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INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

INFLUÊNCIA DE METAIS TÓXICOS NAS ENZIMAS DO SISTEMA PURINÉRGICO E NA ACETICOLINESTERASE EM SISTEMA NERVOSO CENTRAL DO PEIXE ZEBRA (*Danio rerio*)

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Com motivação, perseverança e determinação

fazemos o impossível se tornar possível.

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APRESENTAÇÃO

De acordo com as normas do Programa de Pós Graduação em Ciências Biológicas: Bioquímica, esta tese está subdividida em três partes.

A **PARTE I** traz uma breve INTRODUÇÃO, onde é feita uma pesquisa bibliográfica atualizada acerca dos temas contidos nesta tese, seguido dos objetivos deste estudo.

Na **PARTE II** são apresentados os RESULTADOS, os quais são apresentados na forma de três artigos científicos, que foram publicados ou estão submetidos à comissão editorial de periódicos científicos.

A **PARTE III** compreende uma DISCUSSÃO GERAL e a CONCLUSÃO dos dados obtidos nesta tese de doutorado, assim como as REFERÊNCIAS e os ANEXOS.

SUMÁRIO

PARTE I

I.1. Resumo..2

I.2. Abstract...3

I.3. Lista de Abreviaturas...4

I.4. Introdução...6

I.4.1. Metais Tóxicos...6

I.4.1.1. Zinco e Cádmio...8

I.4.1.2. Alumínio..9

I.4.1.3. Mercúrio...10

I.4.2. Zebrafish (*Danio rerio*)..11

I.4.3. Sistema purinégico..15

I.4.3.1. Ectonucleotidases...18

I.4.3.2. Adenosina deaminase...23

I.4.4. Sistema Colinérgico...25

I.4.4.1. Acetilcolinesterase..27

I.5. Objetivos...29

PARTE II

II. 1. Capítulo 1- SENGER, M. R., ROSEMBERG, D. B., RICO, E. P., ARIZI, M. B., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2006. ***In vitro*** **effect of zinc and**

cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain. Toxicology *in vitro* 20, 954-958............................31

Abstract..32

1. Introduction...32
2. Materials and methods…………………………………………………………..33
    2.1. Animals……………………………………………………………..33
    2.2. Chemicals…………………………………………………………..33
    2.3. *In vitro* experiments………………………………………….………33
    2.4. Biochemical measurements of AChE activity………………………..33
    2.5. Determination of ectonucleotidase activities…………………………33
    2.6. Protein determination…………………………………………………..34
    2.7. Statistical analysis……………………………………………..…….34
3. Results………………………………………………………………………….34
4. Discussion……………………………………………………………………...34

Acknowledgements……………………………………………………………….36

References……..36

II. 2. Capítulo 2- SENGER, M. R., SEIBT, K. J., GHISLENI, G., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2009. **Aluminum exposure alters behavioral parameters and increase acetylcholinesterase activity in zebrafish *(Danio rerio)* brain.** Manuscrito submetido ao periódico Aquatic Toxicology....................37

Abstract..39

1. Introduction...40
2. Methods…………………………………………………………………….…...42

2.1. Animals……………………………………………………………..42

2.2. Chemicals…………………………………………………………..42

2.3. *In vivo* treatments…………………………………………………42

2.4. *In vitro* treatments…………………………………………………43

2.5. Determination of AChE activity……………………………………..43

2.6. Protein determination………………………………………………..44

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)………44

2.8. Behavioral analysis…………………………………………………..…45

2.9. Statistical analysis……………………………………………………46

3. Results…………………………………………………………………46

4. Discussion……………………………………………………………...47

Acknowledgements……………………………………………………………50

References……………………………………………………………………51

II. 3. Capítulo 3- SENGER, M. R., ROSEMBERG, D. B., SEIBT, K. J., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2009. **Influence of trace metals on adenosine deaminase activity in zebrafish brain.** Manuscrito submetido ao periódico Toxicology……………………………………………………………………..……63

