View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Toxicology in Vitro 25 (2011) 1493–1500

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Cellular responses of *Prochilodus lineatus* hepatocytes after cylindrospermopsin exposure

S. Liebel [a], C.A. Oliveira Ribeiro [a], R.C. Silva [c], W.A. Ramsdorf [b], M.M. Cestari [b], V.F. Magalhães [c], J.R.E. Garcia [d], B.M. Esquivel [e], F. Filipak Neto [a,*]

[a] *Departamento de Biologia Celular, Universidade Federal do Paraná, Caixa Postal 19031, CEP 81.531-990 Curitiba, PR, Brazil*
[b] *Departamento de Genética, Universidade Federal do Paraná, Caixa Postal 19031, CEP 81.531-990 Curitiba, PR, Brazil*
[c] *Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Bloco G, Ilha do Fundão, Universidade Federal do Rio de Janeiro, CEP 21949-900 Rio de Janeiro, RJ, Brazil*
[d] *Estação de Piscicultura Panamá, Paulo Lopes, SC, Brazil*
[e] *Campus Laranjeiras do Sul, Universidade Federal da Fronteira Sul, CEP 85303-775 Laranjeiras do Sul, PR, Brazil*

A R T I C L E I N F O

Article history:
Received 17 March 2011
Accepted 7 May 2011
Available online 13 May 2011

Keywords:
Cylindrospermopsin
Hepatocytes primary culture
Prochilodus lineatus
Multixenobiotic resistance
Oxidative stress

A B S T R A C T

Cylindrospermopsin is a potent toxicant for eukaryotic cells produced by several cyanobacteria. Recently, primary hepatocyte cultures of Neotropical fish have been established, demonstrating to be a quite efficient in vitro model for cellular toxicology studies. In the current study, a protocol for culture of *Prochilodus lineatus* hepatocytes was established and utilized to investigate the cellular responses to purified cylindrospermopsin exposure. Hepatocytes were successfully dissociated with dispase, resulting in a cell yield of 6.36×10^7 cells g^{-1} of liver, viability of 97% and attachment on uncoated culture flasks. For investigation of cylindrospermopsin effects, hepatocytes were dissociated, cultured during 96 h and exposed to three concentrations of the toxin (0.1, 1.0 or 10 $\mu g\,l^{-1}$) for 72 h. Cylindrospermopsin exposure significantly decreased cell viability (0.1 and 1 $\mu g\,l^{-1}$) and multixenobiotic resistance mechanism, MXR (all exposed groups), but increased reactive oxygen/nitrogen species levels (all exposed groups) and lipid peroxidation (10 $\mu g\,l^{-1}$). On the other hand no significant alterations were observed for other biochemical biomarkers as 2GSH/GSSG ratio, protein carbonyl levels and DNA strand breaks or glutathione *S*-transferase and glucose 6-phosphate dehydrogenase activities. In conclusion, hepatocytes might be made sensitive to cylindrospermopsin, at least in part, due to reduction of xenobiotics and endobiotics efflux capacity by MXR. Additionally, the toxin exposure suggests important issues regarding hepatocytes survival at the lowest cylindrospermopsin concentrations.

© 2011 Elsevier Ltd. Open access under the Elsevier OA license.

1. Introduction

Several cyanobacteria produce a diverse array of toxic metabolites, which can pose a serious threat to humans and aquatic organisms due to contamination of water and food (Berry, 2010; Chorus et al., 2000; Rao et al., 2002). Cylindrospermopsin, a cyanobacterial alkaloid toxin, was first identified following its implication as the causative agent in an outbreak of severe hepatoenteritis on Palm Island in 1979 (Hawkins et al., 1985). Recently, studies showed that cylindrospermopsin is a potent inhibitor of eukaryotic protein synthesis (Froscio et al., 2008; Terao et al., 1994) and the liver is the major target organ, although heart, thymus, spleen and kidneys may be affected (Falconer et al., 1999; Hawkins et al., 1997). Among the effects in mammal cells, genotoxicity, activation of different isoforms of cytochrome P450 (CYP), reduction of glutathione synthesis and endocrine disruption have been reported (Bain et al., 2007; Froscio et al., 2009; Humpage et al., 2005; Neumann et al., 2007). However, few data are available for fishes about cylindrospermopsin despite of the high exposure in natural environment and fish farms.

The teleost *Prochilodus lineatus* curimbatá is a freshwater detritivore fish widely distributed in South America and considered one of the most important species for human consumption in Southern and Southeastern Brazil (Jensch-Junior et al., 2005). This species is of great potential for fish farming due to good accommodation for different aquatic environments, ease of artificial fertilization, management and rapid growth, as well as high resistance to temperatures and pH variations (Fontenele, 1953; Winkaler et al., 2007). Although the highest fish biodiversity in the World is found in Brazil we did not find published data about cylindrospermopsin effects to Brazilian fishes or fish cells. In addition, the data about primary hepatocytes culture of Brazilian fish species are restricted for *Hoplias malabaricus* (Filipak Neto et al., 2006) and *Hypostomus commersoni* (Bussolaro et al., 2010). In vitro studies with intact cells have the potential of answering important questions about

* Corresponding author. Tel.: +55 41 3361 1680; fax: +55 41 3266 2042.
E-mail address: filipak@ufpr.br (F. Filipak Neto).

0887-2333 © 2011 Elsevier Ltd. Open access under the Elsevier OA license.
doi:10.1016/j.tiv.2011.05.010

the effects and mechanistic aspects of toxicants, and are useful for both biomedical and toxicological research (Fent, 2001; Filipak Neto et al., 2007). Primary cultured hepatocytes are particularly important to investigate xenobiotics effects, since the metabolism of these cells is comparable with intact hepatocytes in vivo (Chong et al., 2002; Fastner et al., 2003).

The aim of the present study was therefore to establish a protocol for isolation and culture of *P. lineatus* hepatocytes, as well as to investigate the toxic effects of purified cylindrospermopsin on this model, assessing the viability, redox milieu and multixenobiotic resistance system.

