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AVALIAÇÃO DA TOXICIDADE INDUZIDA PELA EXPOSIÇÃO À MICROCISTINA-LR SOBRE AS NEUROTRANSMISSÕES COLINÉRGICA E PURINÉRGICA EM *ZEBRAFISH* (*Danio rerio*)

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Tese apresentada como requisito para a obtenção do título de Doutora pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Prof. Dr. Maurício Reis Bogo

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Aos meus pais.

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RESUMO

Microcistinas (MCs) constituem uma família de toxinas, com mais de 80 variantes. Estas toxinas são capazes de induzir hepatotoxicidade em diversos organismos, principalmente através da inibição das fosfatases PP1 e PP2A e geração de estresse oxidativo. Evidências recentes mostram que MCs podem acumular no cérebro e alterar padrões comportamentais em diferentes espécies de peixes. Portanto, a presente tese teve por objetivo estudar os efeitos da exposição à MC-LR (com os aminoácidos variáveis leucina (L) e arginina (R)) sobre parâmetros bioquímicos em *zebrafish*, enfatizando os sistemas colinérgicos e purinérgicos, bem como, avaliar padrões comportamentais e níveis de cortisol corporal. Resultados do estudo *in vivo* mostraram que a exposição a 100 µg/L de MC-LR durante 24 h causaram um aumento significativo na atividade da AChE (27%) quando *zebrafish* foi exposto à toxina dissolvida em água; porém, a toxina não causou mudanças significativas quando injetada intraperitonealmente. Além disso, a análise semiquantitativa de RT-PCR demonstrou que a exposição à 100 µg/L de MC-LR também aumentou os níveis de RNAm da *ache* em cérebro de *zebrafish*. Os ensaios *in vitro* não revelaram nenhuma alteração significativa na atividade da AChE. Nós também avaliamos padrões comportamentais e níveis de cortisol corporal de *zebrafish* adultos expostos à cultura de células de *Microcystis aeruginosa* produtoras de MC-LR. A exposição à MC-LR (100 µg/L) diminuiu em 63% a distância viajada e aumentou três vezes o tempo de imobilidade quando comparado ao grupo controle. Interessantemente, não houve alteração no número de linhas cruzadas na mesma concentração e tempo de exposição à MC-LR. Quando animais foram expostos a 50 e 100 µg/L, a MC-LR promoveu um aumento significante (aproximadamente 93%) no tempo gasto na porção inferior do aquário teste, sugerindo um efeito ansiogênico. Adicionalmente, os resultados também mostraram que nenhuma das concentrações de MC-LR testadas promoveu alterações significativas nas mudanças de ângulo, eficiência de rota e interação social ou, no nível de cortisol corporal total. Além disso, também foi avaliado o efeito de diferentes concentrações de MC-LR na atividade das NTPDases (nucleosídeo trifosfato difosfoidrolase) e 5'nucleotidase em membranas cerebrais de *zebrafish* (*Danio rerio*) adultos. Os resultados mostraram que não houve alteração nas hidrólises de ATP, ADP e AMP. Nos experimentos *in vitro* também não houve alteração nas hidrólises dos nucleotídeos nas concentrações testadas. Estes achados mostram que exposição aguda à MC-LR não modulou a atividade das ectonucleotidases nas condições testadas. Ademais, fornecem a primeira evidência que a AChE cerebral é um outro alvo potencial das MCs e que exposição à MC-LR prejudica significativamente a performance exploratória do animal. Estudos futuros incluindo exposição de longo prazo dever ser feitos para um melhor entendimento dos mecanismos de toxicidade das MCs no Sistema Nervoso Central.

Palavras-chave: microcistina-LR, neurotoxicidade, *zebrafish*, AChE, padrões comportamentais, neurotransmissão purinérgica.

ABSTRACT

Microcystins (MCs) constitute a family of cyanobacterial toxins, with more than 80 variants. These toxins are able to induce hepatotoxicity in several organisms, mainly through the inhibition of protein phosphatases PP1 and PP2A and oxidative stress generation. Recent evidence shows that MCs can either accumulate in brain or alter behavior patterns of fish species. Thus, this thesis aimed to study the effects of MC-LR (with the variable amino acids leucine (L) and arginine (R)) exposure on biochemical parameters in zebrafish, emphasizing the cholinergic and purinergic signaling, as well as to evaluate the behavioral patterns and whole-body cortisol levels. *In vivo* studies showed that 100 µg/L MC-LR for 24 h led to a significant increase in the AChE activity (27%) when zebrafish were exposed to the toxin dissolved in water, but did not cause any significant changes when injected intraperitoneally. In addition, semiquantitative RT-PCR analysis demonstrated that 100 µg/L MC-LR exposure also increased ache mRNA levels in zebrafish brain. The *in vitro* assays did not reveal any significant changes in AChE activity. We also assessed behavioral patterns and whole-body cortisol levels of adult zebrafish exposed to cell culture of the microcystin-producing cyanobacterium *Microcystis aeruginosa*. MC-LR exposure (100 μg/L) decreased by 63% the distance traveled and increased threefold the immobility time when compared to the control group. Interestingly, no significant alterations in the number of line crossings were found at the same MC-LR concentration and time of exposure. When animals were exposed to 50 and 100 μg/L, MC-LR promoted a significant increase (around 93%) in the time spent in the bottom portion of the tank, suggesting an anxiogenic effect. In addition, the results also showed that none of the MC-LR concentrations tested promoted significant alterations in absolute turn angle, path efficiency, social behavior, or whole-body cortisol level. Moreover, we evaluated the acute effects of different concentrations of MC-LR on NTPDases (nucleoside triphosphate diphosphohydrolases) and 5'-nucleotidase in adult zebrafish (*Danio rerio*) brain membranes. The results have shown no significant changes in ATP, ADP and AMP hydrolysis in zebrafish brain membranes. MC-LR *in vitro* also did not alter ATP, ADP and AMP hydrolysis in the concentrations tested. These findings show that acute exposure to MC-LR did not modulate ectonucleotidases activity in the tested conditions. Taken together these findings provide the first evidence that brain AChE is another potential target for MCs and that MC-LR exposure significantly impairs animal`s exploratory performance. Nevertheless, further studies including long-time exposure should be performed in order to achieve a better understanding about MC-LR toxicity mechanisms in the central nervous system.

Keywords: microcystin-LR, neurotoxicity, zebrafish, AChE, behavior patterns, purinergic neurotransmission.

LISTA DE SIGLAS E ABREVIATURAS

Acetil CoA - acetil coenzima A

ACh - acetilcolina

AChE - acetilcolinesterase

Adda - Ácido 3-amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienóico

ADP - adenosina 5´-difosfato

AMP - adenosina 5´-monofosfato

AMPc- adenosina 5´-monofosfato cíclico

ATP - adenosina 5´-trifosfato

BuChE - butirilcolinesterase

Ca^{2+} - cálcio

CAT - catalase

CD39 - antígeno de ativação celular linfóide

CD73 - proteína de superfície de linfócitos

CDP - citidina 5'-difosfato

CTP - citidina 5'-trifosfato

CHT - transportador de colina

DAG - diacilglicerol

E-NPP - ectonucleotídeo pirofosfatase/fosfodiesterase

GABA - ácido γ-aminobutírico

GDP - guanosina 5'-difosfato

GMP - guanosina 5'-monofosfato

GPI - glicosilfosfatidilinositol

GPx - glutationa peroxidase

GR - glutationa redutase

GST - glutationa S-transferase

HPLC - High Performance Liquid Chromatography (Cromatografia Líquida de Alta Eficiência)

IDP - inosina 5´-difosfato

IP3 - inositol 1,4,5-trifosfato

K^+ - potássio

K_M - Constante de Michaelis

Mdha - N-metildehidroalanina

MC - microcistina

MCs - microcistinas

MC-LR - microcistina-LR

Na^+ - sódio

NTPDase - Nucleosídeo Trifosfato Difosfoidrolase

OATPs - Organic Anion Transporting Polypeptides

Pi - fosfato inorgânico

PP1 - proteína fosfatase 1

PP2A - proteína fosfatase 2

ROS - Espécies Reativas de Oxigênio

SNC - Sistema Nervoso Central

SNP - Sistema Nervoso Periférico

SOD - superóxido dismutase

TCDD - 2,3,7,8-tetraclorodibenzeno-p-dioxina

TTP - timidina 5´-trifosfato

UDP - uridina 5'- difosfato

UMP - uridina 5'-monofosfato

UTP - uridina 5´-trifosfato

ZFIN - *Zebrafish* Information Network (Rede Internacional de Dados do *Zebrafish*)

SUMÁRIO

Capítulo I

Capítulo II

Capítulo III

Capítulo IV

Capítulo V

Capítulo I

INTRODUÇÃO

1. Microcistina (MC)

Cianobactérias são produtoras de uma variedade de potentes toxinas, incluindo neurotoxinas, citotoxinas, hepatotoxinas e dermatotoxinas (Chorus e Bartram, 1999; Wiegand e Pflugmacher, 2005). Microcistinas (MCs) são primariamente hepatotoxinas produzidas por cianobactérias dos gêneros *Microcystis*, *Anabaena*, *Nodularia*, *Oscillatoria* (*Planktothrix*) e *Nostoc* durante os eventos de floração (Carmichael, 1994; Chorus e Bartram, 1999; Malbrouck e Kestemont, 2006).

Atualmente, são conhecidas mais de 80 isoformas de MCs que se distinguem através do grau de metilação, de hidroxilação e de epimerização, além da sequência peptídica e grande toxicidade (Svrcek e Smith, 2004). MCs são heptapeptídeos monocíclicos contendo ambos D- e L-aminoácidos mais Mdha (N-metilhidroalanina) e um único β-aminoácido de grupo lateral, Adda (Ácido 3-amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienóico) (Dawson, 1998; Svrcek e Smith, 2004). O aminoácido Adda é essencial para a expressão da atividade biológica e, mudanças estruturais resultam na abolição da toxicidade (Dawson, 1998). A MC-LR é a mais comum e foi a primeira microcistina quimicamente identificada sendo considerada a que ocorre com maior frequência nas florações de cianobactérias e, também, a mais tóxica, onde os aminoácidos variáveis são leucina (L) e arginina (R) (Rinehart et al.,1994; Watanabe et al., 1996; Dawson, 1998; Sivonen e Jones, 1999). Outras variantes que também ocorrem frequentemente são MC-RR (aminoácidos variáveis arginina (R)), MC-YR (aminoácidos variáveis tirosina (Y) e arginina (R)) e MC-LA (aminoácidos variáveis leucina (L) e alanina (A)). (Figueiredo et al. 2004).

A concentração das MCs dissolvidas em água no meio ambiente varia desde traços chegando a 1.800 μg/L ou mais, imediatamente após a floração (Chorus e Bartram, 1999; Svrcek e Smith, 2004); entretanto, já foram encontrados níveis muito mais altos, como por exemplo, de 29 mg/L em São Lourenço do Sul (RS) e de 44 mg/L na Barragem Santa Bárbara (RS) (Leão, 2008). Células intactas, como também as toxinas liberadas após a lise celular podem ser responsáveis pelos efeitos tóxicos observados em muitos organismos, de microalgas a mamíferos (Figueiredo et al., 2004; Jos et al., 2005).

As MCs podem afetar organismos aquáticos e terrestres, inclusive os seres humanos, e a contaminação por esta toxina tem sérias consequências ecológicas e de saúde pública (Carmichael, 1994; Wiegand e Pflugmacher, 2005). O caso mais grave no Brasil ocorreu em Caruaru-PE, em 1996,

quando em uma unidade de hemodiálise foi utilizada água contaminada com MCs que resultou na morte de 60 dos 126 pacientes (Jochimsen et al., 1998; Pouria et al., 1998).

A temperatura influencia o tipo de toxina produzida; temperaturas altas (>25ºC) aumentam a produção de MC-RR e temperaturas mais baixas favorecem a síntese de MC-LR (Rapala et al., 1997; Rapala e Sivonen, 1998). Devido a sua estrutura química, as MCs são extremamente estáveis em água e podem tolerar mudanças químicas radicais, incluindo variações de pH (Harada et al., 1996; Harada e Tsuji, 1998).

A toxicidade das MCs depende da relação entre sua acumulação e metabolismo (Ito et al., 2002). As MCs agem principalmente inibindo irreversivelmente as fosfatases PP1 e PP2A (Mackintosh et al., 1990; Ding e Ong, 2003; Prieto et al., 2007). Ao inibir essas enzimas (PP1 e PP2A), as MCs levam a um aumento da fosforilação protéica, que está relacionada com sua atividade citotóxica e promotora de tumores (Humpage et al., 2000; Zegura et al., 2004). Essas toxinas não parecem iniciar a formação de um câncer; porém, se as alterações iniciais já tiverem ocorrido, elas podem acelerar o desenvolvimento de tumores (Carmichael, 1994).

O perigo de promoção de tumores pela exposição crônica à microcistina em água para consumo humano foi a razão principal para a definição de níveis aceitáveis para esta toxina pela Organização Mundial de Saúde (OMS). Ficou estabelecido que o limite máximo aceitável de MCs em águas para consumo humano é de 1 µg/L de MC-LR (WHO, 1998; Fitzgerald, 2001). Países como Brasil, Nova Zelândia e Reino Unido adotaram este valor como referência em água para consumo; entretanto, o Canadá propôs um nível de 1,5 µg/L e a Austrália propôs níveis variando de 1,3 a 10 µg/L (Usepa, 2001). No Canadá, também foi proposto um nível de 10 µg/L para exposição a curto prazo (Fitzgerald, 2001).

Em consequência do alto peso molecular (900-1100 Da), as MCs são incapazes de penetrar facilmente através de membranas biológicas e bioconcentrar (Svrcek e Smith, 2004). Entretanto, alguns tipos de células expressam transportadores específicos de membranas, os “Organic Anion Transporting Polypeptides” (animais: oatps; humanos: OATPs) que possibilitam uma acumulação tóxica. Estes transportadores estão primeiramente presentes no fígado de mamíferos. Por esta razão, as MCs são reconhecidas como hepatotoxinas (Fischer et al., 2005), mas foram também descritas em fígado de outros vertebrados, como por exemplo, no peixe *Leucoraja erinacea* (Meier-Abt et al., 2007).

Dependendo da dose, tempo de exposição e do organismo, as MCs podem provocar tanto um aumento (Li et al., 2003; Gehringer et al., 2004; Jos et al., 2005; Pinho et al. 2005), como também uma redução (Moreno et al., 2005; Cazenave et al., 2006) na atividade de enzimas antioxidantes como

a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx), a glutationa redutase (GR) e a glutationa S-transferase (GST). A exposição às MCs pode resultar em uma produção excessiva de espécies reativas de oxigênio (ROS do inglês Reactive Oxygen Species), um dos fatores desencadeadores de estresse oxidativo (Ding et al., 2000a, 2000b; Li et al., 2003; Zegura et al., 2004), alterar níveis de antioxidantes (Ding e Ong, 2003; Jos et al., 2005; Pinho et al., 2005), chegando inclusive a causar morte celular por apoptose (Ding e Ong, 2003) e câncer de fígado (Hu et al., 2008; Hernández et al., 2009; Li et al., 2009).

Em peixes, baixas concentrações de MC-LR causaram danos no hepatopâncreas e rins de carpa européia (*Cyprinus carpio*) (Fischer e Dietrich, 2000). A hepatotoxicidade pela acumulação de MC-LR conduz a mudanças na morfologia celular, inibição de fosfatases protéicas e necrose de fígado em trutas (*Oncorhynchus mykiss*) (Fischer et al., 2000). Um estudo utilizando embriões de *zebrafish* (*Danio rerio*) mostrou que MC-LR é absorvida pelos embriões (Wiegand e Pflugmacher, 2001) e também induz malformação e letalidade dos embriões e larvas de forma dose e tempo-dependente (Wang et al., 2005). Em outro estudo, embriões e larvas de *Misguruns mizolepis*, um pequeno peixe de água-doce, mostraram ser afetados pela toxicidade de MC-LR, cujos alvos são o fígado e coração (Liu et al., 2002). Oberemm (2001a) também mostrou que nos estágios iniciais de vida, os peixes são mais sensíveis a efeitos hepatotóxicos causados pela exposição às MCs do que peixes adultos ou jovens (Oberemm, 2001a), devido ao seu epitélio fino, a relação entre a superfície do corpo e o volume do embrião e vulnerabilidade dos processos envolvidos no desenvolvimento (Oberemm, 2001b). O peixe de água-doce *Oreochromis niloticus* acumula MC em intestinos, fígado e rins (Mohamed et al., 2003). Porém, existem peixes que também podem acumular estas toxinas no tecido muscular, resultando em altos riscos para humanos que consumirem peixes contaminados (Magalhães et al., 2001).

