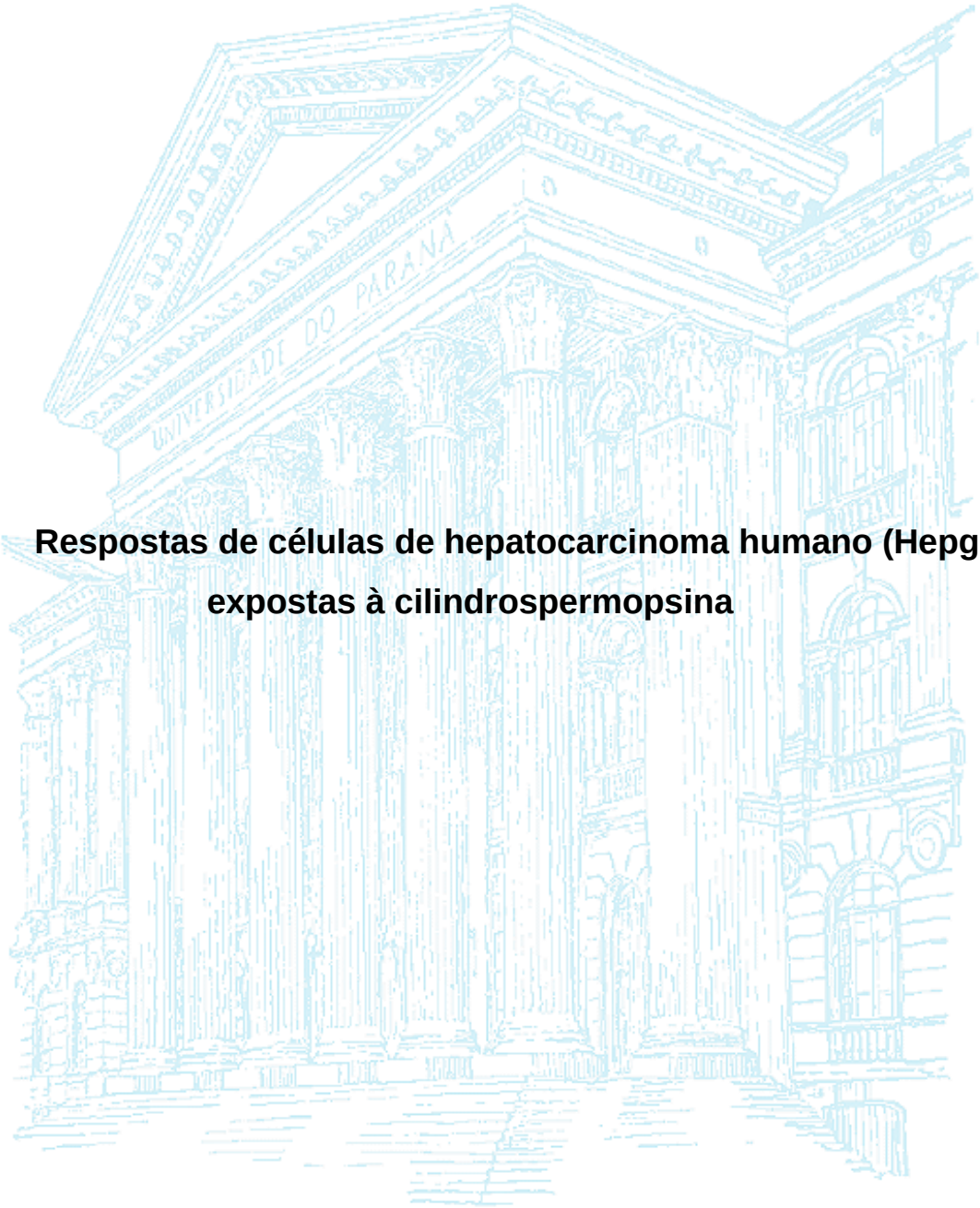
UNIVERSIDADE FEDERAL DO PARANÁ

SAMUEL LIEBEL



Respostas de células de hepatocarcinoma humano (Hepg2) expostas à cilindrospermopsina

CURITIBA

2015

SAMUEL LIEBEL

Respostas de células de hepatocarcinoma humano (Hepg2) expostas à cilindrospermopsina

Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, Departamento de Biologia Celular, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito parcial à obtenção de título de Doutor em Biologia Celular e Molecular.

Orientador: Dr. Francisco Filipak Neto

Coorientador: Dr. Ciro Alberto de Oliveira Ribeiro

CURITIBA

2015

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Departamento de Biologia Celular e Departamento de Fisiologia
Setor de Ciências Biológicas - Universidade Federal do Paraná
Instituto Carlos Chagas (ICC/FIOCRUZ)

PARECER

A banca examinadora, instituída pelo colegiado do Programa de Pós-Graduação em Biologia Celular e Molecular, do Setor de Ciências Biológicas, da Universidade Federal do Paraná, composta por:

Prof. Dr. Francisco Filipak Neto
Orientador e presidente da banca
Universidade Federal do Paraná - UFPR

Prof. Dr. Rubens Bertazolli Filho
Universidade Federal do Paraná - UFPR

Prof. Dr. Daniel Bussolaro
Instituto Federal do Paraná - IFPR

Profa. Dra. Maria Eliane Merlim Rocha
Universidade Federal do Paraná – UFPR

Prof. Dr. Marcos Luiz Pessatti
Universidade do Vale do Itajaí - UNIVALI

E tendo como suplentes,

Prof. Dr. Edvaldo da Silva Trindade
Universidade Federal do Paraná - UFPR

Profa. Dra. Lia Sumie Nakao
Universidade Federal do Paraná - UFPR

Após arguir o candidato ***Samuel Liebel,*** em relação ao seu trabalho intitulado: "Respostas de células de hepatocarcinoma humano (HepG2) expostas à cilindrospermopsina", são de parecer favorável à...APROVAÇÃO..... do acadêmico, habilitando-o ao título de Doutor em Biologia Celular e Molecular, área de concentração em Biologia Celular e Molecular.
A obtenção do título está condicionada à implementação das correções sugeridas pelos membros da banca examinadora, bem como ao cumprimento integral das exigências estabelecidas no Regimento Interno deste Programa de Pós-Graduação.

Curitiba, 27 de Fevereiro de 2015.

Prof. Dr. Ciro Alberto de Oliveira Ribeiro
Coord. do Programa de Pós-Graduação
Em Biologia Celular e Molecular
Matr./UFPR 104132

AGRADECIMENTOS

Ao Prof.º Dr. Francisco (Chico) mais que um orientador, um amigo, pela confiança no meu trabalho, pela dedicação em transmitir seus conhecimentos, e por sempre estar pronto para ajudar no que for necessário;

Ao Prof.º Dr. Ciro, por abrir as portas do laboratório possibilitando o meu ingresso na vida científica. Agradeço pela sua disponibilidade, orientação e paciência sempre demonstrada;

A Prof.ª Sônia, uma amiga integra e generosa, pela ajuda com as técnicas de imunofluorescência, sempre disposta a colaborar de forma irrefutável no que for preciso.

Aos Professores Rubens, Cláudia e Luiza, por terem contribuído com suas avaliações ano a ano durante os eventos dos doutorandos, contribuindo com o enriquecimento do projeto e obtenção dos resultados.

A minha estimada família, por sempre me apoiar e incentivar, pelo seu exemplo e por não medir esforços em contribuir nesta trajetória, em especial a minha esposa Sandra, que esteve sempre ao meu lado, por todo seu amor, carinho, respeito, compreensão, paciência, e por me transmitir força e coragem para superar os desafios;

Aos amigos do laboratório de Toxicologia celular, Inês, Andressa, Daniele, Dandie, Ana, Flávia, Renata, Yvanna, Marcelle, Ludi, Eduardo, Gis, Mayla, Débora entre outros, por poder contar com vocês sempre que precisei. Obrigado pelo apoio, e também por compartilharem os sorrisos e as brincadeiras;

Um agradecimento especial a Andressa, Renata, Ludi, Marcelle, Yvanna, colegas do cultivo celular, obrigado por compartilharem os momentos de sucesso e insucesso que a cultura celular proporciona.

A Dani pela ajuda no processamento das amostras para obtenção dos géis 2D.

A Prof.ª Valeria e ao Rodrigo pelo fornecimento da toxina purificada utilizada neste estudo.

Ao Prof.º Edvaldo por ceder as alíquotas de anticorpo utilizadas neste estudo.

Ao Prof.º Emanuel, e a Caroline e o Gláucio, pelo suporte na realização das analises com espectrometria de massa.

Ao Israel pelo ajuda na obtenção das imagens no microscópio de alta resolução confoccal.

Ao Programa de Pós Graduação em Biologia Celular e Molecular (Coordenação, professores, secretaria), por dar o suporte e possibilitar a conclusão do meu doutorado.

Ao Conselho Nacional Científico Tecnológico (CNPQ) e a CAPES pelo suporte na realização do trabalho.

A Deus, por estar sempre presente na minha vida e no meu coração;

Enfim, a todos aqueles que de alguma forma contribuíram com a realização deste trabalho.

RESUMO

O aumento das atividades antropogênicas vem sendo causa de uma crescente eutrofização dos ambientes aquáticos, levando a floração de cianobactérias, dentre as quais algumas produtoras de cianotoxinas potencialmente tóxicas à vida animal e à saúde humana. Desta forma, fazem-se necessárias a avaliação dos principais danos causados pelas toxinas assim como a investigação os mecanismos de toxicidade da cilindrospermopsina (CYN) em células. Particularmente, modelos *in vitro* empregando a linhagem de hepatocarcinoma humano (HepG2) são importantes para a investigação dos mecanismos de toxicidade das cianotoxinas nas células. O objetivo do presente estudo foi investigar as respostas de células HepG2 frente à exposição a diferentes concentrações da cilindrospermopsina (CYN) através de ensaios de viabilidade celular; atividade das enzimas de biotransformação de fase II (Glutationa *S*-transferase - GST)*;* sistema de resistência a multidrogas (MDR); ambiente redox celular, quantificação da produção de espécies reativas de oxigênio e nitrogênio; danos oxidativos a biomoléculas; e alterações na morfologia das células. Além disso, determinou-se se a indução de isoformas do citocromo P450 aumenta a sensibilidade das células à CYN e avaliou-se a expressão diferencial de proteínas (proteoma). A CYN não se apresentou tóxica na concentração de 10 µg l^{-1}, levando a um aumento da viabilidade e toxicidade celular em células cultivadas com 10% de soro bovino fetal (SBF). A redução da concentração de SBF para 2% e a indução das isoformas do citocromo P450 (CYP) tornaram o metabolismo das células HepG2 mais próximas das células “normais”. Após a indução, poucos parâmetros foram alterados, como a peroxidação lipídica. No entanto, baixas concentrações de CYN (inferior ou igual a 10 µg l^{-1}) induziram um aumento da proliferação e toxicidade celular. Analisando o proteoma das células expostas a CYN, identificaram-se 26 proteínas, as quais estão envolvidas em diferentes processos biológicos como enovelamento de proteínas, efluxo de xenobióticos, defesa antioxidante, metabolismo energético, anabolismo, sinalização, potencial tumorigênico e estrutura do citoesqueleto. Este perfil de proteínas indica que a exposição à CYN leva a um aumento da absorção e oxidação da glicose para proporcionar energia às células responder ao estresse químico. Aumento da proteína G (GPCRs), *heterogeneous nuclear ribonucleoprotein* (hnRNP) e dos níveis das espécies reativas de oxigênio (EROs) podem estar provocando o aumento da proliferação celular, e aumento do potencial tumorigênico das células HepG2. O excesso de espécies reativas de oxigênio ativou mecanismos de proteção celular como a *multidrug resistance protein* (MRP3) e a glutationa peroxidase. As alterações provocadas pela CYN nas proteínas do citoesqueleto também podem estar associadas com a proliferação celular bem como a reposição das proteínas danificadas pelas EROs.

Palavras-chave: cianotoxina, cilindrospermopsina, HepG2, CYPs, proteômica, imunofluorescência

ABSTRACT

The increase of anthropogenic activities has been causing eutrophication of aquatic environments, leading to bloom of cyanotoxin-producer cyanobacteria that can thread wildlife and human health. The evaluation of the toxic effects and the mechanisms of toxicity of cyanotoxins such as cylindrospermopsin (CYN) are important issues in toxicology. For these purposes, cell lines such as human hepatocellular carcinoma (HepG2) are very useful *in vitro* models. The aim of the current study was to investigate the responses of HepG2 cells after exposure to different concentrations of CYN through assays of cell viability; activities of glutathione S-transferase (GST, a phase II biotransformation enzyme) and the efflux transporters involved in multidrug resistance phenotype (MDR); oxygen and nitrogen reactive species levels; oxidative damage to biomolecules; and alterations of cell morphology. In addition, the possibility of increased toxicity to CYN due to cytochrome P450 (CYP) induction and the differential expression of proteins (proteome) have been investigated. CYN at 10 μg l^{-1} was not toxic to HepG2 cells, leading to increased cell viability and metabolism in cells cultured with 10% fetal bovine serum (FBS). Decrease of FBS concentration to 2% and induction of CYP isoforms have made HepG2 cells more sensitive to exposure to CYN. After induction, few parameters have changed, e.g., lipid peroxidation. However, CYN at low concentrations (bellow or equal 10 μg l^{-1}) has induced increases of cell proliferation and metabolism. A total of 26 proteins have been differentially expressed in CYN-exposed cells *versus* control. These proteins are involved in different biological processes like protein folding, xenobiotic efflux, antioxidant defense, energy metabolism, anabolism, cell signaling, tumorigenic potential and cytoskeleton structure. This protein profile indicates that the exposure to CYN leads to an increased absorption and breakdown of glucose as to provide energy for the cells to respond to chemical stress. Increased G protein (GPCRs), nuclear ribonucleoprotein heterogeneous (hnRNP) and the levels of reactive oxygen species (ROS) may be associated with increased cell proliferation, and increased tumorigenic potential of HepG2 cells. The excess ROS has activated cellular protection mechanisms such as MRP3 and glutathione peroxidase. The changes caused by CYN in cytoskeletal proteins may also be associated with cell proliferation as well as replacement of proteins damaged by ROS.

Keywords: cyanotoxin, Cylindrospermopsin, HepG2, CYP, proteomics, immunofluorescence

SUMÁRIO

1. INTRODUÇÃO

O enriquecimento da água com nutrientes provenientes de esgoto urbano, agropecuária e indústria, assim como o despejo impróprio de resíduos sólidos têm afetado consideravelmente os ambientes aquáticos. Como resultado, essa contaminação ambiental leva à eutrofização dos ecossistemas atingidos e, consequentemente, ao crescimento acelerado de organismos fotossintéticos simples como as cianobactérias. As cianobactérias conseguem sobreviver em ambientes eutrofizados e até inóspitos a organismos estruturalmente mais complexos, o que tem levado à contaminação de ecossistemas aquáticos próximos às cidades e de grandes reservas de água destinadas ao abastecimento público das cidades e devido à ocorrência de proliferação descontrolada destes microrganismos, caracterizando as florações (DE LA CRUZ, *et al.*, 2013; MERAL *et al.*, 2013).

Como certas espécies de cianobactérias produzem toxinas de grande potencial tóxico para os organismos aquáticos e para as populações humanas que são supridas com essa água ou que se alimentam destes organismos (RAO *et al.*, 2002; BERRY, 2010). Há a necessidade de se conhecer os mecanismos de toxicidade das cianotoxinas a fim de se estabelecer limites de segurança, cada vez mais discutíveis, assim como conhecer as espécies que estariam em risco e até estabelecer tratamentos no caso de exposição ou intoxicação com as cianotoxinas. Vários gêneros de cianobactérias como *Microcystis*, *Anabaena, Nodularia, Oscillatoria, Planktothrix, Cylindrospermopsis* e *Nostoc* produzem toxinas (MOLICA e AZEVEDO, 2009), as quais têm sido caracterizadas de acordo com o órgão-alvo ou

sistema-alvo em mamíferos, sendo as hepatotoxinas como a cilindrospermopsina (CYN) um grupo importantíssimo e de interesse no presente projeto.

A cilindrospermopsina tem uma distribuição generalizada, tanto em zonas tropicais quanto temperadas (HUSZAR *et al.*, 2000; PONIEDZIALEK *et al.*, 2012.), é um alcaloide hepatotóxico guanidínico cíclico altamente solúvel em água (Figura 1), cujas informações sobre propriedades tóxicas são escassas comparadas com outras toxinas como a microcistina. No entanto, a estabilidade desta substância é bem conhecida, resistindo a altas temperaturas (não degradada a 100 °C por 15 min) e a vários valores de pH (25% degradada em valores de pH 4, 7 e 10 somente após 8 semanas; MASTEN e CARSON, 2000), o que aumenta o risco de exposição dos organismos nos ambientes aquáticos, onde condições mais amenas de temperatura e pH permitem a persistência da cilindrospermopsina por tempo prolongado.

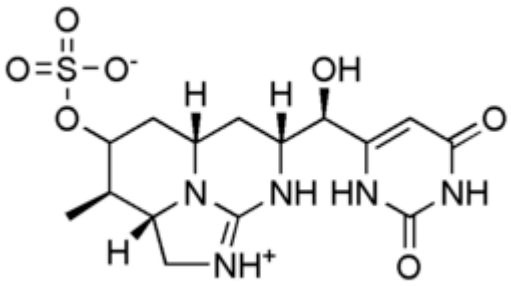


FIGURA 1 – Estrutura química da cilindrospermopsina ($C_{15}H_{21}N_5O_7S$, massa molecular = 415,43 Da). (FONTE: MOLICA e AZEVEDO, 2009)

A cilindrospermopsina foi identificada pela primeira vez após a sua implicação como agente causador de um grave surto de hepatoenterites em seres humanos de *Palm Island,* Austrália, em 1979 (HAWKINS *et al*., 1985). No Brasil, o caso mais conhecido foi à intoxicação de pacientes em uma clínica de hemodiálise

em Pernambuco em 1996. Neste caso, os pacientes tratados na clínica apresentaram perturbações visuais, náuseas e vômitos após o tratamento de hemodiálise de rotina; dos 131 pacientes, 100 desenvolveram insuficiência hepática aguda, sendo registradas posteriormente 76 mortes como consequência da exposição. Investigações na unidade de tratamento de água da clínica detectaram contaminação dos filtros por dois tipos de cianobactérias produtoras das toxinas microcistina e cilindrospermopsina (CARMICHAEL *et al*., 2001).

O limite aceitável de cilindrospermopsina em água potável estabelecido pelo Ministério da Saúde foi reduzido de 15 $\mu g.l^{-1}$ para 1 $\mu g.l^{-1}$ (BRASIL, 2011). No entanto, alguns pesquisadores como FALCONER e HUMPAGE (2005 e 2006) relatam que ainda não há dados suficientes para se definir um limite máximo admissível para a cilindrospermopsina em água destinada ao consumo humano. Segundo estes, com base nos dados provenientes da exposição oral subcrônica em camundongos, o valor não deveria passar de 1 $\mu g.l^{-1}$, valor que já vinha sendo adotado como máximo aceitável por outros países, como na Austrália, antes da alteração de limite no Brasil. O alto potencial citotóxico pode ser a razão para a alteração do limite atual, mas poucos estudos têm focado nos efeitos da cilindrospermopsina em órgãos-alvo como o fígado ou em células-alvo como os hepatócitos.

Toxicologicamente, a cilindrospermopsina é um potente inibidor da síntese de proteínas eucarióticas (FROSCIO *et al.*, 2009), sendo o fígado um dos principais órgãos-alvo (GRIFFITHS e SAKER, 2003; HUMPAGE e FALCONER, 2003), embora o comprometimento renal, hemorragias nos pulmões e no coração, assim como atrofia do timo e necrose no baço também tenham sido relatados (SEAWRIGHT *et*

al., 1999; BERNARD *et al.*, 2003). Além disso, efeitos genotóxicos, ativação do sistema de biotransformação mediado por monooxigenases mistas do citocromo P450 (CYP), redução da síntese de glutationa (GSH) e desregulação endócrina têm sido relatados em células de mamíferos (HUMPAGE *et al.*, 2005; BAIN *et al.*, 2007; FROSCIO *et al.*, 2009). Estes efeitos reforçam a importância da realização de estudos visando desvendar os mecanismos envolvidos com as respostas celulares e toxicidade da cilindrospermopsina, visto que os estudos não relatam, satisfatoriamente, como essas alterações são geradas do ponto de vista molecular.

Os organismos dispõem de duas vias principais de eliminação de agentes tóxicos, a excreção na sua forma química original e a biotransformação que pode levar a destoxificação ou bioativação do composto (VAN DER OOSTER *et al.*, 2003). Além disso, a biotransformação pode determinar parcialmente a taxa de eliminação dos compostos tóxicos das células através de sistemas de transporte transmembrânicos que, em alguns casos, reconhecem apenas os produtos da biotransformação de contaminantes e xenobióticos. Estes transportadores desempenham um papel-chave no fenótipo de resistência a multidrogas, MDR (BARD, 2000). Na célula, um dos principais sistemas envolvidos no processo de biotransformação de xenobióticos é o citocromo P450 (CYP), pertencente à fase I – funcionalização - do metabolismo de drogas e xenobióticos. Vários trabalhos descrevem que a toxicidade da cilindrospermopsina é mediada pela atividade deste complexo enzimático (NORRIS *et al.*, 2002; FROSCIO *et al.*, 2003; HUMPAGE *et al.*, 2005). Neste aspecto, estudos têm relatado que inibidores do citocromo P450 diminuem a toxicidade da cilindrospermopsina, reforçando a ideia de que esta cianotoxina é bioativada por este sistema nos modelos biológicos analisados, o que

pode estar contribuindo para a citotoxicidade hepática (WIEGAND e PFLUGMACHER, 2005; LANKOFF, *et al.*, 2007; PUERTO, *et al.*, 2011; ZEGURA *et al.*, 2011). No entanto, não foi esclarecido quais isoformas do citocromo P450 estão envolvidas, assim como as possíveis vias de biotransformação pela glutationa S-transferase (GST) ou outras enzimas de fase II (conjugação).