2. Materials and methods

2.1. Fishes

A number of 40 adult specimens of *P. lineatus* (500–800 g) were obtained from a commercial fish farm (Paulo Lopes City, Santa Catarina State, Brazil; http://www.pisciculturapanama.com.br) and maintained collectively in 3.000 l water tank with dechlorinated tap water for a period of 3–4 weeks before hepatocytes isolation. Constant aeration was performed by submerged pumps and food was supplied through commercial pelleted fish food (Supra Acqua Line®, 28% of protein) twice a week.

2.2. Hepatocytes isolation and culture

Fishes were anesthetized with benzocaine (200 ppm in water), injected with 0.5 ml of heparin ($5000\ U\ l^{-1}$) through the caudal vein and maintained during 5 min in dechlorinated water; then, fishes were anesthetized again and killed by spinal cord section for liver removal. The liver was kept in phosphate buffered saline (PBS, pH 7.6, 4 °C) supplemented with amphotericin-B ($25\ \mu g\ l^{-1}$), streptomycin ($100\ \mu g\ ml^{-1}$) and penicillin ($100\ U\ ml^{-1}$) during 10 min for antibiotic shock and perfused through the portal vein and arterial system with ice-cold PBS-EDTA solution (2 mM EDTA, $1.0\ g\ l^{-1}$ D-glucose in phosphate buffered saline – PBS, pH 7.6) for blood removal. After perfusion, the liver was aseptically minced with stainless steel blades in PBS containing dispase ($1.0\ U\ l^{-1}$) and $1.0\ g\ l^{-1}$ D-glucose, and incubated for 3 h (30 °C) for the hepatocytes dissociation. The cell suspension was forced through a stainless-steel mesh (60–60 mesh) for additional mechanical disruption. Cells were collected, centrifuged at low speed (100–120 g, 3–5 min), washed four times with PBS for debris removal and suspended to a density of 1.0×10^6 cells per ml in RPMI 1640 medium ($2.0\ g\ l^{-1}$ D-glucose, pH 7.6) supplemented with $NaHCO_3$ (25 mM), human insulin ($0.1\ U\ ml^{-1}$), gentamycin ($40\ mg\ l^{-1}$), streptomycin (10 µg ml-1), penicillin ($10\ U\ ml^{-1}$), amphotericin-B ($2.5\ \mu g\ l^{-1}$) and fetal bovine serum (5% $v.v^{-1}$). Finally, 2.0×10^5 and 1.0×10^6 cells (viability ⩾97%) were, respectively, seeded onto 96- and 24-well microplates (TTP® or Biofil®) and kept at 24 °C in a CO_2 incubator (1.7% of pCO_2). For each cell culture, a pool of cells from three fishes was utilized.

Before establishing this protocol, non-enzymatic dissociation and several enzymatic digestions were tested: EDTA (2 mM in PBS), trypsin–EDTA (0.05% tripsin, 2 mM EDTA in PBS), pancreatin (0.25% in PBS, 30 min, room temperature), collagenase IV ($0.25\ U\ ml^{-1}$ in PBS, 30 min, 30 °C), collagenase IV ($0.15\ U\ ml^{-1}$ in PBS) associated with dispase ($0.5\ U\ ml^{-1}$ in PBS, 30 min, 30 °C), and dispase ($1\ U\ l^{-1}$ in PBS, 30 min, 30 °C). After selecting the best dissociation protocol, cells were seeded in culture plates of 24 and 96 wells from three different brands (TPP®, Biofil® and Corning®), with and without prior treatment with matrigel ($10\ \mu g\ ml^{-1}$) or denatured collagen type I (gelatin, 1.5%) in order to determine the best attachment protocol.

2.3. Experimental design

After seeding, cells were cultured during four days to allow attachment and recovery from the isolation procedure. Then, culture medium was replaced by medium containing 0 (control), 0.1, 1.0 or $10\ \mu g\ l^{-1}$ of purified cylindrospermopsin (obtained at the Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Federal University of Rio de Janeiro, Brazil) and exposed during 72 h. After this period of exposure, cell viability, multixenobiotic resistance and oxidative stress biomarkers were determined.

2.4. Cell viability

The culture medium was replaced by 200 µl of fresh medium containing $50\ \mu g\ ml^{-1}$ of neutral red dye. After 3 h, cells were washed three times with solution I (15% formaldehyde, $100\ g\ l^{-1}$ of calcium chloride in water), and the dye was released from cells by addition of 300 µl of solution II (1% acetic acid, 50% ethanol in water). Then, 200 µl of supernatant were transferred to another 96-well microplate and read at 540 nm.

2.5. Multixenobiotic resistance mechanism (MXR) activity

Cells were incubated with 200 µl PBS containing rhodamine B (1 µM) for 30 min (at 24 °C and protected from light) and washed twice with PBS. Then, 250 µl of PBS was added to the 96-well microplate, which was frozen at −76 °C to cause cell lyses, and subsequently thawed. The cell lysate was transferred to a black microplate and fluorescence intensity resulting from accumulated rhodamine B was determined, using the excitation wavelength of 485 nm and the emission wavelength of 530 nm (Pessatti et al., 2002, with modifications).

2.6. Reactive oxygen/nitrogen species (RONS)

Cells were incubated with 200 µl of culture medium containing 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; 10 µM in 0.1% DMSO) for 15 min (at 25 °C and protected from light), washed twice with PBS and suspended with 250 µl of PBS-EDTA. The 96-well microplate was frozen at −76 °C, and 200 µl of cell lysate was transferred to a black microplate for fluorescence measurement using the excitation wavelength of 488 nm and the emission wavelength of 530 nm (Benov et al., 1998).

2.7. Glutathione S-transferase (GST) activity

Cells cultured in 96-well microplates were washed with PBS and frozen at −76 °C. Cell lysate was suspended in 150 µl of ice-cold PBS per well and microplates were centrifuged at 2800*g* for 10 min at 4 °C. Then, 30 µl of supernatant (PBS for blank) was placed in another 96-well microplate. Reaction was started by addition of 170 µl of reaction medium (1.5 mM GSH, 2.0 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 0.1 M potassium phosphate buffer, pH 6.5) and absorbance increase was measured at 340 nm for 2 min for enzyme activity determination using CDNB molar extinction coefficient of $9.6\ mM^{-1}\ cm^{-1}$ (Keen et al., 1976).