Evidências indicam que as MCs podem acumular em cérebro de peixes. Fischer e Dietrich (2000) detectaram MC-LR por Western Blot em cérebro de *Cyprinus carpio* (carpa), quando foi administrada por gavagem uma dose de 400 μg/Kg de *M. aeruginosa* (PCC 7806) após 72 horas de administração. Recentemente, Cazenave et al. (2005) reportaram a presença de MC-RR em cérebro de *Jenynsia multidentata* por Cromatografia Líquida de Alta Eficiência (HPLC do inglês High Performance Liquid Chromatography), quando exposto a 50 μg/L da toxina dissolvida em água por um período de 24 horas. Ainda que os efeitos tóxicos da acumulação das MCs em cérebro de peixes não tenham sido estudados, alterações em padrões de comportamento já foram registradas e incluem mobilidade reduzida, taxas aumentadas de atividade à noite, atividade reduzida durante o período de desova, e

“reação reduzida” durante a alimentação quando expostos a longo prazo a doses subletais de MC-LR (Baganz et al. , 2001).

2. Neurotransmissão Colinérgica

A acetilcolina (ACh) é o neurotransmissor mais importante do sistema colinérgico (Descarries et al., 1997). A neurotransmissão colinérgica é fundamental para o correto funcionamento do SNC, e representa o sistema neurotransmissor mais antigo filogeneticamente (Gotti e Clementi, 2004). Os neurônios colinérgicos inervam a musculatura voluntária do sistema somático e também são encontrados no Sistema Nervoso Central (SNC) e Sistema Nervoso Periférico (SNP) (Soreq e Seidman, 2001).

A ACh desempenha um papel fundamental no SNC e está relacionada ao comportamento, ao aprendizado, à memória, à organização cortical do movimento e ao controle do fluxo sanguíneo cerebral (Mesulam et al., 2002; Moretto et al., 2004). Além de sua ação neurotransmissora, a ACh possui função neuromoduladora, pois os níveis da mesma podem regular a concentração de outros neurotransmissores no cérebro (Cooper et al., 1991).

A síntese da ACh ocorre a partir de Acetil Coenzima A (Acetil CoA), formada durante o metabolismo celular mitocondrial e, da colina, um importante produto do metabolismo dos lipídios. A etapa final da síntese da ACh ocorre no citoplasma, sendo o neurotransmissor transportado para o interior de vesículas sinápticas (Kapczinski et al., 2000; Soreq e Seidman, 2001). A colina usada na síntese de ACh pode vir diretamente da reciclagem da ACh, que é hidrolisada pela acetilcolinesterase (AChE) na fenda sináptica ou a partir da fosfatidilcolina. Essas duas fontes de colina são particularmente importantes para o SNC, porque a colina presente no plasma não ultrapassa a barreira hematoencefálica (Taylor e Brown, 1994).

A liberação de ACh depende das variações no potencial elétrico das membranas dos terminais nervosos sendo este processo dependente da concentração de cálcio intracelular. Ao ser liberada, a ACh interage com receptores específicos causando despolarização e propagação do potencial de ação na célula pós-sináptica (Oda, 1999).

A ACh que permanece na fenda sináptica é hidrolisada por uma colinesterase específica em ácido acético e colina. Grande parte da colina resultante é captada pelo terminal do axônio colinérgico por um transportador de colina (CHT) e reutilizada na síntese de nova ACh (Mesulam et al., 2002).

Baseando-se em suas diferentes afinidades por agentes que mimetizam a ação da acetilcolina, os receptores colinérgicos são divididos em duas classes distintas: muscarínicos e nicotínicos (Tinsley et al., 2004). Os receptores muscarínicos são metabotrópicos e ligam-se à acetilcolina e à muscarina, um alcalóide presente em certos cogumelos venenosos. Estes receptores são encontrados em gânglios do SNP e nos órgãos efetuadores autonômicos, como coração, musculatura lisa, cérebro e glândulas exócrinas (Sarter e Parikh, 2005). Existem cinco subtipos de receptores muscarínicos (M1-M5) que foram clonados e identificados farmacologicamente. Os receptores M1, M3 e M5 estão acoplados a uma proteína Gq/11 e alteram a atividade celular pela estimulação da fosfolipase C e pela geração do segundo mensageiro IP3, o qual induz a liberação de cálcio intracelular e diacilglicerol (DAG). Contudo, os receptores M2 e M4 estão acoplados a uma proteína Gi que induz sua reposta via inibição da adenilato ciclase (Caulfield e Birdsall, 1998; Uchiyama e Chess-Williams, 2004).

Os receptores nicotínicos são ionotrópicos e reconhecem a acetilcolina e a nicotina. Tais receptores se localizam no SNC, na medula adrenal, nos gânglios autonômicos e na junção neuromuscular (Sarter e Parikh, 2005). Os receptores nicotínicos estão ligados a canais catiônicos e possuem uma estrutura pentamérica (McKay e Placzek, 2007). Estes receptores pertencem a uma família heterogênea que consiste em diferentes subtipos, os quais formam combinações homoméricas ou heteroméricas a partir de 12 diferentes subunidades (α2-α10, β2-β4) (Gotti e Clementi, 2004).

2.1. Acetilcolinesterase (AChE, E.C.3.1.1.7)

As colinesterases hidrolisam a ACh na fenda sináptica e desempenham um papel muito importante na neurotransmissão colinérgica, além de outras funções fisiológicas. São classificadas de acordo com suas propriedades catalíticas, especificidade de inibidores e distribuição nos tecidos: A AChE (E.C.3.1.1.7) hidrolisa preferencialmente ésteres com grupamento acetil, estando presente principalmente nas sinapses do SNC, SNP parassimpático e junção neuromuscular; e a butirilcolinesterase (E.C.3.1.1.8, BuChe) hidrolisa outros tipos de ésteres como a butirilcolina. Ambas as colinesterases são amplamente distribuídas no organismo (Taylor e Brown, 1999). A AChE é uma importante enzima regulatória que controla a transmissão de impulsos nervosos através da sinapse colinérgica pela hidrólise do neurotransmissor excitatório ACh (Quinn, 1987; Milatovic e Dettbarn, 1996). Os níveis de AChE parecem ser controlados pela interação da ACh com seus receptores, sendo que quando a interação é acentuada, aumentam os níveis de AChE. No entanto, a AChE pode ser

usada como um marcador da função colinérgica e mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (Fernandes e Hodges-Savola, 1992).

O domínio catalítico da AChE é composto por uma tríade serina-histidina-glutamato que está localizado no fundo de uma estrutura estreita (gorge) que vai da superfície da proteína até o seu centro (Shafferman et al., 1992; Zimmerman e Soreq, 2006). Esta estrutura está flanqueada por 14 resíduos aromáticos localizados nos *loops* entre diferentes folhas β-pregueadas. Um sítio aniônico periférico composto por cinco resíduos está ligado na entrada estreita da "gorge" e está cercado por dez resíduos ácidos denominados "motivo anular eletrostático" (Silman e Sussman, 2008).

A AChE é uma glicoproteína e sua forma estrutural divide-se em globular ou assimétrica. Estas formas são geradas por processamento alternativo seguido por modificações pós-traducionais (Silmann e Sussman, 2008). O SNC contém principalmente as formas globulares, enquanto as formas assimétricas são encontradas principalmente no SNP e músculos (Rakonczay et al. 2005). Existem três formas globulares, G1 (monômero), G2 (dímero) e G4 (tetrâmero) da subunidade catalítica. A unidade G1 está ligada ao citosol, enquanto que G4 à membrana da célula. O tipo G4 é o mais observado no sistema nervoso e muscular (Xie et al., 2007). No sangue, predominam as formas G2 (glóbulos vermelhos) e G4 (plasma) (Skau, 1985). As formas homoméricas são encontradas como espécies solúveis na célula, provavelmente para a exportação, ou se apresentam associadas à membrana celular externa através de uma sequência de aminoácidos hidrofóbicos intrínsecos ou de um glicofosfolipídio acoplado (Taylor e Brown, 1999). As formas heteroméricas da AChE estão associadas à lâmina basal externa por proteínas que determinam sua localização sináptica. Os tipos assimétricos consistem de um (A4), dois (A8) e três (A12) tetrâmeros catalíticos ligados ao colágeno Q (ColQ) que é predominante nas junções musculares. O tetrâmero ligado à âncora de membrana rica em prolina (PRIMA) é abundante nas sinapses cerebrais (Zimmerman e Soreq, 2006).

O gene que codifica para a AChE do *zebrafish* foi clonado e sequenciado por Bertrand e colaboradores em 2001, revelando que esta enzima é codificada por somente um gene e que sua sequência de 634 aminoácidos apresenta 62% de similaridade em relação aos mamíferos. Neste estudo, não foi verificada a presença de um gene que codifique à butirilcolinesterase, indicando que possivelmente não há atividade desta enzima no *zebrafish*.

A inibição da atividade da AChE foi observada quando *zebrafish* foi exposto aos agentes tóxicos paration (Roex et al., 2003) e metanol (Rico et al., 2006), enquanto que o etanol promoveu um aumento significativo desta atividade (Rico et al., 2007).

3. Neurotransmissão Purinérgica

A hipótese de neurotransmissão purinérgica foi proposta pela primeira vez por Geoffrey Burnstock, em 1972, após a análise de evidências que o nucleotídeo ATP era a molécula transmissora em nervos não-adrenérgicos e não-colinérgicos de intestino e bexiga (Burnstock et al., 1970; Burnstock, 1972; Burnstock, 2007). Ainda na década de 70, houve a identificação e caracterização dos purinoceptores, enfatizando ainda mais a sinalização mediada por nucleotídeos (Burnstock, 1976). Atualmente, a sinalização purinérgica é amplamente reconhecida como um sistema de sinalização primitivo no reino animal e vegetal envolvido em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo resposta imune, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (Burnstock, 2004; Burnstock e Verkhratsky, 2009).

A sinalização purinérgica utiliza purinas extracelulares, ATP (adenosina 5´-trifosfato) e adenosina, e pirimidinas como moléculas sinalizadoras que exercem seus efeitos por meio da interação com receptores de membrana específicos, denominados receptores purinérgicos ou purinoceptores (Abbracchio, 2008). Diversas evidências têm demonstrado o importante papel desempenhado por essas moléculas, entre elas o ATP e a adenosina, no SNC (Ralevic e Burnstock, 1998; Dunwiddie e Masino, 2001).

O ATP é uma importante molécula sinalizadora no espaço extracelular e desempenha papéis fundamentais em condições fisiológicas e patológicas (Zimmermann, 2001). Como neurotransmissor, o ATP é liberado para o meio extracelular através de vesículas pré-sinápticas dependentes de cálcio (Phillis e Wu, 1981) e é principalmente coliberado com outros neurotransmissores, como acetilcolina (Richardson e Brown, 1987), noradrenalina (Rathbone et al., 1999), serotonina (Potter e White, 1980) e ácido γ-aminobutírico (GABA) (Di Iorio et al., 1998; Burnstock, 2004).

Ao ser liberado na fenda sináptica, este nucleotídeo pode modular a atividade sináptica através dos receptores purinérgicos ou purinoceptores divididos em dois grandes grupos: P1 e P2. Os purinoceptores do tipo P1 (A_1, A_{2A}, A_{2B} e A_3) são mais eficientemente ativados por adenosina, enquanto que os purinoceptores P2 (P2X e P2Y) são ativados por ATP (Ralevic e Burnstock, 1998; Burnstock, 2007; Abbracchio et al., 2008).

Os purinoceptores do tipo P2 são divididos em duas subclasses: P2X e P2Y. A família P2X consiste em receptores ionotrópicos (acoplados a canais) que apresentam permeabilidade rápida e seletiva para cátions (Na^+, K^+ e Ca^{2+}), e está dividida em sete membros ($P2X_{1-7}$), distribuídos em

neurônios, células gliais e no músculo liso (Burnstock, 2004). A família P2Y consiste em receptores metabotrópicos (acoplados à proteína G), e foram funcionalmente descritos oito membros ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) (Ralevic e Burnstock, 1998; Burnstock, 2006).

Os receptores P2Y apresentam uma ampla distribuição nos tecidos e sistemas, tais como: vascular, nervoso e cardíaco (Burnstock, 2004). Dessa forma, dependendo do subtipo de receptor P2 e da via de sinalização envolvida, esses receptores podem desencadear e mediar processos de curto prazo (agudo) que afetam o metabolismo celular, neurotransmissão, neuromodulação, secreção endócrina e exócrina, agregação plaquetária e vasodilatação. Além disso, a sinalização purinérgica também provoca profundo impacto sobre outras respostas mais prolongadas, incluindo proliferação celular, diferenciação e apoptose (Weisman et al., 1998; Burnstock e Knight, 2004).

A clonagem e caracterização molecular dos receptores P2X do *zebrafish* já foi realizada (Egan et al., 2000; Diaz-Hernandes et al., 2002). A subunidade P2X possui nove membros, sendo destes, seis ortólogos aos genes dos receptores P2X de mamíferos, dois parálogos e um gene ainda precisa ser devidamente classificado (Kucenas et al., 2003). Os subtipos dos receptores P2X do *zebrafish* contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos. Até o momento, na família de receptores P2Y foram identificadas oito proteínas funcionais (Ralevic e Burnstock, 1998; Illes e Ribeiro, 2004), e apenas foram identificados receptores P2Y1 em trombócitos de *zebrafish* (Gregory e Jagadeeswaran, 2002).

Uma vez liberado no espaço extracelular, o ATP pode ser metabolizado pela ação de ectoenzimas que fazem a conversão deste nucleotídeo até adenosina (Zimmermann, 2001; Robson et al., 2006). A adenosina não é descrita classicamente como neurotransmissor, pois não há indícios de que é armazenada em vesículas sinápticas, sendo classificada como neuromodulador (Agranoff et al., 1999). A adenosina é um neuromodulador endógeno que influencia muitas funções do SNC (Cunha, 2001), sendo reconhecida como um importante modulador da neurotransmissão excitatória e agente neuroprotetor em diferentes patologias relacionadas ao SNC, tais como na isquemia, hipóxia (Fredholm, 1997; Ribeiro et al., 2003) e epilepsia (Vianna et al., 2005). A concentração extracelular de adenosina é um fator determinante dos efeitos neuromoduladores desta molécula.

A adenosina exerce seus efeitos através da ativação de receptores de membrana específicos P1, subdivididos em A_1, A_{2A}, A_{2B} e A_3, todos acoplados à proteína G e exibindo sete domínios transmembrana formados por aminoácidos hidrofóbicos (Ralevic e Burnstock, 1998; Fredholm et al., 2001; Ribeiro et al., 2003; Yaar et al., 2005). Os receptores A_1 e A_{2A} apresentam alta afinidade pela adenosina, enquanto os receptores A_{2B} e A_3 são de baixa afinidade (Ribeiro et al., 2003).

Na sinalização purinérgica, existe um eficiente mecanismo de inativação, no qual ATP, ADP (adenosina 5´-difosfato) e AMP (adenosina 5´-monofosfato) são hidrolisados a adenosina por uma cascata enzimática constituída pela via das ectonucleotidases (Ribeiro e Sebastião, 2000). Além de ser formada a partir da hidrólise do ATP através da ação dessas enzimas, a adenosina pode ser produzida no meio intracelular e transportada para o meio extracelular através de transportadores específicos bidirecionais, que mantêm os níveis intracelulares e extracelulares de adenosina em equilíbrio. A adenosina extracelular também pode ser formada a partir da degradação do AMPc (adenosina 5´-monofosfato cíclico) (Latini e Pedata, 2001).

3.1. Ectonucleotidases

Ectonucleotidases são ectoenzimas que hidrolisam nucleotídeos extracelulares ao seu respectivo nucleosídeo e fosfato livre (Zimmermann, 2000). Esta cascata é composta por uma variedade de enzimas que estão localizadas na superfície celular, chamadas de ectonucleotidases. Estas enzimas são responsáveis pelo controle dos níveis extracelulares de ATP e adenosina (Zimmermann, 2001).