Estudos prévios verificaram que hepatócitos do teleósteo *Prochilodus lineatus* expostos à cilindrospermopsina apresentam um aumento nos níveis de espécies reativas de oxigênio (EROs) e nitrogênio além da diminuição da atividade de transportadores de efluxo de xenobióticos envolvidos com o fenótipo MDR, com a existência de riscos importantes à homeostasia celular, corroborada pela diminuição da viabilidade celular nos grupos expostos às menores concentrações da cianotoxinas (0,1 e 1 μg l^{-1}) (LIEBEL *et al.*, 2011). Contudo, na maior concentração (10 μg l^{-1}) de cilindrospermopsina, ocorreu a manutenção da viabilidade celular devido à ativação de mecanismos de defesa celulares, que não foram identificados no trabalho (LIEBEL *et al.*, 2011). Estes dados despertam a curiosidade e necessidade de se conhecer um pouco melhor o mecanismo de toxicidade da cilindrospermopsina e de *hormesis,* caso está de fato ocorra, particularmente porque esta toxina pode ser encontrada em reservatórios destinados ao abastecimento de água para humanos. Desta forma, o objetivo principal do trabalho proposto neste estudo é dar continuidade aos estudos com a cilindrospermopsina, para a qual encontramos resultados muito interessantes e importantes do ponto de vista toxicológico, através do emprego de modelos *in vitro*. Os estudos ao nível celular complementam de forma significativa os dados obtidos com os estudos *in vivo*, sobretudo quando se empregam culturas primárias (BAKSI e FRAZIER, 1990;

SEGNER, 1998). Contudo, as limitações e dificuldades em obter e cultivar células recém-isoladas e a pressão para a redução do emprego de animais inviabiliza, em muitos casos, estudos com algumas finalidades específicas.

Neste contexto, o uso de linhagens celulares permite contornar estes obstáculos, fornecendo resultados adequados para o estabelecimento do modo de ação e dos mecanismos de toxicidade de diferentes xenobióticos, inclusive em células de origem humana.

Dentre os vários tipos de células de vertebrados, os hepatócitos são provavelmente as mais versáteis metabolicamente, controlando e participando de muitos dos eventos responsáveis pela manutenção da homeostase em vertebrados (SEGNER, 1998), além de constituir importante sítio de biotransformação e alvo para ação da cilindrospermopsina. Para a realização do presente estudo, a linhagem celular derivada de hepatoma humano HepG2 foi selecionada, por ser um modelo bastante empregado na literatura científica em estudos do metabolismo de xenobióticos e toxicidade hepática, uma vez que estas células mantêm muitas das funções especializadas de hepatócitos humanos normais (KNASMULLER *et al.*, 2004;. MERSCH-SUNDERMANN *et al.*, 2004). Em particular, as células HepG2 retêm a atividade de muitas enzimas de fase I e II, embora em níveis mais baixos do que hepatócitos humanos recém isolados (RUEFF *et al.* 1996, WALTER *et al.*, 2007). Além disso, as células HepG2 têm níveis mais baixos de NADPH-citocromo P450 redutase e do citocromo b5 quando comparado com o células presentes no fígado humano (RODRIGUEZ-ANTONA *et al.*, 2002, YOSHITOMI *et al.*, 2001). Assim, alguns autores têm sugerido a necessidade de estimular a atividade de receptores nucleares (AhR (*aryl hydrocarbon receptor*), PXR (*Pregnane X receptor*)

e CAR (*constitutive androstane receptor*)) empregando ativadores (WESTERINK & SCHOONEN, 2007a, b) ou o tratamento com indutores seletivos de CYPs, tais como β-naftoflavona, rifampicina, fenobarbital e etanol (MATSUDA *et al.*, 2002, LIU *et al.*, 2009, BOEHME *et al.*, 2010) antes de testes de citotoxicidade, o que exige uma interpretação mais cuidadosa dos resultados. Logo, a exposição das células HepG2 a várias concentrações de cilindrospermopsina permitem uma varredura das várias alterações de metabolismo possíveis (dados apresentados no capítulo I) e direcionam uma investigação mais específica do mecanismo pelo qual se dá sua toxicidade e as respostas celulares (dados apresentados no capítulo II).

2. OBJETIVOS

2.1 Objetivo Geral

Investigar as respostas de células HepG2 frente a exposição à cilindrospermopsina.

2.2 Objetivos Específicos

- Avaliar os efeitos de diferentes concentrações da cilindrospermopsina sobre a viabilidade celular; sobre as atividades das enzimas de biotransformação de fase II (Glutationa *S*-transferase - GST)*;* sobre o sistema de efluxo de xenobióticos; sobre o ambiente redox celular através da quantificação da produção de espécies reativas de oxigênio e nitrogênio, e danos oxidativos a biomoléculas;
- Determinar se a indução de isoformas do citocromo P450 aumenta a sensibilidade das células à cilindrospermopsina, o que seria um indício de bioativação;
- Avaliar as respostas das células expostas a cilindrospermopsina após a indução com fenobarbital, a fim de se determinar os efeitos da ativação do sistema P450.
- Avaliar a expressão diferencial de proteínas (proteoma) nos hepatócitos expostos a uma das concentrações da cilindrospermopsina selecionada com base nos resultados provenientes dos ensaios supramencionados;
- Determinar as possíveis alterações na morfologia das células utilizando imunofluorescência.

CAPÍTULO I

Low concentrations of cylindrospermopsin induce increases of reactive oxygen species levels, metabolism and proliferation in human hepatoma cells (HepG2)

Toxicology in Vitro 29 (2015) 479–488

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Low concentrations of cylindrospermopsin induce increases of reactive oxygen species levels, metabolism and proliferation in human hepatoma cells (HepG2)

Samuel Liebel [a,*,1], Ciro Alberto de Oliveira Ribeiro [a], Valéria Freitas de Magalhães [c], Rodrigo de Cássio da Silva [c], Stéfani Cibele Rossi [b], Marco Antônio Ferreira Randi [a], Francisco Filipak Neto [a,*,1]

[a] Departamento de Biologia Celular, Universidade Federal do Paraná, Cx. Postal 19031, CEP 81.531-990, Curitiba, PR, Brazil
[b] Departamento de Farmacologia, Universidade Federal do Paraná, Cx. 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

ARTICLE INFO

Article history:
Received 29 May 2014
Accepted 27 December 2014
Available online 6 January 2015

Keywords:
Cyanotoxin
Cylindrospermopsin
Cell lineage
HepG2
CYPs

ABSTRACT

Human hepatoma cells (HepG2) were exposed to purified cylindrospermopsin (CYN), a potent toxicant for eukaryotic cells produced by several cyanobacteria. CYN was not toxic at concentrations up to 10 μg l^{-1}, leading to increased viability and metabolism in cells cultured with 10% fetal bovine serum (FBS). Reduction of FBS concentration to 2% and induction of cytochrome P450 (CYP) isoforms were performed in order to make xenobiotic-metabolizing capacity of HepG2 cells closest to that of 'normal' cells. HepG2 cells proliferated less after CYPs-induction, and this induction has lead to similar results of non-induced cells, except for few individual parameters such lipid peroxidation. Foremost, low concentrations of CYN (below or equal 10 μg l^{-1}) have induced HepG2 cells proliferation and metabolism increase, which was not expected.

1. Introduction

Eutrophication of water bodies due to the water nutrients enrichment may lead to cyanobacteria blooms, typical on countries with poor domestic sewage treatment such as Brazil. Several cyanobacteria produce a diverse array of toxic metabolites that can pose a serious threat to humans and aquatic organisms due to contamination of water and food (Berry and Lind, 2010; Rao et al., 2002). Among these cyanotoxin cylindrospermopsin (CYN) has been recognized as a potent eukaryotic protein synthesis inhibitor (Froscio et al., 2008; Terao et al., 1994), genotoxic, activation of different isoforms of cytochrome P450 (CYPs) or even the reduction of glutathione synthesis as reported for mammal cells (Bain et al., 2007; Froscio et al., 2009; Humpage et al., 2005; Neumann et al., 2007). Among the target organs to CYN the liver has been described as the most important, but the effects also occur in the heart, thymus, spleen and kidneys (Falconer and Humpage, 2006; Hawkins et al., 1985).

Cylindrospermopsin effects determination is of great concern for human health, since this toxin is present in eutrophicated freshwater bodies, including reservoirs for water supply to human population. According to Who (2011) the deficit of people without access to clear or treated water even for drinking is still considerable in the world, making the knowledge about the potential effects of this toxins essential to understand the risk of exposure. In Brazil, the Health Ministry has diminished the established limit of CYN for drinking water from 15.0 μg l^{-1} to 1.0 μg l^{-1} as a destined for public supply (Brasil, 2011). However, values lower than 1 μg l^{-1} has been suggested by Falconer and Humpage (2005, 2006). In a previous study a decreases of cell viability and multidrug resistance transporters activity on primary cultured fish hepatocytes exposed to CYN (1–10 μg l^{-1}) were described (Liebel et al., 2011). Additionally, the same authors showed an increase of reactive oxygen and nitrogen species concentration related with significant lipid peroxidation in the same cells. These data brought up the question of whether relative low concentrations of CYN would potentially cause toxic effects also to human cells.

The HepG2 cells, a human hepatoma cell line, has been considered a good *in vitro* model for the studies of xenobiotics metabolism and hepatic toxicity, since these cells retain many of the

* Corresponding authors. Tel.: +55 41 3361 1680; fax: +55 41 3266 2042.
E-mail addresses: samliebel@yahoo.com.br (S. Liebel), filipak@ufpr.br (F. Filipak Neto).
[1] The authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.tiv.2014.12.022
0887-2333/

specialized functions of normal human hepatocytes (Knasmüllerm et al., 2004; Mersch-Sundermann et al., 2004). In particular, HepG2 cells retain the activity of many phase I and II enzymes, although at lower levels than fresh isolated human adult hepatocytes (Rueff et al., 1996; Walter and Willem, 2007) also described to NADPH-cytochrome P450 reductase and cytochrome b5 (Rodríguez-antona et al., 2002; Yoshitomi et al., 2001). Because of this some authors have suggested the need of cells stimulation with nuclear receptors (AhR, PXR and CAR) activators (Westerink and Schoonen, 2007a,b) or treatment with selective CYP inducers such as β-naphtoflavone, rifampin, phenobarbital and ethanol (Boehme et al., 2010; Liu and Zeng, 2009; Matsuda et al., 2002) before cytotoxicity tests.

The present study, therefore, reports the results of a series of experiments aiming to reveal the effects of purified CYN on human HepG2 cells using biomarkers as: (1) cell viability, (2) cell proliferation, (3) cytotoxicity, (4) biotransformation, (5) xenobiotics efflux and (6) oxidative stress. In addition, the influence of factors such as fetal bovine serum concentration and pre-induction of CYPs on CYN effects were included.

2. Methods

2.1. Cyanobacteria culture and cylindrospermopsin purification

CYN-producer strain CYP011K (*Cylindrospermopsis raciborskii* – kindly provided by Dr. Andrew Humpage from Australian Water Quality Center – Australia) was cultured in ASM-1 medium, 12:12 h photoperiod and continuous aeration.

To prepare semi purified CYN, CYP011K strain was centrifuged (40 g/10 min); the pellet was freeze-dried and extracted with ultra-pure water according to Welker et al. (2002). The extracts were stirred for 1 h, centrifuged (110 g/20 min.) and the supernatant was filtered in 0.45 μm regenerated cellulose filters – Sartorius. Then, extract was analyzed by HPLC-PDA (Shimadzu) using a Lichrospher 100 RP-18 reverse phase analytic column (5 μm, MERCK®). The chromatography conditions were carried out under gradient and isocratic conditions according to Welker et al. (2002). There was a single peak corresponding to CYN, which was collected using a semi-preparative column (MERK LiChroCART® 250 × 10 10 μm), frozen, freeze-dried and analyzed by LC–MS/MS (Eaglesham et al., 1999) based on extracted ion chromatograms and MS/MS spectra of CYN standards and CYN purified (Fig. 1). The absorption spectrum and retention time was compared to a commercial standard (NRC CYN – Certified Reference) and showed high similarity ($r^2 \geqslant 0.99$).

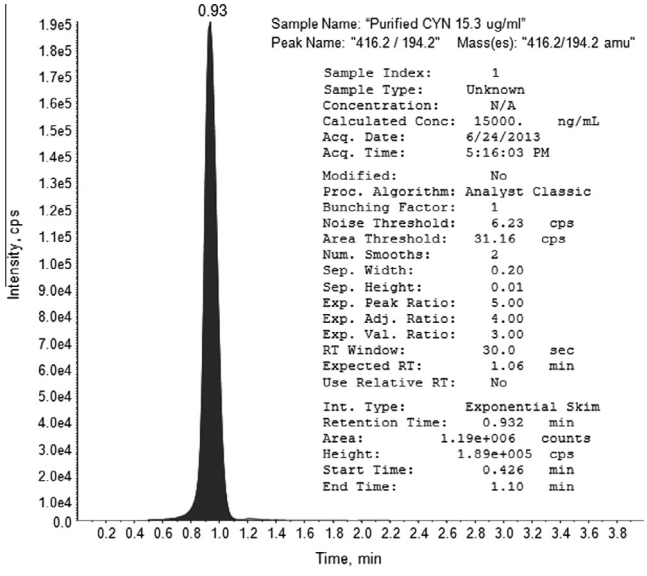


Fig. 1. LC–MS/MS chromatogram obtained for purified CYN.

2.2. HepG2 cells culture

Human hepatoma cells HepG2 were obtained from Rio de Janeiro Cell bank (Brazil) at passage 85. Cells were expanded and utilized in all experiments at passage 86–90 as follow. Cells (10^6 cells) were thawed on ice, suspended with warm (37 °C) high glucose DMEM supplemented with 50% inactivated fetal bovine serum (FBS) and antibiotics (10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin), seeded onto 25 cm^2 flasks and incubated at 37 °C and 5% CO_2. After 2 h, the medium was replaced by fresh DMEM medium with antibiotics and 10% fetal bovine serum. Cell cultures were maintained at 37 °C and 5% CO_2 with medium replacement every 2 days until cells confluence has reached 80–90%. Cells were trypsinized (0.25% trypsin, 0.02% EDTA in PBS, pH 7.2) and re-seeded onto 75 cm^2 flasks following the same procedures aforementioned and cultured until reaching appropriate confluence. Then, cells were trypsinized, resuspended in culture medium and stored for posterior passages (at liquid nitrogen) or seeded onto 96-well microplates (2×10^5 cells ml^{-1}) for experiments. After 24 h of culture, cells were exposed to either cylindrospermopsin (*experiment 1 and 2*) or CYP inducers and cylindrospermopsin (*experiment 3 and 4*). Note that the initial 24 h of culture was kept for in all the experiments.

2.3. Experimental designs

2.3.1. Experiment 1

HepG2 cells exposure to CYN for 48 h with 10% FBS: after 24 h culture, culture medium was replaced by medium containing 0 (control), 0.001, 0.01, 0.1, 1, 10 and 100 μg l^{-1} of semi purified cylindrospermopsin, antibiotics and 10% FBS. After 48 h exposure, biomarkers were determined.

2.3.2. Experiment 2

HepG2 cells exposure to CYN for 4, 12, 24 and 48 h, but with 2% FBS: based on the results of experiment 1, we thought fetal bovine serum could be interfering with cell's responses. Thus, we decided to test different FBS concentrations (1%, 2%, 5% and 10%) to determine the lowest one in which cell viability would not be affected after 48 h culture so as to design a second experiment. For experiment 2, the FBS concentration of 2% was utilized. In this experiment, cells were exposed to CYN as in experiment 1, but utilizing medium supplemented with 2% FBS instead of 10% and during 4, 12, 24 and 48 h, for viability and cytotoxicity determination.

2.3.3. Experiment 3

HepG2 cells exposure to CYN after CYPs induction: since literature reports that *HepG2* cells expresses low levels of biotransformation enzymes, including CYP isoforms, we selected potent CYPs inducers (Liu and Zeng, 2009) and tested their effects on cell viability and cytotoxicity, in the absence and presence of cylindrospermopsin. For this screening, cells were cultured for 24 h and the culture medium was replaced with DMEM medium containing 10% bovine serum, antibiotics and CYPs inducers (rifampicin at 30 μM in DMSO 0.1%, phenobarbital at 500 μM in DMSO 0.1% and ethanol at 250 mM in PBS; see Fig. 2 for CYP isoforms information), with appropriate negative controls (DMSO and PBS). This medium was replaced every 24 h. After 3 days, medium was replaced by DMEM medium supplemented with 2% FBS, antibiotics and CYN (1 and 10 μg l^{-1}) or vehicle (DMSO and PBS).

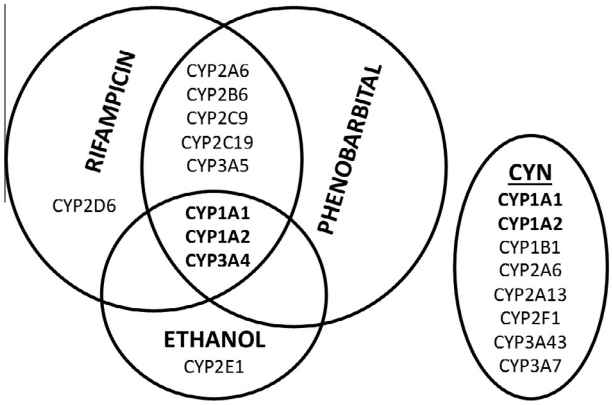


Fig. 2. Main CYP isoforms induced by rifampicin, phenobarbital, ethanol and cylindrospermopsin (CYN) in HepG2 cells and human hepatocytes, according to Humpage et al. (2005), Flockhart (2007), Walter and Willem (2007), Straser et al. (2013). (mRNAs or protein levels.)

2.3.4. Experiment 4

HepG2 cells exposure to CYN after CYPs induction with phenobarbital: based on results from experiment 3, phenobarbital was selected for CYPs induction in *HepG2* cells, according to the procedure previously described. After induction, culture medium was replaced by fresh DMEM medium supplemented with 2% FBS, antibiotics and CYN (0.1, 1 and 10 μg l^{-1}) and cells were exposed for 12 and 24 h (based on results from experiment 2), with a negative control group and phenobarbital group kept in parallel.

2.4. Biomarkers

Cell viability: culture medium was replaced by 200 μl of fresh medium containing 50 μg ml^{-1} of neutral red dye, and cells were incubated for 3 h. Then, the cells were washed three times with solution I (15% formaldehyde, 100 g l^{-1} of calcium chloride in water), the dye was extracted with 300 μl of solution II (1% acetic acid, 50% ethanol in water) and 200 μl of supernatant were transferred to another 96-well microplate for absorbance determination at 540 nm.

Cytotoxicity: culture medium was replaced by 200 μl of fresh medium containing 0.5 mg ml^{-1} of MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and cells were incubated for 2 h. Then, the cells were washed three times with PBS and formazan was solubilized with 100 μl of dimethyl sulfoxide (DMSO). Volume was increased to 300 μl by the addition of 100 μl of PBS and absorbance was measured at 560 nm (Mosmann, 1983, with modifications).

Cell proliferation: cells were washed with PBS and fixed with 4% paraformaldehyde (in potassium phosphate buffer, pH 7.4) for 30 min. Then, 50 μl of 0.25 mg ml^{-1} of crystal violet solution (prepared in water) was added to each well. After 10 min, the cells were washed with PBS to remove the dye and incubated with 100 μl of 33% acetic acid during 30 min with constant shaking. The absorbance was measured at 570 nm (Vega-Avila and Pugsley, 2011, with modifications).

Multixenobiotic resistance (MXR) transporters activity: culture medium was replaced by 200 μl of PBS containing 1 μM of rhodamine B for 30 min at 24 °C and protected from light. Cells were washed twice with PBS and frozen at −76 °C in 250 μl of PBS per well. Then, cells were thawed and 200 μl of the lysate were transferred to a black microplate for fluorescence quantification at 540 nm (excitation) and 580 nm (emission) (Pessatti et al., 2002, with modifications).

Reactive oxygen species (ROS): culture medium was replaced by 200 μl of fresh medium containing 10 μM of 2′,7′-dichlorodihydrofluorescein diacetate (in 0.1% DMSO) for 15 min at 25 °C and protected from light. Cells were washed twice with PBS, suspended in 250 μl of PBS and fluorescence was measured at 488 nm (excitation) and 530 nm (emission) (Benov et al., 1998).

Glutathione S-transferase (GST) activity: cells 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 transferred to another 96-well microplate and 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) was added. Absorbance was immediately measured at 340 nm for 2 min and activity was calculated using CDNB molar extinction coefficient of 9.6 mM^{-1} cm^{-1} (Keen et al., 1976 with modifications).

Lipid peroxidation: culture medium was replaced by 200 μl of fresh medium containing 50 μM of pyrenylphosphine-1-diphenyl (prepared in DMSO and diluted in PBS; DMSO final concentration = 0.01%; Okimoto et al., 2000). After 15 min of incubation at 37 °C, the cells were washed twice with PBS, 200 μl of PBS were added per well, and fluorescence was measured at 351 nm (excitation) and 380 nm (emission).

Alanine aminotransferase/glutamic pyruvic transaminase (ALT/GPT) and Aspartate Aminotransferase (AST) activities: after cells exposure, culture medium was transferred to another microplate and frozen at −76 °C for subsequent analysis. The procedures were developed according to the protocols of commercial kits (Labor-clin®), and the gradual increase of absorbance was recorded during 8 min at 340 nm. The culture medium with the same supplementation, but not used by cells, was utilized as blank.

Superoxide production in mitochondria: culture medium was replaced by 200 μl of fresh medium containing 5 μM of MitoSOX™ (Invitrogen). After 15 min of incubation at 37 °C, the cells were washed three times with PBS, 200 μl of PBS was added per well and fluorescence was measured at 514 nm (excitation) and 580 nm (emission).

2.5. Statistical procedures

Three independent cell cultures were performed for each biomarker per experiment and 12 replicates per group per culture were utilized for each biomarker. Shapiro–Wilk normality test was utilized to verify data distribution (Gaussian distribution). Data without normal distribution was log transformed. One-way ANOVA followed by Dunnett post test was performed for comparisons *vs.* appropriate controls, and t test for comparison of CYP inducer *vs.* control. Multivariate correlation analysis was performed for all biomarkers. A value of $p < 0.05$ was assumed as statistically significant.