Abstract..65

1. Introduction...66

2. Materials and methods……………………………………………..………67

2.1. Chemicals…………………………………………………………..67

2.2. Experimental animals………………………………………………..68

2.3. Preparation of soluble and membrane fractions……………………68

2.4. Determination of adenosine deaminase activity……………………….69

2.5. *In vitro* assays…………………………………………………………...70

2.6. *In vivo* assays…………………………………………………………...71

2.7. Protein determination…………………………………………….....…71

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)………71

2.9. Statistical Analysis…………………………………………………...72

3. Results…………………………………………………………………………….72

4. Discussion……………………………………………………………………...73

Acknowledgements……………………………………………………………….77

References...78

PARTE III

III.1. Discussão..92

III.2. Conclusão Final...95

III.3. Referências...96

III.4. Anexos.. 125

III.4.1. Índice de Figuras..125

III.4.2. Cartas de submissão...129

PARTE I

i.1. Resumo

A poluição ambiental causada por resíduos de metais tóxicos é muito relevante pelo seu amplo uso em processos industriais e agrícolas, sendo que muitos efluentes chegam ao ambiente aquático sem qualquer tratamento. A contaminação da água com estes poluentes tornaram-se iminentes e, consequentemente efeitos adversos são inevitáveis em humanos, plantas e animais, onde um dos táxons mais atingidos são os peixes. O peixe zebra (*Danio rerio*) é uma espécie muito utilizada como modelo experimental em diversas áreas, como neurociências e toxicologia. Evidências demonstram que nucleotídeos e nucleosídeos da adenina exercem efeitos sinalizadores no espaço extracelular por meio da ativação de receptores específicos. A inativação do sinal mediado pelo ATP extracelular é realizada por uma família de enzimas denominadas ectonucleotidases, na qual se destacam as NTPDases (nucleosídeo trifosfato difosfoidrolases) e a ecto-5'-nucleotidase. Após hidrólise pelas ectonucleotidases, o ATP produz o nucleosídeo adenosina. A adenosina pode ser desaminada pela enzima adenosina desaminase (ADA), gerando inosina. Evidências demonstram que o ATP é coliberado com a acetilcolina em terminais colinérgicos. A acetilcolina é uma molécula transmissora que age nos receptores muscarínicos e nicotínicos e seu catabolismo é promovido pela enzima acetilcolinesterase (AChE). Nosso laboratório já demonstrou a presença de diferentes membros da família das NTPDases e da família da ADA, além de uma ecto-5'-nucleotidase em cérebro de peixe zebra. A presença da enzima AChE também já foi descrita nesta espécie. No capítulo I foi testado o efeito *in vitro* do zinco e cádmio na atividade da AChE e das ectonucleotidases em cérebro de peixe zebra. Ambos os metais não alteraram a atividade da AChE. A hidrólise de ATP teve um aumento na presença de 1 mM de zinco (17 %) e a hidrólise de AMP teve um aumento dose dependente nas concentrações 0,5 e 1 mM de zinco (188 % e 199 %). Após a exposição a 0,5 e 1 mM de cádmio, a atividade ATPásica foi aumentada significativamente (53 % e 48 %). O cádmio na faixa de 0,25 a 1 mM inibiu a hidrólise de ADP de forma dose-dependente (13,4 - 69 %). A atividade da ecto-5'-nucleotidase foi inibida (38 %) apenas na presença de 1 mM de cádmio. No capítulo II foi investigado o efeito da exposição ao alumínio na atividade da AChE cerebral e em parâmetros comportamentais em peixe zebra. A exposição *in vivo* a 50 μg/L de $AlCl_3$ durante 96 h a pH 5,8 aumentou significativamente (36 %) a hidrólise de acetiltiocolina em cérebro de peixe zebra. Não foram observadas mudanças na atividade da AChE quando os peixes foram expostos a mesma concentração de $AlCl_3$ em pH 6,8. RT-PCR semi-quantitativo não demonstrou alterações significativas nos níveis de expressão do RNAm do gene da *ache* em cérebro de peixe zebra. Concentrações in vitro de $AlCl_3$ variando de 50 a 250 μM aumentaram a atividade da AChE (28 a 33 %). Além disso, animais expostos ao $AlCl_3$ em pH 5,8 apresentaram uma diminuição na atividade locomotora, avaliada pelo número de cruzamentos (25 %), distância viajada (14,1 %) e velocidade máxima (24 %). No capítulo III, foi verificado o efeito de metais tóxicos na atividade e expressão da ADA em frações solúveis e de membrana em cérebro de peixe zebra. Dos metais testados, apenas o mercúrio foi capaz de inibir a desaminação da adenosina em ambas as frações *in vitro*. A inibição foi observada de 5 a 250 μM de $HgCl_2$ (84,6 – 92,6 %) na fração solúvel enquanto nas frações de membrana a inibição variou de 50 a 250 μM (20,9 – 26 %). A exposição *in vivo* do peixe zebra a 20 μg/L de $HgCl_2$ foi avaliada após exposição aguda (24 h) e subcrônica (96 h). A atividade da ADA na fração solúvel foi inibida após a exposição aguda (24,5%) e subcrônica (40,8%) enquanto que a atividade da ADA na fração de membrana foi inibida somente após a exposição subcrônica (21,9 %). Em contraste, não foram observadas mudanças na expressão dos genes da ADA após os tratamentos. Nossos resultados apresentados demonstraram que as enzimas envolvidas na degradação de nucleotídeos e nucleosídeos (NTPDase, 5'-nucleotidase e ADA) e a acetilcolinesterase são afetadas por metais tóxicos em cérebro de peixe zebra. Além de serem possíveis alvos da neurotoxicidade dos metais, a alteração destas atividades enzimáticas pode ser utilizada como bioindicador da presença ambiental destes poluentes.