2.8. Glucose-6-phosphate dehydrogenase (G6PDH) activity

Cells cultured in 96-well microplates were washed with PBS and frozen at −76 °C. Cell lysate was suspended in 150 µl of ice-cold PBS and centrifuged at 2800*g* for 10 min at 4 °C. Then, 50 µl of supernatant (PBS for blank) and 150 µl of reaction medium (1.0 mM of β-$NADP^+$, 2.0 mM D-glucose-6-phosphate, 0.1 M of Tris–HCl, 10 mM of $MgCl_2$, pH 8.0, 25 °C) were added to a 96-well microplate for absorbance measurement at 340 nm for 3 min.

NADPH molar extinction coefficient of 6.22 $mM^{-1} cm^{-1}$ was utilized for enzyme activity calculation (Glock and Mclean, 1953).

2.9. Glutathione (2GSH/GSSG) ratio

Cells cultured in 24-well plates were washed with PBS and frozen at −76 °C. Cell lysate was suspended in 100 μl of ice-cold PBS, and 200 μl of lysate suspension (from two wells) was transferred to 1.5 ml tubes and centrifuged at 10,000*g* for 10 min at 4 °C. A volume of 50 μl of the supernatant was separated for protein determination and the remaining volume (150 μl) was mixed with 30 μl of 50% trichloroacetic acid for protein precipitation after centrifugation at 10,000*g* (10 min, 4 °C). Then, 150 μl of this supernatant was transferred to a new tube and the pH was neutralized with 390 μl of Tris (0.4 M, pH 8.9). Finally, 200 μl of the neutralized solution was separated in two tubes of 1.5 ml for quantification of total glutathione and reduced glutathione, respectively. In the first tube, 26 μl of solution containing 0.9 U ml^{-1} of glutathione disulfide reductase and 1.8 mM of NADPH was added; the second tube received the 26 μl of Tris buffer (0.4 M, pH 8.9). After 10 min incubation at room temperature, 200 μl of tubes' content were added to a 96-well microplate. Finally, 20 μl DTNB solution (2.5 mM of 5,5′-dithiobis (2-nitrobenzoic acid) in 25% methanol) were added to microplate wells, and after 5 min, the absorbance was measured at 415 nm. GSH concentration was calculated by comparison with a standard curve of GSH (Sedlak and Lindsay, 1968; Griffith, 1980 with modifications). Glutathione disulfide concentration was calculated through the difference between total glutathione and GSH.

2.10. Protein carbonylation (PCO)

Cells cultured in 24-well plates were washed with PBS and frozen at −76 °C. Cell lysate were suspended with 300 μl of ice-cold PBS, transferred to a 1.5 ml tube and centrifuged (12,000*g*, 20 min, 4 °C). A volume of 200 μl of supernatant was transferred to a 2.0 ml tube and mixed with 500 μl of DNPH solution (10 mM of 2,4-dinitrophenylhydrazine in 2.0 M of hydrochloric acid). For the blank, 2.0 M hydrochloric acid (without DNPH) was utilized. Samples were incubated at 30 °C during 90 min, proteins were precipitated with 1.0 ml of 28% trichloroacetic acid and centrifugation at 9000*g* for 10 min, and pelleted proteins were washed three times by suspension in ethanol/ethyl acetate (1:1) followed by centrifugation. Proteins were solubilized in 6.0 M of guanidine hydrochloride and tubes were centrifuged at 9000*g* for 5 min to remove any trace of insoluble material. The carbonyl content was determined spectrophotometrically at 360 nm using the molar absorption coefficient of $2.1 \times 10^4 M^{-1} cm^{-1}$ for hydrazones and normalized by total protein content quantified in an aliquot reserved from the first centrifugation procedure (Levine et al., 1994; Quinlan and Gutteridge, 2000).

2.11. Lipid peroxidation (LPO)

Cells cultured in 24-well plates were washed with PBS and frozen at −76 °C. Cell lysate were suspended in 250 μl of ice-cold PBS, transferred to a 1.5 ml tube and centrifuged (10,000*g*, 5 min, 4 °C). A volume of 200 μl of supernatant (PBS for blank) was transferred to another tube and mixed with 500 μl of reaction solution (0.1 mM of xylenol orange, 25 mM of H_2SO_4, 4.0 mM of BHT (butylated hydroxytoluene) and 0.25 mM of $FeSO_4 \cdot NH_4$ (ammonium ferrous sulfate) in 100% grade methanol). After 20 min incubation at room temperature, tubes were centrifuged at 10,000*g* for 5 min at 4 °C and supernatant absorbance was measured in a 96-well microplate at 570 nm. The molar extinction coefficient for H_2O_2 and cumene hydroperoxide of $4.3 \times 10^4 M^{-1} cm^{-1}$ was used (Jiang et al., 1992).

2.12. DNA damage

Cells were trypsinized (0.05% tripsin, 2 mM of EDTA) at room temperature, washed with PBS and suspended in 0.5% low melting point agarose. Cell suspension was added onto glass slides, followed by agarose solidification and cell membranes disruption in lyses solution (220 mM of NaCl, 9 mM of EDTA, 0.9 mM of Tris, 1% Triton X-100, 10% dimethylsulfoxide (DMSO), 0.9% sodium sarcosianate, pH 10) for 24 h at 4 °C. DNA was denatured (10 M of NaOH, 200 mM of EDTA, pH > 13 for 20 min) and electrophoresis was performed at 300 mA and 25 V for 25 min. Then, slides were neutralized with 0.4 M Tris (pH 7.5), fixed in ethanol during 10 min and stained with 0.02 $g \cdot ml^{-1}$ of ethidium bromide (Singh et al., 1988). DNA damage was classified according to comet tail length (damage class: 0, 1, 2, 3 or 4), and scores were calculated according to Collins et al. (1997).

2.13. Protein content

Total proteins were quantified following Bradford (1976). Supernatant (10 μl) and 250 μl of Bradford reagent ("Coomassie brilliant blue" BG-250) were placed in a 96-well microplate and absorbance was measured at 595 nm. Protein content was calculated through comparison with a standard curve of bovine serum albumin.