3. Results

3.1. Experiment 1 – HepG2 cells exposure to CYN for 48 h with 10% FBS

CYN was not toxic to HepG2 cells after 48 h of exposure, except for the higher concentration (100 μg l^{-1}) (decrease of 11%), as observed through cell viability (Neutral red assay; Fig. 3A). Indeed, cell viability increased at CYN concentrations ⩽10 μg l^{-1}, as indicated by the increases observed in the neutral red assay and the decreases in ALT activity; ALT is a cytosolic enzyme that is found in culture medium when cell dies by necrosis (Fig. 3A–C). Likewise, cell metabolism increased at CYN concentrations ⩽10 μg l^{-1}, thus not revealing cytotoxicity (Fig. 3D). This increase on mitochondrial metabolism (NADH dehydrogenase activity) was partially accom-

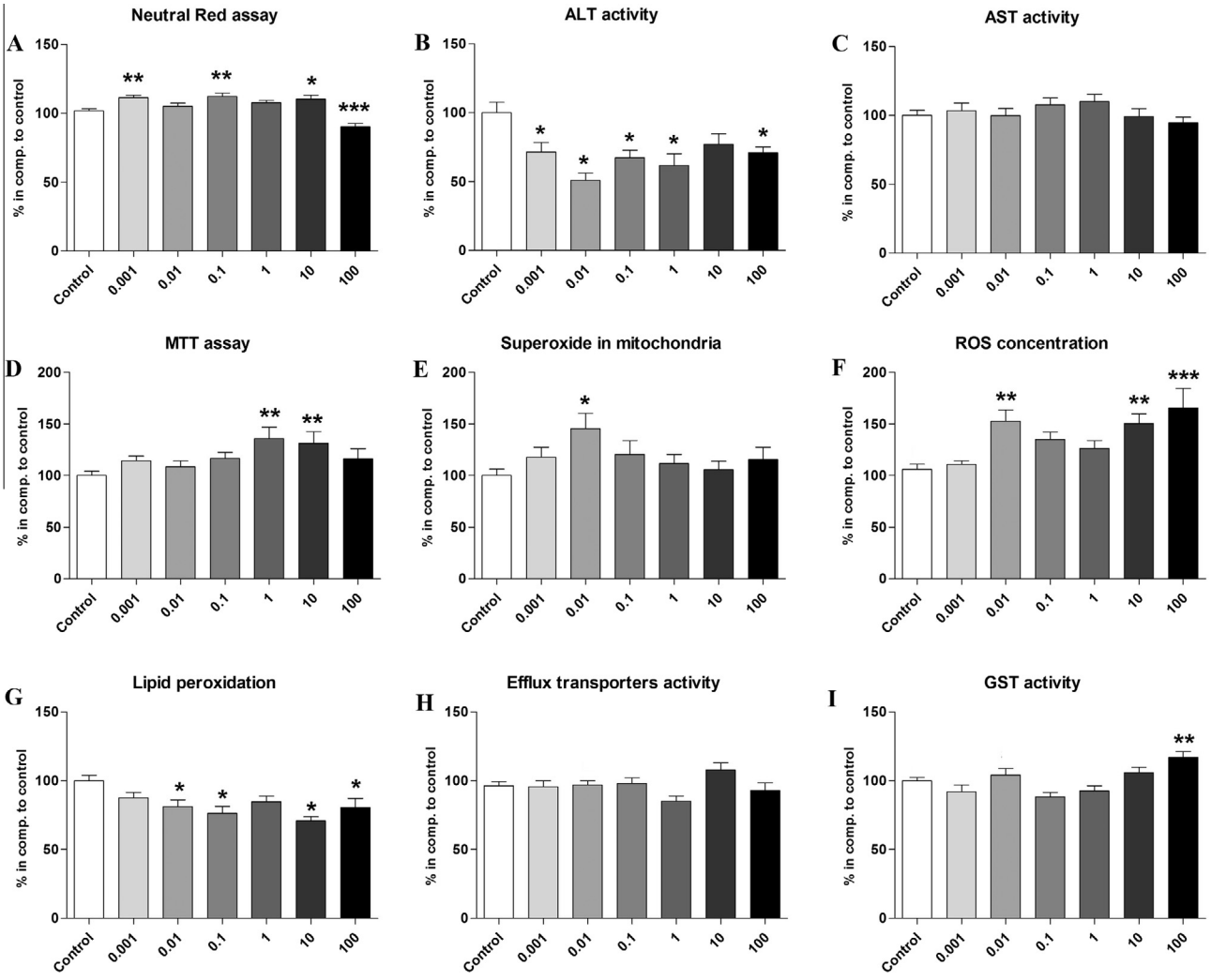


Fig. 3. Neutral red (A). Alanine aminotransferase activity (B). Aspartate Aminotransferase activity (C). MTT assay (D). Mitochondrial superoxide concentration (E). ROS concentration (F). Lipid peroxidation (G). Efflux transporters activity (H). GST activity (I). All analyses are in percentage of the control group. Groups: control and 0.001–100 μg l^{-1} of CYN. Asterisks (∗) indicate differences of CYN groups in comparison to the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

panied by an increase of superoxide levels (Fig. 3E), but there was evidence of additional ROS sources other than mitochondria in low, intermediate and high CYN concentrations (Fig. 3F). Those increases of ROS have not resulted in lipid peroxidation increase, since it decreased in most CYN exposed groups (Fig. 3G). Finally, cell efflux transporters activity was not affected by CYN exposure (Fig. 3H), whereas a 17% increase of glutathione *S*-transferase (GST) activity was observed only at CYN concentration of 100 μg l^{-1} (Fig. 3I).

3.2. Experiment 2 – HepG2 cells exposure to CYN for 4, 12, 24 and 48 h, but with 2% FBS

HepG2 cells have been successfully cultured for 48 h in DMEM medium supplemented with 2% and 5% of FBS, but not 1%, without viability decrease in comparison to 10% of serum (Fig. 4).

After performing a new experiment utilizing 2% of FBS at several exposure periods and CYN concentrations, viability still increased in some groups exposed to CYN during 4 and 12 h (>41%). But, the increases observed for 48 h of exposure at 10% FBS (Fig. 3A) did not occur at 2% (Fig. 5A). Cell viability was influenced by exposure time, i.e., usually increased at 4 and 12 h and then decreased to control levels; only exposure to very high CYN

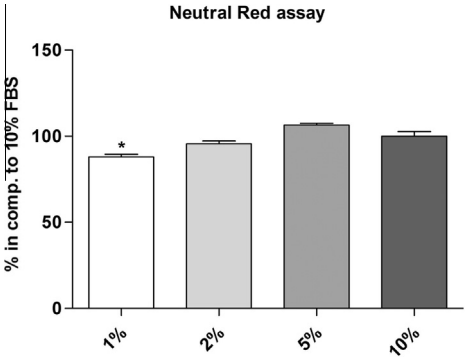


Fig. 4. Cell viability by neutral red assay in percentage of 10% FBS group. Groups: 1%, 2%, 5% and 10% of FBS. Asterisks (∗) indicate differences in comparison 10% FBS group. $^{*}p < 0.05$.

concentration (100 μg l^{-1}) during 48 h resulted in slight cell viability decrease (12%; Fig. 5A). There was no indication of cytotoxicity by MTT test, but cells metabolism increased at intermediate exposure times, i.e., 12 and 24 h (>43%; Fig. 5B). Unlike for cell viability, the increases of cell metabolism observed for 48 h and 10% FBS were not reversed at 2% FBS (Fig. 3D).

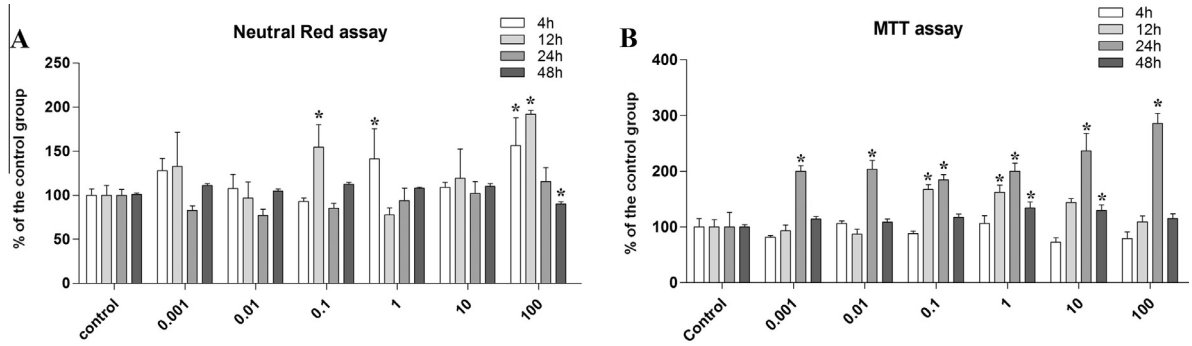


Fig. 5. Neutral red (A, $p < 0.05$) and MTT assay (B, $p < 0.05$) of cells exposed to CYN at 4, 12, 24 and 48 h. Both analyses are in percentage of the control group. Groups: control and 0.001–100 μg l^{-1} of CYN. Asterisks (∗) indicate differences of cells exposed to CYN in comparison to the control at the same period of exposure. $^{*}p < 0.05$.

3.3. Experiment 3 – HepG2 cells exposure to CYN after CYPs induction

Cell viability decreased more than 26% in rifampicin/CYN, phenobarbital/CYN and Ethanol/CYN groups after 12 h of exposure to CYN (Fig. 6A–B). For 24 h of exposure, cell viability was not affected, except for a decrease observed in ethanol/CYN group at 10 μg l^{-1} of CYN (Fig. 6B). For 4 and 48 h of exposure, cell viability was generally not affected, except for increases at 1 μg l^{-1} of CYN in rifampicin/CYN (4 h) and phenobarbital/CYN (48 h) groups (Fig. 6A).

CYN-induced cytotoxicity, i.e. decrease in MTT assay values, was generally not observed in CYPs-induced cells (Fig. 6C and D). The increase of cell metabolism, which has been observed in non-induced cells at experiment 2 (Fig. 5B), was now impaired after CYPs induction (Fig. 6C and D), with the exception of two particular situations for the ethanol/CYN group: an increase of MTT after 48 h of exposure to 1 μg l^{-1} of CYN (Fig. 6C) and a decrease of MTT after 12 h of exposure to 10 μg l^{-1} of CYN (Fig. 6D).

3.4. Experiment 4 – HepG2 cells exposure to CYN after CYPs induction with phenobarbital

Cell viability increased at CYN concentration of 0.1 μg l^{-1} at 12 and 24 h of exposure with and without prior CYPs induction, but this increase was impaired at intermediate and highest CYN concentrations (Fig. 7A and B). However, CYPs induction was neces-

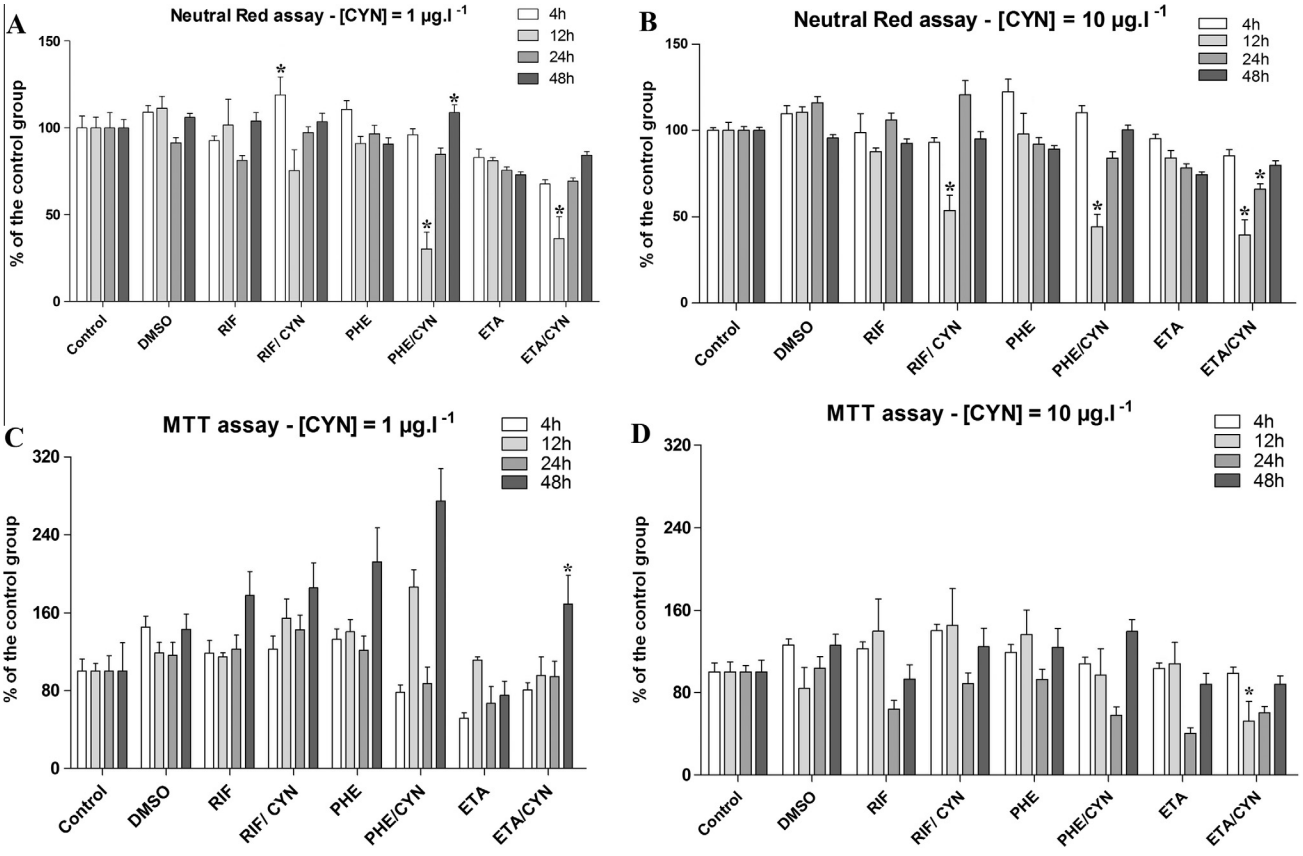


Fig. 6. Neutral red assay of cells exposed to 1 μg l^{-1} (A, $p < 0.05$) and 10 μg l^{-1} of CYN (B, $p < 0.05$). MTT assay of cells exposed to 1 μg l^{-1} (C, $p < 0.05$) and 10 μg l^{-1} of CYN (D, $p < 0.05$). Both analyses are in percentage of the control group. Groups: control, DMSO, RIF (rifampicin), RIF/CYN, PHE (phenobarbital), PHE/CYN, ETA (ethanol), ETA/CYN. Asterisks (∗) indicate differences of CYP-induced cells exposed to CYN and the respective CYP inductor (eg., ETA/CYN *vs.* ETA) at the same period of exposure. $^{*}p < 0.05$.

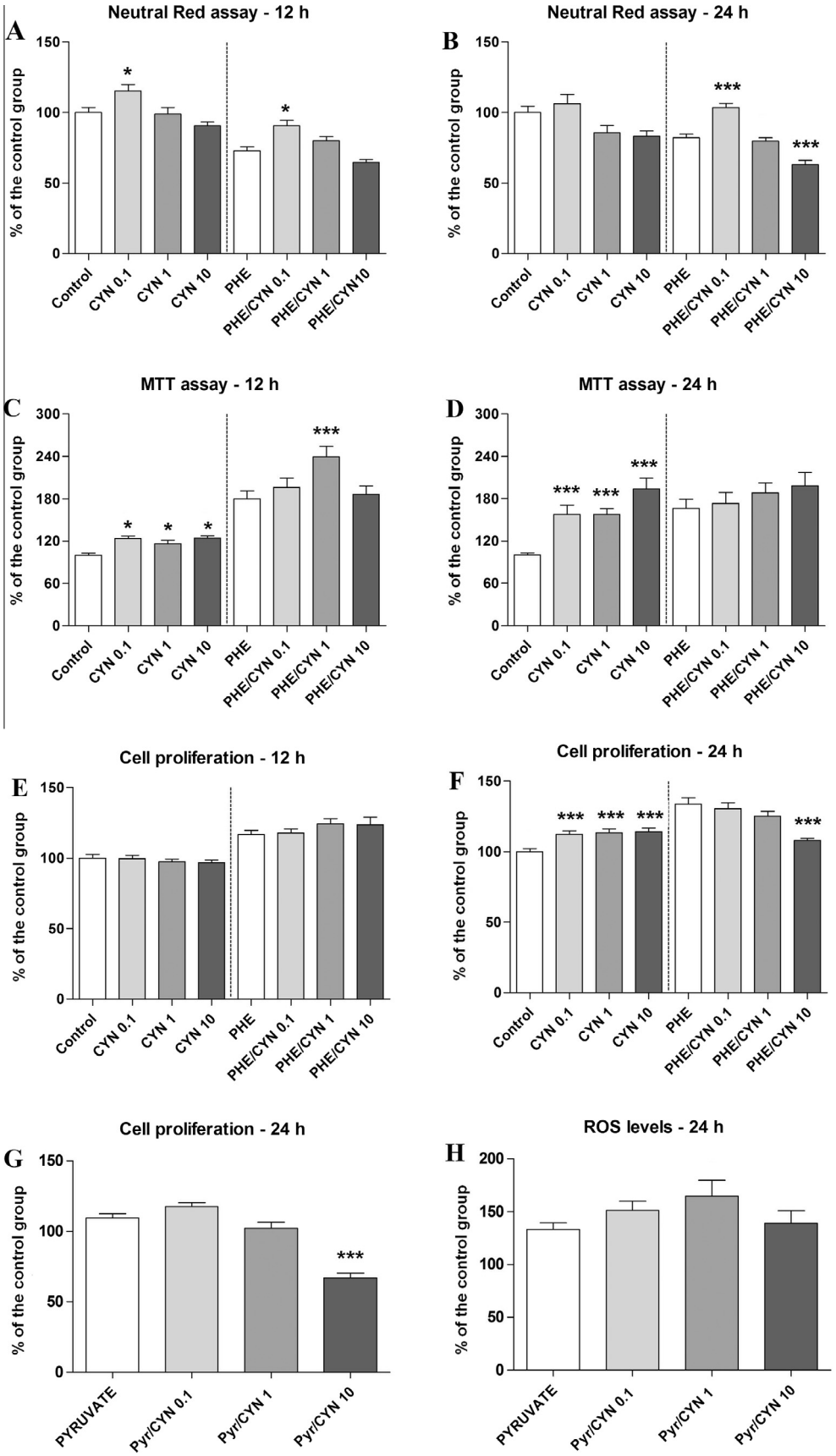


Fig. 7. Neutral red (A and B), MTT (C and D) and cell proliferation assays (E and F) after 12 and 24 h of CYN exposure. Cell proliferation (G) and ROS levels (H) in non-induced cells after incubation with CYN and sodium pyruvate (Pyr) for 24 h. All experiments are in percentage of the control group. Groups: control, 0.1, 1 and 10 $\mu g\, l^{-1}$ of CYN; phenobarbital (PHE), PHE/CYN 0.1, PHE/CYN 1, PHE/CYN 10; and pyruvate (Pyr), Pyr/CYN 0.1, Pyr/CYN 1, Pyr/CYN 10. Two independent comparisons were performed (Control *vs.* CYN groups; and PHE *vs.* PHE/CYN groups) and differences are indicated by asterisks (∗). $^{*}p < 0.05$, $^{***}p < 0.001$.

sary for viability decrease at CYN concentration of 10 μg l^{-1} (Fig. 7A and B). Cell metabolism increased at all CYN concentrations ⩽10 μg l^{-1} at 12 h (>9%); and 24 h (>69%) of exposure (Fig. 7C and D). However, this effect was impaired in cells pre-treated with phenobarbital for CYPs induction (Fig. 7C and D), except for 1 μg l^{-1} of CYN after 12 h exposure.

Cell proliferation was induced by CYN exposure after 24 h (>13%), but not 12 h of exposure (Fig. 7E and F), which makes sense since HepG2 cell cycle usually takes about 20 h (Natarajan and Darroudi, 1991). Cell proliferation partially explains the increased cell metabolism observed at 24 h of exposure (Fig. 7D). As expected, phenobarbital induced cell proliferation in both exposure periods, except for CYP-induced cells exposed to the highest CYN concentration during 24 h (Fig. 7E and F), in which a decrease of 26% of cell number was observed.

Reactive oxygen species levels increased at both exposure times in an approximate concentration–response pattern, with and without prior CYPs induction (>28%; Fig. 8A and B). ROS levels seemed to be related to cell proliferation, since incubation with 5 mM of sodium pyruvate (a ROS scavenger) during CYN exposure for 24 h avoided the increases of ROS ($p = 0.2032$) and proliferation in non-induced cells (Fig. 7G and H). Indeed, cell number decreased 39% in the group exposed to 10 μg l^{-1} (Fig. 7G) and in CYN-induced cells exposed to the same CYN concentration (Fig. 7F).

Lipid peroxidation response was very variable; it decreased in non-induced cells exposed to CYN for 12 h and increased in the cells exposed to the highest CYN concentration for 24 h (28%; Fig. 8C–D). For CYPs-induced cells, lipid peroxidation increased at 12 h (17%) and 24 h (48%) of exposure to 10 μg l^{-1} of CYN (Fig. 8C and D). This increase at 12 h was accompanied by induction of GST activity (14%; Fig. 8E) and decrease of efflux transporters activity (13%; Fig. 8G). However, at 24 h the efflux transporters activity was not affected by CYN (Fig. 8H) and GST activity decreased in CYPs-induced cells exposed to 10 μg l^{-1} (13%; Fig. 8E and F), probably due to the lowest cell number (Fig. 7F).

4. Discussion

Water contamination with CYN is an important issue concerning human health, particularly in countries with inefficient or insufficient sewage treatment, where eutrophication can lead to cyanobacteria blooms. After algal blooms, cyanobacteria lyses can release great amounts of CYN, but the effects of low CYN concentrations are still very relevant in terms of risk of exposure (Who, 2011), since exposure to low levels of contaminated water, i.e. with contaminants below the "safe limits", may be a continuous process during human life. Thus, the effects of low concentrations of CYN were investigated in the current study.

Differently than expected and reported for other models (Kinnear, 2010; Lankoff et al., 2007; Liebel et al., 2011) HepG2 cells were not deleteriously affected by CYN exposure at concentrations up to 10-fold the safe limit of 1 μg l^{-1} as these concentrations were not cytotoxic and, surprisingly, promoted metabolism increase and cell proliferation. These controversy data are then discussed in order to highlight that CYN at low concentrations promote a proliferation of human hepatoma cells (HepG2) and CYP-induction slightly increase CYN toxicity.

4.1. Fetal bovine serum must be adjusted for cell toxicology studies

Fetal bovine serum (FBS) concentration of 10% is often utilized in studies with HepG2 and other cell lineages, since FBS promotes cells attachment, survival and proliferation in culture, but it also influences cell response to CYN. In this sense, an experiment was performed to evaluate the effectiveness of different FBS concentrations in culture of HepG2, since FBS can decrease test-compounds bioavailability and toxicity (Valk et al., 2010). FBS at 2% showed be the appropriate FBS concentration for toxicology approaches with HepG2 cells, since cells remained viable and may proliferate less. In addition, HepG2 cells may not suffer from toxic effects of CYN concentrations ⩽10 μg l^{-1} when cultured with 10% FBS, as demonstrated by viability (neutral red, ALT and AST activities), cytotoxicity, lipid peroxidation, efflux transporters and GST activities (see Fig. 3). Both compensatory proliferation as well as reduced CYN toxicity could explain these findings.