I.2. Abstract

The environmental pollution caused by toxic metals is relevant due to its widespread use in industrial and agricultural process, and many effluents enter in the aquatic environment without treatment. The contamination of water by toxic metals has become imminent and, consequently, adverse effects are inevitable in humans, plants, and animals, which fish are one of the most affected taxons. Zebrafish (*Danio rerio*) is a specie consolidated as a model system in many research areas, including neuroscience and toxicology. Evidence has show that nucleotides and nucleosides exert signaling effects at the extracellular space by the activation of specific receptors. The inactivation of extracellular ATP signaling is promoted by a family of enzymes named ectonucleotidases, whivh inlude NTPDases (nucleoside triphosphate diphosphohydrolases) and ecto-5'-nucleotidase. After hydrolysis promoted by ectonucleotidases, ATP forms the nucleoside adenosine. The adenosine could be deaminated by the enzyme adenosine deaminase (ADA). Evidence demonstrates that ATP is coreleased with acetylcholine in cholinergic terminals. Acetylcholine is a transmitter molecule that acts on muscarinic and nicotinic receptors and its catabolism is promoted by acetylcholinesterase (AChE) activity. Our laboratory has already characterized NTPDases, ecto-5'-nucleotidase and ADA activity in zebrafish brain. The AChE activity has been reported in this specie. In chapter I we tested the *in vitro* effect of zinc and cadmium on AChE and ectonucleotidase (NTPDase and ecto-5-nucleotidase) activities in zebrafish brain. Both zinc and cadmium treatments did not alter significantly the zebrafish brain AChE activity. ATP hydrolysis presented a significant increase at 1 mM zinc (17 %) and the AMPase activity had a dose-dependent increase at 0.5 and 1mM zinc exposure (188 % and 199 %). After cadmium treatment, ATPase activity was significantly increased (53 % and 48 %) at 0.5 and 1 mM, respectively. Cadmium, in the range 0.25–1 mM, inhibited ADP hydrolysis in a dose-dependent manner (13.4–69 %). Ecto-5-nucleotidase activity was only inhibited (38 %) in the presence of 1 mM cadmium. In chapter II we have investigated the effect of aluminum exposure on brain AChE activity and behavior parameters in zebrafish. *In vivo* exposure of zebrafish to 50 μg/L $AlCl_3$ during 96 h at pH 5.8 significantly increased (36 %) acetylthiocholine hydrolysis in zebrafish brain. There were no changes on AChE activity when fish were exposed to the same concentration of $AlCl_3$ at pH 6.8. Semi-quantitative RT-PCR has not shown significant alterations on the expression levels of *ache* mRNA gene in zebrafish brain. *In vitro* concentrations of $AlCl_3$ varying from 50 to 250 μM have increased AChE activity (28 to 33 %, respectively). Moreover, we observed that animals exposed to $AlCl_3$ at pH 5.8 presented a significant decrease in locomotor activity, as evaluated by the number of line crossings (25 %), distance traveled (14.1 %) and maximum speed (24 %). In chapter III we have investigated the effects of toxic metals on soluble and membrane ADA activity and gene expression in zebrafish brain. Regarding the metals tested, only $HgCl_2$ was able to inhibit the adenosine deamination *in vitro* in both fractions. The inhibition was observed from 5 to 250 μM $HgCl_2$ (84.6 – 92.6 %) in soluble fraction while in membrane fractions the inhibition varied from 50 to 250 μM (20.9 – 26 %). The *in vivo* exposure of zebrafish to 20 μg/L of $HgCl_2$ was evaluated after acute (24 h) and subchronic (96 h) treatments on ADA activity in soluble and membrane fractions. The ADA activity from soluble fraction was inhibited after both acute (24.5 %) and subchronic (40.8 %) exposures whereas the ADA activity from brain membranes was inhibited only after subchronic exposure (21.9 %). In contrast, semi-quantitative RT-PCR analysis showed that $HgCl_2$ was not able to modulate the ADA gene expression. Our results have show that the enzymes involved in the degradation of nucleotides and nucleosides (NTPDase, 5'-nucleotidase and ADA) and AChE are modulated by toxic metals in zebrafish brain. Besides been a possible target in the neurotoxicity mediated by metals, the alteration of these enzyme activities can be used as bioindicator of the environmental presence of these pollutants.