2.14. Scanning electron microscopy (SEM)

SEM was utilized to evaluate the morphology and arrangement of clusters of cells after 7 days of culture. Cells were cultured and fixed in the own 24-well culture plate by 3% glutaraldehyde for 1 h and preserved in 70% ethanol at 4 °C. The bottom of the plates was carefully cut in small pieces (~1 cm^2) and the cells were dehydrated in ethanol series (50%, 70%, 80%, 90% and 100% for 5 min) and in liquid CO_2, coated with gold powder and observed under the scanning electron microscope JEOL JSM – 6360 LV SEM (Electron Microscopy Center of Federal University of Parana, Brazil).

2.15. Statistical procedures

Three independent cell isolations were performed for each biomarker analyzed. A number of 24 replicates per cell isolation were utilized for cell viability, MXR, GST, G6PDH and RONS determination, totalizing 72 replicates. For glutathione concentration, lipid peroxidation, protein carbonylation and DNA damage, 6 replicates per cell isolation were utilized, totalizing 18 replicates. Kruskal–Wallis test (non-parametric ANOVA) was performed for comparing DNA damage scores, whereas One-way analysis of variance (parametric ANOVA) and Tukey post test was performed (when appropriate) for the other biomarkers, considering these replicates. $P < 0.05$ was assumed as statistically significant.

3. Results

3.1. Method establishment

Six protocols were considered to establish the hepatocytes dissociation method for *P. lineatus*. The non-enzymatic dissociation with EDTA (2 mM in PBS) was unsuccessful due to lyses of most cells during the procedure. The same drawback happened after using the trypsin (0.05%) and pancreatin (0.25%) enzymes. However, collagenase IV (0.25 U ml^{-1}), collagenase IV (0.15 U ml^{-1})

Table 1
Cell yield and viability for the procedures tested for isolation of *P. lineatus* hepatocytes.

Procedure	Number of cells per gram of liver	Viability
EDTA	Nd	Nd
Tripsin	Nd	Nd
Pancreatin	Nd	Nd
Collagenase IV	$1.01 \pm 0.3 \times 10^7$	88% ± 0.5%
Collagenase IV + dispase	$3.58 \pm 0.5 \times 10^{7**}$	90% ± 2.0%
Dispase	$6.36 \pm 0.6 \times 10^{7**}$	97% ± 1.0%*

Mean ± standard deviation (data from three independent experiments).
Nd – not determined due lyses of the majority of cells obtained after dissociation.
ANOVA followed by Tukey post test.
* $p < 0.05$.
** $p < 0.01$.

associated with dispase (0.5 U ml^{-1}), and only dispase (1 U ml^{-1}) presented satisfactory results considering cell yield and viability (Table 1). The protocol using collagenase IV resulted in 88% of cell viability and 1.01×10^7 hepatocytes per gram of liver, whereas collagenase IV and dispase enzymes resulted in about 3-fold increase in hepatocytes yield (Table 1) maintaining similar cell viability. However, 97% of cell viability and 6.36×10^7 hepatocytes per gram of liver were obtained using dispase (Table 1). On this way, the latter protocol was selected for further tests of cell attachment. Hepatocytes adhered properly on two of the three culture flask brands tested, TTP® and Biofil®. However, there was no improvement in hepatocyte attachment with coat pretreatments and four days were necessary for cell attachment after seeding, as evidenced by the clustering of hepatocytes in large groups (Figs. 1 and 2). On this way, we adopted the following protocol for investigation of cylindrospermopsin effects. Hepatocytes were dissociated with dispase, seeded on TTP® flasks, cultured during four days for cell recovery and attachment, and then exposed to cylindrospermopsin through replacement of culture medium.

3.2. Effects of cylindrospermopsin

Cell viability decreased 8% in hepatocytes exposed to the two lowest concentrations of purified cylindrospermopsin (0.1 and 1 μg l^{-1}), but not at the highest concentration of 10 μg l^{-1} (Fig. 3A). Cells exposed to the three concentrations of cylindrospermopsin have similar GST and G6PDH activities in comparison to the control group (Fig. 3B and C), although Tukey post test indicated that the GST activity of the hepatocytes exposed to 10 μg l^{-1} was 12% lower than of those exposed to 1 μg l^{-1} (Fig. 3B). Similarly, this post test showed that the G6PDH activity of the hepatocytes exposed to cylindrospermopsin at 10 μg l^{-1} was 19% lower than of those exposed to 0.1 and 1 μg l^{-1} (Fig. 3C). No significant alterations were observed for GSH concentration (53.6 ± 15.8 μmoles of non protein thiols per mg of protein) and also for the 2GSH/GSSG ratio ($p > 0.7188$) after exposure (Fig. 3D), despite of the 25% increase of reactive oxygen/nitrogen species levels (mainly hydrogen peroxide) in all cylindrospermopsin-exposed groups in comparison to the control group (Fig. 3E). Likewise, MXR activity decreased in about 22% in exposed groups, but without a concentration–response relation (Fig. 3F), demonstrating that cylindrospermopsin may be able to make hepatocytes of *P. lineatus* sensitive to another chemical stressor, even at the lowest concentration. No macromolecular damages were observed after exposure, considering the protein carbonylation ($p > 0.4909$; Fig. 3G) and DNA comet test ($p > 0.0505$; Table 2). However, an increase of 35% occurred for lipid peroxidation after exposure to the highest cylindrospermopsin concentration (10 μg l^{-1}) in comparison with the control group (Fig. 3H).

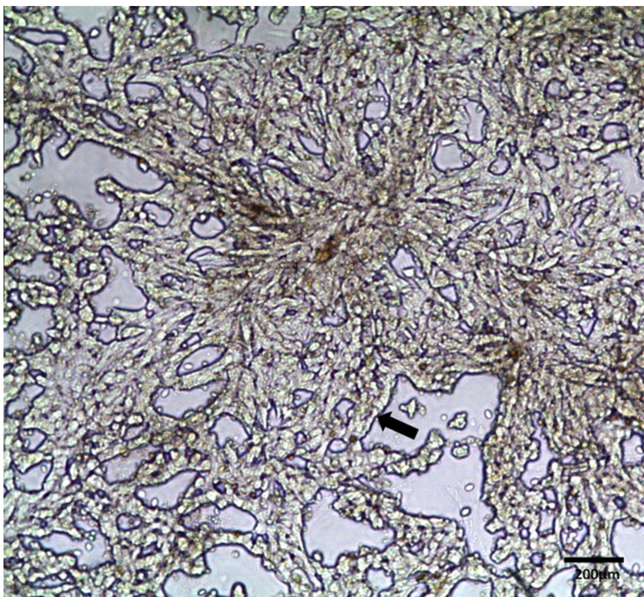

Fig. 1. *P. lineatus* hepatocytes after seven days in culture under phase contrast microscope. Observe the clusters of hepatocytes (arrow). Scale bar = 200 μm.