4.2. CYPs induction can make HepG2 cells more sensitive to CYN toxic effects

Investigations have revealed effects of low concentrations of CYN in *in vitro* (genotoxicity at CYN concentration of 20 μg l^{-1} or above) and *in vivo* (intraperitoneal injections of CYN at 32 μg kg^{-1} or above were lethal to mice) approaches (Humpage et al., 2005; Rogers et al., 2007). For HepG2, no evident cytotoxic effects were observed for CYN concentrations <10 μg l^{-1} for 12-h exposure; for example, lipid peroxidation has decreased even after increases of metabolism and ROS production observed at this period in the current study. Cytochrome P450s (CYPs) appear to have a role in the toxicity of CYN (Chuang and Albert, 2001; Walter and Willem, 2007) since broad-spectrum CYPs inhibitors have protective effects against the toxicity (Froscio et al., 2003; Norris et al., 2002; Runnegar et al., 1995). Alteration on the expression of genes for CYP1A1, CYP1B1, CYP2A6, CYP2A13, CYP3A43, UDP-glucuronosyltransferase (UGT) 1A6, thioredoxin reductase 1, glutathione *S*-transferase M3, and catalase was reported after CYN exposure (Straser et al., 2013), providing evidence for the involvement of phase I (CYP1A1, CYP1B1, aldehyde dehydrogenase 1A2 and carboxylesterase-2) and phase II (UGT1A6, UGT1A1, *N*-acetyltransferase 1 and GSTM3) enzymes in the biotransformation of CYN (Straser et al., 2013). HepG2 express low levels of biotransformation enzymes, which can be by-passed through prior CYPs induction with drugs (Liu and Zeng, 2009). Therefore, the next step on the current study was to utilize CYPs-inducers to verify whether they could make the cells more sensitive to CYN exposure.

Comparison of rifampicin, ethanol and phenobarbital (CYPs inducers) revealed that in phenobarbital pre-incubated cells CYN exposure could reduce cell viability and impair the increase of metabolism after 12 and 24 h (see Fig. 6). In principle, it may be an indication that the activity of CYPs are involved in the toxicity of CYN at very low concentrations, even though cells may recover from CYN insult at 48 h of exposure. Phenobarbital has proved to be a very effective CYP inducer in HepG2 in other studies (Boehme et al. 2010; Turpeinen et al., 2009; Walter and Willem, 2007), particularly for isoforms CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5 (Fig. 2). Based on results, the study proceeded using this CYP inducer at 12 and 24 h of exposure.

CYPs-induced HepG2 cells are more sensitive to CYN exposure, as observed through the viability and efflux transporters activity decreases after 24 h of exposure to 10 μg l^{-1}. Unlike in these cells, non-induced cell's viability only decreased at a 10-fold higher concentration of CYN and longer period of exposure (100 μg l^{-1}, 48 h; see Fig. 5). This higher sensitivity is corroborated by the impairment of the increase of metabolism after CYPs induction. Despite cellular metabolism has not increased in CYPs-induced cells, the ROS levels has still increased, which could be involved in the increased lipid peroxidation after exposure to 10 μg l^{-1} of CYN.

GST activity was generally not affected by CYN exposure, except for non-induced HepG2 cells exposed to a very high concentration (100 μg l^{-1}) and for CYPs-induced cells. In the latter, GST activity has increased at 10 μg l^{-1} of CYN and 12 h, but then decreased later at 24 h. These results may be due to the variability of cell number.

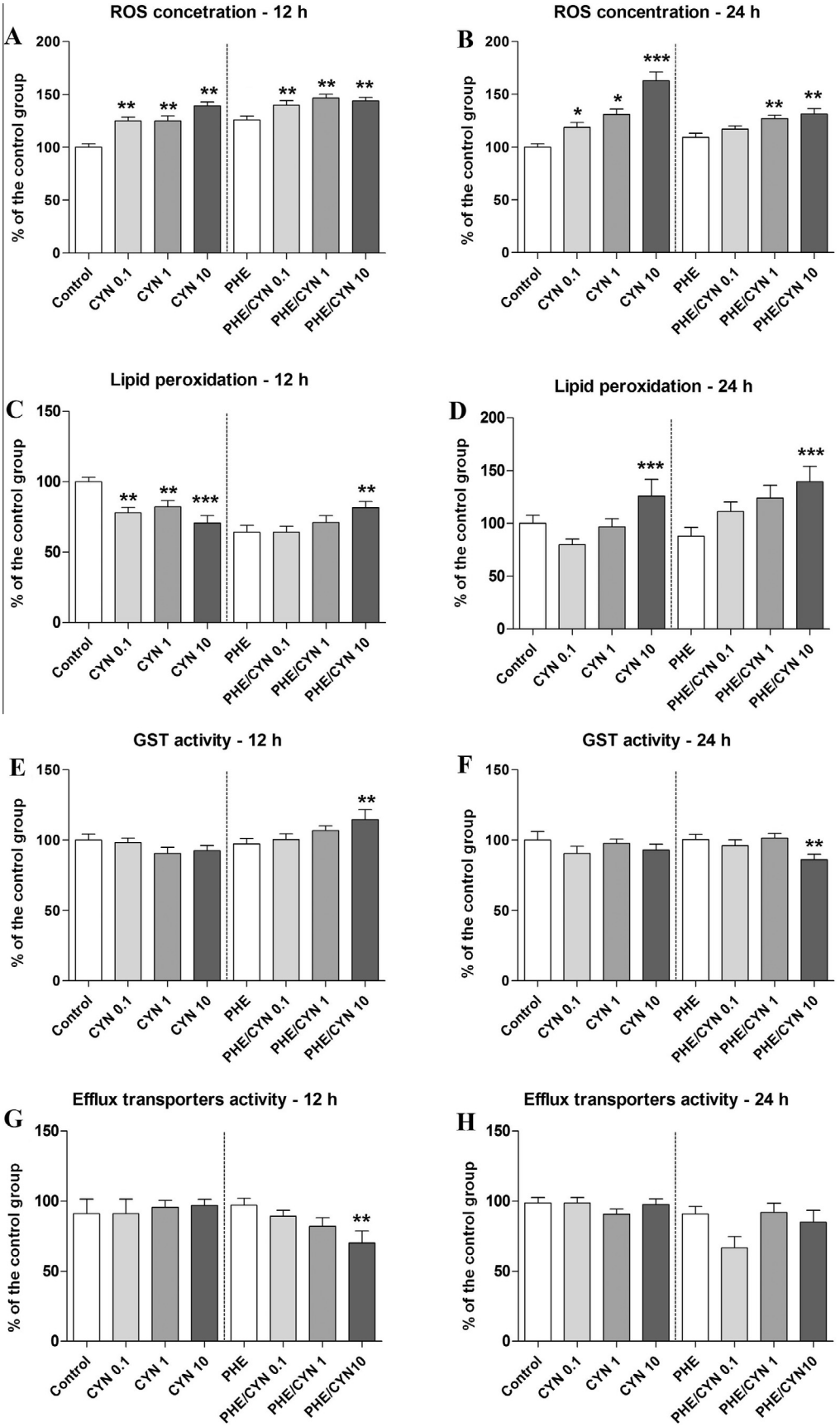


Fig. 8. ROS concentration (A and B), lipid peroxidation (C and D), GST activity assays (G and H), and efflux transporters activity (E and F) after 12 and 24 h of CYN exposure. All experiments are in percentage of the control group. Groups: control, 0.1, 1 and 10 $\mu g\, l^{-1}$ of CYN; phenobarbital (PHE), PHE/CYN 0.1, PHE/CYN 1, PHE/CYN 10. Two independent comparisons were performed (Control *vs.* CYN groups; and PHE *vs.* PHE/CYN groups) and differences are indicated by asterisks (∗). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

We have previously reported efflux transporters activity decrease in primary cultured fish hepatocytes, which may express levels of biotransformation enzymes very close to the *in vivo* levels (Liebel et al., 2011). These cells appear to be more sensitive to CYN than HepG2, since efflux transporters activity was generally not affected by CYN exposure, except for 10 $\mu g\ l^{-1}$ in 12 h-exposed CYPs-induced cells. These transporters are involved with efflux of endobiotic as well as toxic compounds, such as some toxins and toxin-conjugation products (glutathione, glucuronate and sulfate conjugates), thus being directly involved with multixenobiotic resistance (MXR) phenotypes (Bard, 2000; Van der Oost et al., 2003). However, it is not characterized which transporters are involved with CYN efflux. Likewise, the pathway of CYN uptake is poorly understood as great effort has been invested in toxicity determination instead of accumulation potential. Runnegar et al. (2002) showed that the sulfate group of CYN is not required for cell entry. The hydrophilic nature decreases the possibility of CYN to efficiently cross lipid bilayers and so endocytosis as well as transport through bile acid transporters may be important candidate systems for CYN uptake (Chong et al., 2002; Wiegand and Pflugmacher, 2005).

4.3. Low concentrations of CYN induce HepG2 cells proliferation and metabolism increase

Phenobarbital can promote cell proliferation (Li et al., 2011; Pitot et al., 1987), which may explain the increased number of cells in CYPs-induced cells cultured with 2% FBS compared to non-induced cells. In addition, CYPs-induction impaired the effects of CYN on cell proliferation and so may have important consequences concerning tumor cells. Tumor cells usually express low levels of xenobiotic-metabolizing enzymes (Boehme et al., 2010; Liu and Zeng, 2009; Matsuda et al., 2002) in comparison to normal cells, so that CYN can favor proliferation of these cells. Only a few published studies have reported CYN influence on the cell cycle and proliferation. Lankoff et al. (2007) described that CYN significantly decreased the mitotic index and proliferation in CHO-K1 cells at 0.05; 0.1; 0.2; 0.5; 1; and 2 $\mu g\ ml^{-1}$ during 3, 16 and 21 h in a dose and time dependent, whereas it decreased the nuclear division index in HepG2 cells exposed to 0.5 $\mu g\ ml^{-1}$ of CYN for 24 h (Straser et al., 2013) and in human peripheral lymphocytes at 0.1 $\mu g\ ml^{-1}$ for 24 h (Zegura et al., 2004). The decrease of cell proliferation on these works occurred at relative high concentrations of CYN ($\geqslant 100\ \mu g\ l^{-1}$), in which CYN has induced a viability decrease of HepG2 cells cultured with 10% FBS. In HepG2 cells, proliferation was positively correlated with metabolism at 12 h ($r^2 = 0.276$, $p < 0.0001$) and to ROS concentration at 24 h for non-induced cells ($r^2 = 0.118$, $p = 0.0044$) and so, part of the metabolism increase observed in the current study may be a preparation for cell division. Considering the last correlation, although it is very weak, incubation with sodium pyruvate (a ROS scavenger) avoided the increases of ROS production and cell proliferation in non-induced HepG2 cells after CYN exposure. Low levels of ROS like hydrogen peroxide can signalize for cell proliferation (Li et al., 2009; Pan et al., 2011).

4.4. Final comments

CYN has been included in the revision of WHO "Guidelines for Drinking-water Quality, chemical hazards in drinking-water" and classified on the list of compounds with highest priority for hazard characterization by the US Environmental Protection Agency (EPA, 2010). In the current study, we demonstrated that the toxicity of low concentrations of CYN (<10 $\mu g\ l^{-1}$) is limited in human hepatoma cells (HepG2), since these concentrations of the toxin have induced increases of viability, proliferation and metabolism. There are evidences on literature that CYN toxicity and genotoxicity depend on CYPs-metabolism as different broad-spectrum CYPs inhibitors can protect cells against the toxicity, but it is not clear which isoforms are involved (Froscio et al., 2003; Norris et al., 2002; Runnegar et al., 1995). Crossing the information about CYP isoforms induced by ethanol, rifampicin and phenobarbital may give a clue of the possible CYPs involved in CYN biotransformation (Fig. 2). For testing the HepG2 cell's responses to organic compounds subject to biotransformation, it may be useful to pre-induce the expression of xenobiotic metabolism enzymes such as CYPs. However, CYPs-induction with phenobarbital has led generally to similar results as those observed in non-induced cells for the tested biomarkers. It may also be useful to reduce FBS concentration to 2% in order to reduce cell proliferation and induce cells to a metabolic state more close to the 'normal' hepatocyte, since this reduction does not affect cell viability.

Increases of metabolism and proliferation were not expected results and may have important consequences for tumor cells like HepG2. Although we performed our research with *in vitro* model, the question of the influence of low concentration of pollutants, which are below the limits established by legislation and whose toxicity are not very evident, on established diseases such as cancer should be explored in future investigations.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

This research was supported by CNPq (Brazilian Agency for Science and Technology) and CAPES (PhD scholarship).

References

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

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357–389.

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., Lind, O., 2010. First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, LagoCatemaco, and apparent bioaccumulation of the toxins in "tegogolo" snails (Pomacea patula catemacensis). Toxicon 55, 930–938.

Boehme, K., Dietz, Y., Hewitt, P., Mueller, S.O., 2010. Activation of P53 in HepG2 cells as surrogate to detect mutagens and promutagens in vitro. Toxicol. Lett. 198, 272–281.

Brasil, 2011. Ministério da Saúde Portaria n. 2914, 1ed. Ministério da Saúde, Brasília.

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

Chuang, L., Albert, P.L., 2001. Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague–Dawley rat, minipig, and beagle dog. Chem. Biol. Interact. 134, 271–281.

Eaglesham, G.K., Norris, R.L., Shaw, G.R., Smith, M.J., Chiswell, R.K., Davis, B.C., Neville, G.R., Seawright, A.A., Moore, M.R., 1999. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue–green algal toxin, for public health purposes. Environ. Toxicol. 14, 151–154.

Epa (Environmental Protection Agency), 2010. Creating a Cyanotoxin Target List for the Unregulated Contaminant Monitoring Ruley. Technical Service Center, USEPA Press, Cincinnati.

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.
Flockhart, D.A., 2007. Drug Interactions: Cytochrome P450 Drug Interaction Table. Indiana University School of Medicine, http://medicine.iupui.edu/clinpharm/ddis/clinical-table/ (accessed 10.11.13).
Froscio, S.M., Humpage, A.R., Burcham, P.C., Falconer, I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ. Toxicol. 18, 243–251.
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.
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. Microbiol. 50, 1292–1295.
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.
Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the glutathione Stransferases. J. Biol. Chem. 251, 6183–6188.
Kinnear, S., 2010. Cylindrospermopsin: a decade of progress on bioaccumulation research. Mar. Drugs 8, 542–564.
Knasmüllerm, S., Mersch-sundermann, V., Kevekordes, S., Darroudi, F., Huber, W.W., Hoelzl, C., Bichler, J., Majer, B.J., 2004. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. Toxicology 198, 315–328.
Lankoff, A., Wojcik, A., Lisowska, H., Bialczyk, J., Dziga, D., Carmichael, W.W., 2007. No induction of structural chromosomal aberrations in cylindrospermopsin-treated CHO-K1 cells without and with metabolic activation. Toxicon 50, 1105–1115.
Li, M., Zhao, L., Liu, J., Liu, A.L., Zeng, W.S., Luo, S.Q., Bai, X.C., 2009. Hydrogen peroxide induces G2 cell cycle arrest and inhibits cell proliferation in osteoblasts. Anat. Rec. 292, 1107–1113.
Li, J., Harp, C., Tharappel, J.C., Spear, B.T., Glauert, H.P., 2011. Effect of vitamin E on hepatic cell proliferation and apoptosis in mice deficient in the p50 subunit of NF-jB after treatment with phenobarbital. Food Chem. Toxicol. 49, 2706–2709.
Liebel, S., Oliveira Ribeiro, C.A., Silva, R.C., Ramsdorf, W.A., Cestari, M.M., Magalhães, V.F., Garcia, J.R.E., Esquivel, B.M., Filipak Neto, F., 2011. Cellular responses of Prochilodus lineatus hepatocytes after cylindrospermopsin exposure. Toxicol. in Vitro 25, 1493–1500.
Liu, Z.H., Zeng, S., 2009. Cytotoxicity of ginkgolic acid in HepG2 cells and primary rat hepatocytes. Toxicol. Lett. 187, 131–136.
Matsuda, H., Kinoshita, K., Sumida, A., Takahashi, K., Fukuen, S., Fukuda, T., Takahashi, K., Yamamoto, I., Azuma, J., 2002. Taurine modulates induction of cytochrome P450 3A4 mRNA by rifampicin in the HepG2 cell line. Biochim. Biophys. Acta (BBA) – Mol. Cell Res. 1593, 93–98.
Mersch-sundermann, V., Knasmüller, S., Wu, X., Darroudi, F., Kassie, F., 2004. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicology 198, 329–340.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Natarajan, A.T., Darroudi, F., 1991. Use of human hepatoma cells for in vitro metabolic activation of chemical mutagens/carcinogens. Mutagenesis 6, 399–403.
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.
Okimoto, Y., Watanabe, A., Niki, E., Yamashita, T., Noguchi, N., 2000. A novel fuorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes. FEBS Lett. 474, 137–140.
Pan, Q., Qiu, W., Huo, Y., Yao, Y., Lou, M.F., 2011. Low levels of hydrogen peroxide stimulate corneal epithelial cell adhesion, migration, and wound healing. Invest. Ophthalmol. Vis. Sci. 52, 1723–1734.
Pessatti, M.L., Resgalla Jr., 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. Br. J. Biol. 62, 651–656.
Pitot, H.C., Goldsworthy, T.L., Moran, S., Kennan, W., Glauert, H.P., Maronpot, R.R., Campbell, H.A., 1987. A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. Carcinogenesis 8, 1491–1499.
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.
Rodríguez-antona, C., Donato, M.T., Boobis, A., Edwards, R.J., Watts, P.S., Vicente castell, J., Gómez-lechón, M.J., 2002. Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. Xenobiotica 32, 505–520.
Rogers, E.H., Zehr, R.D., Gage, M.I., Humpage, A.R., Falconer, I.R., Marr, M., Chernoffa, N., 2007. The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon 49, 855–864.
Rueff, A., Dawson, A.J.L.R., Mendell, L.M., 1996. Characteristics of nerve growth factor induced hyperalgesia in adult rats: dependence on enhanced bradykinin-1 receptor activity but not neurokinin-1 receptor activation. Pain 66, 359–372.
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.
Runnegar, M.T., Xie, C., Snider, B.B., Wallace, G.A., Weinreb, S.M., Kuhlenkamp, J., 2002. In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol. Sci. 67, 81–87.
Straser, A., Filipic, M., Zegura, B., 2013. Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells. Toxicol. in Vitro 27, 1809–1819.
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.
Turpeinen, M., Tolonen, A., Chesne, C., Guillouzo, A., Uusitalo, J., Pelkonen, O., 2009. Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. Toxicol. in Vitro 23, 748–753.
Valk, J., Brunner, D., De Smet, K., Fex Svenningsen, Å., Honegger, P., Knudsen, L.E., Lindl, T., Noraberg, J., Price, A., Scarino, M.L., Gstraunthaler, G., 2010. Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods. Toxicol. in Vitro 24, 1053–1063.
Van Der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.
Vega-Avila, E., Pugsley, M.K., 2011. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proc. West. Pharmacol. Soc. 54, 10–14.
Walter, M.A., Willem, G.E.J., 2007. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved. Toxicol. in Vitro 21, 1581–1891.
Welker, M., Bickel, H., Fastner, J., 2002. HPLC-PDA detection of cylindrospermopsin – opportunities and limits. Water Res. 36, 4659–4663.
Westerink, W.M.A., Schoonen, W.G.E.J., 2007a. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. Toxicol. in Vitro 21, 1581–1591.
Westerink, W.M.A., Schoonen, W.G.E.J., 2007b. Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2. Toxicol. in Vitro 21, 1592–1602.
Who (World Health Organization), 2011. Guidelines for Drinking-Water Quality, fourth ed. Who Press, Geneva.
Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol. Appl. Pharmacol. 203, 201–218.
Yoshitomi, S., Ikemoto, K., Takahashi, J., Miki, H., Namba, M., Asahi, S., 2001. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. Toxicol. in Vitro 15, 245–256.
Zegura, B., Lah, T.T., Filipic, M., 2004. The role of reactive oxygen species in microcystin -LR-induced DNA damage. Toxicology 200, 59–68.

CAPÍTULO II

Cylindrospermopsin affects protein profile of HepG2 cells

Cylindrospermopsin affects protein profile of HepG2 cells

Samuel Liebel[a1], Sonia Regina Grötzner[a], Ciro Alberto de Oliveira Ribeiro[a], Daniele Dietrich Moura Costa[a], Marco Antônio Ferreira Randi[a], Francisco Filipak Neto[a*]

[a]Departamento de Biologia Celular, Universidade Federal do Paraná, Cx. Postal 19031, CEP 81.531-990, Curitiba, PR, Brazil.

[*] The authors contributed equally to this paper and are corresponding authors.

Abstract

[1] Corresponding Authors:
Francisco Filipak Neto
e.mail: filipak@ufpr.br
Samuel Liebel
e.mail: samliebel@yahoo.com.br
Universidade Federal do Paraná - Departamento de Biologia Celular
PO box : 19031
Zip code : 81.531-980 Curitiba - PR Brasil
Phone: 55 41 3361-1680
Fax: 55 41 3266-2042

Human hepatoma cells (HepG2) were exposed to purified cylindrospermopsin (CYN), a potent toxicant for eukaryotic cells produced by several cyanobacteria. Exposure to 10 µg l^{-1} of CYN resulted in alteration of expression of 48 proteins, from which 26 were identified through mass spectrometry. These proteins are implicated in different biological processes: protein folding, xenobiotic efflux, antioxidant defense, energy metabolism and cell anabolism, cell signaling, tumorigenic potential, and cytoskeleton structure. Protein profile indicates that CYN exposure led to increased glucose uptake and breakdown to provide useful energy to cells to respond to chemical stress. Increase, of G proteins–coupled receptors (GPCRs), heterogeneous nuclear ribonucleoproteins (hnRNP) and reactive oxygen species (ROS) levels can enhance cell proliferation and tumorigenic potential of HepG2 cells. Excess of ROS activated protective mechanisms provided by MRP3 and glutathione peroxidase. Cytoskeleton proteins expression was also altered by CYN, which may be associated with cell proliferation as well as replacement of ROS-damaged proteins.

Keywords: xenobiotic, cylindrospermopsin, proteomic, immunofluorescence, HepG2

1. Introduction

The progressive occurrence and global expansion of harmful cyanobacteria blooms have been forecasted as consequences of eutrophication and climate change (Elliott, 2012; O'Neil *et al.*, 2012). Among freshwater cyanobacteria, *Cylindrospermopsis raciborskii* has a substantial widespread distribution, both in tropical and temperate zones (Huszar et al, 2000; Poniedzialek *et al.*, 2012).