I.3. Lista de abreviaturas

Acetil CoA – acetil coenzima A

ACh – acetilcolina

AChE- acetilcolinesterase

ADA – adenosina deaminase

ADA 1 – adenosina deaminase 1

ADA 2 – adenosina deaminase 2

ADA 2-1 – parálogo 1 da adenosina deaminase 2

ADA 2-2 – parálogo 2 da adenosina deaminase 2

ADA L – adenosina deaminase "like"

ADO - adenosina

ADP – adenosina 5'- difosfato

Al - Alumínio

AMP – adenosina 5'- monofosfato

AMPc – adenosina 3',5'- monofosfato cíclico

ATP – adenosina 5'- trifosfato

cDNA – ácido desoxirribonucléico complementar

CD39 – antígeno de ativação celular linfóide

CD73 – proteína de superfície de linfócitos

DAG – diacilglicerol

Ecto-ADA – ecto-adenosina deaminase

EHNA – hidrocloreto de eritro-9-(2-hidroxi-3-nonil) adenina

EST's – "expressed sequence tags" (genes expressos)

GABA – ácido γ –aminobutírico

INO – inosina

IP3 – inositol 1,4,5-trifosfato

K_M – constante de Michaelis

RNAm – ácido ribonucléico mensageiro

5'-NC – 5'- nucleotidase

NTPDase – nucleosídeo trifosfato difosfoidrolase

PCR – "polymerase chain reaction" (reação em cadeia da polimerase)

RNA – ácido ribonucléico

RT-PCR – "reverse transcription-polymerase chain reaction" (reação em cadeia da polymerase com transcrição reversa)

PKA – proteína quinase A (proteína quinase dependente de AMP cíclico)

SNC – sistema nervoso central

SNP – sistema nervoso periférico

ZFIN – "zebrafish international network" (rede internacional de dados do zebrafish)

I.4. INTRODUÇÃO

I.4.1 Metais Tóxicos

Atualmente, nossa economia depende largamente de produtos industrializados e colheitas produtivas, e em consequência disso, dejetos industriais e agrícolas tornaram-se um grande problema para a saúde humana e do meio ambiente. A poluição ambiental causada por resíduos de metais tóxicos é muito relevante pelo seu amplo uso em processos antrópicos, sendo que muitos efluentes chegam ao meio ambiente sem qualquer tratamento (KUNO et al., 1999; SCHERER et al., 2003). A contaminação da água por estes poluentes tornou-se iminente e, conseqüentemente efeitos adversos são inevitáveis em humanos, plantas e animais, onde um dos táxons mais atingidos são os peixes (MACHADO et al. 2002).

Os metais ocorrem naturalmente no meio ambiente, mas desde a revolução industrial esta ocorrência aumentou muito com a atividade agrícola e industrial (FOUNTAIN & HOPKIN, 2004). Os metais pesados possuem diversos mecanismos de ação, exercendo seus efeitos tóxicos ao combinar-se com um ou mais grupos reativos essenciais para funções fisiológicas normais. O principal efeito tóxico desde grupo de elementos é a sua ação sobre a estrutura das proteínas, muitas delas com atividade enzimática. Ao alterarem as atividades enzimáticas, os metais pesados afetam o metabolismo, membranas celulares e organelas. A influência destas substâncias se dá por mecanismos complexos, tais como: interação com metais essenciais por similaridade eletrônica, formação de complexos metal-proteína, inibição enzimática de proteínas com grupos sulfidrilas

(–SH) e comprometimento na função de organelas celulares como mitocôndrias, lisossomas e microtúbulos (FERRER, 2003).

Devido ao amplo efeito exercido pelos metais tóxicos, não são conhecidos todos os mecanismos de ação destes compostos (SANDHIR et al., 1994; MYERS et al., 2000). Além disso, muitos fatores afetam os efeitos patofisiológicos dos metais pesados, tais como a sua forma química, via de entrada no organismo, duração da exposição, concentração, idade e espécie do animal (KOSTIAL et al., 1978; MOLLER-MADSEN, 1990). VAN STRAALEN et al. (1987) sugeriram que a principal diferença da ação tóxica dos metais ocorre devido à sua essencialidade versus sua não essencialidade nos organismos, sendo que níveis de metais essenciais são regulados e metais não essenciais são acumulados e estes se tornam tóxicos em determinadas concentrações.