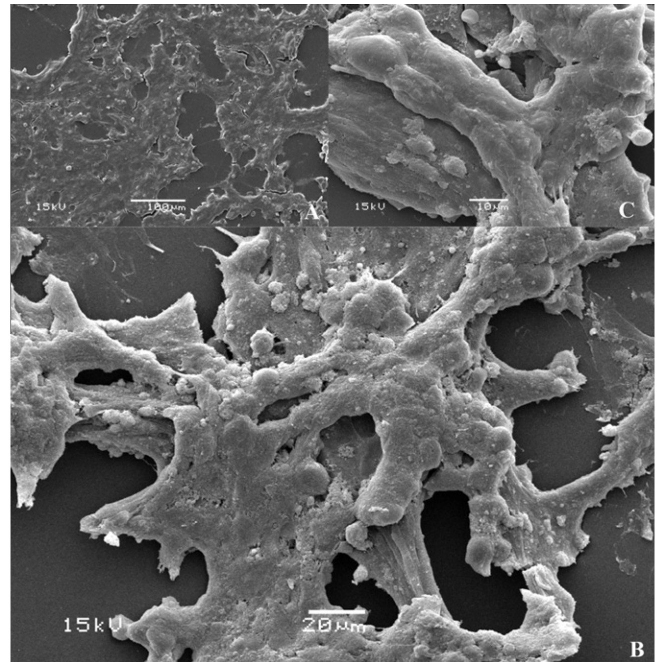

Fig. 2. *P. lineatus* hepatocytes after seven days in culture under scanning electron microscopy. General aspect of hepatocytes culture (A); hepatocyte aggregates (B); (C) details of groups of hepatocyte aggregated.

4. Discussion

4.1. Hepatocytes isolation and culture

The liver is the major site of xenobiotic metabolism, being involved in the maintenance of homeostasis in vertebrates. When freshly isolated and cultured, intact hepatocytes retain metabolic and functional characteristics that are closer to the in vivo situation than those of established cell lines (Segner, 1998; Zucco et al., 2004). Therefore, primary hepatocyte culture represents a valuable model for mechanistic and toxicity studies. Currently,