Cylindrospermopsin (CYN)-producing cyanobacteria are spread in regions of different climate despite intense efforts to decrease the main cause of eutrophication, nutrient loading, into water bodies (Hudnell, 2010; Lehman *et al.*, 2013). CYN has been identified in reservoirs aimed for water supply and is highly toxic to eukaryotes including humans. For these reasons, it receives broad interest in both, toxicological and ecological studies (Bittencourt-Oliveira *et al.*, 2003; Poniedzialek *et al.*, 2012; Lei *et al.*, 2014).

Potential routes of CYN exposure include consumption of contaminated water and food, as well as recreational activities (Ibelings and Chorus, 2007; Poniedziałek *et al.*, 2012).

The safety guidelines for CYN in drinking water is 1.0 $\mu g.l^{-1}$ (Humpage and Falconer, 2003; Brasil, 2011), although some countries, such as Australia and France, have adopted even stricter regulations (AFSSA, 2006). During massive cyanobacteria proliferation, CYN concentration can exceed 500 $\mu g.l^{-1}$ in water (Saker and Eaglesham, 1999). At the same time, the high stability of CYN under different pH and temperature conditions has been demonstrated (Chiswell *et al.*, 1999; Wörmer *et al.*, 2010). These two aspects increase the risk of organisms' exposure in aquatic environments, where milder conditions of temperature and pH permit CYN to persist

for a long time. Therefore, its potential harmful effects on human health require substantial studies and evaluation.

CYN has been recognized as a potent eukaryotic protein synthesis inhibitor (Froscio *et al.* 2008; Terao *et al.* 1994), genotoxic agent, activator of different isoforms of cytochrome P450 (CYPs) and oxidative stress inducer through reduction of glutathione synthesis, as reported for mammal cells (Bain *et al.* 2007; Froscio *et al.* 2009; Humpage *et al.* 2005; Neumann *et al.* 2007). Among the target organs of CYN, the liver has been described as the most important, but the effects also occur in the heart, thymus, spleen and kidneys (Falconer and Humpage 2006; Hawkins *et al.* 1985). These effects reinforce the importance of conducting studies to unravel deeper into the mechanisms involved in cellular responses and CYN toxicity, since the studies do not satisfactorily report how these changes are generated from the molecular point of view.

Techniques such as proteomics provide effective strategies and tools for toxicological studies and are regarded as a powerful tool to investigate the cellular responses to toxicants (Dowling and Sheehan, 2006). The proteomic approaches have proved to be valuable in identifying early responses to toxins and, concomitantly, identifying the mechanisms of toxicity involved and the effects on organisms, including humans (Gazzah *et al.*, 2013).

We have observed in a previous work that exposure to low concentrations of CYN promotes HepG2 cell proliferation associated with increases of cell metabolism and reactive oxygen species (ROS) production, which may have important consequences for tumor cells (Liebel *et al.*, 2015). In the current study, proteins profile of human hepatoma HepG2 cells was investigated after exposure to CYN in

order to determine which proteins have their expression affected by CYN and propose possible molecular and target processes. In addition, the effects of CYN on microtubules and actin filaments cytoskeleton were determined through confocal fluorescence microscopy.

2. Methods

2.1 Cyanobacteria culture and cylindrospermopsin purification

Cyanobacteria culture and cylindrospermopsin purification procedures were performed according to previously published protocols (Liebel *et al.*, 2015).

2.2 HepG2 cells culture

Human hepatoma cells (*HepG2 cells*) were obtained from Rio de Janeiro Cell bank (Brazil). Cells were expanded and maintained according to Liebel *et al.* (2015) and utilized at passage 86-90 in all experiments. After reaching 80-90% confluence in culture flasks, cells were trypsinized (0.25% trypsin, 0.02% EDTA in PBS, pH 7.2) and seeded either onto 25 cm^2 flasks ($2x10^5$ cells ml^{-1}) for proteomics analysis or onto 13 mm-glass coverslips placed in 24-well microplates ($5x10^5$ cells ml^{-1}) for immunofluorescence. After complete attachment and recovery, during 24 h for proteomics and 48 h for immunofluorescence, cells were exposed for 24 h to 10 and 100 $\mu g.l^{-1}$ of cylindrospermopsin in high-glucose DMEM culture medium supplemented with 2% FBS and antibiotics (10 U ml^{-1} penicillin and 10 µg ml^{-1} streptomycin) and maintained at the incubator (5% CO_2, 37°C)

2.3 Analytical procedures

Proteomic experiment: after 24 h culture, culture medium was replaced by medium containing 0 (control) and 10 µg l^{-1} of semi purified cylindrospermopsin,

antibiotics and 2% FBS. Protein extraction: After 24 h exposure, the cells were centrifuged (120g) to remove cell culture medium. Then, cells were frozen and homogenized in lyses buffer (40 mM dithiothreitol (DTT), 7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG Buffer (carrier ampholytes)), and the supernatant was removed by centrifugation at (120g) at 4 ◦C. The pellet containing proteins was washed with 2D clean up kit (GE Helthcare) following manufacturer's instructions. The protein content in the pellets was quantified using the Bradford´s method. Then, 400 µg of pelleted protein were dissolved in rehydration buffer (8 M urea, 2% CHAPS, 2.8 mg ml^{-1} DTT, 0.5% immobilized pH gradient (IPG) buffer and a trace of bromophenol blue). Finally, 3-10 linear strips were rehydrated in the IPHphor (GE Helthcare) during 12 h at 20^{o}C and50 V per strip for subsequent isoelectric focusing conducted with the Ettan IPGphor III Isoelectric Focusing System (Amersham Biosciences, USA).

Isoelectric focusing was performed in the following manner: 1) Step and hold - 100 V - 0.5 W/h; 2) Gradient – 1000 V - 0.8 W/h; 3) Gradient – 8000 V - 11.3 W/h; 4) Step and hold – 8000 V - 4.4 W/h. After the first dimension was run, each strip was equilibrated with about 15 ml equilibration buffer (50 mM Tris,pH 8.8; 6 M urea, 30% glycerol, 2% SDS, 1% DTT and a trace amount of bromophenol blue) for 20 min. The strip was placed in fresh equilibration buffer containing 2.5% iodoacetamide (instead of DTT) for another 20 min. Then a 10% SDS–PAGE second dimension was performed. Electrophoresis was carried out at 10 mA/gel for 30 min, followed by a 5-6 h run at 35 mA/gel.

Colloidal Coomassie staining was carried out as follows. Briefly, the gel was fixed for 1 h in a fixation solution containing 20% (v/v) methanol and 1.3% (v/v) phosphoric acid, followed by four Milli-Q water washes (15 min each). The gel was

stained overnight with the working colloidal “blue silver” solution containing 0.12% Coomassie Brilliant Blue G-250, 10% ammonium sulfate, 10% phosphoric acid and 20% methanol. Then, the gels were neutralized in 0.1 M of tris-base solution and stabilized in 4% of ammonium sulfate solution. Finally, the gels were destained for approximately 48 h via Milli-Q water washes.

Image analysis: the gels were scanned and the images were analyzed using the ImageMaster 2D Platinum v7.0 software (GE Healthcare).

Mass spectrometry: the differentially expressed spots, determined through images comparison of scanned gels of the control and CYN groups, were removed from SDS-PAGE gels for MALDI-ToF analysis; the excised bands spots destained, reduced and alkylated (Schevchenko *et al.*, 1996) as follows. Briefly, proteins were reduced with 10 mM DTT in 25 mM NH_4HCO_3 (for 45 min at 56 °C) and then alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 (for 30 min at room temperature and protected from light). The excess of incubation solution was removed and the pieces of gel were washed twice with 50 mM NH_4HCO_3 solution/acetonitrile (1:1) and partially dehydrated with acetonitrile (for 10 min at room temperature); complete dehydration was achieved via SpeedVac. Finally, proteins were digested with trypsin (Tripsin-Gold – Promega; 12 ng μl^{-1}) for 24 h 37 ºC into peptides. Trypsin used in the protocol has been modified to prevent its autolytic digestion. The peptides were desalted and concentrated using perfect pure C18 Zip-Tips, according to the manufacturer´s instructions (Millipore®). Then, the desalted peptides were eluted directly onto the MALDI-ToF-ToF target (scout MTP MALDI ion source 384 target – Bruker Daltonics, GmbH) with 2 µl of matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA) and

allowed to air dry. The peptide mass fingerprints were obtained using MALDI-ToF-ToF mass spectrometer (Ultraflex Bruker Daltonics, GmbH), in positive reflector mode and the spectra were analyzed using FlexControl 2.0 (Bruker Daltonics). Mass spectrometer was externally calibrated using known peptide masses (angiotensin, P substance, ACTH and somatostatin - from Sigma). Measured monoisotopic mass of tryptic peptides were analyzed using the Mascot search engine (www.matrixscience.com) consulting *Homo sapiens* taxonomy in NCBInr data base. The modifications considered were carbamidomethylation of cysteine residues (fixed) and methionine oxidation (variable), with 100 ppm mass error tolerance for protein positive identification. Contaminant peptides were manually removed using FlexAnalysis 2.0 software database (Bruker, Daltonics).

Immunofluorescence experiment: after exposure to CYN at 100 µg l^{-1} during 24 h, cells were washed in PBS (pH 7.4), fixed 2% paraformaldehyde (Electron Microscopy Sciences, Washington, NY, USA) prepared in PBS for 30 min, blocked for 30 min with two blocking buffers: blocking buffer I (1% albumin, 0.01% saponin in PBS) for nonspecific proteins interaction sites, and blocking buffer II (0.1 M glycine) for aldehyde groups. Immunolabeling was performed for tubulin proteins /microtubules, and actin filaments. For microtubules, cells were incubated with mouse anti-α-tubulin (Thermo Scientific; 1: 200 in blocking buffer I) for 1 h, washed with PBS and incubated with Alexa Fluor® 488-conjugated rabbit anti-mouse antibodies (Jackson Immuno Research; 1: 200 in PBS with 0.01% saponin) for 40 min. For actin filaments, the cells were incubated with Alexa Fluor® 546-conjugated Phalloidin (Life Technology; 1: 200 in blocking buffer I) for 40 min. For both imunolabeling procedures, the slides were mounted using Vectashield mounting medium with DAPI

(4', 6-diamidino-2-phenylindole; Vector Laboratories), which stains DNA. All incubation procedures were performed under slight agitation and at room temperature. Cells were observed under laser scanning confocal microscope multiphoton, model A1 MP+ (NIKON Instruments Inc., Tokyo, Japan) and the same image capture parameters were used in the control and CYN groups in order to allow statistical comparisons of fluorescence intensity.

Quantification of fluorescence: the intensity of fluorescence emitted by the fluorophore (Alexa Fluor® 488 and 546) and the area and circularity of the cell nuclei were determined by Imaging Software Nis Elements 4.20 (NIKON, Tokyo, Japan). For these analyzes 7 images per experimental group (control and CYN) with the same magnification and in full projection were selected. Kolmogorov-Smirnov normality test was utilized to verify data distribution (Gaussian distribution), and the data was compared through t-student test.

3. Results

3.1 Proteomics

Proteins were visualized as well-defined colloidal Coomassie Blue stained spots in 2D electrophoresis gels (Fig. 1). Differential protein expression of CYN *versus* control group comprised 48 proteins (spots). Twenty-six (26) proteins were identified by mass spectrometry, representing 54% of the total number of spots considered of interest in this study (Table 1). From these, seventeen (17) proteins were upregulated and nine (09) were downregulated in the presence of CYN; the latter detected only in the control group (Table 1). These proteins normally function in the plasma membrane, cytosol, endoplasmic reticulum and mitochondria (Figure 2).

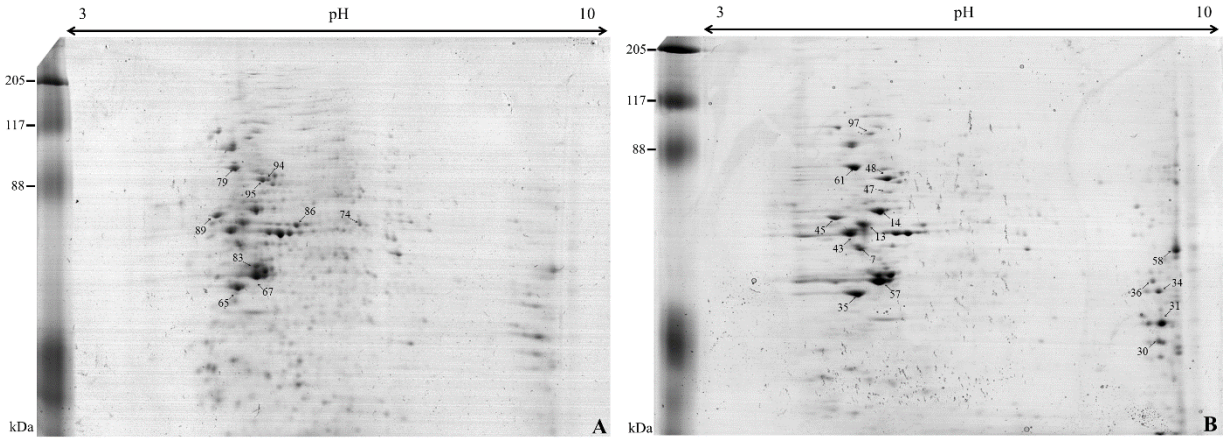


Fig.1. Two-dimensional gel electrophoresis of proteins from HepG2 cells. Control group (A) and CYN group (B). Isoelectric focusing was carried out in 13 cm immobiline IEF gel strips, pH range 3–10, loaded with 300 µg of protein. The second dimension (SDS-PAGE) was performed in 10% (w/v) polyacrylamide gels. Gels were stained with Colloidal Coomassie G-250 and proteins were identified by MALDI- TOF/TOF. Arrows and numbers indicate the identified proteins, presented in table 1.

In the plasma membrane, two proteins were upregulated: multidrug resistance protein 3 and a seven transmembrane helix receptor.

In the cytosol, a total of sixteen proteins were identified, from which seven play a role on cell cytoskeleton (keratin, α-actin, β-actin, ACTB protein, α-spectrina, α-tubulin, β-5 tubulin). Only α-actin and ACTB protein were downregulated; the other five cytoskeleton proteins were upregulated in response to CYN exposure. From the remaining cytosolic proteins (nine proteins) , the protein kinase A catalytic subunit β and heat shock protein (HSP) 90-β isoform c were downregulated, whereas glyceraldehyde-3-phosphate dehydrogenase, fructose 1,6-bisphosphate aldolase complexed with fructose 1,6-bisphosphate, heterogeneous nuclear ribonucleoprotein A2/B1, lactate dehydrogenase variant A, plasma glutathione peroxidase (GSHPx plasma), heat shock protein 71 kDa isoform 1 and heat shock protein 70kDa isoform 8 variant 2 were all upregulated (Table 1).

Table 1. Differential protein expression in HepG2 cells exposed to 10 µg.l^{-1} of CYN during 24 h.

Spot	*Match ID*	*Proteins name*	*Cell localization*	*MW*	*pI*	*Score*	*Method*	*Expression in CYN*
5822	65	40-kDa keratin protein, partial	cytosol	44065	5.04	336	ms	-
5778	67	alpha-actin	cytosol	42480	5.23	41	ms/ms	-
5755	83	ACTB protein, partial	cytosol	40536	5.55	88	ms	-
607	57	mutant beta-actin (beta'-actin)	cytosol	42128	5.22	43	ms/ms	+
16479	43	tubulin 5-beta	cytosol	50055	4.81	64	ms/ms	+
16416	47	alpha-spectrin, partial	cytosol	54468	5.72	76	ms	+
526	13	alpha-tubulin	cytosol	50810	5.02	122	ms	+
16664	31	glyceraldehyde-3-phosphate dehydrogenase	cytosol	36202	8.26	30	ms/ms	+
5634	74	protein kinase A catalytic subunit beta	cytosol	1479	5.91	29	ms	-
16615	34	Human muscle fructose 1,6-bisphosphate aldolase complexed with fructose 1,6-bisphosphate	cytosol	39720	8.39	72	ms	+
16696	30	Heterogeneous nuclear ribonucleoproteins A2/B1	cytosol	32524	8.74	138	ms	+
16696	30	lactate dehydrogenase A variant	cytosol	36951	7.63	121	ms	+
16606	35	plasma glutathione peroxidase, plasma GSHPx {EC 1.11.1.9}	cytosol	3547	4.95	28	ms/ms	+
16392	48	heat shock cognate 71 kDa protein isoform 1	cytosol	71082	5.37	115	ms	+
5509	95	heat shock 70kDa protein 8 isoform 2 variant	cytosol	53580	5.62	83	ms	-
5401	97	heat shock protein HSP 90-beta isoform c	cytosol	82611	4.98	175	ms	+
356	61	78 kDa glucose-regulated protein precursor	ER	72402	5.07	221	ms	+
5485	79	GRP78 precursor, partial	ER	72185	5.03	151	ms/ms	-
5678	7	protein disulfide isomerase-related protein 5	ER	46512	4.95	95	ms	+
16453	45	protein disulfide-isomerase precursor	ER	57480	4.76	55	ms/ms	+

5629	86	Chain A, tapasin ERP57 heterodimer	ER	54541	5.61	34	ms/ms	-
5600	89	glutathione-insulin transhydrogenase (216 AA)	ER	25107	4.5	52	ms/ms	-
16592	36	multidrug resistance protein 3, partial	membrane	4430	8.15	67	ms	+
537	58	seven transmembrane helix receptor	membrane	37808	9.19	18	ms/ms	+
504	14	mitochondrial heat shock 60kD protein 1 variant 1	mitochondria	60181	5.59	135	ms	+
5510	94	Chain A, crystal structure of the human mortalin (grp75) Atpase domain in the Apo form	mitochondria	41511	6.54	96	ms	-

(+) indicates the protein is present only in the CYN group. (-) indicates the protein is absent in the CYN group, i.e., expressed only in the control group.

.

In the endoplasmic reticulum (ER), six proteins were identified: 78 kDa glucose-regulated protein precursor, protein disulfide isomerase-related 5 and protein disulphide isomerase precursor were upregulated, whereas GRP78 precursor, tapasin ERP57 heterodimer and glutathione-insulin transhydrogenase were downregulated as a consequence of CYN exposure.

Finally, two mitochondrial proteins were identified: mitochondrial heat shock 60 kDa protein 1 variant 1, which was upregulated by CYN exposure, and a crystal structure of the Human mortalin (Grp75) ATPase domain in the Apo form, which was downregulated.

All localization of the identified proteins is represented in the schematic image of the HepG2 cell (Fig. 2).

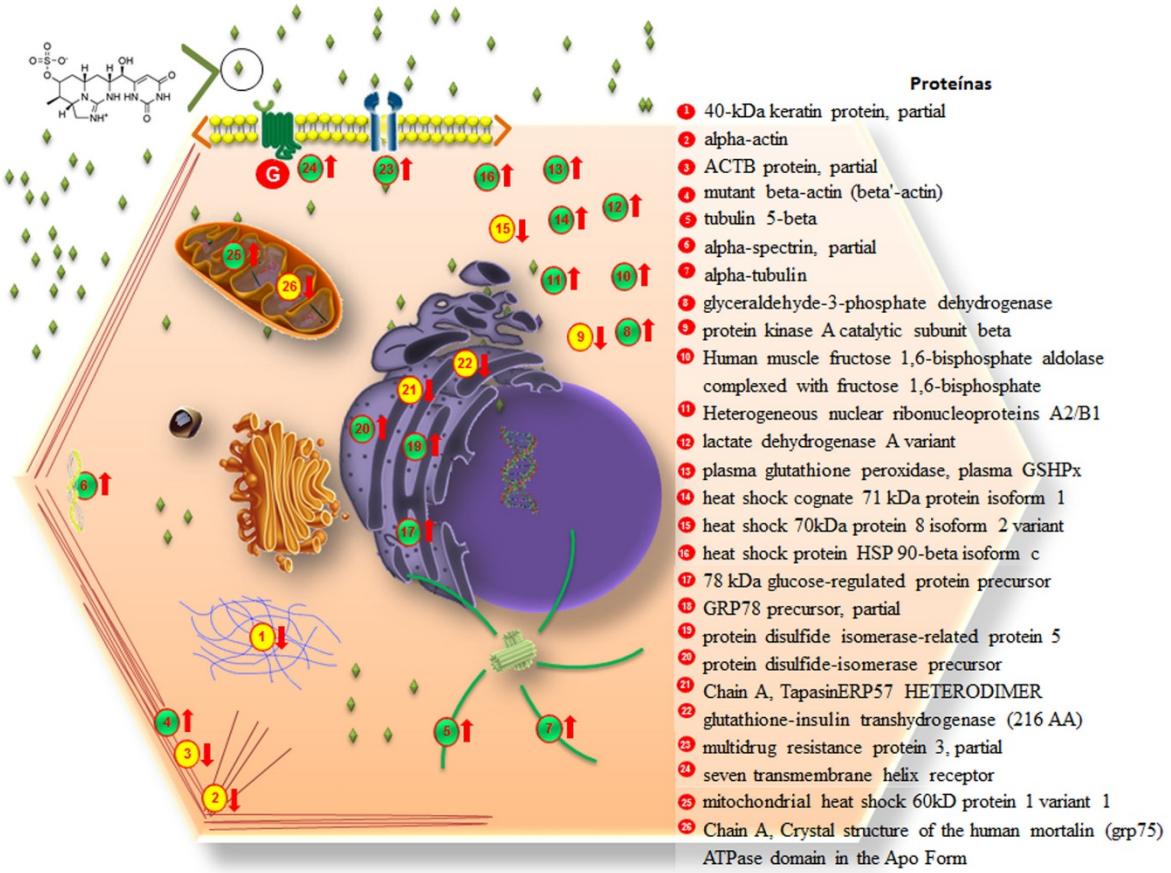


Fig. 2. Schematic representation of a HepG2 cell with the localization of the identified proteins. Up-arrows indicate protein upregulation (presents only in CYN group) and down-arrows indicate downregulation (presents only in control group).

3.2 Immunofluorescence

In order to determine how CYN affected the integrity of the cytoskeleton, microtubules and actin filaments were labeled and observed under confocal microscope. Microtubules distribution was very homogeneous and similar between control and CYN group (Fig. 3A and D), without statistical differences of fluorescence intensity between these groups (Fig. 4A). However, actin filaments fluorescence intensity decreased in cells exposed to CYN (Fig. 3B and 3E, 4B).

Since we have previously reported an increase of cell proliferation in response to CYN exposure (Liebel *et al.*, 2015), the nuclei of cells were also analyzed. There was a decrease of the mean nuclear area following CYN exposure (Fig 4 C) without interference on nucleus circularity (Fig. 4D). Considering the area of recently divided cells as a parameter, there was about 4.1 % of recently divided cells in the CYN group *versus* 1.6% in the control, indicating an increased mitotic index in former group. The presence of mitosis can be observed in figure 3C.