A contaminação de peixes por metais tóxicos é muito relevante, pois estes animais estão no topo da cadeia alimentar do ambiente aquático e podem afetar diretamente a saúde dos seres humanos quando se alimentam destes animais. Estudos têm observado níveis mais elevados de arsênio, chumbo e zinco em peixes criados em cativeiro em comparação com peixes obtidos da natureza (CALVI et al., 2006). Em outro estudo, concentrações de mercúrio foram avaliadas em peixes obtidos comercialmente no Japão e foi observado que os peixes criados em cativeiro apresentavam níveis mais elevados de mercúrio e metil mercúrio, devido ao fato de que a alimentação destes animais com outras espécies de peixes que são amplamente predatórios poderia contruibuir para os altos níveis de metais observados (YAMASHITA et al., 2005). Os peixes são amplamente utilizados no biomonitoramento da poluição aquática (ZHOU et al.,

2008). A análise da bioacumulação e de biomarcadores em peixes tais como parâmetros hematológicos, imunológicos, reprodutivos endócrinos, histológicos e morfológicos estão envolvidos na determinação do nível de contaminação ou dos efeitos da poluição ambiental promovida por metais em ambientes aquáticos (VAN DER OOST et al., 2003). Respostas comportamentais dos peixes também são utilizados para avaliar a toxicidade de poluentes, assim como a taxa aguda letal, crescimento, reprodução, metabolismo e fecundidade (CARNS, 1981).

I.4.1.1. Zinco e Cádmio

Os metais zinco e cádmio diferem em termos de importância metabólica para os organismos. O zinco é um metal essencial e desempenha um papel importante em vários processos metabólicos (ODENDAAL & REINECKE, 1999; DIGIROLAMO et al., 2009). O zinco participa do metabolismo de ácidos nucléicos, replicação celular e do reparo e crescimento de tecidos (MURAKAMI & HIRANO, 2008). SMART et al (2004) demonstraram que baixas concentrações deste metal são importantes na neurotransmissão, além de participar na estrutura de receptores excitatórios de glutamato. Entretanto, este metal pode ser potencialmente tóxico para os organismos quando em altas concentrações (PAVLICA ET al., 2009). Considerando os seus efeitos toxicológicos, o zinco é um metal que já foi testado em diversos organismos com respostas bioquímicas e fisiológicas variáveis. Por exemplo, BROCARDO et al. (2005) demonstraram um efeito inibitório do zinco na atividade da AChE em córtex cerebral e hipocampo de ratos. Outro estudo apresentou resultados diferentes: a atividade colinesterásica

do molusco bivalve *Adamussium colbecki* não foi alterada pela exposição *in vivo* ao zinco (CORSI et a., 2004).

O cádmio é um metal não essencial e geralmente tóxico aos organismos (DE SOUZA DAHM et al., 2006). Este metal afeta a estrutura de ácidos nucléicos e a atividade de certas enzimas e os níveis de vários neurotransmissores (COOPER & MANALIS, 1984). Estudos já demonstraram o efeito *in vivo* do cádmio na AChE cerebral de outros peixes. Em *Barbus conchonius*, uma exposição de 12,6 mg/L de cádmio durante 48 horas estimulou a atividade da AChE cerebral (GILL, et al., 1991). Por conseguinte, DE LA TORRE et al. (2000) demonstraram que a AChE da carpa (*Cyprinos carpio*) não apresentou mudanças após sua transferência para água contaminada com uma solução de cádmio (1,6 mg/L).

I.4.1.2. Alumínio

O alumínio (Al) é um metal tóxico não essencial extremamente comum no meio ambiente, sendo um dos elementos mais abundantes na crosta terrestre na forma de óxido de Al (Al_2O_3), e com a capacidade de se ligar a diversas macromoléculas biológicas (GANROT, 1986). O alumínio é um dos poucos elementos abundantes na natureza que parecem não apresentar nenhuma função biológica significativa. Entretanto, a exposição a altas concentrações pode causar problemas de saúde, principalmente quando na forma de íons em que o Al é solúvel em água. A solubilidade do Al inorgânico aumenta como resultado direto da diminuição do pH aumentando assim sua biodisponibilidade (FINN, 2007). O Al é praticamente inócuo em pH alcalino e neutro, mas em ambientes acidificados