A família das ectonucleotidases é composta pelas ectonucleotídeo pirofosfatase/fosfodiesterase (E-NPP), nucleosídeo trifosfato difosfoidrolase (NTPDase), fosfatases alcalinas e a ecto-5-nucleotidase (EC 3.1.3.5) (Robson et al., 2006; Zimmermann, 2006). Essas enzimas possuem características em comum, tais como: todas são proteínas de membranas com isoformas intracelulares clivadas e solúveis; suas atividades de hidrólise são dependentes de cátions divalentes; são ativadas em pH alcalino; e possuem o K_M na faixa de micromolar (Zimmermann, 2001).

As NTPDases (nucleosideo trifosfato difosfoidrolases) são enzimas capazes de hidrolisar nucleotídeos tri e difosfatados em seus respectivos nucleotídeos monofosfatados e fosfato inorgânico (Zimmermann, 2001). Nesta hidrólise, ocorre a liberação de 2 mols de Pi (fosfato inorgânico) por mol de nucleotídeo trifosfatado e 1 mol de Pi por mol de nucleotídeo difosfatado (Plesner, 1995).

Além das características comuns ao grupo das ectonucleotidases, as NTPDases apresentam as seguintes características: uma subunidade catalítica glicosilada; um sítio de hidrólise de nucleotídeos, voltado para o espaço extracelular ou para o lúmen das organelas citoplasmáticas; atividade dependente de cátions divalentes (principalmente cálcio e/ou magnésio); insensibilidade a inibidores específicos de ATPases do tipo P, F, V; e habilidade para hidrolisar uma ampla variedade de

nucleotídeos púricos e pirimídicos tri e difosfatados (Plesner, 1995; Zimmermann et al., 1998; Goding, 2000; Robson et al., 2006).

Oito enzimas já foram descritas e caracterizadas dentro dessa família, a NTPDase1 (CD39, ATPDase, ecto-apirase) (Wang e Guidotti, 1996; Sevigny et al., 1997), a NTPDase2 (CD39L1, ecto-ATPase) (Vlajkovic et al., 1999; Kegel et al., 1997; Mateo et al., 1999; Heine et al., 1999), a NTPDase3 (CD39L3, HB6) (Chadwick e Frischauf, 1998), a NTPDase4 (UDPase, LALP70) (Wang e Guidotti, 1996; Biederbick et al., 2000), a NTPDase5 (CD39L4, ER-UDPase, PCPH) (Mulero et al., 1999; Paez et al., 2001), a NTPDase6 (CD39L2) (Yeung et al., 2000; Hicks-Berger et al., 2000), a NTPDase7 (LALP1) (Shi et al., 2001) e a NTPDase8 (Bigonnesse et al., 2004). As NTPDases1, 2, 3, 8 estão localizadas na superfície das células, com um sítio catalítico extracelular, as NTPDases5 e 6 apresentam localização intracelular e as NTPDases4 e 7 são enzimas intracelulares cujos centros ativos estão direcionados para o lúmen das organelas citoplasmáticas (Zimmermann, 2001; Robson et al., 2006).

Em termos de hidrólise de nucleotídeos, a NTPDase1 hidrolisa ATP e ADP igualmente, sendo a proporção da hidrólise destes dois substratos de 1:1 (Heine et al., 1999). A enzima NTPDase2 hidrolisa 30 vezes mais ATP do que o ADP (Kirley et al., 1997). A NTPDase3 e a NTPDase8 tem maior afinidade por ATP em relação ao ADP numa razão de hidrólise de aproximadamente 3:1 e 2:1, respectivamente (Chadwick et al., 1998; Bigonnesse et al., 2004). NTPDase4α tem uma alta preferência por UTP (uridina 5´-trifosfato) e TTP (timidina 5´-trifosfato), enquanto que a NTPDase4β apresenta alta preferência por CTP (citidina 5'-trifosfato) e UDP (uridina 5'- difosfato). A função destas NTPDases ainda não é clara (Zimmermann, 2001). A NTPDase5 tem uma preferência na hidrólise de nucleotídeos na seguinte ordem: UDP>GDP = IDP>>ADP = CDP, enquanto que a NTPDase6 tem a seguinte ordem de afinidade: GDP>IDP>>UDP = CDP>>ADP. Acredita-se que a NTPDase5 e a NTPDase6 participam das reações de glicosilação envolvidas nos processos de dobramento de glicoproteínas (Zimmermann, 2001). A NTPDase7 prefere nucleotídeos trifosfatados como substratos (Zimmermann, 2001). Estas enzimas hidrolisam tanto ATP como ADP, formando AMP na presença de íons Ca^{+2} e Mg^{+2} (Chan et al., 1986; Zimmermann, 2001). O AMP formado é então convertido a adenosina pelas 5´-nucleotidase (Zimmermann, 2001; Robson et al., 2006). A família das NTPDases tem modulado alguns processos de sinalização ou biossintéticos nos quais os nucleotídeos extracelulares desempenham um importante papel, incluindo a homeostase vascular, a sinalização celular, a função imune e a modificação de proteínas e lipídeos (Burnstock, 1998; Gayle et al., 1998; Marcus et al., 2003).

A ecto-5´-nucleotidase, também conhecida como a proteína linfocitária CD73 em associação à NTPDase, realiza a hidrólise do AMP até a produção de adenosina (Zimmermann, 1992). Esta atividade enzimática é dependente de cátions divalentes, como cálcio e magnésio. A ecto-5´-nucleotidase é uma enzima ancorada à membrana plasmática por glicosilfosfatidilinositol (GPI), sendo que formas solúveis da enzima podem ser originadas mediante a ação de uma fosfolipase específica (Zimmermann, 1992). Esta enzima encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleotídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, aos seus respectivos nucleosídeos, sendo o AMP o nucleotídeo hidrolisado com maior eficiência com valores de K_M na faixa de micromolar (Zimmermann, 1996). O ATP e o ADP são inibidores competitivos da 5´-nucleotidase com valores de Ki também na faixa de micromolar (Zimmermann, 1996). Em SNC, a ecto-5´-nucleotidase está predominantemente associada à glia, mas várias evidências têm demonstrado que sua atividade também está associada a neurônios (Zimmermann, 1996; Zimmermann et al., 1998; Zimmermann, 2001). A ecto-5´-nucleotidase exerce um papel modulador sobre a produção de adenosina extracelular, sendo a enzima marcapasso desta cascata enzimática (Zimmermann, 1996; Cunha, 2001).

Em membranas cerebrais de *zebrafish* estudos demonstraram a presença de uma NTPDase (Rico et al., 2003; Appelbaum et al., 2007) e uma ecto-5'-nucleotidase (Senger et al., 2004), a qual hidrolisa o AMP até adenosina. Estas duas enzimas foram caracterizadas como cátion-dependentes, apresentando atividade máxima à temperatura de 37 °C, pH ótimo entre 7,2 e 8,0, K_M na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (Rico et al., 2003; Senger et al., 2004). Estudo feito por Rosemberg et al. (2010) realizou a identificação molecular e avaliou os padrões de expressão de diferentes genes relacionados à família das NTPDases em *zebrafish*. Os genes ortólogos que codificam para as NTPDase1-6 e NTPDase8 foram identificados por similaridade de sequência. A análise filogenética mostrou a presença de três genes parálogos que codificam para NTPDase2 e dois genes parálogos para NTPDase5. A análise molecular revelou a existência de um perfil de expressão dos genes *entpd1-6* e *entpd8* em cérebro, fígado, e coração. Estes resultados indicam que diversos membros das NTPDases podem contribuir para uma estreita regulação da hidrólise de nucleotídeos em tecidos de *zebrafish* (Rosemberg et al., 2010).

As ectonucleotidases desempenham uma função essencial na neurotransmissão purinérgica, controlando a disponibilidade e os níveis de nucleotídeos e nucleosídeos extracelulares e, consequentemente a ativação dos purinoceptores P2 e P1 (Zimmermann 2001).

4. *Zebrafish*

O *zebrafish* ou peixe-zebra é um pequeno teleósteo (3-4 cm) de água doce, da família Cyprinidae, que vem sendo considerado um modelo ideal para estudos de numerosas doenças humanas (Sloman et al., 2003; Best e Alderton, 2008). O pioneiro a estudar esta espécie foi George Streisinger que, no final da década de 1960, aplicou as técnicas de análise mutacional para estudar o desenvolvimento embrionário do *zebrafish* (Grunwald e Eisen, 2002).

O *zebrafish* como um organismo modelo para pesquisa apresenta diversas características favoráveis, tais como: baixo custo, requer pouco espaço para manutenção, rápido desenvolvimento e ciclo biológico, fácil manipulação, seu comportamento pode ser facilmente observado e quantificado em um ambiente controlado (Sloman et al., 2003), e ainda, seu pequeno tamanho, a sensibilidade para fármacos, e o rápido metabolismo (Karlovich et al., 1998; Goldsmith, 2004).

Nos últimos anos, houve um progresso considerável no conhecimento sobre características genéticas e informações genômicas do *zebrafish* (Postlethwait et al., 2000; Amatruda e Patton, 2008; Milan e MacRae, 2008). Em 2001, o Instituto Sanger começou o sequenciamento do genoma desta espécie (Stern e Zon, 2003) que atualmente está em fase final de revisão. A sequência do genoma mitocondrial já é conhecida, servindo de base para estudos filogenéticos (Broughton et al., 2001). Os genes deste teleósteo são evolutivamente conservados e apresentam um alto grau de similaridade com os genes humanos e de camundongo (Barbazuk et al., 2000; Lieschke e Currie, 2007).

Foi criada uma rede de informações na web sobre o *zebrafish*, o ZFIN (http://zfin.org), na qual laboratórios de pesquisa do mundo inteiro depositam informações sobre esta espécie (Sprague et al., 2003). Além disso, existe um excelente, compreensivo e frequentemente atualizado manual de manutenção e controle das condições de criação deste teleósteo em laboratórios (Westerfield, 2000). O *zebrafish* se tornou o principal modelo experimental para estudos envolvendo o desenvolvimento de vertebrados (Anderson e Ingham, 2003). As características de sua embriogênese são bem conhecidas, assim como o destino celular durante o seu desenvolvimento (Kimmel e Warga, 1988; Kimmel, 1989).

Atualmente, a utilização do *zebrafish* vem sendo expandida para outras áreas do conhecimento e incluem por exemplo, bioquímica (Taylor et al., 2004), neurociências (Edwards e Michel, 2002), toxicologia (Hill et al., 2005), farmacologia (Goldsmith et al., 2004) e biologia do comportamento (Gerlai, 2003; Guo, 2004; Egan et al., 2009; Blaser et al., 2010; Cachat et al., 2010; Blaser e Peñalosa 2011; Piato et al., 2011). Devido às suas peculiaridades reprodutivas e às suas características morfológicas e fisiológicas, esta espécie desperta o interesse pela oportunidade de

acelerar o processo da descoberta de novos fármacos (Stern e Zon, 2003). Este teleósteo é capaz de absorver de forma rápida os compostos que são diretamente adicionados na água e acumulá-los em diferentes tecidos, incluindo no SNC (Grosell e Wood, 2002). Entre os estudos envolvendo aspectos toxicológicos, a exposição a diferentes contaminantes ambientais, tais como a 2,3,7,8-tetraclorodibenzeno-p-dioxina (TCDD) (Dong et al., 2002; Hill et al., 2003), pesticidas carbamatos e organofosforados (Senger et al., 2005), metanol (Rico et al., 2006), etanol (Rico et al., 2008) e metais pesados (Senger et al., 2006a; Rosemberg et al., 2007) já foi estudada no SNC de *zebrafish*.

Atualmente, muitos projetos são realizados nesta espécie para estudar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (Guo, 2004; Eddins et al., 2009; Gerlai et al., 2009). Diferentes sistemas de neurotransmissão já foram identificados nesta espécie tais como: glutamatérgico (Edwards e Michel, 2002; Tabor e Friedrich, 2008), colinérgico (Behra et al., 2002; Clemente et al., 2004; Arenzana et al., 2005; Senger et al., 2006b; Edwards et al., 2007), dopaminérgico (Boehmler et al., 2004; Ryu et al., 2006; Russek-Blum et al., 2008), serotoninérgico (Rink e Guo, 2004; Lillesaar et al., 2007; Norton et al. 2008), histaminérgico (Kaslin e Panula, 2001), gabaérgico (Kim et al., 2004; Delgado e Schmachtenberg, 2008) e purinérgico (Kucenas et al., 2003; Rico et al., 2003; Senger et al., 2004; Low et al., 2008).

5. Estudos comportamentais

Alterações comportamentais refletem como um animal se sente e responde ao seu ambiente e constituem a primeira linha de defesa quando o animal é exposto a uma perturbação ambiental (Scherer, 1992; Paul e Simonin, 1996; Campbell et al., 2002; Begout e Lagardére, 2004). *Zebrafish* tem também sido utilizado como organismo modelo em estudos comportamentais e, muitas vezes, os testes empregados são adaptações de protocolos já estabelecidos em roedores, como por exemplo, labirinto em cruz elevado, campo aberto, exposição ao predador, preferência por claro-escuro (Levin et al., 2007; Saverino e Gerlai, 2008; Sison e Gerlai, 2010; Champagne et al., 2010; Grossman et al., 2010; Maximino et al., 2010; Stewart et al., 2011).

Estudos avaliando características comportamentais em *zebrafish* incluem comportamento social (Pyron, 2003; Engeszer et al., 2004; Bass e Gerlai, 2008), comportamentos relacionados ao olfato (Mann et al., 2003; Vitebsky et al., 2005), ansiedade (Barcellos et al.,2007), drogadição (Darland e Dowling, 2001; Ninkovic e Bally-Cuif, 2006; Kily et al., 2008), sono (Cirelli e Tononi, 2000),

aprendizagem e memória (Williams et al., 2002; Colwill, 2005), e preferência por claro-escuro (Serra et al., 1999; Blank et al., 2009).

Ainda existem poucos estudos avaliando os efeitos de MCs no comportamento de peixes. Baganz et al. (1998) verificou que a exposição à MC-LR causou mudanças de efeito dose dependente na atividade locomotora espontânea de *zebrafish*. Enquanto exposições à baixas concentrações (0.5 e 5 µg/L) causaram um aumento na mobilidade no período diurno, concentrações altas (15 e 50 µg/L) levaram a uma diminuição significativa da mobilidade. Além disso, na maior dose de exposição (50 µg/L) também houve diminuição na atividade da desova. Ao contrário das atividades diárias, as atividades noturnas de nado foram significativamente maiores nas maiores concentrações da exposição à MC-LR. Em outro estudo, Baganz et al. (2004) mostrou mudanças no comportamento de locomoção espontânea de *zebrafish* e *L. delineatus* após exposição a MC-LR em concentrações de 0.5, 5, e 15 µg/L por 17 dias e 50 µg/L por seis dias. Durante o dia, a mobilidade de *zebrafish* assim como de *L. delineatus* aumentou significativamente pela exposição às menores concentrações, enquanto que altas concentrações levaram a uma significativa diminuição da mobilidade. Adicionalmente, Cazenave et al. (2008) reportou mudanças na atividade natatória de *J. multidentata* tratada com pellets de comida contaminada com MC-RR. Baixos níveis (0.01 μg/g) aumentaram o nado, enquanto que a maior dose (1 µg/g) usada produziu alterações significativas em relação ao grupo controle quando a atividade natatória foi diminuída.

JUSTIFICATIVA

O sistema colinérgico, um dos mais importantes sistemas de neurotransmisão, tem sido reconhecido por desempenhar papéis chave em muitas funções no SNC, incluindo o controle da atividade locomotora, comportamento emocional, processos cognitivos, aprendizado e memória (Pepeu e Giovannini, 2004; Martins-Silva et al., 2011). ACh, o principal neurotransmissor do sistema colinérgico, é co-liberado juntamente com ATP, molécula sinalizadora do sistema purinérgico, na fenda sináptica e estudos têm demonstrado que o ATP pode controlar a liberação de ACh (Cunha e Ribeiro, 2000; Burnstock, 2004).

Levando-se em consideração que os possíveis efeitos neurotóxicos e os mecanismos da toxicidade em cérebro estão longe de serem completamente elucidados é importante estudar os efeitos causados pela exposição as MCs sobre diferentes sistemas de neurotransmissão e sobre padrões comportamentais.