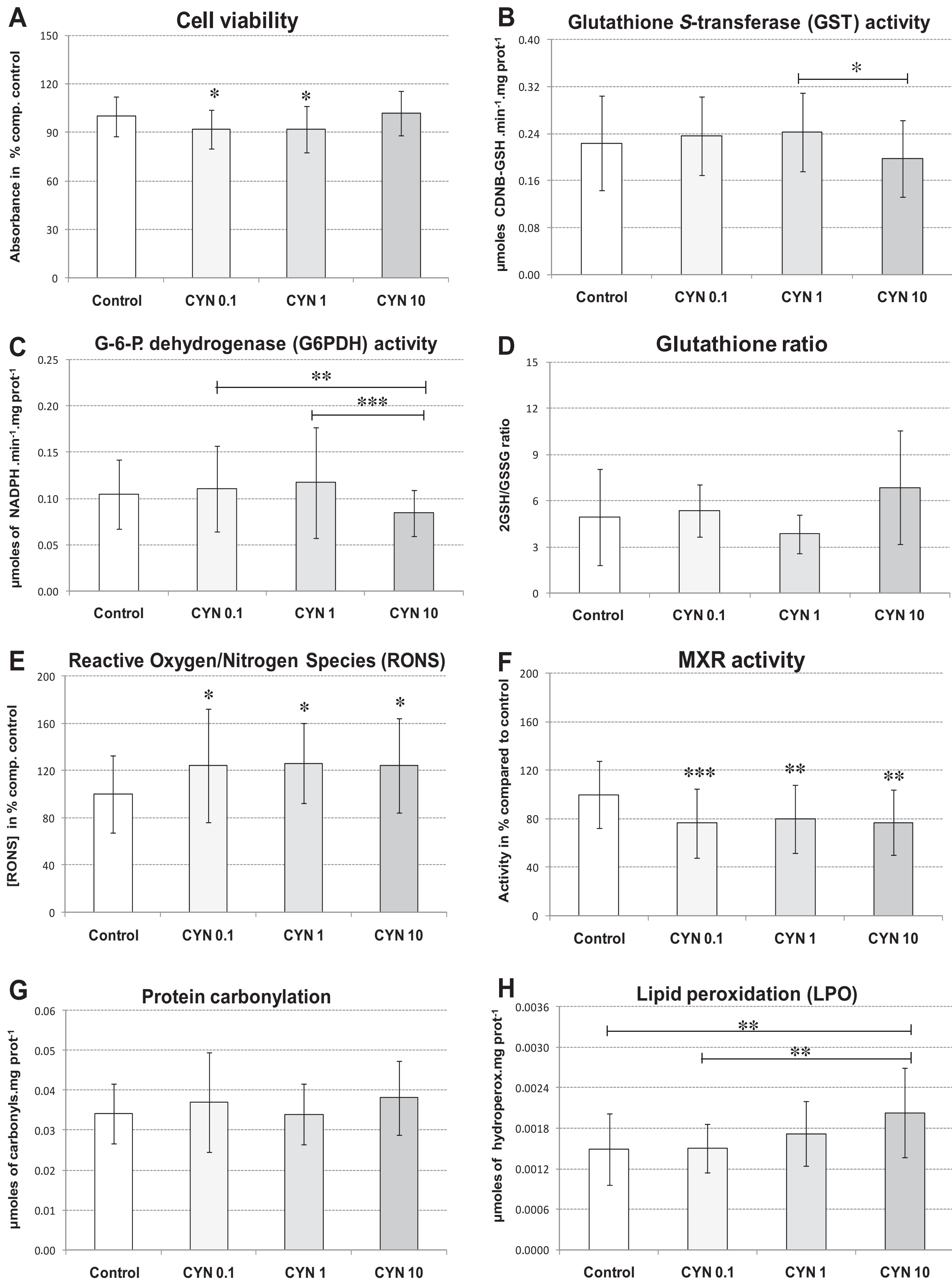

Fig. 3. Cell viability (A) in percentage compared to the control; GST activity (B) in micromoles of CDNB-GSH produced per min per mg of protein; activity of G6PDH (C) in micromoles of NADPH produced per min per mg of protein; glutathione ratio (D) in 2GSH/GSSG ratio; RONS (E) in percentage compared to the control; MXR activity (F) in percentage compared to the control; protein carbonylation (G) in micromoles of protein carbonyls per mg of protein; lipid peroxidation (H) in micromoles of hydroperoxides per mg of protein. Groups: control, 0.1, 1.0 and 10 µg l^{-1} of cylindrospermopsin. ANOVA followed by Tukey test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

there are few protocols for isolation of Neotropical fish hepatocytes (Bussolaro et al., 2010; Filipak Neto et al., 2006). In the current study, six isolation procedures with variations on the presence and type of protease were tested. Although two step perfusion

Table 2
Percentage of nucleoids with DNA damage classes (0, 1, 2, 3 and 4) and scores.

	0	1	2	3	4	Scores
Control	3.5 ± 3.1	5.5 ± 2.9	27.6 ± 15.1	52.2 ± 11.8	11.1 ± 8.1	261.9 ± 23.5
CYN 0.1	4.8 ± 5.2	7.2 ± 6.7	24.8 ± 8.8	52.2 ± 15.4	11.0 ± 5.5	257.4 ± 36.9
CYN 1.0	8.7 ± 7.7	7.6 ± 5.0	38.8 ± 14.6	37.5 ± 12.6	7.4 ± 9.0	227.1 ± 38.2
CYN 10	6.0 ± 3.3	7.0 ± 4.3	27.1 ± 6.5	50.2 ± 7.2	9.7 ± 5.3	251.1 ± 20.6

Scores = $\sum$ (damage class × percentage of incidence).

with a Ca^{2+} chelating agent such as EDTA and collagenase has been the most used protocol to obtain high yields of viable liver cells from different species of mammals and fishes (Naik et al., 2007; Yanhong et al., 2008), the protocol using dispase at 1 U ml^{-1} was the most efficient for *P. lineatus* hepatocyte isolation. Importantly, cell yield was enough to allow biochemical and other analyses to be performed with cells obtained from a single adult fish, although *P. lineatus* cell yield had been lower than that reported for other Brazilian teleosts, *H. malabaricus* (Filipak Neto et al., 2006) and *H. commersoni* (Bussolaro et al., 2010), probably due to interspecies differences in the degree of cell attachments. Additionally, incubation of liver pieces for an extended period of up to 3 h did not decrease cell viability. Concluding, the strong attachment of liver cells from *P. lineatus* made difficult to dissociate the hepatocytes comparatively with those two Neotropical fish species, and so the non-enzymatic protocol has not worked out.

Another important issue to be considered in hepatocyte primary culture is cell density, since it can affect the functioning and maintenance of hepatocyte viability and liver-specific functions (Nakamura et al., 1983; Hayashi and Ooshiro, 1986). For *P. lineatus* hepatocytes, 1.0×10^6 cells ml^{-1} of culture medium or 4×10^5 cells cm^{-2} cell culture flask/plate surface was the appropriated density for attachment and maintenance of viable cells for up to 7 days. Attachment was not improved by pretreatments of the culture plates as observed in phase contrast microscopy, and intercellular contacts were recreated with and without any pretreatment; these contacts are required for hepatocytes survival in vitro (Filipak Neto et al., 2006; Bussolaro et al., 2010).

4.2. Effects of cylindrospermopsin

Despite of the limit of 15 μg l^{-1} established by Brazilian Health Ministry in 2004 for cylindrospermopsin in water destined for human consumption (Brasil, 2005), recent studies have suggested lowest acceptable limits (<1 μg l^{-1}) based on subchronic oral mouse exposure (Falconer and Humpage, 2005, 2006). The high potential cytotoxicity means to be the reason for changing the present limit, but few studies have investigated it concerning aquatic vertebrates like fish. More specifically, studies focusing the effects of this compound to a target tissue such as the liver or the hepatocytes are scarce.

The current study established a primary hepatocyte culture model from *P. lineatus*, which was utilized to investigate the cellular responses for realistic concentrations of purified cylindrospermopsin. The reduced MXR activity found in this work showed that the first-line defense mechanism, responsible for efflux xenobiotics, toxins, drugs and endobiotic metabolites (Kurelec et al., 1992) was affected. Since cells normally respond to many forms of chemical stresses by increasing MXR activity in order to facilitate the efflux of toxic substances (Gottesman and Pastan, 1993), the decreased MXR activity of hepatocytes suggests an possible cellular accumulation of substances up to toxic levels. As a consequence, cellular sensitization to other endobiotic or xenobiotic stressors could disturb the cellular homeostasis. Then, hepatocytes exposed to cylindrospermopsin were significantly more sensitive and may succumb more rapidly to eventual exposure to other xenobiotics or metabolites that are substrates to some of these ABC transporters. The liver depends on this system for xenobiotic efflux, and sensitization of hepatic cells increases the potential of liver failure. Also, the establishment of MXR system as a biomarker in cultured hepatocytes represents a valuable tool for investigation of complex mixtures effects.

Higher production of reactive oxygen/nitrogen species (RONS) due to cylindrospermopsin exposure may increase the potential damage to biomolecules such as lipids, proteins and even DNA. Particularly, lipid peroxidation can alter membrane fluidity, permeability, transport and electric potential (Abuja and Albertini, 2001; Kühn and Borchert, 2002; Prieto et al., 2007). As reported in the present study, the increased lipid peroxidation on hepatocytes exposed to the highest cylindrospermopsin concentration in comparison to the control group did not seem to be the cause of cell death. Indeed, cells may support a slight lipid peroxidation due to the robust protective mechanisms present in hepatocytes that may be activated to maintain cell viability. Apparently, other defense mechanisms besides the glutathione *S*-transferase (one enzyme responsible for lipid hydroperoxides degradation) and the glucose 6-phosphate dehydrogenase may be involved in this finding, since there were no differences in these enzymes activities in relation to the control group.