pode induzir diversos efeitos adversos na biota aquática (BRODEUR et al., 2001). Estudos vêm relacionando a diminuição de populações de peixes com a exposição ao Al em pH ácido (MONETTE & MCCORMICK, 2008). Os mecanismos precisos pelo qual o Al exerce seus efeitos neurotóxicos ainda não estão completamente elucidados. Entretanto, a literatura sugere que este metal interage com o sistema colinérgico, agindo como uma colinotoxina (GULYA et al., 1990). Estudos sugerem que o Al interage com o sistema colinérgico em sistemas *in vitro* e *in vivo* (KAIZER et al., 2007; KAIZER et al., 2008). Os resultados são paradoxais, porque alguns autores encontraram uma inibição da atividade da AChE (HETNARSKI, et al., 1980; KUMAR, 1998) enquanto a maioria dos estudos, encontraram um aumento na atividade da AChE na presença do Al (PENG et al., 1992; SARKARATI et al., 1999; ZATTA, et al., 1994; 2002).

I.4.1.3. Mercúrio

O mercúrio é um dos metais não essenciais mais tóxicos aos seres vivos. Existem três formas de mercúrio: elementar, inorgânico e orgânico sendo que cada uma destas formas possui seu próprio perfil tóxico (GUZZI & LA PORTA, 2008). O mercúrio inorgânico (sais de mercúrio) é uma fonte significativa de contaminação aquática, devido a sua presença em numerosos produtos industrializados (GUZZI & LA PORTA, 2008). Entretanto, a via mais comum de exposição humana ao mercúrio ocorre pela ingesta de alimentos contaminados, principalmente de peixes (EKINO et al., 2007). O primeiro caso bem documentado de envenenamento agudo por metil mercúrio pelo consumo de peixes ocorreu em Minamata, Japão, em 1953 (EKINO et al., 2007). O quadro clínico foi oficialmente

reconhecido e denominado doença de Minamata. Animais expostos a este metal pesado apresentam, principalmente, respostas adversas no SNC (SENGER et al., 2006; EKINO et al., 2007). Muitas respostas celulares são afetadas pela exposição ao mercúrio e consequentemente, desequilíbrios neuroquímicos e a transmissão sináptica são alterados (CHUU et al., 2007). Mudanças na liberação, metabolismo extracelular e/ou recaptação e expressão de componentes dos sistemas de neurotransmissão já foram relatados em animais expostos ao mercúrio (SIROIS & ATCHISON, 1996). Nosso laboratório demonstrou que a atividade da NTPDase e da 5'-nucleotidase são inibidas por mercúrio e chumbo em cérebro de peixe zebra, sugerindo que esta inibição pode ser um dos alvos da neurotoxicidade mediada por estes metais tóxicos (SENGER et al., 2006a).

I.4.2. Peixe zebra ou Zebrafish (*Danio rerio*)

O peixe zebra, zebrafish ou paulistinha (*Danio rerio*) é um pequeno teleósteo (3-4 cm) dulceaquícola da família Cyprinidae, sendo uma espécie bastante conhecida pelos aquariofilistas (Fig 1). O pioneiro a estudar esta espécie foi George Streisinger que, no final da década de 60, aplicou as técnicas de análise mutacional para estudar o desenvolvimento embrionário do peixe zebra (GRUNWALD & EISEN, 2002). Atualmente, este peixe é um modelo experimental consolidado em diversas áreas da ciência, tais como: genética e genômica, desenvolvimento, teratologia, comportamento, toxicologia e neurociências (VASCOTTO et al., 1997; CHAKRABORTY ET AL., 2009; INGHAM, 2009). O interesse pela espécie pode ser observado pelo número crescente de laboratórios que tem utilizado este teleósteo em suas pesquisas científicas e pelo crescimento

exponencial do número de estudos publicados que envolvem esta espécie (BARBAZUK et al., 2000; CARVAN III et al., 2000; ZON & PETERSON, 2005; LIESCHKE & CURRIE, 2007; GERLAI et al., 2009).

Figura 1: Peixe zebra (*Danio rerio*)

Este peixe possui muitas características que o tornam um modelo de estudo bastante atrativo em muitas áreas da Ciência, as quais podemos citar: pequeno custo e espaço requerido para manutenção, rápido desenvolvimento e ciclo biológico, grande prole, embriões translúcidos e suscetíveis à manipulação e microinjeção (LELE & KRONE, 1996). Foi criada uma rede de informações na internet sobre o peixe zebra (http://zfin.org), na qual laboratórios do mundo inteiro podem depositar 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 em laboratórios deste teleósteo (WESTERFIELD, 2000).