OBJETIVOS

Objetivo geral

A presente tese teve por objetivo avaliar a participação dos sistemas colinérgico e purinérgico na toxicidade causada por microcistinas utilizando-se como modelo experimental o *zebrafish* (*Danio rerio*).

Objetivos específicos

Os objetivos específicos deste estudo foram:

- Avaliar o efeito da exposição *in vitro* à microcistina-LR sobre a atividade da acetilcolinesterase em homogenato cerebral de *zebrafish*;

- Avaliar o efeito da exposição *in vivo* à microcistina-LR sobre a atividade da acetilcolinesterase em homogenato cerebral de *zebrafish*. Verificar a expressão do gene que codifica para acetilcolinesterase após a exposição *in vivo* à microcistina-LR;

- Avaliar o efeito da exposição *in vitro* à microcistina-LR sobre as atividades das NTPDases e da 5´-nucleotidase em membranas cerebrais de *zebrafish*;

- Avaliar o efeito da exposição *in vivo* à microcistina-LR sobre as atividades das NTPDases e da 5´-nucleotidase em membranas cerebrais de *zebrafish*. Verificar a expressão dos genes que codificam para as enzimas do sistema purinérgico após a exposição *in vivo* com microcistina-LR.

- Avaliar o efeito da exposição à microcistina-LR sobre parâmetros comportamentais de *zebrafish*, como atividade locomotora e interação social.

Capítulo II

Microcystin-LR acute exposure increases AChE activity via transcriptional *ache* activation in zebrafish (*Danio rerio*) brain

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Microcystin-LR acute exposure increases AChE activity via transcriptional *ache* activation in zebrafish (*Danio rerio*) brain

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ABSTRACT

Microcystins (MCs) constitute a family of cyanobacterial toxins, with more than 80 variants. These toxins are able to induce hepatotoxicity in several organisms mainly through the inhibition of protein phosphatases PP1 and PP2A and oxidative stress generation. Since recent evidence shows that MCs can either accumulate in brain or alter behavior patterns of fish species, in this study we tested the *in vitro* and *in vivo* effects of MC-LR at different concentrations on acetylcholinesterase (AChE) activity in zebrafish brain. *In vivo* studies showed that 100 μg/L MC-LR led to a significant increase in the AChE activity (27%) when zebrafish were exposed to the toxin dissolved in water, but did not cause any significant changes when injected intraperitoneally. In addition, semiquantitative RT-PCR analysis demonstrated that 100 μg/L MC-LR exposure also increased *ache* mRNA levels in zebrafish brain. The *in vitro* assays did not reveal any significant changes in AChE activity. These findings provide the first evidence that brain AChE is another potential target for MCs and suggest that the observed increases in AChE enzymatic activity and in *ache* transcript levels after MC-LR exposure depend, at least partially, on branchial uptake or ingestion.

1. Introduction

Microcystins (MCs) are potent hepatotoxins produced by the cyanobacteria of the genera *Planktothrix*, *Microcystis*, *Aphanizomenon*, *Nostoc* and *Anabaena* (Dai et al., 2008). They are among the most frequently detected toxins in fresh waters, and produce potent hepatotoxic effects on terrestrial animals (for example, Puschner et al., 1998) and fish (Malbrouck and Kestemont, 2006). There are over 80 different variants of this molecule, including amino acid variations and modifications, among which microcystin-LR (MC-LR) is one of the most common and toxic forms, and which occurs more often in cyanobacterial blooms (Dawson, 1998). MCs inhibit phosphatases PP1 and PP2A and they appear to interact with the mitochondria of animal cells triggering oxidative stress and apoptosis (for review see Campos and Vasconcelos, 2010). This pathway is considered to be one of the main mechanisms of MCs toxicity. Nevertheless, MCs toxicity is a multi-pathway process and, regardless of recent achievements, the molecular mechanisms underlying MCs toxicity are still not completely understood (Campos and Vasconcelos, 2010).

Studies have demonstrated that MCs accumulate in several fish tissues, such as liver (Williams et al., 1995; Sipiä et al., 2001; Malbrouck et al., 2003; Mohamed et al., 2003), intestine (Williams et al., 1995; Mohamed et al., 2003; Xie et al., 2004, 2005), gills (Carbis et al., 1997; Cazenave et al., 2005), kidney (Williams et al., 1995; Mohamed et al., 2003), muscle (Bury et al., 1998; Magalhaes et al., 2001, 2003; Xie et al., 2004), gallbladder (Sahin et al., 1996), and blood (Tencalla and Dietrich, 1997; Xie et al., 2004, 2005). Studies have shown that MCs can also accumulate in fish brain. Fischer and Dietrich (2000) detected MC-LR in *Cyprinus carpio* brain after gavage with the toxin, and Cazenave et al. (2005) reported the presence of microcystin-RR

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(MC-RR) in the brain of *Jenynsia multidentata* exposed to the toxin dissolved in water for 24 h. In addition, changes in the spontaneous locomotor behavior of zebrafish (*Danio rerio*) and *Leucaspius delineatus* after MC-LR exposure (Baganz et al., 2004), in the swimming activity and in the glutathione S-transferase activity of *J. multidentata* fed with MC-RR (Cazenave et al., 2008), and the induction of lipid peroxidation in the brain of *Corydoras paleatus* after MC-RR exposure (Cazenave et al., 2006) indicate probable neurotoxic effects of MCs. More recently, Wang et al. (2010) using proteomic analysis revealed that MC-LR neurotoxicity induced oxidative stress and a dysfunction of cytoskeleton assembly and macromolecule metabolism. These findings suggest that MC-LR toxicity to the brain is complex and diverse.

The zebrafish has long been considered a powerful animal model because of its tractable genetics and embryology, but it has more recently become a model of choice in environmental studies, pharmaceutical screening, and physiologic analysis (Hernández and Allende, 2008). Its greatest assets are its small body size, sensitivity to drugs, and the ability to rapidly absorb chemicals from the water and then to accumulate them in several tissues (Goldsmith, 2004; Hill et al., 2005).

The cholinergic system is one of the most important modulatory neurotransmitter systems and has long been recognized to play key roles in many functions in the central nervous system (CNS), including the control of locomotor activity, emotional behavior, and cognitive processes (Pepeu and Giovannini, 2004; Martins-Silva et al., 2011). In cholinergic neurotransmission, choline acetyltransferase (ChAT) is responsible for the synthesis of acetylcholine (ACh) in the presynaptic neuron. After its release into the synaptic cleft, cholinesterases rapidly cleave ACh into choline and acetate. Two different types of cholinesterases are able to carry out this hydrolysis: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8) (Soreq and Seidman, 2001). It has been demonstrated that BuChE is not encoded in the zebrafish genome, but AChE is encoded by a single gene that has already been cloned, sequenced and functionally detected in zebrafish brain (Bertrand et al., 2001). AChE enzymes in vertebrates exist as soluble monomers (G1), dimers (G2), and tetramers (G4). The tetramers may be associated with structural subunits, a collagenic tail in neuromuscular junctions, or a membrane protein in brain (Massoulié et al., 1998, 2008). Moreover, zebrafish AChE shows a transition, at the time of hatching, from the globular G4 form to an asymmetric form containing a collagenic subunit that becomes prominent in adults (Bertrand et al., 2001).

AChE activity has been widely used as a bioindicator of environmental exposure. For example, the inhibition of AChE as a biomarker for assessment of the exposure of organisms to organophosphate and carbamate insecticides is well known (Weiss, 1958; O'Brien, 1976; for review see Van Dyk and Pletschke, 2011), including in fish species (Bretaud et al., 2000; Roex et al., 2003). The inhibition of zebrafish brain AChE activity by toxic substances such as methanol (Rico et al., 2006) and the heavy metals mercury and lead (Richetti et al., 2011) also has been well established. On the other hand, AChE activation has also been demonstrated as a consequence of exposure to neurotoxic compounds such as aluminum (Kaizer et al., 2010; Senger et al., 2011) and ethanol (Rico et al., 2007).

Considering that: (i) MCs synthesized by cyanobacteria can either accumulate in brain and change locomotor behavior and swimming activity in fish; (ii) ACh is known to play a major role in the regulation of locomotor control; (iii) measurement of AChE activity in organisms is used worldwide as a biomarker of environmental contamination and that (iv) the zebrafish is a model organism to study drug mechanisms and toxicology, the aim of this study was to evaluate the *in vivo* and *in vitro* effects of different concentrations of MC-LR on AChE activity in zebrafish brain. Furthermore, when alterations in kinetics occurred after MC-LR exposure, we also determined the *ache* gene expression level in zebrafish brain.

2. Materials and methods

2.1. Animals

Adult wild-type zebrafish (*Danio rerio*, Cyprinidae) of both sexes (3–6 months old) were obtained from a specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept at a density of up to five animals per liter in 50 L housing tanks with tap water that was previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O_2/L) at 26 ± 2 °C, pH 7.0, under a 14/10 h light/dark controlled photoperiod. Animals were acclimated for at least two weeks before the experiments and were fed three times a day with TetraMin Tropical Flake fish food®. The fish were maintained healthy and free of any signs of disease and were used according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. All procedures in the present study were approved by the Animal Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), protocol number 10/00142-CEUA.

2.2. Chemicals

MC-LR (purity >95%) isolated from *Microcystis aeruginosa* (strain RST9501) were produced by Unidade de Pesquisas em Cianobactérias (Universidade Federal de Rio Grande — FURG, BR). The amount of MC-LR (strain RST9501) was detected by a Quantitative Antibody Immunoassay (Elisa) provided by Envirologix (Portland, USA), within a range of detection from 0.05 to 2.5 μg/L MCs. A suitable dilution was applied to the sample to provide detection within the range. Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis(beta amino ethylether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA). TRIzol® reagent, SuperScript™ III First-Strand Synthesis SuperMix, Platinum® Taq DNA Polymerase and GelRed® were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were of analytical grade.

2.3. In vivo treatment

The *in vivo* exposures were performed in 1-L aquariums (10 fish per aquarium). Fish were exposed to different MC-LR concentrations (50 and 100 μg/L) dissolved in water for 24 h and, immediately after the exposure, they were euthanized by decapitation. For the control group the animals were exposed only to water for 24 h, after which they were euthanized by decapitation. The MC-LR concentrations and the *in vivo* time of exposure were chosen based on a previous study using *J. multidentata* (Cazenave et al., 2005). In addition, such concentrations are commonly encountered in cyanobacterial bloom events (Oberholster et al., 2009; Backer et al., 2010).

2.4. In vitro treatment

In vitro assays were performed as previously described (Seibt et al., 2009; Siebel et al., 2010). Briefly, MC-LR was added to the reaction medium before the preincubation with the enzyme-containing lysate from zebrafish brain homogenate and maintained during the enzyme assays. MC-LR was tested at a final concentration of 10, 25, 50, 100, 500, 1000, and 5000 μg/L. For the control group, the enzyme assay was performed in the absence of MC-LR (i.e. no toxin was added to the reaction medium).

2.5. Intraperitoneal injection

Intraperitoneal injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31 G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. (2009). Briefly, each fish was weighed prior to the intraperitoneal injection and the volume injected (10 μL) into the animal was adjusted to achieve a dose of 50 and 100 μg/kg of MC-LR, based on previously studies (Carbis et al., 1996; Fournie and Courtney, 2002). Anesthesia of the animals prior to the injection was obtained by its immersion in a solution of benzocaine (1 mM in MeOH 1%) until the animal showed a lack of motor coordination and reduced respiratory rate. The anesthetized animal was gently placed in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine in the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted in such a way as to guarantee that the animal did not spend more than 10 s out of the water. After the injection the animals were placed in a separate tank with highly aerated unchlorinated tap water (26 ± 2 °C) to facilitate recovery from the anesthesia. Saline solution was used as control. All the animals that recovered within 2–3 min following the injection continued in the experiment, while animals that did not recover during this period were discarded. Twenty-four hours after the injection the animals were euthanized by decapitation and AChE activity was determined.

2.6. Determination of AChE activity (EC 3.1.1.7)

Zebrafish were euthanized and their whole brains were removed by dissection. The brains (two whole brains for each sample) were homogenized on ice in 60 vol. (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, and 2 mM EGTA, pH 7.4, adjusted with citric acid), in a glass-Teflon homogenizer. The rate of acetylthiocholine hydrolysis (ACSCh, 0.88 mM) was assessed in a final volume of 300 μL with 11 mM phosphate buffer, pH 7.5, and 0.22 mM DTNB using a method previously described (Ellman et al., 1961). Before the addition of substrate, samples containing protein (5 μg) and the reaction medium described above were preincubated for 10 min at 25 °C. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) in a microplate reader. Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. The linearity of absorbance against time and protein concentration was previously determined. The AChE activity was expressed as micromoles of thiocholine (SCh) released per h per mg of protein. All enzyme assays were evaluated in triplicate and at least four independent experiments were performed.

2.7. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Forward (5′-CCAAAAGAATAGAGATGCCATGGACG-3′) and reverse (5′-TGTGATGTTAAGCAGACGAGGCAGG-3′) *ache* primers and the optimal conditions for RT-PCR were used according to Rico et al. (2006). The *β-actin* forward (5′-GTCCCTGTACGCCTCTGGTCG-3′) and reverse (5′-GCCGGACTCATCGTACTCCTG-3′) primers were used as described previously (Chen et al., 2004).

Immediately after *in vivo* exposure to MC-LR (described above), the animals were euthanized by decapitation. For each sample, a pool of two zebrafish whole brains was used. Total RNA from zebrafish brain was isolated using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1.0% agarose gel. Afterwards, all samples were adjusted to 160 ng/μL and cDNA species were synthesized using SuperScript III™ First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions. One microliter of RT reaction mix was used as a template for each PCR. For *ache*, the reaction was performed in a total volume of 25 μL using 0.08 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). PCR for the *β-actin* gene was performed in a total volume of 20 μL using 0.1 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). PCR parameters, including $MgCl_2$, cDNA template concentration and PCR cycles (25–45 cycles), were first optimized (data not shown) and reactions were performed using optimal conditions that allowed product detection within the linear phase of band densitometry analyzed. PCRs were conducted for 1 min at 94 °C, 1 min at 60 °C (*ache*) or at 54 °C (*β-actin*), and 1 min at 72 °C for 35 cycles. A post-extension step was performed for 10 min at 72 °C. For each PCR set, a negative control was included. PCR products were analyzed on a 1% agarose gel, containing GelRed® and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as a molecular marker and samples were normalized by employing *β-actin* as a constitutive gene. The band intensities were measured by optical densitometry analysis and the *enzyme*/*β-actin* mRNA ratios were established for each treatment using the freeware Image J 1.37. Each experiment was repeated at least four times, using RNA isolated from independent extractions.

2.9. Statistical analysis

AChE activity were expressed as means ± S.E.M. and analyzed by one-way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey's test considering $p \leq 0.05$ as statistical significance. Molecular data were expressed as means ± S.E.M. and analyzed by Student's *t*-test considering $p \leq 0.05$ as statistical significance.

3. Results

The effect of different MC-LR concentrations on brain AChE activity was demonstrated by performing *in vivo* (50 and 100 μg/L) and *in vitro* (10, 25, 50, 100, 500, 1000 and 5000 μg/L) experiments using adult zebrafish. The *in vivo* analysis demonstrated that animals treated with the concentration of 100 μg/L for 24 h presented a significant increase in AChE activity (31.14 ± 1.39; 27%; $p<0.05$) when compared to the control group (24.50 ± 1.80) (Fig. 1). On the other hand, MC-LR intraperitoneal injection did not alter the brain AChE activity (data not shown).

The up-regulation of brain AChE activity after exposure to 100 μg/L MC-LR could be a consequence of transcriptional control and/or post-translational modifications. In order to determine if transcriptional regulation of *ache* has occurred, semiquantitative RT-PCR analysis was performed. The results demonstrated that the relative levels of *ache* mRNA were significantly increased (1.07 ± 0.01; 17%; $p<0.05$) after exposure to 100 μg/L MC-LR (Fig. 2).

To verify whether the toxin might have a direct effect on the enzyme, we tested the *in vitro* effect of MC-LR on AChE activity in zebrafish brain. The results showed that MC-LR did not bring about any alteration in AChE activity ($p>0.05$; Fig. 3).