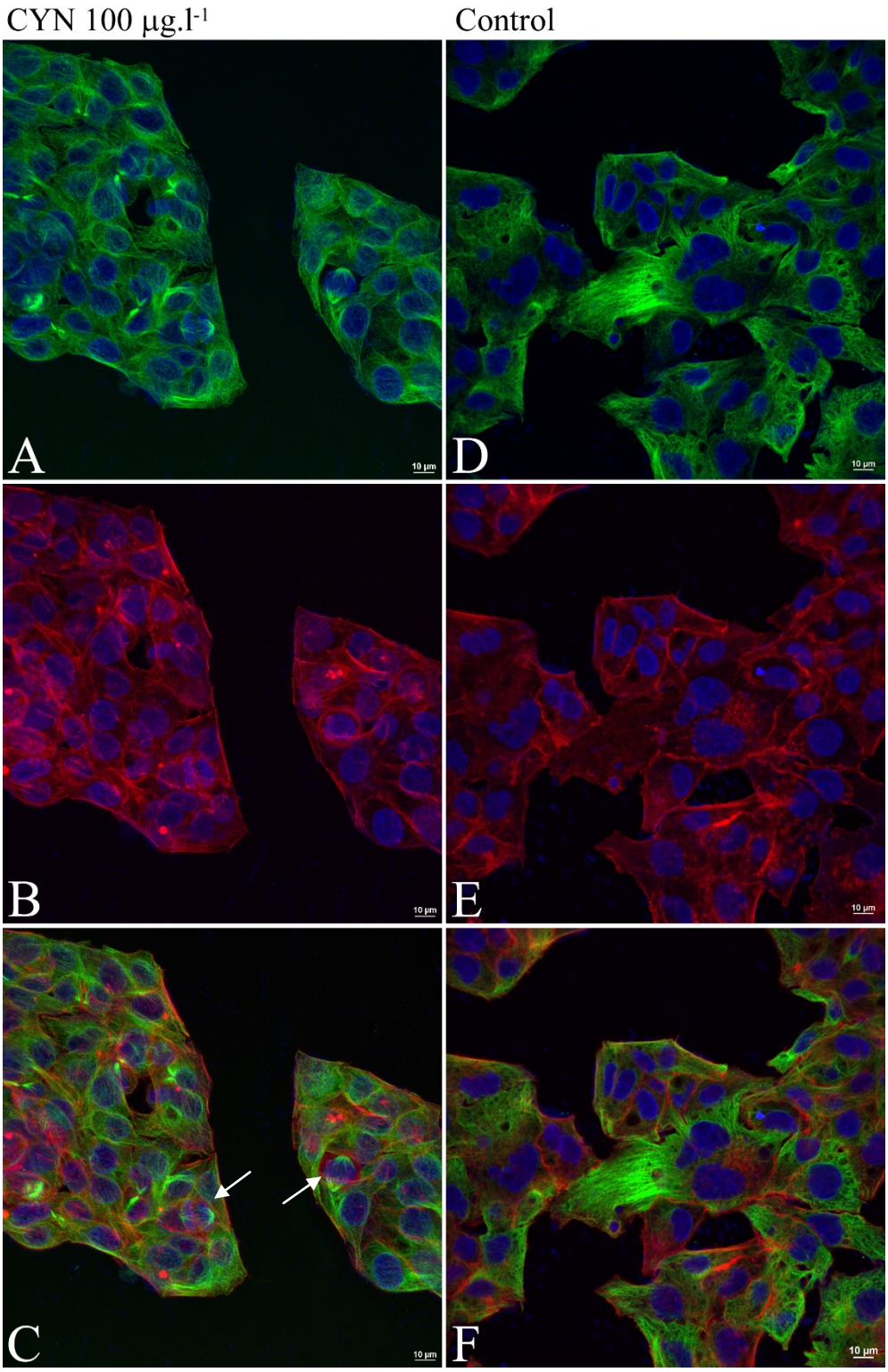


Fig. 3. Confocal images of HepG2 cells exposed to CYN and the control group. DAPI (blue), tubulin and microtubules (green, **A** and **D**) and actin filaments (red, **B** and **E**). Fluorescent markers overlap (**C** and **F**). Arrows point to cells in division (**C**). Scale bar = 10 µm.

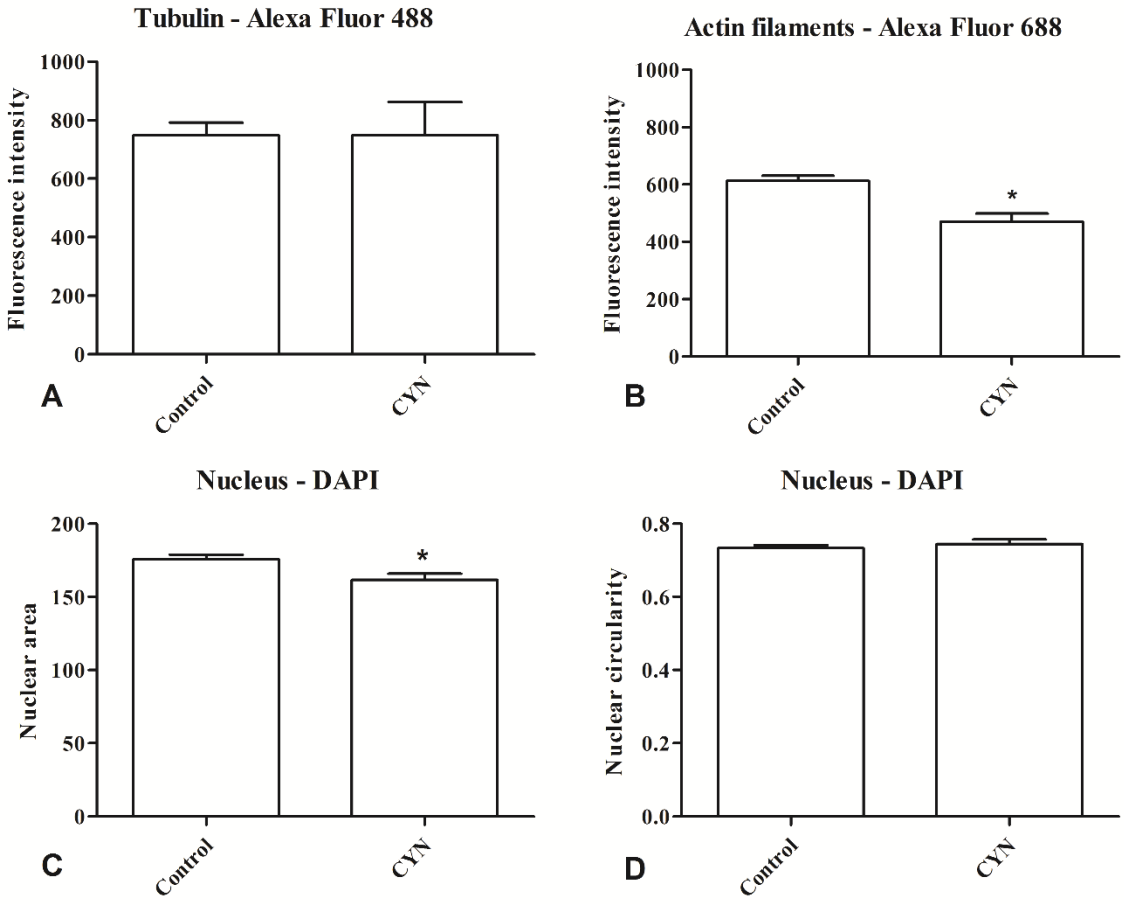


Fig. 4. Fluorescence analysis. Fluorescence intensity emitted in tubulin (**A**) and actin filaments (**B**) labeling. Nuclear area (**C**) and circularity (**D**) quantification. Asterisks (*****) indicate statistical difference between the control and CYN groups. $p < 0.05$.

4. Discussion

The investigation of proteins' profiles may help to identify proteins that are altered by CYN exposure and provides new data about the toxic mechanisms of cylindrospermopsin. A total of 48 proteins have their expression altered by CYN, but 26 proteins were identified. These proteins are implicated in different biological processes, such as protein folding, xenobiotic efflux, antioxidant defense, energy

metabolism and cell anabolism, cell signaling, tumorigenic potential, and cytoskeleton.

Protein folding

Chaperones are proteins dedicated to proper protein folding and translocation. CYN provoked dual effects on chaperones, decreased expression of heat shock protein 70 (HSP70) and of 75 kDa glucose regulated protein (GRP75) as well as increased expressions of HSP71, HSP90, protein disulfide isomerase (PDI), GRP78 (BiP) and HSP60.

HSPs 70 and 71 assist protein folding and re-folding (Glover and Lindquist, 1998; Hendrick and Hartl, 1993). Their expression is boosted during the unfolded protein response, and the decreased expression is generally correlated with early cytotoxic events and is a secondary consequence of damages that affect cellular integrity (Singh *et al.*, 2009). Mild unfolded protein response results in the reduction of general protein synthesis and selective expression of chaperones that facilitate survival of damaged cells (Frydman, 2001; Young *et al.*, 2004). Furthermore, the up-regulation of HSP71 in leukemic cells contributes to cell cycle disruption, and HSC71 binding to cyclin D1 in nuclei leads to the stabilization of the cyclin D1/cyclin dependent kinase 4 complex, inducing cell proliferation (Young *et al.*, 2004). Cell proliferation has been previously reported for HepG2 cell (Liebel *et al.*, 2015).

The 75 kDa glucose regulated protein (GRP75) is a mitochondrial matrix protein that is generally recognized as a member of the heat shock protein 70 (HSP70) class of proteins. GRP75 is induced under conditions of low glucose and other nutritional and environmental stresses. But GRP75 expression decreased in response to CYN. Closely related proteins have been identified in the immune

system (PBP74; CSA), liver (mtHsp70), and cell lines. GRP75 is involved in various chaperoning functions in protein translocation, folding and function in mitochondria. It also appears to be involved in antigen recognition, cell proliferation, and senescence.

HSP90 can facilitate protein synthesis and early folding events preventing aggregation of non-folded proteins, and cell cycle regulation. Particularly, HSP90 plays a role in vesicular transport and protein trafficking. Because cell proliferation is critically dependent on membrane trafficking, impairment of this process would inhibit cell growth (Gazzag *et al.*, 2013). However, Hsp90 has been reported to be overexpressed in various types of cancer cells and may become a new target for cancer therapy. Moreover, Hsp90 can bind to and stabilize multiple autophagy-related proteins or kinases, such as Beclin1, Bcl-2, Raf-1, which may assist tumor cells ‘adapting’ to stressful conditions (Wanga et al, 2014).

Members of the protein disulfide isomerase (PDI) family are predominately located within the endoplasmic reticulum (ER) where they are involved in the oxidative folding of newly synthesized membrane and secretory proteins (Kober *et al.*, 2012) by catalyzing the formation and breakage of disulfide bonds (Ellgaard *et al.*, 2005).

The 78 kDa glucose-regulated protein (GRP78), also known as binding immunoglobulin protein (BiP), is a HSP70 chaperone located in the lumen of ER that binds newly synthesized proteins as they are translocated into the ER, and maintains them in a state competent for subsequent folding and oligomerization. BiP is also an essential component of the translocation machinery and plays a role in retrograde transport across the ER membrane of aberrant and misfolded proteins destined for degradation by proteasomes. BiP is an abundant protein under all growth conditions,

but its synthesis is markedly induced under conditions that lead to the accumulation of unfolded polypeptides in the ER. When Chinese hamster K12 cells are starved of glucose, the synthesis of several proteins, called glucose-regulated proteins (GRPs), is markedly increased. The level of GRP78 is strongly correlated with the amount of secretory proteins within the ER (Reddy *et al.*, 2003, Kober *et al.*, 2012).

Finally, the mitochondrial chaperone HSP60 is involved in the synthesis and transport of essential mitochondrial proteins by promoting conformational and structural changes (Koll *et al.*, 1992). This family of proteins is usually upregulated in situations of stress (Santoro, 2000), such as that previously observed in HepG2 cells exposed to the same concentration of CYN through increases of ROS and lipid peroxidation (Liebel *et al.*, 2015),.

Xenobiotic efflux

The multidrug resistance protein 3 (MRP3) is situated at the basolateral membrane of epithelial cells such as hepatocytes and functions as an efflux pump of a broad spectrum of molecules such as glutathione, glucuronide and sulfate conjugates into the extracellular space and thereafter to the blood (for elimination toxic molecules through urine in the case of hepatocytes). Particularly, it mediates the efflux of cytotoxic drugs leading to chemotherapeutics resistance (Gillet *et al.*, 2007; Yu *et al.*, 2014). CYN exposure provoked an increase of MRP3 expression, although changes of general efflux activity in HepG2 through rhodamine efflux assay had not been observed (Liebel *et al.*, 2015), probably due to limitations of the method utilized. Increased levels of ROS are usually associated with increases of cell antioxidant defense mechanisms, cell signaling and damage to biomolecules,

depending on the intensity of the increase (Li *et al.* 2009; Wojewod et al, 2010; Liebel *et al.* 2011).

Antioxidant defense

Glutathione peroxidase (GSHPx) is involved in the reduction of lipid hydroperoxides and the hydrogen peroxide (Chu *et al.*, 1992; Zahara *et al.*, 2005), and so induction of GSHPx is an important defense mechanism against oxidative stress caused by CYN. However, this increase has not been enough to avoid the increase of ROS levels in HepG2 cells.

Energy metabolism

CYN exposure increased the expression of several proteins involved in cell energy metabolism (fructose 1,6-bisphosphate aldolase complexed with fructose 1,6-bisphosphate, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, glucose-regulated protein (GRP78).These proteins are involved in a chain of reactions, as follows.

Fructose 1,6-bisphosphate aldolase catalyzes the conversion of fructose 1,6-bisphosphate in dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) in glycolysis, gluconeogenesis, and the Calvin cycle; the latter exclusive to plant cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate in glycolysis. GAPDH is a multifunctional enzyme involved in numerous processes (Sirover, 2009). The levels of GAPDH have been increased in Clone 9 cells exposed to 5 mM of CYN (24 and 48 h), but not in cells exposed to 1 mM of CYN (Fernandez *et al.* 2014). Lactate dehydrogenase catalyzes the conversion of pyruvate, the final product of glycolysis, to lactate in anaerobic conditions in hepatic

cells. Anaerobic metabolism predominates in cultured cell (Gnaiger *et al.*, 1990; Zhang *et al.*, 2013) and increases of these three glycolysis enzymes is a clear indication of increased energy demand for cells to respond to stress caused by CYN and to perform anabolic reactions required to proliferate.

Cell anabolism

Glutathione-insulin transhydrogenase is located primarily in the ER, but it is also associated with plasma membrane and other intracellular compartments. Glutathione-insulin transhydrogenase inactivates insulin by catalyzing the cleavage of the disulfide bonds of insulin in the presence of a simple sulfhydryl compound such as GSH (glutathione) by way of sulfhydryl-disulfide interchange (Varandani, 1972). The enzyme is apparently regulated by many mechanisms, which include the feedback regulation by insulin on its protein concentration and the regulation of its enzymatic activity by phospholipids, divalent cations and certain hormones (Varandani *et al*., 1974).

The decrease of this protein expression may indicate an attempt of the HepG2 cells to maintain more active insulin and thus increase liver anabolic pathways, since insulin is responsible for maintaining glucose homeostasis (by decreasing gluconeogenesis and glycogenolysis) and cell growth and differentiation (Boulton *et al.*, 1991; Cross *et al.*, 1995). Insulin also stimulates lipogenesis in the liver and adipocytes and reduces lipolysis, as well increases the synthesis and inhibits protein degradation (Anthonsen *et al.*, 1998).

Cell signaling

An important energy metabolism correlated protein that responded to CYN exposure was protein kinase A (PKA). The PKA is present both in the cytosol and

nucleus of cells, and takes part in many cellular processes such as cell cycle progression, gene expression, cell growth and differentiation, embryogenesis, circadian rhythms and apoptosis (Pinna and Allende, 2009; St-Denis and Litchfield, 2009). PKA is a cAMP-dependent protein kinase that stimulates glycogenolysis and gluconeogenesis, and inhibits glycogenesis and glycolysis in hepatocytes (Pilkis and Granner, 1992). Thus, decrease of PKA expression in response to CYN can be associated with increase of energy demand of cells, supplied by glycolysis.

The seven transmembrane helix receptor also known G protein–coupled receptors (GPCRs) represent by far the largest family of cell-surface molecules involved in cell signaling. These receptors control key physiological functions, including neurotransmission, hormone and enzyme release from endocrine and exocrine glands, immune responses, cardiac and smooth-muscle contraction and blood pressure regulation (Gutkind, 1998; Rozengurt, 2002). Overexpression of many GPCRs in various cancer types contributes to cancer cell proliferation (Dorsam & Gutkind, 2007), and so their increased expression in response to CYN may be direct associated with the cell proliferation previously reported for HepG2 cell (Liebel *et al.*, 2015) and may have important consequences to the tumorigenic potential of cells

Tumorigenic potential

In this study, CYN exposure resulted in decreased expression of the heterodimer tapasin ERP57. ERP57 is associated with a major histocompatibility complex (MHC I) molecules (Peaper *et al.*, 2005). MHC I itself is an inhibitory ligand for natural killer cells (NKs), so that decrease of normal surface class MHC I levels is an important mechanism employed by some viruses during immune evasion. Several

human cancers also show down-regulation of MHC I, giving transformed cells the same survival advantage of being able to avoid normal immune surveillance designed to destroy any infected or transformed cells (Wang *et al.* 2008). Therefore, CYN modulation of the expression of ERP57 may facilitate HepG2 cells evasion from NK immune cells, which could have important consequences if one considers the *in vivo* situation.

Heterogeneous nuclear ribonucleoproteins (hnRNP) are a family of proteins which share common structural domains, and extensive research has shown that they have central roles in DNA repair, telomere biogenesis, cell signaling and in regulating gene expression at both transcriptional and translational levels. Through these key cellular functions, individual hnRNPs have a variety of potential roles in tumor development and progression, including the inhibition of apoptosis, angiogenesis and cell invasion (Carpenter *et al.*, 2006). Thus, the increase of hnRNP in cells exposed to CYN can increase HepG2 tumorigenic potential.

Cytoskeleton

Several differentially expressed proteins identified in the current study are structural proteins, e.g. belongs to the cytoskeleton. Alterations of actin microfilaments structure and distribution have long been reported in situations of cellular stress and apoptosis (Alvarez and Sztul, 1999; Kanlaya *et al.*, 2009). Gácsi *et al.* (2009) described alterations of cytoskeletal structures and apoptosis in Chinese hamster ovary cells (CHO-K1) mediated by CYN. At the proteomic level, correlations were found between the changes of the expression of the cytoskeletal proteins and stress factors in different biological systems including bovine kidney and blood cells (Riedmaier *et al.*, 2009; Zhang *et al.*, 2009), human cell lines (Ou *et al.*, 2008), in the

fish *Sparus aurata* (Ibarz *et al.*, 2010), the fresh water bivalve *Corbicula fluminea* (Martins *et al.*, 2009) and in rabbits (Almeida *et al.*, 2010). Taking these data in consideration, it is possible to infer that the changes in actin and tubulin isoforms expression, observed in this work, are indicative of stress and a possible cellular injury in HepG2 exposed to CYN. These results were corroborated by immunofluorescence analysis, where a decrease of the fluorescence intensity of actin filaments was observed. Changes in cytoskeleton morphology are reported in the cells exposed to different CYN concentrations (Gácsi *et al.*, 2009), which reinforces their cytotoxic potential to cytoskeleton.

Several proteins identified in the current work are related to increased proliferation of cells, which has been previously reported (Liebel *et al.,* 2015). Here, CYN group presented a higher percentage of newly divided nuclei (marked with DAPI) than the control and so a higher mitotic index.

Final comments

CYN has been included in the revision of WHO ‘‘Guidelines for Drinking-water Quality, chemical hazards in drinking-water” and classified on the list of compounds with highest priority for hazard characterization by the US Environmental Protection Agency (EPA, 2010)

In the current study, the expression of several proteins was altered by CYN exposure, providing new data about the toxic mechanisms of CYN. These proteins are implicated in different biological processes. HSPs are correlated with cytotoxic events, cell proliferation and tumor cells ‘adaptation’ to stressful conditions, processes that are interlinked in a remarkable complex net, as described in short. CYN appeared to increase glucose uptake, since the expression of GRP75 and

GRP78 decreased, and the expression of three enzymes involved in glycolysis increased. Glucose uptake and breakdown is necessary to provide useful chemical energy to cells, in order to respond to the CYN stress, synthesizes macromolecules (anabolism reactions) and to proliferate; decreases of the expressions of PKA and glutathione-insulin transhydrogenase protein may allow increase of glycolysis and anabolism reaction, respectively. Increase of GPCRs is associated with cell proliferation and it, together with increases of hnRNP expression and ROS levels, can enhance CYN-associated HepG2 tumorigenic potential. Alteration of redox milieu by mild accumulation of ROS can increase cell proliferation, but excess of ROS are controlled by protective mechanisms such as MRP3 and GSHPx. Else, ROS can damage biomolecules such as cytoskeleton protein, that must be synthesized to replaced oxidized proteins (Allani et al, 1994; Santa-Maria et al, 2005) as well as to provide new building blocks necessary for proliferating cells.

Finally, the results reported in the current work complement a previous study (Liebel *et al.*, 2015) and provide new evidence of molecular targets of CYN.

Highlights

Cylindrospermopsin affected the expression of at least 48 proteins in HepG2 cells;
Proteins are involved in energy metabolism, signaling and tumorigenic potential;
Others participate in protein folding, efflux, antioxidant defense and cytoskeleton;
Increase of ROS and cell proliferation can partially explain protein profile;
The identified proteins provide useful information about CYN toxic mechanism.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

6. Acknowledgements

This research was supported by CNPq (Brazilian Agency for Science and Technology) and CAPES (PhD scholarship). The authors thank Valéria Freitas Magalhães and Rodrigo Cássio da Silva for providing purified cylindrosprmopsin, and Emanuel Maltempighi de Souza, Caroline Kukolj and Israel Henrique Bini for technical support in mass spectrometry and high resolution confocal microscopy.

7. References

Afssa, 2006. Rapport sur l'évaluation des risques liés à la présence de cyanobactéries et de leurs toxines dans les eaux destinées à l'alimentation, à la baignade et autres activités récréatives pp. 300 <http://www.afssa.fr/ftp/afssa/37423-37424.pdf>.

Allani, P. K, Sum, T., Bhansali, S. G., Mukherjee, S. K., Sonee, M., 2004. A comparative study of the effect of oxidative stress on the cytoskeleton in human cortical neurons. Toxicol. App. Pharmacol. 196, 29–36.