Nos últimos anos, está acontecendo um progresso considerável na genética e genômica do peixe zebra (POSTLETHWAIT et al., 2000; AMATRUDA & PATTON, 2008; MILAN & MACRAE, 2009). Em 2001, o Instituto Sanger começou o sequenciamento do genoma total desta espécie (VOGEL, 2000; STERN & ZON

2003). Paralelamente com o sequenciamento do genoma total, também está ocorrendo o sequenciamento e identificação dos seus genes expressos “ESTs”. A seqüência do genoma mitocondrial já está conhecida nesta espécie e pode ser base para estudos filogenéticos (BROUGHTON et al., 2001). O estudo do genoma do peixe zebra pode servir como um complemento funcional para o projeto genoma humano, o qual produz enormes quantidades de seqüências, mas carece de informações funcionais para a maioria dos genes identificados (DOOLEY & ZON, 2000). Além disso, os genes deste teleósteo são evolutivamente conservados e apresentam alto grau de similaridade, quando comparados em sua seqüência, com genes de humanos e de camundongos (BARBAZUK et al., 2000; LISCHKE & CURRIE, 2007).

O peixe zebra se tornou o principal modelo experimental para o estudo do desenvolvimento de vertebrados (ANDERSON & INGHAM, 2003). As características básicas de sua embriogênese são bem conhecidas, assim como o destino celular durante o seu desenvolvimento (KIMMEL & WARGA, 1988; KIMMEL, 1989).

Recentemente, estudos avaliando características comportamentais do peixe zebra foram desenvolvidos (GUO, 2004; EMRAN et al.,2008;SPENCE et al., 2008). A maioria dos trabalhos avaliou o efeito de pesticidas, drogas, metais e xenobióticos na atividade comportamental desta espécie (STEINBERG et al., 1995; LEVIN & CHEN, 2004; LEVIN et al. 2004; SWAIN et al., 2004; KOKEL & PETERSON, 2008; GERLAI et al., 2009). Alguns estudos também observaram a importância do comportamento inato e adquirido em modelos de agressão,

sociabilidade e sua preferência por ambientes claros ou escuros (SERRA et al., 1999).

Devido às vantagens de se usar o peixe zebra em experimentos, o efeito agudo e crônico de diversas substâncias tóxicas pode ser avaliado facilmente. Devido ao pequeno espaço requerido por estes animais, uma quantidade menor de toxinas é empregada nos testes toxicológicos. Além disso, o efeito e a acumulação de diversas substâncias químicas vêm sendo testados no peixe zebra desde o final dos anos 70 (LELE & KRONE, 1996). Muitos compostos, tais como pesticidas, metais pesados, fenóis e misturas complexas já foram avaliados em diversos órgãos deste peixe, o que indica o crescente interesse nesta espécie para estudos ecotoxicológicos, toxicologia ambiental e bioindicação (CARVAN et al., 2000; YAMAZAKI et al., 2002; SENGER et al., 2005; SENGER et al., 2006; KORBAS et al., 2008; SEOK et al., 2008; FROEHLICHER et al., 2009).

Atualmente, muitos estudos 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). Muitos sistemas de neurotransmissão já foram identificados no peixe zebra tais como: glutamatérgico (PATTEN & ALI, 2007 & TABOR FRIEDRICH, 2008;), colinérgico (SENGER et al. 2006b; EDWARDS et al, 2007), dopaminérgico (RUSSEK-BLUM et al., 2008), serotoninérgico (NORTON et al. 2008), gabaérgico (DELGADO & SCHMACHTENBERG, 2008) e purinérgico (RICO et al., 2003; SENGER et al., 2004; LOW et al., 2008).

I.4.3. Sistema Purinérgico

Além do seu já consolidado papel como molécula energética, o ATP desempenha diversas funções no espaço extracelular. A sinalização purinérgica utiliza purinas extracelulares (principalmente ATP e adenosina) e pirimidinas como moléculas sinalizadoras que exercem seus efeitos por meio da interação com receptores de membrana específicos, denominados receptores purinérgicos ou purinoreceptores (ABBRACCHIO et al., 2008).

O conceito da neurotransmissão purinérgica foi proposto em 1972 (BURNSTOCK, 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; 2007). Ainda na década de 70, houve a identificação e caracterização dos purinoreceptores, enfatizando ainda mais a sinalização mediada por nucleotídeo (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 (BURNSTOCK & VERKHRATSKY, 2009). Uma vez no espaço extracelular, os nucleotídeos servem como ligantes para um grande número de receptores de superfície celular denominados purinoreceptores (Fig. 2). Os receptores P2 (P2X e P2Y) são ativados predominantemente por nucleotídeos da adenina, e os receptores P1 (A_1, A_{2A}, A_{2B} e A_3) são ativados por principalmente pelo nucleosídeo adenosina (BURNSTOCK, 2007; ABBRACCCHIO et al., 2008).