4. Discussion

The results presented herein demonstrated the influence of MC-LR on AChE activity and *ache* gene expression in zebrafish brain. We

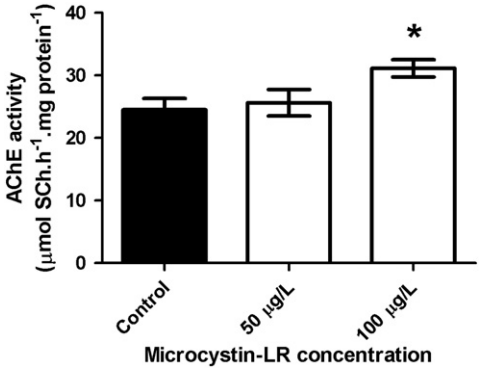

Fig. 1. *In vivo* AChE activity in zebrafish brain after 24 h of microcystin-LR exposure at distinct concentrations (50 and 100 μg/L). Data were expressed as means ± S.E.M. of four independent experiments, each one performed in triplicate. The specific enzyme activity is reported as micromoles of thiocholine released per hour per milligram of protein. The asterisk (*) indicates a significant difference when compared to the control group (one way ANOVA, followed by Tukey's test as post-hoc, $p<0.05$).

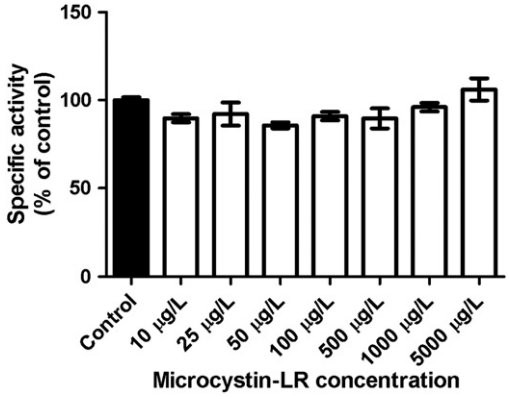

Fig. 3. *In vitro* effect of different concentrations of microcystin-LR (10–5,000 μg/L) on ACh hydrolysis in zebrafish brain. Data were expressed as means ± S.E.M. of four independent experiments, each one performed in triplicate. The control acetylcholinesterase activity was 26.19 ± 1.69 μmol SCh h^{-1} mg $protein^{-1}$.

found that AChE activity was significantly enhanced when zebrafish were exposed to 100 μg/L of MC-LR dissolved in water but not when intraperitoneal injected. The *ache* mRNA levels were significantly increased after MC-LR exposure, suggesting that, MC-LR could also modulate *ache* gene expression. The results also showed that none of the MC-LR concentrations tested caused significant changes in the AChE activity in zebrafish brain when added directly to the enzyme assays, as in the *in vitro* experiments, suggesting that MC-LR did not act directly on the enzyme. These findings provide further evidence for toxic effects on the brain caused by exposure to MCs.

The first evidence that MC-LR can accumulate in fish brain was reported by Fischer and Dietrich (2000), who detected the toxin by Western Blot 48 h after bolus dosing of freeze dried algae equivalent to 400 μg/kg of *C. carpio*. Later, Cazenave et al. (2005) showed the presence of MC-RR in the brain of *J. multidentata* exposed to the toxin. Fish were exposed for 24 h to 50 μg/L of MC-RR dissolved in water and were then analyzed by high performance liquid chromatography (HPLC). Recently, Papadimitriou et al. (2010) used ELISA to show toxin accumulation in brain tissue of *Carassius gibelio* collected from Greek Lakes.

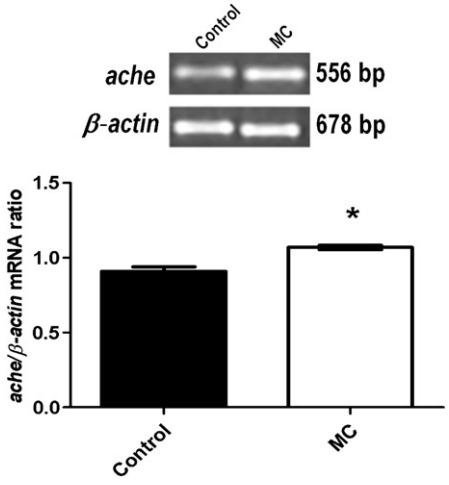

Fig. 2. Effect of exposure to 100 μg/L of microcystin-LR for 24 h on *ache* mRNA transcripts from zebrafish brain. The PCR products were subjected to electrophoresis on a 1% agarose gel, using *β-actin* as a constitutive gene. The figure shows a representative gel and the *ache*/*β-actin* mRNA ratio (expressed as arbitrary units) obtained by optical densitometry analysis of three independent experiments, with entirely consistent results. Data were expressed as means ± S.E.M. The asterisk (*) indicates a significant difference when compared to the control group (Student's *t* test, $p<0.05$).

There is a large body of evidence in the literature associating changes in normal behavioral patterns with neurotoxic effects of exposure to pollutants. In this sense, although the effects of MCs on the behavior of fish are still mostly unknown some aspects have already been addressed. For example, changes in the spontaneous locomotor behavior of *D. rerio* and *L. delineatus* were observed after exposure to MC-LR at concentrations of 0.5, 5, and 15 μg/L for 17 days and 50 μg/L for six days. During the daytime, the motility of *D. rerio* as well as *L. delineatus* was significantly increased by exposure to the lowest concentrations, whereas higher concentrations led to significantly decreased motility. Furthermore, under the influence of MC-LR, the period of swimming activity in *L. delineatus* was reversed, going from a predominantly diurnal activity to a nocturnal one; *D. rerio* remained active during the daytime (Baganz et al. 2004). Additionally, Cazenave et al. (2008) reported changes in the swimming activity of *J. multidentata* fed with contaminated food pellets containing MC-RR. Low levels (0.01 μg/g) increased the swimming activity, while the highest dose (1 μg/g) produced significant changes with respect to the control group only after approximately 20 h of exposure, when the swimming activity was decreased.

As seen by other authors, the hepatotoxicity of MC depends on the route of uptake (for review see Malbrouck and Kestemont, 2006). Thus, different exposure routes have been used to examine the toxic effects of MCs on fishes including intraperitoneal injection, oral ingestion and immersion in water containing the dissolved toxin (Carbis et al., 1996; Landsberg, 2002). In general, injected MC has stronger effects than fed toxin whereas exposure via immersion causes the mildest effects in liver (Malbrouck and Kestemont, 2006). Therefore, an alternative route of exposure was subsequently tested to determine whether it also lead to a change in brain AChE activity as occurred when zebrafish were exposed to the toxin dissolved in water. Interestingly, when MC-LR (50 or 100 μg/kg) was injected intraperitoneally there was no change in AChE activity suggesting that the observed increases in brain AChE depend, at least partially, on branchial uptake or ingestion. It is important to highlight that doses used in immersion and intraperitoneal MC-LR exposures are not equivalents. These findings appear to be particularly relevant to better understanding of the molecular mechanisms of MC toxicity in brain since the toxins are synthesized during the growth phase of the cyanobacteria, and large quantities of MC are released directly into the water during the collapse of a bloom (Berg et al., 1987) or from actively growing cyanobacterial populations (Sivonen et al., 1990).

AChE has been well studied for its classical functions in terminating neurotransmission at cholinergic synapses and neuromuscular junctions (Taylor and Radic, 1994). In this context, AChE is a primary target of organophosphorus and carbamate insecticides, which are structural

analogues of acetylcholine. Upon binding of these insecticides to the enzyme, AChE is inactivated due to phosphorylation or carbamoylation of the serine residue in the active site. As a result, the post-synaptic membrane remains depolarized and synaptic transmission fails (Matsumura, 1985).

However, more recently AChE has been redefined as an important regulator of apoptosis, because it can be induced by a variety of apoptotic stimuli (Zhang et al., 2002; for review see Jiang and Zhang, 2008). Thus, it has been demonstrated, for example, that over-expression of AChE is able to inhibit cell proliferation and promote apoptosis (Jin et al., 2004).

It is well known that apoptosis underlies the neurotoxic effects of various compounds. For instance, the apoptotic basis of the neurotoxic activity of β-amyloid and prion protein fragments was confirmed by ultrastructural examination of rat brain hippocampal neurons (Forloni et al., 1993). Moreover, brain AChE activation has also been demonstrated as a consequence of exposure to known neurotoxic compounds. For example, aluminum caused up-regulation of AChE activity in different mouse brain regions (Kaizer et al.; 2005), in rat lymphocytes (Kaizer et al., 2010) and in zebrafish brain (Senger et al., 2011). In addition, a significant increase in zebrafish brain AChE activity was established after acute ethanol exposures (Rico et al., 2007).

MCs are strong inhibitors of the PP1 and PP2A and this is probably the main mechanism of action of these toxins in the liver (Campos and Vasconcelos, 2010). Another reasonable possibility is that MC-LR may influence brain AChE indirectly via inhibition of a serine/threonine phosphatase. However, to our knowledge, until now, the phosphatases PP1 and PP2A have not been described in zebrafish's brain. Calcineurin, a Ca^{2+}/calmodulin-activated phosphatase seems to be a good candidate once it has specifically been implicated in AChE regulation in mammalian cells (Pregelj et al., 2007) and it is present in the zebrafish brain (Yoshida et al., 2009).

In summary, the results presented in this article provide experimental evidence that brain AChE is another potential target of MCs, and that the observed increases in AChE enzymatic activity together with *ache* transcript levels after MC-LR exposure depend, at least partially, on branchial uptake or ingestion. It might also be speculated that the neurotoxicity resulting from MC-LR exposure is mediated by apoptosis. Accordingly, microcystin-induced apoptosis in a variety of mammalian non-brain cell types was already demonstrated (McDermott et al., 1998; Ding et al., 2000). Further studies must be performed in order to reinforce these findings.

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Capítulo III

Microcystin-LR acute exposure does not alter *in vitro* and *in vivo* ATP, ADP and AMP hydrolysis in adult zebrafish (*Danio rerio*) brain membranes

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Microcystin-LR acute exposure does not alter *in vitro* and *in vivo* ATP, ADP and AMP hydrolysis in adult zebrafish (*Danio rerio*) brain membranes

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Abstract

Microcystins are toxins produced by cyanobacteria during the blooms that could accumulate in aquatic animals and be relocated to higher trophic levels. ATP acts as an excitatory neurotransmitter and/or a neuromodulator in the extracellular space playing important roles in physiological and pathological conditions. For this reason, the aim of this study was to

evaluate the acute effects of different concentrations of MC-LR on NTPDases (nucleoside triphosphate diphosphohydrolases) and 5'-nucleotidase in adult zebrafish (*Danio rerio*) brain membranes. The results have shown no significant changes in ATP, ADP and AMP hydrolysis in zebrafish brain membranes. MC-LR *in vitro* also did not alter ATP, ADP and AMP hydrolysis in the concentrations tested. These findings show that acute exposure to MC-LR didn't modulate ectonucleotidases activity in the conditions tested. However additional studies including chronic exposure should be performed in order to achieve a better understanding about MC-LR toxicity mechanisms in the central nervous system.

Keywords: microcystin-LR, zebrafish, ectonucleotidases, neurotoxicity.

Introduction

Microcystins (MCs) are heptapeptides primarily known as potent hepatotoxins released into water during cyanobacterial blooms.[1] More recently, it was demonstrated that MCs are also potential neurotoxic compounds.[2,3,4] MCs inhibit serine/threonine-specific protein phosphatases (PPs) such as PP1 and PP2A, induce oxidative stress, and generate cell apoptosis.[5]

ATP is an important signaling molecule in the extracellular space which plays key roles in physiological and pathological conditions. Once released into the extracellular space, the ATP can be metabolized by the action of ectoenzymes that make the conversion of this nucleotide to adenosine.[6] The ATP and metabolites, including ADP and adenosine affect several crucial biological events including neurotransmission and neuromodulation, platelet aggregation, heart contraction,[7] the normal and abnormal cell growth and apoptosis,[8] and intracellular signaling.[9]

NTPDases hydrolyze triphosphonucleosides and diphosphate with different abilities. The NTPDase1 (CD39) hydrolyzes ATP and ADP in the same way (1:1) while NTPDase2 (CD39L1) prefers trifosfonucleosídeos (in a ratio of 30:1), being known as ecto-ATPase. The NTPDase3 (CD39L3, HB6) and NTPDase8 prefer ATP then ADP at a ratio of 3:1 and 2:1 respectively. Other members of the NTPDase family are associated with membranes of intracellular organelles (NTPDase4-7) (for review see 10).

Zebrafish (*Danio rerio*) is a small teleost that has long been considered a successful animal model for studying diverse biological actions. More recently, zebrafish has also become a valuable model for toxicological and neurochemical studies including those performed to investigate the toxic effects generated by MC-LR exposure in the liver[11,12] and in the brain.[2,3,4,13]

Therefore, considering that: (i) MCs synthesized by cyanobacteria can either accumulate in the brain and change locomotor behavior in fish, (ii) the neurotoxic effects of MCs exposure are far from being completely understood, (iii) zebrafish is a well-established organism model for toxicological analysis and that (iv) the purinergic system has been extensively studied in this species, the objective of this study was to investigate whether MC-LR acute exposure was able to modulate ATP, ADP and AMP (*in vitro* and *in vivo*) hydrolysis in the brain of adult zebrafish, contributing to a better understand of the complete scenario of MC-LR toxicity.

Materials and Methods

Animals

Wild-type adult (<8 months old) zebrafish (*Danio rerio*) of both sexes were obtained from specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O_2/L) at 26 ± 2 ºC, under a 14–10 h light/dark photoperiod in a density of up to five animals per liter. Animals were acclimated for at least two weeks before the experiments. They were fed three times a day with TetraMin Tropical Flake fish®.

The procedures were previously approved by the Animal Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the protocol number 10/00142-CEUA.

Reagents

Microcystin-LR was purchased from BioVision (California, USA). Trizma base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium chloride, magnesium chloride, and

nucleotides (ATP, ADP, and AMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

In vivo treatment

The *in vivo* exposures were performed in 1-L aquariums (10 fish per aquarium). Fish were exposed to different MC-LR concentrations (50 and 100 µg/L) dissolved in water for 24 hours and, immediately after the exposure, they were euthanized by decapitation. For the control group the animals were exposed only to water for 24 hours, after which they were euthanized.

In vitro treatment

In vitro assays were performed as previously described.[14] Briefly, MC-LR was added to the reaction medium before the preincubation with the enzyme-containing lysate from zebrafish brain homogenate and maintained during the enzyme assays. MC-LR was tested at a final concentration of 50, 100, 500, and 1000 µg/L. For the control group, the enzyme assay was performed in the absence of MC-LR (i.e. no toxin was added to the reaction medium).

Membrane preparation

Brain membranes were prepared as described previously.[15] Zebrafish were euthanized by decapitation and their whole brains were removed from the cranial skull by the dissection technique. For each sample (membrane preparation), a pool of five zebrafish brains was used, which were briefly homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 1000 g for 10 min and the pellet was discarded. The supernatant was then centrifuged for 25 min at 40 000 g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged for 20 min at 40 000 g. This freeze–thaw–wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2-4 °C throughout preparation.

Nucleotide hydrolysis assay

Zebrafish brain membranes (3 μg protein for NTPDase and 5 μg protein for ecto-5'-nucleotidase) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5mM $CaCl_2$ (for NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP, ADP, AMP) to a final concentration of 1mM. The reaction was stopped after 30 min with the addition of 200 μL of 10% trichloroacetic acid and immediately placed on ice for 10 min. The inorganic phosphate (Pi) released was determined by colorimetric assay.[16] To ensure that the concentration of Pi was within the linear range, aliquots of 13 and 50 μL were diluted to a final volume of 100 μL for assaying the ATP and ADP hydrolysis, respectively, whereas aliquots of 100 μL were performed for AMP substrate. Each sample was mixed to 250 μL of Malachite Green solution and the nucleotide hydrolysis was measured in a microplate reader at 630 nm after 20 min. Controls with the addition of the enzyme preparation after incubation period were used to correct non-enzymatic hydrolysis of substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays run in duplicate of at least four independent experiments.

Protein determination

Protein was measured by the Coomassie blue method[17] using bovine serum albumin as standard.