Almeida, A.M., Campos, A., Francisco, R., Harten, S.V., Cardoso, L.A., Coelho, A.V., 2010. Proteomic investigation of the effects of weight loss in the gastrocnemius muscle of wild and NZW rabbits via 2D-electrophoresis and MALDI-TOF MS. Anim. Genet. 41, 260–272.

Alvarez, C., Sztul, E. S., 1999. Brefeldin A (BFA) disrupts the organization of the microtubule and the actin cytoskeletons. Europ. J. Cell Biol. 78, 1-14.

Anthonsen, M.W., Ronnstrand, L., Wernstedt, C., Degerman, E., Holm, C., 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. J Biol. Chem. 273, 215-221.

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

Bittencourt-Oliveira, M. C., 2003. Detection of potential microcystin-producing cyanobacteria in Brazilian reservoirs with a mcyB molecular marker. Harm. Alg. 2, 51-60.

Boulton, T.G., Nye S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., et al., 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65, 663-75.

Brasil. 2011. Ministério da Saúde Portaria n. 2914, 1ed. Ministério da Saúde , Brasília.

Carpenter, B., MacKay, C., Alnabulsi, A., MacKay, M., Telfer, C., Melvin, W.T., Murra, G.I., 2006. The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. Biochim. Biophys. Acta, 1765, 85–100.

Chiswell, R. K., Shaw, G. R., Eaglesham, G., Smith, M. J., Norris, R. L., Seawright, A. A., Moore, M. R., 1999. Stability of cylindrospermopsin, the toxin from the cyanobacterium, Cylindrospermopsis raciborskii: Effect of pH, temperature, and sunlight on decomposition. Environ. Toxicol. 14, 155–161.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785-789.

Dorsam, R. T., Gutkind, J. S., 2007. G-protein-coupled receptors and cancer. Nature Reviews Cancer 7, 79-94.

Dowling, V.A., Sheehan, D., 2006. Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics 6, 5597-5604.

Ellgaard, L., Ruddock, L.W., 2005. The human protein disulphide isomerase family: substrate interactions and functional properties. EMBO Reports 6, 28-32.

Elliott, J. A, 2012. Is the future blue-green? A review of the current model predictions of how climate change could affect pelagic freshwater cyanobacteria. Water Res. 46, 1364–1371.

Epa (Environmental Protection Agency). 2010. Creating a Cyanotoxin Target List for the Unregulated Contaminant Monitoring Ruley. Technical Service Center, USEPA Press, Cincinnati.

Chu, F.F., Esworthy, S., Doroshow, J.H., Doan, K., Liu, X.F., 1992. Expression of plasma glutathione peroxidase in human liver in addition to kidney, heart, lung, and breast in humans and rodents. Blood 79, 3233–3238.

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

Fernandez, D. A., Louzao, M. C., Vilarino, N., Begoña Espiña, M. F., Vieytes, M. R., Botana, L. M., 2014. Evaluation of the intestinal permeability and cytotoxic effects of cylindrospermopsin. Toxicon 91 23-34.

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.

Gácsi, M., Antal, O., Vasas, G., Máthé, C., Borbély, G., Saker, M.L., Gyori, J., Farkas, A., Vehovszky, A., Bánfalvi, G., 2009. Comparative study of cyanotoxins affecting cytoskeletal and chromatin structures in CHO-K1 cells. Toxicol. In Vitro 23, 710-718.

Gazzaha, A. C., Camoinc L., Abida S., Bouaziza C., Ladjimib M., Bachaa H., 2013. Identification of proteins related to early changes observed in Human hepatocellular

carcinoma cells after treatment with the mycotoxin Zearalenone. Experimental and Toxicol. Patho. 65, 809-816.

Gillet, J.P., Efferth, T., Remacle, J., 2007. Chemotherapy-induced resistance by ATP-binding cassette transporter genes, Biochim. Biophys. Acta 1775, 237-262.

Glover, J.R., Lindquist, S. 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94, 73-82.

Gnaiger E., Kemp R., Gutkind B., 1998. Anaerobic metabolism in aerobic mammalian cells: information from the ratio of calorimetric heat flux and respirometric oxygen flux. Biochim. Biophys. Acta 1016, 328-332.

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

Hendrick, J.P., Hart F.U., 1993. Molecular chaperone functions of heat-shock proteins Annu. Rev. Biochem., 62, 349-384.

Hudnell, H.K., 2010. The state of U.S. freshwater harmful algal blooms assessments, policy and legislation. Toxicon 55, 1024–1034.

Humpage, A.R., Falconer, I.R., 2003. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice. determination of no observed adverse effect level for deriving a drinking water guideline value. Environ. Toxicol. 18, 94–103.

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.

Huszar, V. L. M., Silva, L. H. S., Marinho, M., Domingos, P. & Anna, C. L. S. 2000. Cyanoprokaryote assemblages in eight productive tropical Brazilian waters. Hidrobiologia, 424: 67-77.

Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater ‘‘seafood’’ and its consequences for public health: a review. Environ. Pollut. 150, 177–192.

Kanlaya, R., Pattanakitsakul, S.-n., Sinchaikul, S., Chen, S.-T., Thongboonkerd, V., 2009. Alterations in actin cytoskeletal assembly and junctional protein complexes in human endothelial cells induced by dengue virus infection and mimicry of leukocyte transendothelial migration. J. Proteome Res. 8, 2551–2562.

Kober, F.X., Koelmel, W., Kuper, J., Drechsler, J., Mais, C., Hermanns, H.M., Schindelin, H., 2013. The crystal structure of the protein-disulfide isomerase family member ERp27 provides insights into its substrate binding capabilities. J. Biol. Chem. 288, 2029-2039.

Koll, H., Guiard, B., Rassow, J., Ostermann, J., Horwich, A.L., Neupert, W., Hartl, F.U., 1992. Antifolding activity of hsp60 couples protein import into the mitochondrial matrix with export to the intermembrane space. Cell 68, 1163–1175.

Lehman, J.T., Doubek, J.P., Jackson, E.W., 2013. Effect of reducing allochthonous P load on biomass and alkaline phosphatase activity of phytoplankton in an urbanized watershed. Michigan. Lake Reservoir Manage. 29, 116–125.

Lei, L., Peng, L., Huang, X., Han, B.P., 2014. Occurrence and dominance of Cylindrospermopsis raciborskii and dissolved cylindrospermopsin in urban reservoirs used for drinking water supply, South China. Environ. Monit. Assess. 186, 3079-3090.

Li, M., Zhao, L., Liu, J., Liu, A.L., Zeng, W.S., Luo, S.Q., Bai, X.C., 2009. Hydrogen peroxide induces G2 cell cycle arrest and inhibits cell proliferation in osteoblasts. Anat. Rec. 292, 1107-1113.

Liebel, S., Oliveira Ribeiro, C.A., Silva, R.C., Ramsdorf, W.A., Cestari, M.M., Magalhães, V.F., Garcia, J.R.E., Esquivel, B.M., Filipak Neto, F., 2011. Cellular responses of Prochilodus lineatus hepatocytes after cylindrospermopsin exposure. Toxicol. in Vitro. 25, 1493- 1500.

Liebel S., de Oliveira Ribeiro C.A., Magalhães V.F., Silva, R.C., Rossi, S.C., Randi, M.A., Filipak Neto, F., 2015. Low concentrations of cylindrospermopsin induce increases of reactive oxygen species levels, metabolism and proliferation in human hepatoma cells (HepG2). Toxicol. in Vitro 29, 479-488.

Martins, J.C., Leão, P.N., Vasconcelos, V., 2009. Differential protein expression in Corbicula fluminea upon exposure to a Microcystis aeruginosa toxic strain. Toxicon 53, 409–416.

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.

O'Neila, J.M., Davisb, T.W., Burfordb, M.A., Goblerc, C.J., 2012. The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. Harm. Alg. 14, 313–334.

Ou, K., Yu, K., Kesuma, D., Hooi, M., Huang, N., Chen, W., Lee, S.Y., Goh, X.P., Tan, L.K., Liu, J., Soon, S.Y., Bin, A. R. S., Putti, T.C., Jikuya, H., Ichikawa, T., Nishimura, O., Salto-Tellez, M., Tan, P., 2008. Novel breast cancer biomarkers identified by integrative proteomic and gene expression mapping. J. Proteome Res. 7, 1518–1528.

Peaper, D. R, Wearsch, P. A, Cresswell, P., 2005. Tapasin and ERp57 form a stable disulfide-linked dimer within the MHC class I peptide-loading complex EMBO J. 24, 3613–3623.

Pilkis, S. J., Granner, D. K., 1992. Molecular Physiology of the Regulation of Hepatic Gluconeogenesis and Glycolysis. Annual Rev. Physiol. 54, 885-909.

Poniedziałek, B., Rzymski, P., Kokocinski, M., 2012. Cylindrospermopsin: waterlinked potential threat to human health in Europe. Environ. Toxicol. Pharmacol. 34, 651–660.

Reddy, R.K., Mao, C., Baumeister, P., Austin, R.C., Kaufman, R.J., Lee, A.S., 2003. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis

induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem. 278, 20915-20924.

Riedmaier, I., Tichopad, A., Reiter, M., Pfaffl, M.W., Meyer, H.H.D., 2009. Identification of potential gene expression biomarkers for the surveillance of anabolic agents in bovine blood cells. Anal. Chim. Acta 638, 106-113.

Rozengurt, E., Guha, S., Sinnett-Smith, J., 2002. Gastrointestinal peptide signalling in health and disease. Eur. J. Surg. Suppl. 23–38.

Saker, M.L., Eaglesham, G.K., 1999. The accumulation of cylindrospermopsin from the cyanobacterium Cylindrospermopsis raciborskii in tissues of the Redclaw crayfish Cherax quadricarinatus. Toxicon 37, 1065–1077.

Santa-María, I., Smith, M. A., Perry, G., Hernández, F., Avila J., Moreno, F. J., 2005. Effect of quinones on microtubule polymerization: a link between oxidative stress and cytoskeletal alterations in Alzheimer's disease. Biochim. Biophys. Acta, 1740, 472–480.

Santoro, M.G., 2000. Heat shock factors and the control of the stress response. Biochem. Pharmacol. 59, 55-63.

Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., Mann, M., 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. USA 93, 14440-14445.

Singh, M. P., Krishn, M.M., Reddy, N., Mathur, D.K, Saxena, D., Chowdhuri, K. 2009. Induction of hsp70, hsp60, hsp83 and hsp26 and oxidative stress markers in benzene, toluene and xylene exposed Drosophila melanogaster: Role of ROS generation. Toxicol. App. Pharmacol. 235, 226-243.

Sirover, M.A., 1999. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim. Biophys. Acta 1432, 159–184.

St-Denis, N.A., Litchfield, D.W., 2009. Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. Cell. Mol. Life Sci. 66, 1817–1829.

Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E., Watanabe, M., 1994. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga Umezakia natans. Toxicon 32, 833–843

Varandani, P T., 1972 Insulin degradation. I. Purification and properties of glutathione-insulin transhydrogenase of rat liver. Biochim. Biophys. Acta. 286, 126-35.

Varandani, P. T., Nafz, M. A., Shroyer, L. A., 1974. Insulin Degradation: VI. Feedback Control by Insulin of Liver Glutathione-Insulin Transhydrogenase in Rat. Diabetes 23, 117-125.

Wang, Q., Xie, P., Chen, J., Liang, G., 2008. Distribution of microcystins in various organs (heart, liver, intestine, gonad, brain, kidney and lung) of Wistar rat via intravenous injection. Toxicon 52, 721–727.

Wanga, B., Chenb, L., Nia, Z., Daic, X., Qina, L., Wua, Y., Lia, X., Xud, L., Liana, J., He, F., 2014. Hsp90 inhibitor 17-AAG sensitizes Bcl-2 inhibitor (-)-gossypol by suppressing ERK-mediated protective autophagy and Mcl-1 accumulation in hepatocellular carcinoma cells. Experim. Cell Res. 328, 379–387.

Wörmer, L., Huerta-Fontela, M., Cirés, S., Carrasco, D., Quesada, A., 2010. Natural photodegradation of the cyanobacterial toxins microcystin and cylindrospermopsin. Environ. Sci. Technol. 44, 3002–3007.

Wojewoda, M., Duszyński, J, Szczepanowska, J., 2010. Antioxidant defence systems and generation of reactive oxygen species in osteosarcoma cells with defective mitochondria: effect of selenium. Biochim Biophys Acta. 1797, 890-896.

Young, J.C., Agashe, V.R., Siegers, K., Hart, F.U. 2004. Pathways of chaperone-mediated protein folding in the cytosol. Nature Rev. Mol. Cell Biol. 5, 781-791.

Yu, Z., Zhang, C., Wang, H., Xing, J., Gong, H., Yu, E., Zhang, W., Zhang, X., Cao, G., Fu, C., 2014. Multidrug resistance-associated protein 3 confers resistance to chemoradiotherapy for rectal cancer by regulating reactive oxygen species and caspase-3-dependent apoptotic pathway. Cancer Letters 353, 182–193.

Zachara, B.A., Wlodarczyk, Z., Andruszkiewicz, J., Gromadzinska, J., Wasowicz, W., 2005. Glutathione and glutathione peroxidase activities in blood of patients in early stages following kidney transplantation. Renal Failure 27, 751-755.

Zhang, G., Yang, P., Guo, P., Miele, L., Sarkar, F. H., Wang, Z., Zhou, Q., 2013. Unraveling the mystery of cancer metabolism in the genesis of tumor-initiating cells and development of cancer. Biochim. Biophys. Acta 1836, 49-59.

3. RESULTADOS E DISCUSSÃO

No primeiro capítulo a CYN não se apresentou tóxica nas concentrações de até 10 µg l^{-1}, levando a um aumento da viabilidade e tocidade celular em células cultivadas com 10% de soro bovino fetal (SBF) (Fig. 3, pag. 21). A redução do soro a 2% e a indução das isoformas do citocromo P450 (CYP) tornaram a capacidade metabólicas das células HepG2 próximas das células “normais”. Após a indução poucos parâmetros se mostraram alterados como a peroxidação lipídica (Fig. 8, pag. 25), no entanto baixas concentrações de CYN (inferior ou igual a 10 µg l^{-1}) induziram um aumento da proliferação e toxicidade celular (Fig. 7, pag. 23).

A grande maioria dos artigos utiliza durante os experimentos com células HepG2 a concentração de soro bovino fetal (SBF) em 10%. O SBF tem como função promover o crescimento, proliferação e manutenção das células na cultura celular, mas o fato das células cultivadas nas condições padrão (10% FBS) estarem proliferando em experimentos com tempos de exposição mais longos pode mascarar os resultados. Além disso, a concentração de soro em experimentos deve ser o mínimo necessário para a manutenção da célula em níveis normais de metabolismo e viabilidade (VALK *et al.*, 2010). Os experimentos usando diferentes concentrações de SBF permitem verificar que concentrações menores de soro não afetam o toxicidade e a viabilidade celular. No entanto, a célula apresenta-se menos resistente na presença da cilindrospermopsina, já que não ocorrem alterações nos níveis destes biomarcadores, ao contrário do observado nas células expostas a toxina em meio de cultura contendo 10% de SBF. Assim, há necessidade do ajuste

da concentração de SBF para 2% para estudos de toxicidade com HepG2, a fim de se manter as células numa condição mais próxima de uma condição *in vivo*.

De maneira geral, a exposição à cilindrospermopsina por 12 e 24h levou a resultados similares, com aumento do citotoxicidade e EROs sem efeitos sobre os transportadores de efluxo e a viabilidade celular. No entanto, houve uma redução da peroxidação lipídica em 12h e aumento em 24h nas células expostas a 10 $\mu g.l^{-1}$ de cilindrospermopsina. Com a análise dos resultados, verifica-se uma resistência das células HepG2 à toxina muito superior ao encontrado para outros estudos in *vivo* e *in vitro* (HUMPAGE *et al.* 2005, NEUMANN *et al.* 2007, LIEBEL, *et al.* 2011). Isto provavelmente deve-se ao fato de que entre as enzimas com baixa atividade apresentadas por estas células estão incluídas algumas que agem diretamente no mecanismo de toxicidade da cilindrospermopsina. Particularmente, as CYPs parecem ter um papel na toxicidade desta toxina (CHUANG *et al.*, 2001; WALTER *et al.*, 2007). Estas enzimas são responsáveis por adicionar um grupo funcional ao substrato através de reações de hidroxilação, oxidação ou redução. Esses grupos funcionais adicionados podem ser modificados (ou não) de maneira a aumentar a solubilidade dos substratos e facilitar a excreção, permitindo assim um processo de destoxificação ou bioativação (DONATO et. al., 2008; RODRÍGUEZ-ANTONA et. al., 2002). Nesse sentido, estudos utilizando indutores e inibidores de determinadas CYPs mostram-se importantes para desvendar as possíveis rotas envolvidas na biotransformação de xenobióticos, que podem direcionar ao mecanismo de toxicidade da cilindrospermopsina. Trabalhos com HepG2, utilizando fármacos ativadores das principais famílias de CYPs em humanos (1A, 2A, 2C, 3A), relatam resultados bastante significativos e reforçam a relevância das células HepG2 como

um modelo de estudo, mesmo em estudos nos quais a atividade do sistema P450 mostra-se importante (LIU *et al.*, 2009).

Ao se comparar os indutores utilizados neste estudo (Fig. 6, pg. 22), observa-se que o fenobarbital apresentou, de forma geral, os resultados mais preponderantes, em ambas as concentrações de toxina nos tempos 12 e 24 h, onde redução da viabilidade e alteração no metabolismo celular foram observadas, diferente do grupo controle e dos demais indutores. Ou seja, não está ocorrendo um aumento do metabolismo energético, mas uma redução da viabilidade, confirmando que a atividade das CYPs está envolvida com a toxicidade da cilindrospermopsina. O fenobarbital foi bastante eficiente na indução das CYPs, confirmando assim que a exposição das células a determinados indutores pode ser utilizada como ferramenta para verificar aumento da toxicidade (WALTER *et al.* 2007; TURPEINEN *et al.* 2009). Com a escolha do indutor e dos tempos mais adequados de exposição à cilindrospermopsina, a investigação dos demais biomarcadores tornou-se necessária, a fim de se comparar as diferentes respostas celulares em condições mais próximas de uma situação real.

O fato das células expostas à toxina após a indução pelo fenobarbital apresentarem uma redução da viabilidade celular de forma mais evidente do que quando expostas somente à toxina mostra uma relação direta entre a indução e menor viabilidade das células; isto provavelmente ocorreu devido a um aumento da toxicidade da cilindrospermopsina. Como o método utilizado é baseado na avaliação da integridade das membranas celulares e a capacidade das células em manter o valor de pH dos compartimentos do sistema endolisossomal, a cilindrospermopsina pode estar comprometendo a integridade das membranas celulares. Esta

integridade é um pré-requisito indispensável para a regulação de muitos processos celulares, e a célula pode estar entrando em processo de morte celular. Após a indução, a célula parece não ser capaz de aumentar seu metabolismo observado nos ensaios de MTT, o que poderia ocorrer como uma tentativa de evitar a redução da viabilidade celular. No entanto, apesar da toxicidade celular não estar aumentada, os níveis das EROs apresentaram-se elevados, na presença ou não do indutor, ou seja, aparentemente a indução com fenobarbital parece não estar relacionada com os níveis de EROs.

Estes níveis podem levar a efeitos prejudiciais, tais como danos oxidativos aos lipídios, proteínas, carboidratos e ácidos nucleicos (VALKO *et al.*, 2006), alterações comuns observadas em estudos com células submetidas a algum tipo de estresse, como o químico. Esses resultados são corroborados com o aumento da peroxidação lipídica observada nas células induzidas e expostas à toxina em 12 h, diferente do observado quando as células não foram induzidas com o fenobarbital. Isso reforça a ação do indutor no aumento da toxicidade da cilindrospermopsina em células HepG2. Para as células não induzidas, a ativação de algum mecanismo de defesa antioxidante atuante nas membranas celulares seria o suficiente para contrabalancear o excesso de EROs, evitando a peroxidação lipídica.

Hepatócitos de ratos expostos a CYN apresentaram aumento dos níveis de ROS, concentração e tempo dependente. Como consequência, a transcrição de elementos de resposta antioxidante (AREs), fator de ligação Nrf2, foi induzida. Este, combinado com uma diminuição da transcrição do Keap1 (inibidor de Nrf2), levou a maiores níveis de proteína Nrf2 nas células tratadas com a CYN (ALONSO *et al*,

2012). Estes resultados mostram uma tentativa das células neutralizarem os efeitos deletérios das espécies oxidativas geradas pela exposição a CYN.

Diversos tumores humanos, incluindo melanoma, leucemias e carcinomas, apresentam níveis elevados de EROs (REUTER e*t al.*, 2010). Células tumorais estão comumente expostas a condições de estresse, como hipóxia (baixos níveis de oxigênio), perda de adesão célula-célula e célula-matriz extracelular, provocando um desbalanço no metabolismo oxidativo. As células cancerosas, ao contrário das células normais que são hipersensíveis a EROs, matem altos níveis de antioxidantes (glutationa, superóxido dismutase, catalase, e outros), o que as protege contra os níveis mais elevados de EROs a qual estão submetidas (DHILLON *et al.*, 2007; REUTER e*t al.*, 2010;).

No entanto o comprometimento da membrana celular parece estar relacionado também com o mecanismo de resistência a multidrogas (MDR), visto que ocorreu uma redução da atividade de transportadores de efluxo quando as células foram induzidas pelo fenobarbital e expostas à cilindrospermopsina. Esta redução pode acarretar em um acúmulo intracelular da toxina e consequentemente um aumento de citotoxicidade. Esse resultado é similar ao observado no cultivo primário de hepatócitos de peixes expostos à cilindrospermopsina nas concentrações de 0,1; 1,0 e 10 µg l^{-1} que também apresentaram uma redução da atividade de transportadores envolvidos com o fenótipo MDR/MXR (LIEBEL *et al.*, 2011). Esse mecanismo é considerado como uma fase 0 (efluxo do composto original) ou fase III (efluxo de produtos do metabolismo do composto) quando analisamos os mecanismos de defesa celular contra xenobióticos, sendo as fases I e II etapas de biotransformação. Em altas concentrações, alguns xenobióticos ativam

e são metabolizados por uma ou mais isoformas do citocromo P450. Estes metabólitos biotransformados podem ser removidos por transportadores de efluxo como a glicoproteína P (PgP), ou podem ainda ser modificados pela conjugação da glutationa (GSH) ou outras moléculas endobióticas como ácido glucurônico, sulfato etc, sendo o conjugado transportado para fora da célula por transportadores MRP (*multidrug resistance protein*). Em outros casos, a eliminação do xenobiótico da célula pode ser de forma direta, sem a necessidade de passar pelas enzimas de biotransformação (BARD, 2000; VAN DER OOST *et al.*, 2003). Ou seja, o fígado claramente depende deste sistema de efluxo de xenobióticos, e o grau de interferência das cianotoxinas neste sistema está sendo influenciado possivelmente pela atividade das enzimas do citocromo P450.