Besides lipids, proteins and DNA are important targets of chemical stressors. Protein carbonylation and DNA breaks are common biomolecules damages that can significantly interfere with cell functioning. However, cylindrospermopsin exposure did not alter these biomarkers in *P. lineatus* hepatocytes. Then, cell-type and interspecific cylindrospermopsin toxicity differences may occur, since exposure of mammal cells to the same concentrations of cylindrospermopsin led to concentration-dependent DNA damage (Humpage et al., 2000; Lankoff et al., 2007).

The absence of protein and DNA damage are corroborated by unaltered levels of 2GSH/GSSG ratios. Consequently, there was not impairment of the synthesis and cycling of this important non-enzymatic antioxidant and cofactor for glutathione-dependent enzymes involved in xenobiotic biotransformation and peroxides degradation (Arteel and Sies, 2001; Van Bladeren, 2000). Then, although some authors reported that cylindrospermopsin decreased GSH concentrations in rat hepatocytes (Runnegar et al., 1995), the majority of studies on this issue indicate that impairment of GSH homeostasis is not the primary toxic mechanism of this toxin. Conversely, there is some data that indicate that biotransformation of cylindrospermopsin by cytochrome P450 may play a role in mammals (Norris et al., 2002).

Finally, the increase of both lipid peroxidation in the hepatocytes exposed to highest toxin concentration (10 μg l^{-1}) and RONS levels, and the decrease of cell viability in the two lowest concentrations (0.1 and 1 μg l^{-1}) as well as the decreased of MXR activity in all tested concentrations represent important findings that must be considered in the cylindrospermopsin toxicity. Particularly, the decreased MXR activity might have important consequences for cell survival due to accumulation of metabolites within cells. At the highest concentration, activation of other not investigated protective mechanisms by cylindrospermopsin may maintain the cell viability. However, we expect to observe different results if cells

were exposed to unpurified cylindrospermopsin extracts or to the toxin associated with xenobiotics, since this toxin may make *P. lineatus* hepatocytes sensitive to other chemicals.

In conclusion, the current study introduces the studies of cylindrospermopsin, an important toxin to Brazilian reservoirs, on primary cultured hepatocytes of Brazilian fish. Additionally, this work utilizes for the first time the activity of the MXR system as a 'new biomarker' in fish hepatocytes culture for investigation of cylindrospermopsin effects. The next step is to investigate if cylindrospermopsin can ease the effects of other xenobiotics in vitro. This is an important issue, since cyanobacteria proliferation is associated, at least in part, with the presence of other pollutants like urban dejects.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by IAEA (International Atomic Energy Agency) and CNPq (Brazilian Agency for Science and Technology). The authors acknowledge The Electron Microscopy Center of Federal University of Paraná for the technical support.

References

Abuja, P.M., Albertini, R., 2001. Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. Clin. Chim. Acta 306, 1–17.

Arteel, G.E., Sies, H., 2001. The biochemistry of selenium and the glutathione system. Environ. Toxicol. Pharmacol. 10, 153–158.

Bain, P., Shaw, G., Patel, B., 2007. Induction of p53-regulated genevexpression in human cell lines exposed to the cyanobacterial toxin cylindrospermopsin. J. Toxicol. Environ. Health 70, 1687–1693.

Benov, L., Szteinberg, L., Fridovich, I., 1998. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. Free Radic. Biol. Med. 25, 826–831.

Berry, J.P., 2010. First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, Lago Catemaco, and apparent bioaccumulation of the toxins in "tegogolo" snails (*Pomacea patula catemacensis*). Toxicon 55, 930–938.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brasil, 2005. Ministério da Saúde. Portaria n. 518 de 25 de Março de 2004, first ed., Editora do Ministério da Saúde, Brasília.

Bussolaro, D., Filipak Neto, F., Oliveira Ribeiro, C.A., Collins, A., Dusinská, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S., 2010. Responses of hepatocytes to DDT and methyl mercury exposure. Toxicol. In Vitro 24, 1491–1497.

Collins, A., Dusinská, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslová, K., Vaughan, N., 1997. Comet assay in human biomonitoring studies: reliability, validation, and applications. Environ. Mol. Mutagen. 30, 139–146.

Chong, M.W.K., Wong, B.S.F., Lam, P.K.S., Shaw, F.R., Seawright, A.A., 2002. Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon 40, 205–211.

Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J., 2000. Health risks caused by freshwater cyanobacteria in recreational waters. J. Toxicol. Environ. Health B Crit. Rev. 3, 323–347.

Falconer, I.R., Hardy, S.J., Humpage, A.R., Froscio, S.M., Tozer, G.J., Hawkins, P.R., 1999. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environ. Toxicol. 14, 143–150.

Falconer, I.R., Humpage, A.R., 2005. Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. Int. J. Environ. Res. Public. Health 2, 43–50.

Falconer, I.R., Humpage, A.R., 2006. Cyanobacterial (blue-green algal) toxins in water supplies: cylindrospermopsins. Environ. Toxicol. 21, 299–304.

Fastner, J., Heinze, R., Humpage, A.R., Mischke, U., Eaglesham, G.K., Chorus, I., 2003. Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates. Toxicon 42, 313–321.

Fent, K., 2001. Fish cells lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P450 A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol. In Vitro 15, 477–488.

Filipak Neto, F., Zanata, S.M., Randi, M.A.F., Pelletier, É., Oliveira Ribeiro, C.A., 2006. Hepatocytes primary culture from the Neotropical fish trahira *Hoplias malabaricus* (Bloch, 1794). J. Fish Biol. 69, 1524–1532.

Filipak Neto, F., Zanata, S.M., Silva De Assis, H.C., Bussolaro, D., Ferraro, M.V.M., Randi, M.A.F., Alves Costa, J.R.M., Cestari, M.M., Roche, H., Oliveira Ribeiro, C.A., 2007. Use of hepatocytes from *Hoplias malabaricus* to characterize the toxicity of a complex mixture of lipophilic halogenated compounds. Toxicol. In Vitro 21, 706–715.