Statistical analysis

Data were expressed as means $\pm$ S.E.M. The activity of ectonucleotidases for each substrate (ATP, ADP and AMP) was assessed by analysis of variance one-way (one-way ANOVA), followed by Tukey test, where $p<0.05$ indicated significant difference.

Results

In this study, we analyzed the effects of distinct MC-LR concentrations exposure on ectonucleotidases activities (NTPDases and 5′-nucleotidase) by performing *in vivo* (50 and

100 µg/L) and *in vitro* (50, 100, 500 and 1000 µg/L) experiments using adult zebrafish. The *in vivo* analysis demonstrated that ectonucleotidases activities were not altered in the animals treated with different concentrations ($p>0.05$; Fig. 1).

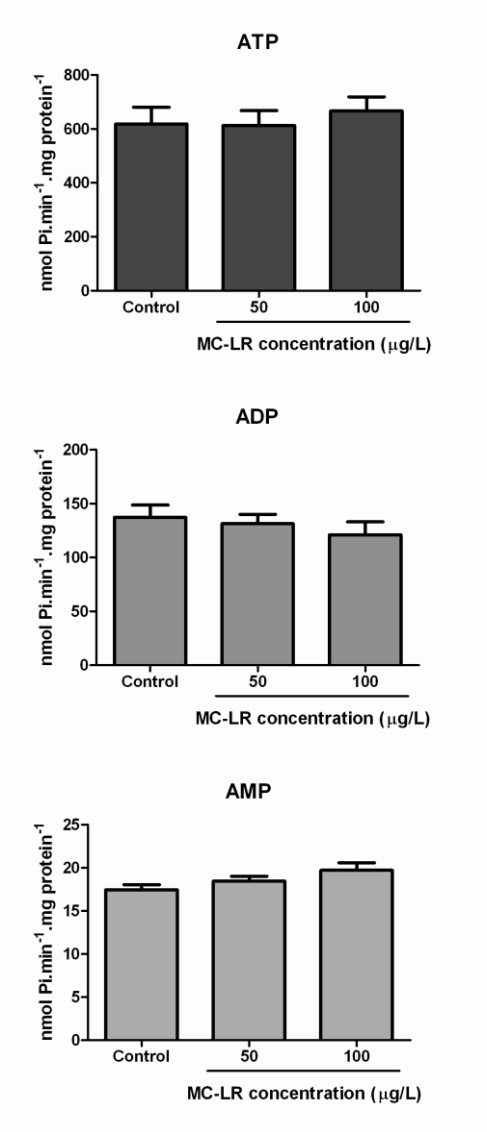

Figure 1 – Kist et al., 2012

To verify whether the toxin might have a direct effect on the enzymes of purinergic signaling, we tested the *in vitro* effect of MC-LR on ectonucleotidases activities in zebrafish brain membranes. The results showed that MC-LR did not cause any alteration in NTPDases and 5′-nucleotidase activities in the conditions tested ($p>0.05$; Fig. 2).

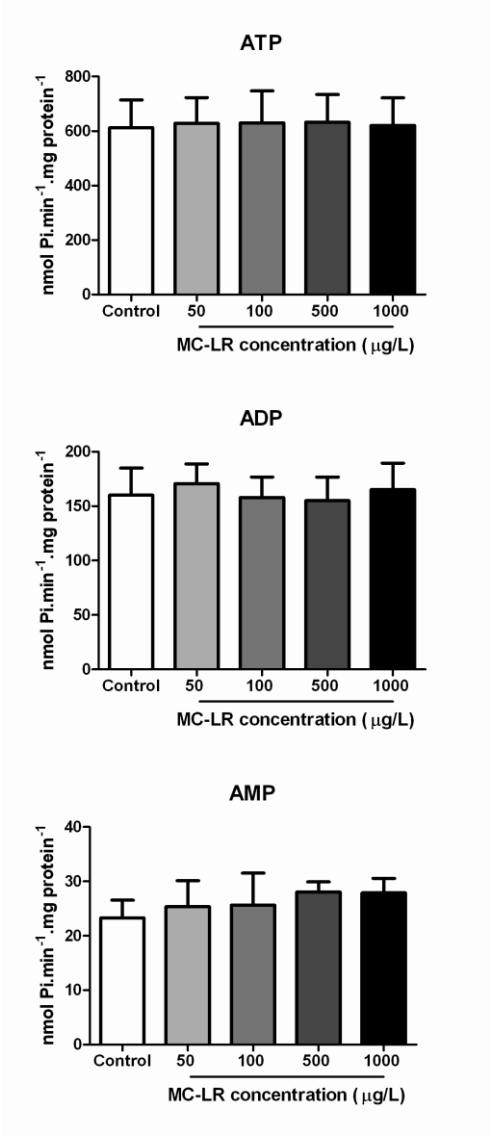

Figure 2 – Kist et al., 2012

Discussion

Recently the cholinergic system was demonstrated as a target to acute exposition to MC-LR in adult zebrafish brain.[4] Acetylcholine (ACh), the main neurotransmitter of cholinergic system, is co-released together with ATP at synaptic cleft[18] and studies have demonstrated that ATP can control the ACh release.[19] In addition, several studies have already demonstrated that toxic compounds to brain such as methanol[20] and ethanol[21,22] affected both cholinergic and purinergic neurotransmission systems. For these reasons, we

investigated the *in vivo* and *in vitro* effects of MC-LR acute exposure over NTPDases and 5'-nucleotidase activities on adult zebrafish brain membranes.

The results presented herein have demonstrated that ATP, ADP and AMP hydrolysis *in vivo* were not altered by the action of MC-LR in any concentration tested on adult zebrafish brain membranes. To our knowledge, the possible toxic effects caused by MC-LR exposure on the enzymes involved in purinergic neurotransmission have not yet been investigated in any organism.

Additionally, a possible direct effect of MC-LR on these enzymes activities was also evaluated. Our results showed that MC-LR did not directly alter NTPDases and 5′-nucleotidase activities on zebrafish brain membranes. These results are in accordance with a previous study where MC-LR did not affect *in vitro* AChE activity.[4]

However, further studies including long-time exposure should be performed in order to achieve a better understanding about MC-LR toxicity mechanisms in the central nervous system.

Acknowledgments

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Figure Legend

Figure 1: *In vivo* effect of acute treatment with MC-LR on ATP hydrolysis (A), ADP hydrolysis (B) and AMP hydrolysis (C) in zebrafish brain membranes. Bars represent the mean ± S.E.M. Data were analyzed by One way ANOVA, followed by Tukey's test as post-hoc, $p < 0.05$.The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

Figure 2: *In vitro* effect of distinct concentrations of MC-LR on ATP hydrolysis (A), ADP hydrolysis (B) and AMP hydrolysis (C) in zebrafish brain membranes. Bars represent the mean ± S.E.M. Data were analyzed by One way ANOVA, followed by Tukey's test as post-hoc, $p < 0.05$.The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

Capítulo IV

Acute exposure to Microcystin-Producing Cyanobacterium *Microcystis aeruginosa* alters adult zebrafish (*Danio rerio*) swimming performance parameters

Kist LW, Piato AL, da Rosa JGS, Koakoski G, Barcellos LJG, Yunes JS, Bonan CD, Bogo MR

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Research Article

Acute Exposure to Microcystin-Producing Cyanobacterium *Microcystis aeruginosa* Alters Adult Zebrafish (*Danio rerio*) Swimming Performance Parameters

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Microcystins (MCs) are toxins produced by cyanobacteria (blue-green algae), primarily *Microcystis aeruginosa*, forming water blooms worldwide. When an organism is exposed to environmental perturbations, alterations in normal behavioral patterns occur. Behavioral repertoire represents the consequence of a diversity of physiological and biochemical alterations. In this study, we assessed behavioral patterns and whole-body cortisol levels of adult zebrafish (*Danio rerio*) exposed to cell culture of the microcystin-producing cyanobacterium *M. aeruginosa* (MC-LR, strain RST9501). MC-LR exposure (100 μg/L) decreased by 63% the distance traveled and increased threefold the immobility time when compared to the control group. Interestingly, no significant alterations in the number of line crossings were found at the same MC-LR concentration and time of exposure. When animals were exposed to 50 and 100 μg/L, MC-LR promoted a significant increase (around 93%) in the time spent in the bottom portion of the tank, suggesting an anxiogenic effect. The results also showed that none of the MC-LR concentrations tested promoted significant alterations in absolute turn angle, path efficiency, social behavior, or whole-body cortisol level. These findings indicate that behavior is susceptible to MC-LR exposure and provide evidence for a better understanding of the ecological consequences of toxic algal blooms.

1. Introduction

Microcystis aeruginosa is a freshwater cyanobacteria, known producer of a family of toxins termed microcystins (MCs) [1, 2]. MCs are hepatotoxic cyclic heptapeptides released into water during or on senescence of cyanobacterial blooms [3]. The peptide rings of MCs contain five nonprotein amino acids, whereas the two-protein amino acids distinguish MCs from one another. MC-LR contains the amino acids leucine and arginine. MC-LR is one of the most commonly

occurring [2, 4] and the most toxic microcystin [5]. The intact cells as well as the toxins released after cellular lysis can be responsible for the toxic effects observed in many organisms, from microalgae [6] to mammals [7] including human [8–10].

Exposure to toxic cyanobacteria or administration of MCs may cause hepatotoxic effects [11–13], oxidative stress [14], kidney damage [15, 16], growth inhibition [17, 18], reproductive injury [19], haematological and biochemical alterations [20–22], apoptosis [23], and even fish death [24].

Alterations in normal behavioral patterns may be the first line of defense when an animal is exposed to an environmental perturbation [25–28]. Additionally, studies have shown important interrelationships between hormones and behavior [29–33]. Thus, alteration in cortisol level may consequently alter normal fish behavior. The effects of MC on fish behavior are still unknown, but some issues have already been addressed. Baganz et al. [34, 35] reported changes in the spontaneous locomotor behavior of zebrafish (*Danio rerio*) and *Leucaspius delineatus* after MC-LR exposure, and Cazenave et al. [36] showed changes in swimming activity of *Jenynsia multidentata* fed with microcystin-RR (MC-RR). In addition, studies using different exposure routes (intraperitoneal injection, oral ingestion, or immersion) have demonstrated that MCs can accumulate in fish tissues, mainly in the liver [21, 36–39], intestine [37, 39–41], gills [42, 43], kidney [37, 39], muscle [40, 41, 44–46], gallbladder [47], blood [40, 41, 48], and brain [43]. Altogether, these findings indicate possible neurotoxic effects of MCs on fish, causing serious risks to the success of fish populations and changes in biodiversity, among other ecological consequences [36].

The zebrafish is rapidly becoming a popular model species in many areas of biological research. Its application includes the fields of developmental biology [49], toxicology [50], neurophysiology, biomedicine, drug discovery [51], human diseases [52–54], pharmacology and behavioral analysis [55–59]. These fish exhibit robust behavioral responses, well-characterized genome, neural and endocrine systems homologous to humans [60–62], and possess all of the "classical" vertebrate neurotransmitters [63, 64]. Additionally, zebrafish are an ideal animal model for laboratory research because they are inexpensive, require low maintenance, and produce abundant offspring [65]. Recently, this fish was also used for proteomic studies on the toxicity of MCs [66, 67].

In order to better understand the neurotoxic effects of MCs on fish and to improve the knowledge of mechanisms underlying the toxicity, the main goal of this study was to assess the effects of MC-LR on zebrafish behavioral parameters and endocrine (whole-body cortisol) response after toxin exposure.

2. Materials and Methods

2.1. Animals. Wild-type adult (<8 months old) zebrafish (*Danio rerio*) of both sexes were obtained from specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O_2/L) at 26 ± 2°C, under a 14–10 h light/dark photoperiod in a density of up to five animals per liter. Animals were acclimated for at least two weeks before the experiments. They were fed three times a day with TetraMin Tropical Flake fish.

The procedures were previously approved by the Animal Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the protocol number 10/00142-CEUA.

2.2. Treatments. The amount of MC-LR in the cell culture of *M. aeroginosa* (strain RST9501) was detected by a Quantitative Antibody Immunoassay (Elisa) against MC-LR provided by Envirologix (Portland, USA), within a range of detection from 0.05 to 2.5 μg/L MCs. A suitable dilution was applied to the culture sample to provide detection within the range. Zebrafish were distributed in three groups: the first group (controls) was exposed to water containing the culture medium of *M. aeroginosa* for 24 hours; the second and third groups were exposed to cell culture in a final MC-LR concentration of 50 μg/L and 100 μg/L during 24 hours, respectively. Immediately after the exposure, animals were tested in tank-diving behavioral test and social interaction. After behavioral tests animals were euthanized by decapitation.

The MC-LR concentrations and the time of exposure were chosen based on a previous study using *J. multidentata* [43]. Besides, such concentrations are commonly encountered in cyanobacterial bloom events [68, 69].

2.3. Tank-Diving Behavioral Test. Behavioral testing took place during the light phase between 10:00 AM and 4:00 PM The animals were individually transferred to a 2.7 L tank (24 cm L × 8 cm W × 20 cm H) with laterals and bottom white covered to avoid any visual disturbances and habituated to the tank for 30 s, as previously described [70]. There was no drug exposure during behavioral experiments. The locomotor activity of the animals was video-recorded using Logitech Quickcam PRO9000 for five minutes after the habituation period and further analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The tank was divided into equal sections with four vertical lines and one horizontal line, and the following behavior patterns were measured: distance traveled (meters), immobility time (seconds), number of crossings, absolute turn angle, path efficiency, and time (seconds) spent in the bottom portion. This task exploits the natural tendency for zebrafish to spend most of the time at the bottom when introduced into a novel environment and then gradually extend the swimming range, over a period of minutes, to include the upper portions of the test tank. A longer time spent in the bottom part of the tank indicates heightened anxiety [71].

2.4. Social Interaction. Zebrafish is a schooling fish that may exhibit preference for its conspecifics under certain circumstances. The social interaction analysis was based on

Gerlai [72]. After 24 hours of exposition to 50 or 100 μg/L of MC-LR, fish were placed in groups of five in a small experimental tank (30 cm L × 15 cm H × 10 cm W). On one side of the experimental tank, an empty fish tank was placed, and, on the other side, there was a tank of identical size containing 15 conspecifics. The experimental fish were allowed to acclimate to the experimental tank for a 30 s period, after which their behavior was analyzed. The next 10 s of this test was analyzed as follows. The experimental tank was virtually divided into two equal sections with one vertical line. The time that all five experimental fish spent on the side of the tank closer to the conspecific school was measured using a stopwatch.

2.5. Acute Restraint Stress (ARS) Protocol. The ARS protocol was based on Piato et al. [73]. Following the habituation period, fish were submitted to the ARS protocol. This experiment consisted in keeping each animal enclosed into microcentrifuge plastic tubes of 2 mL with the cap closed and small openings in both ends to allow free water circulation inside the tube and completely avoid fish locomotion. After 90 min of confinement, animals were gently captured and immediately frozen in liquid nitrogen and stored at −80°C until cortisol extraction. Aeration (8 ppm, Labcom Test Camboriú, SC, Brazil) and water temperature (26 ± 2°C) were controlled throughout the test.

2.6. Measurement of Cortisol. The extraction and measurement of whole-body cortisol from zebrafish have been described in detail by Barcellos et al. [74]. Briefly, zebrafish were distributed in four groups: the first group, which consisted of zebrafish exposed to water containing the culture medium of *M. aeroginosa* for 24 hours, was considered the "negative control"; the second and third groups were exposed to cell culture in a final MC-LR concentration of 50 μg/L and 100 μg/L during 24 hours, respectively; in the fourth group, considered the "positive control," zebrafish were submitted to the ARS protocol. After, zebrafish were captured and immediately frozen in liquid nitrogen and stored at −80°C until whole-body cortisol extraction. Each zebrafish was weighed, and a pool of three fish was minced and placed into a disposable stomacher bag with 2 mL of phosphate buffered saline (PBS, pH 7.4) for 6 min. The contents were transferred to a 10 mL screw top disposable test tube, and 5 mL of laboratory grade ethyl ether was added. The tube was vortexed for 1 min and centrifuged for 10 min at 3000 rpm. The tube was then immediately frozen at liquid nitrogen, and the unfrozen portion (ethyl ether containing cortisol) was decanted. The ethyl ether was transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol. The extract was stored at −20°C until the ELISA was conducted on the samples suspended with 1 mL of PBS buffer. In order to prevent a possible stress response induced by manipulation, the time elapsed between capture and killing was less than 10 s. Whole-body cortisol was measured in duplicate samples of tissue extract with a commercially available high sensitivity salivary cortisol-enzyme immunoassay kit (Salimetrics, USA). The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions of the tissue extracts in PBS (pH 7.4). The standard curve constructed with the human standards ran parallel to that obtained using serial dilutions of zebrafish tissue extracts. In the linear regression test, high positive correlation (R^2 = 0.9818) was found between the curves. The intra-assay coefficient of variation was 3.33–3.65%.

