This effect may be related to lower MC incorporation by the cells, increased GSH levels that help to reduce DNA damage, and/or DNA repair. The reduction in DNA damage to control levels was also reported in the erythrocytes of white fish (*Coregonus lavaretus*) 48 h after MC-LR exposure; it is related to possible DNA repair and/or the loss of apoptotic erythrocytes (Sotton et al., 2012).

After subchronic exposure (CE injection every 72 h during 30 days), the reduced SOD activity, increased GPx activity, unchanged GST activity and GSH levels, and decreased LPO level indicated complex responses under continuous exposure. SOD inhibition may be attributed to covalent linkage between MC and the thiol residues present in the SOD active site (Sicinska et al., 2006) and/or excessive $O_2^{-}\cdot$ production (Pandey et al., 2003). The increased GPx activity suggests possible enzyme activation by low H_2O_2 production or other organic peroxides. In general, the reduced LPO level and absence of DNA damage after subchronic exposure suggest a recovery process from the alterations induced in the fish 12 and 96 h after a single i.p. injection (acute exposure) and even under repetitive doses (subchronic exposure). Two hypotheses may be considered: 1) changes in erythrocyte membrane constituents following continuous exposure and 2) the influence of components present in the CE, other than the MC, that may modulate the MC actions in the erythrocytes. MC is known to be incorporated into the cell by OATP via GSH exchange, which reduces the detoxification capability of the cell (Amado and Montserrat, 2010). After long-term exposure, erythrocytes may reduce OATP production, thereby decreasing MC incorporation by the cells and its toxic effects. MC modulation effects by other components in CE may result in a metabolic pathway that increases MC detoxification and excretion. *R. fernandoi* strain R28 primarily produces MC-RR and MC-YR, but there are also minor concentrations of MC-FR and MC-WR, a demethylated variant of microcystin YR (dMC-YR), one microviridin (MV-1709), and one cyanopeptolin (CY-1071; Pereira et al., 2012; Pereira and Giani, 2014).

5. Conclusions

In conclusion, the *R. fernandoi* strain R28 CE, which contains mostly MC-RR and MC-YR as well as other cellular compounds, induced ROS generation in the erythrocytes of the neotropical fish *H. malabaricus*, and the antioxidant defense responses were not efficient in avoiding oxidative stress at 12 and 96 h post-injection. However, the recovery of blood variables for O_2 transport, and the absence of oxidative stress after subchronic exposure, suggest possible modulation by other biological factors, including reduction in MC uptake by the cells.

Ethical statement

This study was conducted in accordance with national and institutional guidelines for the protection of human and animal welfare. The study was approved by the University's Animal Ethics Committee (Proc. n. 026/2012).

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