Fontenele, O., 1953. Contribuição para o Conhecimento da Biologia da Curimatã Pacu, "*Prochilodus argenteus*", Spix in Spix & Agassiz (Pisces: Characidae, Prochilodinae). Rev. Bras. Biol. 13, 87–102.

Froscio, S.M., Humpage, A.R., Wickramasinghe, W., Shaw, G., Falconer, I.R., 2008. Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. Toxicon 51, 191–198.

Froscio, S.M., Cannon, E., Lau, H.M., Humpage, A.R., 2009. Limited uptake of the cyanobacterial toxin cylindrospermopsin by Vero cells. Toxicon 54, 862–868.

Glock, G.E., Mclean, P., 1953. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. J. Biochem. 55, 400–408.

Gottesman, M.M., Pastan, I., 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. 62, 385–427.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.

Hawkins, P.R., Runnegar, M.T.C., Jackson, A.R.B., Falconer, I.R., 1985. Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenava and SubbaRaju isolated from a domestic water-supply reservoir. Appl. Environ. Microbial. 50, 1292–1295.

Hawkins, P.R., Chandrasena, N.R., Jones, G.J., Humpage, A.R., Falcone, I.R., 1997. Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. Toxicon 35, 341–346.

Hayashi, S., Ooshiro, Z., 1986. Primary culture of the eel hepatocytes in the serum-free medium. Nippon Suisan Gakk 52, 1641–1651.

Humpage, A.R., Fenech, M., Thomas, P., Falconer, I.R., 2000. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutat. Res. 472, 155–161.

Humpage, A.R., Fontaine, F., Froscio, S., Burcham, P., Falconer, I.R., 2005. Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress, J. Toxicol. Environ. Health A 68, 739–753.

Jensch-Junior, B.E., Pressinotti, N., Borges, J.C.S., Silva, J.R.M.C., 2005. Characterization of macrophage phagocytosis of the tropical fish *Prochilodus scrofa* (Steindachner, 1881). Aquaculture 251, 509–515.

Jiang, Z.Y., Hunt, J.V., Wolv, S.P., 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal. Biochem. 202, 384–389.

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the glutathione S-transferases. J. Biol. Chem. 251, 6183–6188.

Kühn, H., Borchert, A., 2002. Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. Free Radic. Biol. Med. 33, 154–172.

Kurelec, B., Lankoff, A., Wojcik, A., Lisowska, H., Bialczyk, J., Dziga, D., Carmichael, W., 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22, 23–43.

Lankoff, A., Wojcik, A., Lisowska, H., Bialczyk, J., Dziga, D., Carmichael, W., 2007. No induction of structural chromosomal aberrations in cylindrospermopsin-treated CHO-K1 cells without and with metabolic activation. Toxicon. 50, 1105–1115.

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233, 346–357.

Naik, B.M., Gay, A.N., Zhu, X., Yu, L., Cass, D.L., Olutoye, O.O., 2007. Age-dependent recruitment of neutrophils by fetal endothelial cells: implications in scarless wound healing. J. Pediatr. Surg. 42, 166–171.

Nakamura, T., Tomita, Y., Ichihara, A., 1983. Density-dependent growth control of adult rat hepatocytes in primary culture. J. Biochem. 94, 1029–1035.

Neumann, C., Bain, P., Shaw, G., 2007. Studies of the comparative in vitro toxicology of the cyanobacterial metabolite deoxycylindrospermopsin, J. Toxicol. Environ. Health A 70, 1679–1686.

Norris, R.L.G., Seawright, A.A., Shaw, G.R., Senogles, P., Eaglesham, G.K., Smith, M.J., Chiswell, R.K., Moore, M.R., 2002. Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon 40, 471–476.

Pessatti, M.L., Resgalla, J.C., Reis, F. R.W., Kuehn, J., Salomão, L.C., Fontana, J.D., 2002. Variability of rates of filtration, respiration and assimilation and multixenobiotic mechanism resistance (MXR) of mussel *Perna perna* under lead influence. Braz. J. Biol. 62, 651–656.

Prieto, A.I., Pichardo, S., Jos, A., Moreno, I., Cameán, A.M., 2007. Time-dependent oxidative stress responses after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (*Oreochromis niloticus*) under laboratory conditions. Aquat. Toxicol. 84, 337–345.

Quinlan, G.J., Gutteridge, J.M.C., 2000. Carbonyl assay for oxidative damage to proteins. In: Taniguchi, N., Gutteridge, J.M.C. (Eds.), Experimental protocols for reactive oxygen and nitrogen species. Oxford University, New York, pp. 257–258.
Rao, P.V., Gupta, N., Bhaskar, A.S., Jayaraj, R., 2002. Toxins and bioactive compounds from cyanobacteria and their implications on human health. J. Environ. Biol. 23, 215–224.
Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Lu, S.C., 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem. Pharmacol. 49, 219–225.
Sedlak, J., Lindsay, R.H., 1968. Estimation of total protein bound and nonprotein sulphydril groups in tissues with Ellman's reagent. Anal. Biochem. 25, 192–205.
Segner, H., 1998. Isolation and primary culture of teleost hepatocytes. Comp. Biochem. Physiol. 120A, 71–81.
Singh, N.P., Mccoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Terao, K., Ohmori, S., Igarashi, K., 1994. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 32, 833–843.
Van Bladeren, P.J., 2000. Glutathione conjugation as a bioactivation reaction. Chem. Biol. Interact. 129, 61–76.
Winkaler, E.U., Santos, T. R.M., Machado-Neto, J.G., Martinez, C. B.R., 2007. Acute lethal and sublethal effects of neem leaf extract on the neotropical freshwater fish *Prochilodus lineatus*. Comp. Biochem. Phys. C 145, 236–244.
Yanhong, F., Chenghua, H., Guofang, L., Haibin, Z., 2008. Optimization of the isolation and cultivation of *Cyprinus carpio* primary hepatocytes. Cytotechnology 58, 85–92.
Zucco, F., Angelis, I., Testai, E., Stammati, A., 2004. Toxicology investigations with cell culture systems: 20 years after. Toxicol. In Vitro 18, 153–163.