2.7. Statistical Analysis. Data of the exploratory assessment, social interaction, and cortisol levels were expressed as mean ± SEM and analyzed by one-way ANOVA, followed by Newman-Keuls post hoc test. A significant difference was attributed to $P < 0.05$. All data were evaluated by SPSS 18.0 for Windows.

3. Results

Distinct parameters of zebrafish swimming activity were evaluated in the 5-min tank-diving behavioral test. MC-LR exposure at 100 μg/L significantly (one-way ANOVA/Newman-Keuls, $P < 0.0081$, $n = 10$) decreased the distance traveled (3.7 ± 0.6 meters) in relation to control animals (10.3 ± 1.7 meters) (Figure 1(a)) and significantly (one-way ANOVA/Newman-Keuls, $P < 0.039$, $n = 10$) increased the immobility time (137.6 ± 27.6 seconds) when compared to the control group (41.5 ± 17.3 seconds) (Figure 1(b)) whereas MC-LR exposure at 50 μg/L did not alter both parameters. No differences in the number of line crossings, absolute turn angle, and path efficiency were observed in both concentrations tested (Figure 1(c), 1D and 1E, resp.).

Control animals spent 58.4% of time (175.0 ± 28.6) in the bottom portion of the test tank. Animals exposed to 50 and 100 μg/L MC-LR significantly (one-way ANOVA/Newman-Keuls, $P < 0.0003$, $n = 10$) increased (93%) the time spent in the bottom portion of the test tank (282.1 ± 7.90 and 282.7 ± 9.7, resp.) when compared with control group (175.0 ± 28.6) (Figure 1(f)).

In relation to social interaction test, the results showed that 50 and 100 μg/L of MC-LR concentrations did not promote any alteration in the animals regarding this behavior (Figure 2).

Levels of whole-body cortisol also were measured. The ARS protocol (positive control) resulted in enhanced whole-body cortisol in relation to control group (one-way ANOVA/Newman-Keuls, $P < 0.005$, $n = 7$; 10.7 ± 1.4 and 6.7 ± 0.7, resp.). Zebrafish treated with both concentrations of MC-LR did not present altered levels of cortisol in relation to control group (Figure 3).

4. Discussion

Behavioral alterations reflect how an animal senses and responds to its environment and is the first line of defense when an animal is exposed to an environmental perturbation [28]. Since it was already demonstrated that the effects

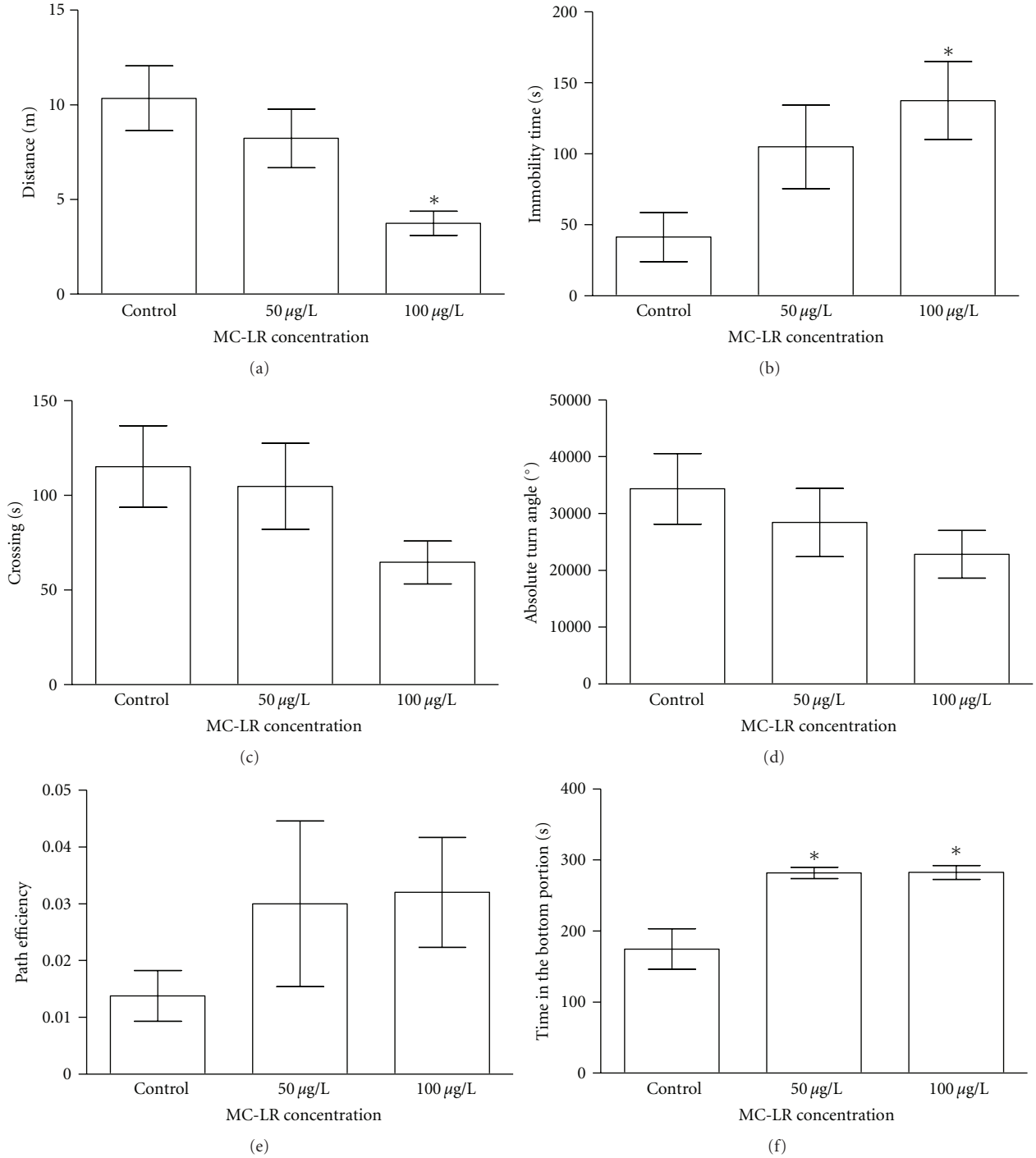

Figure 1: Effect of microcystin-LR exposure on the distance traveled (a), immobility time (b), number of line crossings (c), absolute turn angle (d), path efficiency (e), and time in the bottom portion (f) determined during 5 min of video recording in the tank-diving behavioral test. Data expressed as mean ± SEM. $n = 10$. One-way ANOVA/Newman-Keuls post hoc test. *: $P < 0.05$ compared to control group.

promoted by cyanobacterial crude extracts on aquatic organisms were either more pronounced or different from those observed using pure toxins [75, 76], we used cell culture of the microcystin-producing cyanobacterium *M. aeruginosa* (MC-LR) in order to evaluate the effects of MCs on zebrafish behavior.

The toxin concentration and time period of animals' exposure were chosen based on previous studies that showed MCs accumulation in fish tissues [21, 37–48]. The results presented herein demonstrated that 100 μg/L MC-LR decreased the distance traveled and increased the immobility time. However, no significant alterations were found in the

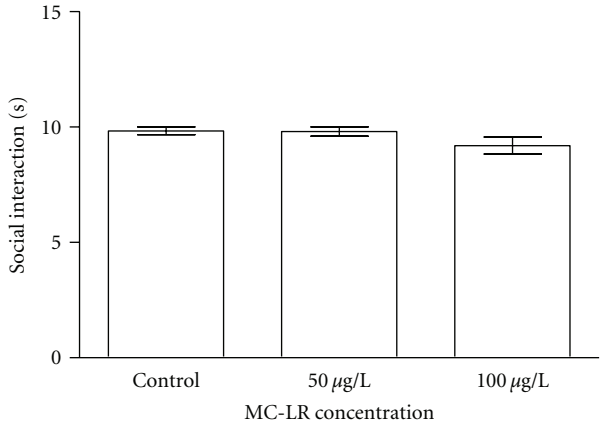

Figure 2: Effect of exposure to microcystin-LR on social interaction. Data expressed as mean ± SEM. $n = 10$. One-way ANOVA/Newman-Keuls post hoc test.

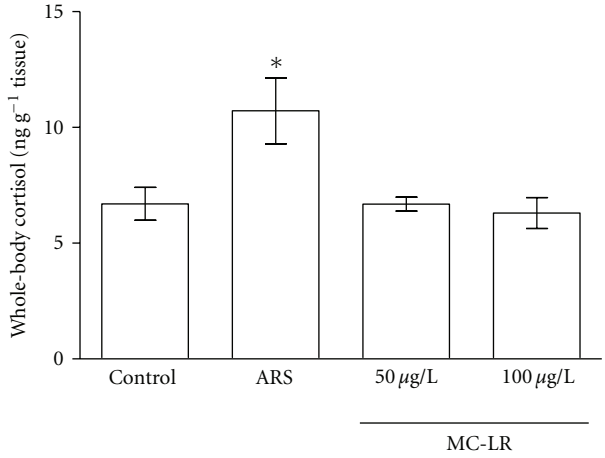

Figure 3: Effect of exposure to microcystin-LR on whole-body cortisol levels. Data expressed as men ± SEM. $n = 7$. One-way ANOVA/Newman-Keuls post hoc test. $^*P < 0.05$ compared to control group.

number of line crossings with both concentrations. When animals were exposed to 50 and 100 μg/L, MC-LR led to a significant increase in the time spent in the bottom portion. The results also showed that none of the MC-LR concentrations tested promoted significant alterations in the absolute turn angle, path efficiency, or social interaction.

Since behavior links physiological function with ecological processes for a given species, it might provide a useful indicator or biomarker for detecting harmful chemical pollutants [77]. The potential of the zebrafish as a model in neurobehavioral research has emerged only recently. Studies have examined behavior in zebrafish larvae [78–81], as well as their responses to different drugs, such as ethanol [82, 83] and fluoxetine [84]. Studies on adult zebrafish include social behavior [85–87], olfactory-related behaviors [88, 89], anxiety [74], addiction [90–92], sleep [93], learning and memory [94, 95].

There are still only few studies evaluating the effects of MCs on fish behavior. Baganz et al. [34] verified that exposure to MC-LR caused dose-effect-related changes in spontaneous locomotor activity in zebrafish. Whereas exposure to lower concentrations (0.5 and 5 μg/L) caused an increase in daytime mobility, elevated exposures (15 and 50 μg/L) led to significantly increased immobility. The highest exposure (50 μg/L) also reduced the spawning activity and reduced spawning success. In contrast to daytime activities, night-time swimming activity was significantly greater at the higher MC-LR exposures. In another study, Baganz et al. [35] showed changes in the spontaneous locomotor behavior of zebrafish and *L. delineatus* after exposure to MC-LR in concentrations of 0.5, 5, and 15 μg/L for 17 days and 50 μg/L for six days. During the daytime, the mobility of zebrafish as well as *L. delineatus* increased significantly by exposure to the lowest concentrations, whereas higher concentrations led to significantly decreased mobility. Influenced by MC-LR, the swimming time of *L. delineatus* reversed, going from a prominently diurnal activity to a nocturnal one; zebrafish remained active during the daytime. Additionally, Cazenave et al. [36] reported changes in the swimming activity of *J. multidentata* fed with contaminated food pellets containing MC-RR. Low levels (0.01 μg/g) increased the swimming activity, while the highest dose (1 μg/g) used produced significant changes with respect to control group (only since approximately 20 hours of exposure), when the swimming activity was decreased.

In this sense, our findings demonstrate that MC-LR at the highest concentration (100 μg/L) caused a decrease in the distance traveled and an increase in the immobility time in zebrafish. Interestingly, no significant alterations in the number of line crossings were found at the same MC-LR concentration and time of exposure, despite the tendency to decrease the number of crossings in greater concentration. It is important to emphasize that these findings are similar to the results published previously by Baganz et al. [35]. However, these authors have used purified toxin whereas a cell culture of the microcystin-producing cyanobacterium *M. aeruginosa* (MC-LR) was used in our experiments. Reduction in swimming capability, resulting in a reduction in the rate of activity, may decrease the ability to gather food and make the fish more vulnerable to predation [96]. Under natural conditions, this reduced overall level of activity will eventually cause disadvantages to the organisms in the ecosystem, and, therefore, influence the biocoenotic structures and functions [34].

Exposure to a novel environment evokes a robust anxiety response in zebrafish [97], as they dive to the bottom (geotaxis) until they feel safe to swim in the upper regions of the tank [58]. Here, MC-LR at 50 and 100 μg/L promoted an increase in the time spent in the bottom portion, suggesting an anxiety behavior.

The zebrafish is a social species and exhibits group preference as well as aggression. Shoaling behavior commences soon after hatching and fish reared in isolation quickly form shoals when placed together [98]. One study has demonstrated that exposure to nonylphenol over a 5-day period decreased shoaling tendency in juvenile rainbow trout

(*Oncorhynchus mykiss*) [99]. Similarly, herbicide-exposed goldfish also showed a decreased aggregation [100]. Locomotor activity, aggressive behavior, and group preference of the male zebrafish and group preference of the females were clearly inhibited when zebrafish were exposed for 60 days to 100 μg/L nonylphenol concentration [101]. For this reason, we evaluated the effect of MC-LR exposure on zebrafish social interaction. However, no significant alteration was found between control and MC-LR-exposed animals.

Studies have shown important interrelationships between stress hormones and behavior [29–33]. An elevated plasma cortisol level is a primary indicator of a stress response in fish [102]. Zebrafish, like humans, employ cortisol as a primary stress response hormone [74]. Considering this, we measured whole-body cortisol in zebrafish to verify if MC could elicit a stress response in treated fish. However, no significant alterations were found in whole-body cortisol levels in animals exposed to both concentrations of MC-LR compared to the control group. Barcellos et al. [74] demonstrated that whole-body cortisol level of zebrafish increases after visual contact with a predator species. Cortisol levels were significantly higher in zebrafish submitted to unpredictable chronic stress (UCS) protocol when compared to control group [103]. Bury et al. [104] reported a significant increase in plasma cortisol levels of the brown trout (*Salmo trutta*) after 1 h and returned to the control level after 24 h of exposure to lysed toxic *Microcystis aeruginosa* cells. Crucian carp (*Carassius auratus*) exposed to sublethal and lethal doses (150 μg/kg and 600 μg/kg, resp.) of *Microcystis* extracts exhibited a significant acute increase in plasma cortisol levels, which suggested that MC elicited a stress response in treated fish. The profiles of cortisol changes in fish treated with MC appeared to be dose dependent, indicating that fish in the high-dose group experienced greater MC-induced disturbance [105].

In summary, behavioral response of fish may be a promising biomarker of sublethal toxicity and water contamination. Several behavioral endpoint measurements, especially locomotor activity and the time spent in the bottom portion, may provide an effective assessment of MCs in aquatic ecosystem.

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Capítulo V

Considerações Finais

CONSIDERAÇÕES FINAIS

Esta tese apresenta os resultados experimentais obtidos do estudo acerca dos efeitos da exposição aguda à microcistina-LR (MC-LR) em parâmetros neuroquímicos e comportamentais de *zebrafish.*

MCs são heptapeptídeos primariamente conhecidas como potentes hepatotoxinas liberadas na água durante eventos de floração (Malbrouck e Kestemont, 2006). Existem mais de 80 diferentes isoformas desta molécula, incluindo variações e modificações nos aminoácidos. Dentre estas, a MC-LR é uma das formas mais comuns e tóxicas (Dawson, 1998). MCs agem inibindo as fosfatases PP1 e PP2A e parecem interagir com a mitocôndria das células animais, desencadeando estresse oxidativo e apoptose. Esta via é considerada o principal mecanismo da toxicidade das MCs. Entretanto, a toxicidade das MCs é um processo de múltiplas vias e, apesar dos achados recentes, o mecanismo molecular da toxicidade das MCs ainda não está completamente entendido (Campos e Vasconcelos, 2010).