De maneira geral, as respostas celulares observadas nas menores concentrações da cilindrospermopsina, isto é, o aumento da citotoxicidade, viabilidade celular, níveis de espécies reativas de oxigênio e nitrogênio, parecem não resultar em efeitos deletérios para essa célula. No entanto, fica claro que para ensaios de toxicidade empregando células HepG2 requerem algumas adaptações, como a redução da concentração de soro bovino fetal além da utilização de indutores de CYPs, fatores que tornam os resultados mais claros e próximos de uma situação *in vivo* dos efeitos citotóxicos da cilindrospermopsina.

No segundo capítulo, investigou-se a expressão diferencial de proteínas na presença da CYN (10 µg l^{-1}) utilizando técnica de proteômica, o que forneceu dados sobre algumas moléculas envolvidas com as respostas celulares e mecanismos de toxicidade da CYN. Ao todo, 48 proteínas apresentaram sua expressão alterada pela CYN, sendo que 26 proteínas foram identificadas. Estas proteínas estão envolvidas

em diferentes processos biológicos: como enovelamento de proteínas, efluxo de xenobióticos, defesa antioxidante, metabolismo energético, anabolismo celular, sinalização celular, potencial tumorigênico e citoesqueleto (Tabela 1, pag. 39). Este capítulo também investigou alterações no citoesqueleto das células expostas a CYN (100 µg l^{-1}) utilizando imunofluorescência, cujos resultados mostraram alterações no citoesqueleto, além de um índice mitótico mais elevado nas células expostas à toxina.

As chaperonas são proteínas dedicadas ao enovelamento correto e a translocação de proteínas. Entre as proteínas *heat shock protein* (HSPs) identificadas, a CYN provocou, a diminuição da expressão da HSP70 e da *75 kDa glucose regulated protein* (GRP75), bem como o aumento das expressões de HSP71, HSP90, proteína dissulfeto isomerase (PDI), *78 kDa glucose regulated protein* (GRP78) e da HSP60, as quais estão correlacionados com eventos citotóxicos, proliferação celular e "adaptação" de células tumorais a condições de estresse, processos que estão interligados em uma rede complexa (Fig. 2). A diminuição na expressão das HSPs geralmente está relacionada a eventos citotóxicos precoces e é uma consequência secundária de danos que afetam a integridade celular (SINGH *et al*., 2009). Já o aumento de Hsp71 pode contribuir para a alteração no controle do ciclo celular, induzindo a proliferação de células (YOUNG *et al.*, 2004), o que corrobora com o resultado encontrado no capítulo I.

A CYN também parece estar aumentando a absorção de glucose, uma vez que a expressão das proteínas GRP75 e GRP78 diminuíram e as expressões das três enzimas (fructose 1,6-bifosfato aldolase, gliceraldeido 3-fosfato (GAP), e lactato desidrogenase) envolvidas na glicólise aumentaram. Tanto uma maior captação de

glicose quanto um aumento da glicólise são necessárias para fornecer energia química útil para as células, a fim de responder ao estresse causado pela CYN, sintetizar macromoléculas (reações de anabolismo) e promover a proliferação celular (Fig. 2).

Houve também redução da expressão da proteína kinase (PKA) e da proteína glutationa-insulina transidrogenase, o que pode aumentar a via da glicólise e de anabolismo celular respectivamente. A redução destas proteínas podem indicar uma tentativa das células HepG2 em manter a insulina mais ativa e assim, aumentar as vias anabólicas do fígado, uma vez que a insulina é responsável pela manutenção da homeostase da glicose, do crescimento e da diferenciação celular (BOULTON *et al*., 1991; CROSS *et al*., 1995).

O aumento de proteínas *G proteins–coupled receptors* (GPCRs) está relacionado com a proliferação celular, isso associado com aumento da expressão das ribonucleoproteínas (hnRNP), as quais têm uma variedade de papéis no desenvolvimento e progressão de tumores, incluindo a inibição de apoptose, angiogênese e invasão celular, aliado aos níveis elevados de EROs pode levar a um aumento do potencial tumorigênico das células HepG2 expostas à CYN (Fig. 2). Sendo assim, EROs podem danificar biomoléculas como as proteínas do citoesqueleto, que devem ser sintetizadas para substituir as proteínas oxidadas (ALANI *et al.*, 1994; SANTA-MARIA *et al.*, 2005), bem como fornecer novas estruturas necessárias para proliferação das células. Várias das proteínas identificadas neste estudo pertencem ao citoesqueleto, sendo que alterações na expressão das isoformas de actina e tubulina são indicativos de estresse e possível dano causado as células HepG2 expostas a CYN (Fig.2). Essas alterações também

foram confirmadas pelas análises de imunofluorescência. A fim de facilitar a interpretação dos resultados, um mapa das possíveis relações entre as proteínas identificadas foi criado (Fig. 2).

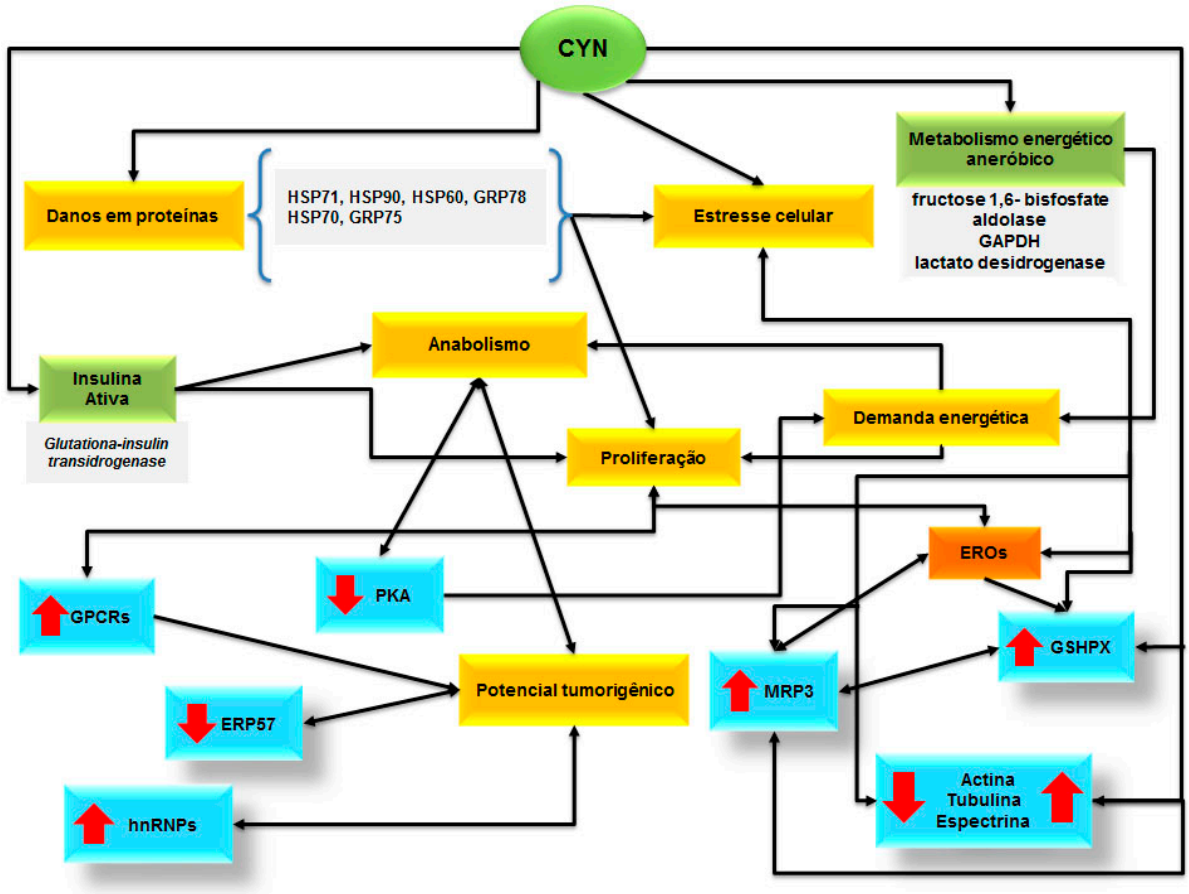


FIGURA 2 – Mapa das possíveis relações entre as proteínas identificadas após exposição à CYN. Células expostas a cilindrospermopsina (CYN) apresentam níveis elevados de proteínas envolvidas com o estresse celular (HSPs). O estresse celular pode ser influenciado por alterações na demanda energética (também provacado pela CYN), e na quantidade de EROs, estes fatores juntamente com a CYN atuam no aumento dos níveis de GSHPx e MRP3, além de influenciar nos níveis dos componentes do citoesqueleto. A CYN também parece atuar na manutenção da insulina ativa, o que pode estar relacinado com o anabolismo, assim como a proliferação celular, fato este que corrobora com o aumento nos níveis de GPCRs, o qual juntamente com as ribonucleoproteínas (hnRNPs) e como os níveis baixos da ERP57 e PKA podem favorecer também o potencial tumorigênico das células.

De forma resumida, os resultados apresentados neste segundo capítulo complementam o estudo anterior (capítulo I) e fornecem evidências de novos alvos moleculares da CYN.

4. REFERÊNCIAS BIBLIOGRÁFICAS

ALLANI, P. K, SUM, T., BHANSALI, S. G., MUKHERJEE, S. K., SONEE, M. A comparative study of the effect of oxidative stress on the cytoskeleton in human cortical neurons. **Toxicol. App. Pharmacol**., v. 196, p. 29–36, 2004.

BAIN, P.; SHAW, G.; PATEL, B. Induction of p53-regulated genevexpression in human cell lines exposed to the cyanobacterial toxin cylindrospermopsin.**J. Toxicol. Environ. Health.**, v.70, p.1687-1693, 2007.

BAKSI, S.M.; FRAZIER, J.M. Isolated fish hepatocytes - model systems for toxicology research: review. **Aquat.Toxicol.**, v.16, p.229-256, 1990.

BARD, S. M. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms **Aquatic. Toxicol.**, v. 48, p. 357-389, 2000.

BERNARD, C.; HARVEY, M.; BRIAND, J.F.; BIRE, R.; KRYS, S.; FONTAINE, J.J. Toxicological comparison of diverse *Cylindrospermopsisraciborskii* strains: evidence of liver damage caused by a French *C. raciborskii* strain. **Environ. Toxicol.**, v.18, p.176–186, 2003.

BERRY, J. P.; LIND, O.First evidence of ‘‘paralytic shellfish toxins’’ and cylindrospermopsin in a Mexican freshwater system, LagoCatemaco, and apparent bioaccumulation of the toxins in ‘‘tegogolo’’ snails. (*Pomaceapatulacatemacensis*).**Toxicon**, v.55, p. 930 – 938, 2010.

BOEHME, K., Dietz, Y., Hewitt, P., Mueller, S. O. Activation of P53 in HepG2 cells as surrogate to detect mutagens and promutagens in vitro. **Toxicol Letters.** v. 198, p. 272-281, 2010.

BRASIL, 2011. Ministério da Saúde. Portaria n. 2914, de 12 de Dezembro de 2011, 1. ed. Brasília: Editora do Ministério da Saúde, 2011.

CARMICHAEL, W. W.; AZEVEDO, S. M. F. O., AN, J. S.; MOLICA, R. J. R.; JOCHIMSEN, E. M.; LAU, S.; RINEHARDT, K. L.; SHAW, G. R.; EAGLESHAM, G. K. Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for Cyanotoxins. **Environ. Health Perspect.**, v.109, p.663-668, 2001.

CHUANG L.; ALBERT, P. L. Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague–Dawley rat, minipig, and beagle dog. **Chem. Biol. Interact**. v.134, p.271 – 281. 2001.

DE LA CRUZ, A.A.; HISKIA, A.; KALOUDIS, T.; CHERNOFF, N.; HILL, D.; ANTONIOU, M.G.;, HE, X.; LOFTIN, K.; O'SHEA, K.; ZHAO, C.; PELAEZ, M.; HAN, C.; LYNCH, T.J.; DIONYSIOU, D.D. A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin. **Environ Sci Process Impacts**. v.15, p.1979-2003, 2013.

DHILLON, A. S.; HAGAN, S.; RATH, O.; KOLCH. W. “MAP kinase signalling pathways in cancer”. **Oncogene**. v. 26, p. 3279–3290, 2007.

DONATO, M. T.; LAHOZ, A.; CASTELL, J. V.; GÓMEZ-LECHÓN, M. J. Cell Lines: A Tool for In Vitro Drug Metabolism Studies. **Current Drug Metab.**, v. 9, p. 1-11, 2008.

FALCONER, I.R.; HUMPAGE, A.R. Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. **Int. J. Environ. Res. Public. Health**, v.2, p.43-50, 2005.

FALCONER, I.R.; HUMPAGE, A.R. Cyanobacterial (blue-green algal) toxins in water supplies: cylindrospermopsins. **Environ. Toxicol**, v.21, p.299- 304, 2006.

FROSCIO,S. M.; HUMPAGE,A. R.; BURCHAM,P. C.; FALCONER, I. R. Cylindrospermopsin-Induced Protein Synthesis Inhibition and Its Dissociation from Acute Toxicity in Mouse Hepatocytes.**Environ Toxicol**, v.18, p. 243–251. 2003.

FROSCIO, S.M.; CANNON, E.; LAU, H.M.; HUMPAGE, A.R. Limited uptake of the cyanobacterial toxin cylindrospermopsin by Vero cells.**Toxicon**, v.54, p.862-868, 2009.

GRIFFITHS, D.J.; SAKER, M.L. The Palm Island Mystery Disease 20 years on: a review of research on the cyanotoxincylindrospermopsin. **Environ. Toxicol.**, v.18, p.78–93, 2003.

HAWKINS, P.R.; RUNNEGAR, M.T.C.; JACKSON, A.R.B.; FALCONER, I.R. 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**, v.50, p.1292-1295,1985.

HUMPAGE, A.R.; FALCONER, I.R. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss Albino Mice: determination of no observed adverse effect level for deriving a drinking water guideline value. **Environ. Toxicol**., v. 18, p.94- 103, 2003.

HUMPAGE, A.R.; FONTAINE, F.; FROSCIO, S.; BURCHAM, P.; FALCONER, I.R. Cylindrospermopsingenotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. **J. Toxicol. Environ. Health A.**, v.68, p.739-753, 2005.

HUSZAR, V. L. M.; SILVA, L. H. S.; MARINHO, M.; DOMINGOS, P; ANNA, C. L. S. Cyanoprokaryote assemblages in eight productive tropical Brazilian waters. **Hidrobiologia**, v. 424, p. 67-77, 2000.

KNASMÜLLER, S., MERSCH-SUNDERMANN, V., KEVEKORDES, S., DARROUDI, F., HUBER, W.W., HOELZL, C., BICHLER, J., MAJER, B.J. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. **Toxicol.**, v. 198, p. 315-328, 2004.

LANKOFF, A.; WOJCIK, A.; LISOWSKA, H.; BIALCZYK, J.; DZIGA, D.; CARMICHAEL, W.W. No induction of structural chromosomal aberrations in cylindrospermopsin-treated CHO-K1 cells without and with metabolic activation. **Toxicon**, v.50, p.1105-1115, 2007.

LIEBEL, S.; OLIVEIRA RIBEIRO, C.A.; SILVA, R.C.; RAMSDORF, W.A.; CESTARI, M.M.; MAGALHÃES, V.F.; GARCIA, J.R.E.; ESQUIVEL, B.M.; FILIPAK NETO, F. Cellular responses of Prochilodus lineatus hepatocytes after cylindrospermopsin exposure.**Toxicol. in Vitro**. v. 25, p. 1493- 1500. 2011.

LIU, Z.H., ZENG, S. Cytotoxicity of ginkgolic acid in HepG2 cells and primary rat hepatocytes. **Toxicol. Letters**. v. 187, p. 131-136, 2009.

MASTEN, S.; CARSON, B. Review of toxicological literature. Final Report. U.S. Environmental Protection Agency. Integrated Laboratory Systems. USA. p.37, 2000.

MATSUDA, H.; KINOSHITAA, K.; SUMIDAA, A.;TAKAHASHIB, K.; FUKUENA, S.; FUKUDAA, T.; TAKAHASHIA, K.; YAMAMOTOA, I.; JUNICHI AZUMA. Taurine modulates induction of cytochrome P450 3A4 mRNA by rifampicin in the HepG2 cell line. **Biochim. Biophys. Acta**. v.1593, p. 93-98, 2002

MEREL, S.; WALKER, D.; CHICANA, R.; SNYDER, S.; BAURÈS, E.; THOMAS, O. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. **Environ Int.** v. 59, p. 303-27, 2013.

MERSCH-SUNDERMANN, V., KNASMÜLLER, S., WU, X., DARROUDI, F., KASSIE, F. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. **Toxicol.**, v.198, p. 329-340, 2004.

MOLICA, R.; AZEVEDO, S. Ecofisiologia de Cianobactérias produtoras de Cianotoxinas. **Oecol.Bras.**, v.13, p. 229-246, 2009.

NEUMANN, C.; BAIN, P.; SHAW, G. Studies of the comparative in vitro toxicology of the cyanobacterial metabolite deoxycylindrospermopsin. **J. Toxicol. Environ. Health A.**, v.70, p.1679-1686, 2007.

NORRIS, R. L. G.; SEAWRIGHT, A. A.; SHAW., G. R.; SENOGLES, P.; EAGLESHAM, G. K.; SMITH, M. J.; CHISWELL, R. K.; MOORE, M. R. Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. **Toxicon**, v.40, p.471-476, 2002.

PONIEDZIAŁEK, B.; RZYMSKI, P.; KOKOCINSKI, M. Cylindrospermopsin: waterlinked potential threat to human health in Europe. **Environ. Toxicol. Pharmacol**. v. 34, p. 651-660, 2012.

PUERTO, M.; CAMPOS, A.; CAMEANA., A.P. A.; ALMEIDA, A. M.; COELHO, A. V.; VASCONCELOS, V. Differential protein expression in two bivalve species; *Mytilusgalloprovincialis* and *Corbiculafluminea;* exposed to *Cylindrospermopsisraciborskii* cells.**Aquatic Toxicol**. v. 101, p.109–116. 2011

RAO, P.V.; GUPTA, N.; BHASKAR, A.S.; JAYARAJ, R. Toxins and bioactive compounds from cyanobacteria and their implications on human health. **J. Environ. Biol.**, v.23, p.215-224, 2002.

REUTER, S.; GUPTA, S. C.; CHATURVEDI, M. M.; AGGARWAL, B. B. “Oxidative stress, inflammation, and cancer: how are they linked?”. **Free Radic. Biol. Med.**, v. 49, p.1603–1616, 2010.

RODRÍGUEZ-ANTONA, C.; DONATO, M. T.; BOOBIS, A.; EDWARDS, R. J.; WATTS, P.S.; VICENTE CASTELL, J.; GÓMEZ-LECHÓN, M.-J. Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. **Xenobiotica**, v. 32, n. 6, p. 505-520, 2002.

RUEFF, A., DAWSON, A.J.L.R., MENDELL, L.M. Characteristics of nerve growth factor induced hyperalgesia in adult rats: dependence on enhanced bradykinin-1 receptor activity but not neurokinin-1 receptor activation. **Pain**, v. 66, p. 359-372, 1996.

SANTA-MARÍA, I.; SMITH, M. A.; PERRY, G.; HERNÁNDEZ, F.; AVILA J.; MORENO, F. J. Effect of quinones on microtubule polymerization: a link between oxidative stress and cytoskeletal alterations in Alzheimer's disease. **Biochim. Biophys. Acta**, v. 1740, p. 472-480, 2005.

SEGNER, H. Isolation and primary culture of teleost hepatocytes.**Comp. Biochem. Physiol.**, v.120A, p.71-81, 1998.

SINGH, M. P.; KRISHN, M.M.; REDDY, N.; MATHUR, D.K; SAXENA, D.; CHOWDHURI, K. Induction of hsp70, hsp60, hsp83 and hsp26 and oxidative stress markers in benzene, toluene and xylene exposed Drosophila melanogaster: Role of ROS generation. **Toxicol. App. Pharmacol.**,v. 235, p. 226-243, 2009.

TURPEINEN, M., TOLONEN, A., CHESNE, C., GUILLOUZO, A., UUSITALO, J., PELKONEN, O. Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. **Toxicol. in Vitro**, v. 23, p.748-753, 2009.

VALK, J., BRUNNER, D., DE SMET, K., FEX SVENNINGSEN, Å., HONEGGER, P., KNUDSEN, L.E., LINDL, T., NORABERG, J., PRICE, A., SCARINO, M.L., GSTRAUNTHALER, G. Optimization of chemically defined cell culture media – Replacing fetal bovine serum in mammalian in vitro methods. **Toxicol. in Vitro**. v. 24, p. 1053-1063, 2010.

VALKO, M.; RHODES, C. J.; MONCOL, J.; IZAKOVIC, M.; MANZUR, M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chem-Biol Interact.**, v.160, p.1–40, 2006.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment : a review. **Environ. Toxicol.Pharmacol.**, v.13, p.57-149. 2003.

WALTER M.A.; WILLEM G.E.J. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved. **Toxicol. in Vitro**. v.21, p. 1581-1891. 2007.

WESTERINK, W.M.A., SCHOONEN, W.G.E.J. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. **Toxicol. in Vitro**. v. 21, p. 1581-1591, 2007a.

WESTERINK, W.M.A., SCHOONEN, W.G.E.J. Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2. **Toxicol. in Vitro**. v. 21, p. 1592-1602, 2007b.

WIEGAND, C.; PFLUGMACHER, S.Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. **Toxicol. App. Pharmacol**. v. 203, p. 201-218. 2005.

YOSHITOMI, S., IKEMOTO, K., TAKAHASHI, J., MIKI, H., NAMBA, M., ASAHI, S. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. **Toxicol. in Vitro**, v. 15, p. 245-256, 2001.

YOUNG, J.C.; AGASHE, V.R.; SIEGERS, K.; HART, F.U. Pathways of chaperone-mediated protein folding in the cytosol. **Nature Rev. Mol. Cell Biol.**, v. 5, p. 781-791, 2004.

ZEGURA, B.; LAH, T.T.; FILIPIC, M. The role of reactive oxygen species in microcystin -LR-induced DNA damage. **Toxicology**, v. 200, p.59–68, 2004.