As MCs podem afetar organismos aquáticos e terrestres, inclusive os seres humanos e, a contaminação por esta toxina tem sérias consequências ecológicas e de saúde pública (Carmichael, 1994; Wiegand e Pflugmacher, 2005). Estudos demonstraram que MCs acumulam em diversos tecidos de peixes como fígado (Williams et al., 1995; Sipiä et al., 2001; Malbrouck et al., 2003; Mohamed et al., 2003; Cazenave et al., 2008) intestino (Williams et al., 1995; Mohamed et al., 2003; Xie et al., 2004, 2005), brânquias (Carbis et al., 1997; Cazenave et al., 2005), rins (Williams et al., 1995; Mohamed et al., 2003), músculos (Bury et al., 1998; Magalhães et al., 2001, 2003; Xie et al., 2004, 2005), sangue (Tencalla e Dietrich, 1997; Xie et al., 2004, 2005) e cérebro (Cazenave et al., 2005). Embora alguns estudos já tenham sido realizados (Baganz et al., 1998; 2004; Cazenave et al., 2008), os efeitos da exposição às MCs sobre padrões de comportamento de peixes ainda estão longe de serem bem compreendidos.

No capítulo II foram avaliados os efeitos da exposição à MC-LR sobre a atividade da AChE cerebral *in vitro* e *in vivo* em *zebrafish* e a contribuição transcricional do gene que codifica para a *ache*. A atividade da AChE tem sido usada como um bioindicador de contaminação ambiental, como por exemplo, para a avaliação da exposição a inseticidas organofosforados e carbamatos, e substâncias tóxicas como metanol e metais pesados (Van Dyk e Pletschke, 2011; Rico et al., 2006; Richetti et al., 2011).

Uma vez que não existem estudos prévios acerca dos efeitos das MCs sobre a atividade da AChE, foram escolhidas condições de exposição descritas por Cazenave et al. (2005) onde foi mostrado o acúmulo de MC-RR em cérebro de *J. multidentata*. Na maior concentração testada (100 µg/L) após um período de 24h, foi observado, que os animais expostos à toxina diluída em água apresentaram um aumento significativo na atividade da AChE quando comparado com os animais do grupo controle. Por outro lado, quando a MC-LR foi injetada intraperitonealmente não houve alteração na atividade da enzima. Para verificar se a regulação da atividade da AChE após a exposição a 100 µg/L de MC-LR poderia ser consequência de controle transcricional ou de modificação pós-traducional, foi realizada a análise de RT-PCR semiquantitativo. Os resultados demonstraram que os níveis relativos de RNAm no gene da *ache* estavam aumentados. Finalmente, não foram observadas mudanças significativas na atividade da AChE na análise *in vitro* nas concentrações testadas (10, 25, 50, 100, 500, 1000, e 5000 µg/L), sugerindo que a MC-LR não atua diretamente sobre a enzima.

No capítulo III, foi avaliado o efeito da MC-LR sobre parâmetros comportamentais e endócrinos (cortisol corporal) em *zebrafish*. Alterações nos padrões normais de comportamento são a primeira linha de defesa de um animal quando exposto a uma perturbação ambiental (Scherer, 1992; Paul e Simonin, 1996; Campbell et al., 2002; Begout Anras e Lagardère, 2004). Adicionalmente, estudos prévios também mostraram a relação entre níveis de hormônios e alterações comportamentais (Oliveira et al., 1996; Contreras-Sánchez et al., 1998) incluindo alterações nos níveis de cortisol (Pottinger e. Carrick, 2001; Sloman et al., 2001; Øverli et al., 2002).

A exposição a 100 µg/L de MC-LR diminuiu significativamente a distância viajada e aumentou significativamente o tempo de imobilidade quando comparado ao controle, enquanto que exposição a 50 µg/L não alterou estes parâmetros. Não foi encontrada nenhuma diferença no número de linhas cruzadas, mudanças de ângulo e eficiência de rota em ambas as concentrações testadas. Foi verificado também que os animais expostos a 50 e 100 µg/L de MC-LR tiveram um aumento significativo do tempo gasto na porção inferior do aquário de teste. Em relação ao teste de interação social, os resultados mostram que 50 e 100 µg/L de MC-LR não promoveram nenhuma alteração nos animais em relação a este comportamento. Os níveis de cortisol corporal dos animais tratados não foram alterados em relação aos animais controles.

Uma vez que a ACh, o principal neurotransmissor do sistema colinérgico, é co-liberada juntamente com o ATP, a principal molécula sinalizadora do sistema purinérgico na fenda sináptica e que estudos têm demonstrado que o ATP pode controlar a liberação de ACh (Cunha e Ribeiro, 2000; Burnstock, 2004) foram analisados no capítulo IV os efeitos de distintas concentrações de exposição à

MC-LR sobre a atividade das ectonucleotidases (NTPDases e 5'-nucleotidase) através da realização de ensaios *in vivo* (50 e 100 μg/L) e *in vitro* (50, 100, 500 e 1000 μg/L) em membranas cerebrais de *zebrafish.*

Os resultados da análise *in vivo* demonstraram que a atividade das ectonucleotidases não foi alterada nos animais expostos às diferentes concentrações de MC-LR. Nos ensaios *in vitro*, nenhuma alteração significativa foi encontrada nas atividades das NTPDases e 5'-nucleotidase nas condições testadas.

O conjunto de resultados apresentados nesta tese proporciona um melhor entendimento dos mecanismos da toxicidade mediada pela exposição aguda às MCs sobre o Sistema Nervoso Central, fornecendo novas evidências do envolvimento do sistema de neurotransmissão colinérgico sugerindo que a AChE cerebral é um novo alvo da ação das MCs e que respostas comportamentais de peixes podem representar um promissor biomarcador de contaminação de águas por cianobactérias. Adicionalmente, os resultados também mostraram que nas condições testadas não há o envolvimento das NTPDases e 5'-nucleotidase na toxicidade induzida pela exposição às MCs.

Estudos futuros envolvendo tempo de exposição prolongado e concentrações mais elevadas devem ser realizados objetivando determinar com maior abrangência os mecanismos da toxicidade mediada pelas MCs no Sistema Nervoso Central.

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Anexos

Artigos científicos não relacionados ao projeto publicados durante o período do doutoramento

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Evidence that acute taurine treatment alters extracellular AMP hydrolysis and adenosine deaminase activity in zebrafish brain membranes

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ABSTRACT

Taurine is one of the most abundant free amino acids in excitable tissues. In the brain, extracellular taurine may act as an inhibitory neurotransmitter, neuromodulator, and neuroprotector. Nucleotides are ubiquitous signaling molecules that play crucial roles for brain function. The inactivation of nucleotide-mediated signaling is controlled by ectonucleotidases, which include the nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5′-nucleotidase. These enzymes hydrolyze ATP/GTP to adenosine/guanosine, which exert a modulatory role controlling several neurotransmitter systems. The nucleoside adenosine can be inactivated in extracellular or intracellular milieu by adenosine deaminase (ADA). In this report, we tested whether acute taurine treatment at supra-physiological concentrations alters NTPDase, ecto-5′-nucleotidase, and ADA activities in zebrafish brain. Fish were treated with 42, 150, and 400 mg L^{-1} taurine for 1 h, the brains were dissected and the enzyme assays were performed. Although the NTPDase activities were not altered, 150 and 400 mg L^{-1} taurine increased AMP hydrolysis (128 and 153%, respectively) in zebrafish brain membranes and significantly decreased ecto-ADA activity (29 and 38%, respectively). *In vitro* assays demonstrated that taurine did not change AMP hydrolysis, whereas it promoted a significant decrease in ecto-ADA activity at 150 and 400 mg L^{-1} (24 and 26%, respectively). Altogether, our data provide the first evidence that taurine exposure modulates the ecto-enzymes responsible for controlling extracellular adenosine levels in zebrafish brain. These findings could be relevant to evaluate potential beneficial effects promoted by acute taurine treatment in the central nervous system (CNS) of this species.

Taurine (2-aminoethanosulfonic acid) is a ubiquitous non-protein amino acid abundant in several tissues. In the brain, intracellular taurine concentration ranges from 3 to 9 mM [1,14], while extracellular taurine reaches micromolar range [18]. Previous study demonstrated that high taurine concentrations can be found in astrocytes and also in neurons [28]. This amino acid has been implicated in different cell protecting events, such as osmolarity regulation [7,8], antioxidant properties [21], and membrane stabilization [19]. In addition, extracellular taurine may act as an inhibitory neurotransmitter via $GABA_A$, glycine, and taurine receptors [20,36]. The control of the levels of taurine at synaptic cleft is exerted by a specific transporter, TAUT, whose sequence homology places it within the gene family of Na^+- and Cl^--dependent neurotransmitter transporters [6]. Taurine has shown neuroprotective properties against excitotoxic cell death [28,37] mainly by regulating cellular levels of Ca^{2+} and its neuromodulatory role, which influences other neurotransmitter signaling pathways [36].

Nucleotides are ubiquitous signaling molecules that play crucial roles for brain function. ATP is a neurotransmitter that elicits its actions by triggering specific P2 receptors [12]. The inactivation of ATP-mediated neurotransmission is controlled

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Ultrastructural features of *Tritrichomonas mobilensis* and comparison with *Tritrichomonas foetus*

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ABSTRACT

Tritrichomonas mobilensis is an intestinal parasite of squirrel monkeys. There are few reports concerning the morphological aspects of this parasite. In addition, the taxonomic relationship between *T. mobilensis* and *Tritrichomonas foetus*, a serious pathogen that causes bovine and feline trichomonosis, has been questioned. For this reason, in the present study, we examined and compared both tritrichomonads with regard to their morphology, ultrastructure, endocytic activity and cytotoxicity when in the presence of host cells. Electron microscopy demonstrated consistent morphological differences between the hydrogenosomes of both parasites. Moreover, *T. mobilensis* and *T. foetus* had striking differences in their endocytic behavior. Thus, this work provides additional data that support the hypothesis that *T. mobilensis* is a distinct species from *T. foetus*.

1. Introduction

Tritrichomonas mobilensis is a common intestinal trichomonad of squirrel monkeys, such as *Saimiri* spp., which have been used in biomedical research (Pindak et al., 1985; Culberson et al., 1986). The role of this parasite in gastrointestinal pathology of monkeys has not been well established. It has been demonstrated that *T. mobilensis* is invasive in natural hosts (Scimeca et al., 1989) as well as in experimental animals (Culberson et al., 1988); however, inflammatory response in the natural host of this parasite has not been observed. Newborn squirrel monkeys are free of this parasite but acquire it at 1–2 months of age either from their mothers or from cage mates (Brady et al., 1988). Sampling of a large number of adults failed to identify trichomonad-free individuals suggesting that squirrel monkeys may be life-long carriers of *T. mobilensis* (Pindak et al., 1988).

Tritrichomonas foetus is the causative agent of cattle trichomonosis, which is one of the most prevalent sexually transmitted disease in cattle. In cows, the infection varies from a mild vaginitis or cervicitis, to endometritis, abortion and infertility. Significant losses may occur because of infertility and abortion (BonDurant, 2005). *T. foetus* is currently recognized as the agent of feline trichomonosis, which is a large bowel disease in domestic cats (Tolbert and Gookin, 2009).

In literature from the 1990s and even more recently, a hypothesis has been raised suggesting that *T. mobilensis*, *T. foetus* and *Tritrichomonas suis* (a gastrointestinal commensal of pigs) are the same species (Felleisen, 1997; Kleina

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Evaluation of Acetylcholinesterase in an Animal Model of Maple Syrup Urine Disease

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Abstract Maple syrup urine disease is an inherited metabolic disease predominantly characterized by neurological dysfunction. However, the mechanisms underlying the neuropathology of this disease are still not defined. Therefore, the aim of this study was to investigate the effect of acute and chronic administration of a branched-chain amino acids (BCAA) pool (leucine, isoleucine, and valine) on acetylcholinesterase (AChE) activity and gene expression in the brain and serum of rats and to assess if antioxidant treatment prevented the alterations induced by BCAA administration. Our results show that the acute administration of a BCAA pool in 10- and 30-day-old rats increases AChE activity in the cerebral cortex, striatum, hippocampus, and serum. Moreover, chronic administration of the BCAA pool also increases AChE activity in the structures studied, and antioxidant treatment prevents this increase. In addition, we show a significant decrease in the mRNA expression of AChE in the hippocampus following acute administration in 10- and 30-day-old rats. On the other hand, AChE expression increased significantly after chronic administration of the BCAA pool. Interestingly, the antioxidant treatment was able to prevent the increased AChE activity without altering AChE expression. In conclusion, the results from the present study demonstrate a marked increase in AChE activity in all brain structures following the administration of a BCAA pool. Moreover, the increased AChE activity is prevented by the coadministration of *N*-acetylcysteine and deferoxamine as antioxidants.

Keywords Maple syrup urine disease · Branched chain amino acids · Antioxidant treatment · Acetylcholinesterase · mRNA levels

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Introduction

Maple syrup urine disease (MSUD) is an inherited metabolic disorder caused by a severe deficiency in branched-chain α-keto acid dehydrogenase complex (E.C. 1.2.4.4) activity. The metabolic defect leads to an accumulation of the branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile), and valine (Val) and the corresponding branched-chain α-keto acids, α-ketoisocaproic acid, α-keto-β-

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NeuroToxicology

Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (*Danio rerio*)

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ABSTRACT

Endosulfan is a broad spectrum organochlorine pesticide that is still widely in use in many developing countries. Following application, endosulfan can get to watercourses through surface runoff from agricultural fields and disturb the non-target aquatic animals including freshwater fish species. Given that the activity of the enzyme acetylcholinesterase (AChE) is one of the most recurrently used biomarkers of exposure to pesticides and there are controversial results concerning the effects of endosulfan exposure and AChE activity in fish, the aim of the present study was to evaluate the effects of endosulfan in brain AChE activity and its gene expression pattern using adult zebrafish (*Danio rerio*) as an animal model. Moreover, we have analyzed the effects of endosulfan exposure in different parameters of zebrafish swimming activity and in long-term memory formation. After 96 h of exposition, fish in the 2.4 μg endosulfan/L group presented a significant decrease in AChE activity (9.44 ± 1.038 μmol SCh h^{-1} mg $protein^{-1}$; $p = 0.0205$) when compared to the control group (15.87 ± 1.768 μmol SCh h^{-1} mg $protein^{-1}$; $p = 0.0205$) which corresponds to approximately 40%. The down-regulation of brain AChE activity is not directly related with the transcriptional control as demonstrated by the RT-qPCR analysis. Our results reinforce AChE activity inhibition as a pathway of endosulfan-induced toxicity in brain of fish species. In addition, exposure to 2.4 μg endosulfan/L during 96 h impaired all exploratory parameters evaluated: decreased line crossings (≈21%, 273.7 ± 28.12 number of line crossings compared to the control group 344.6 ± 21.30, $p = 0.0483$), traveled distance (≈20%, 23.44 ± 2.127 m compared to the control group 29.39 ± 1.585, $p = 0.0281$), mean speed (≈25%, 0.03 ± 0.003 m/s compared to the control group 0.04 ± 0.002, $p = 0.0275$) and body turn angle (≈21%, $69{,}940 \pm 4871$ absolute turn angle compared to the control group $88{,}010 \pm 4560$, $p = 0.0114$). These results suggest that endosulfan exposure significantly impairs animals' exploratory performance, and potentially compromises their ecological and interspecific interaction. Our results also showed that the same endosulfan exposure did not compromise animals' performance in the inhibitory avoidance apparatus. These findings provide further evidence of the deleterious effects of endosulfan exposure in the nervous system.

1. Introduction

Pesticides are chemicals that became indispensable in current agriculture to control pest populations. Three of the main classes of pesticides are organochlorines, organophosphorous and carbamates. Organochlorines are the most commonly found pesticides in the environment including water, sediments, atmospheric air and biotic environment (Chopra et al., 2011). Organochlorine pesticides consist of a variety of chemicals composed primarily of carbon, hydrogen and chlorine that include among others polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), dichlorodiphenyltrichloroethane (DDT), dieldrin, chlordane, heptachlor, toxaphenes, mirex, lindane, dicofol, hexachlorobenzene, chlordecone and endosulfan (for review see Van Dyk and Pletschke, 2011).

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