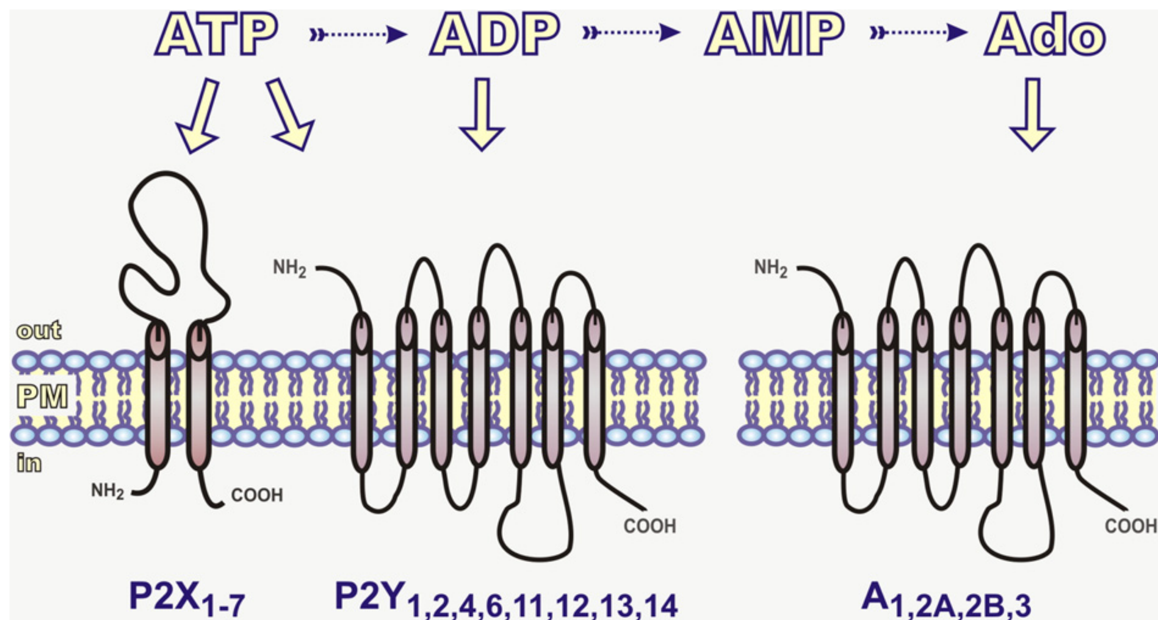

Figura 2: Vias da sinalização purinérgica. Adaptado de YEGUTKIN, 2008

Os receptores P2X são ionotrópicos (acoplados a canais) ativados por ATP que abrem um poro permeável a Na^+, K^+ ou Ca^{2+} e estão divididos em sete subtipos (P2X $_{1\text{-}7}$) (RALEVIC & BURNSTOCK, 1998). A clonagem e caracterização molecular dos subtipos dos receptores P2X no peixe zebra já foram realizadas (DIAZ-HERNANDEZ et al., 2002; BOUÉ-GRABOT et al., 2000; EGAN et al., 2000; NORTON et al., 2000; KUCENAS et al., 2003). A análise da seqüência de nove genes sugere que seis deles são ortólogos a genes dos receptores P2X de mamíferos ($zfP2X_1$, $zfP2X_2$, $zfP2X_3$, $zfP2X_4$, $zfP2X_5$ and $zfP2X_7$), dois são parálogos ($zfP2X_{3.2}$ e $zfP2X_{4.2}$) e um ainda precisa ser devidamente classificado (KUCENAS et al., 2003). Todos os subtipos de receptores P2X do peixe zebra contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos (LOW et al., 2008).

Os receptores P2Y são metabotrópicos (acoplados a proteína G). Estes receptores compartilham sete domínios transmembrana e são subdivididos em $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ (ABBRACCCHIO et al., 2008). Estudos levando em consideração a evolução estrutural de receptores P2Y demonstraram a presença de dois subtipos de receptores GPR34 (receptores acoplados a proteína G sensíveis a ADP) no peixe zebra (SCHULZ & SCHÖNEBERG, 2003). Além disso, GREGORY & JAGADEESWARAN (2002) demonstraram a presença de receptores P2Y(1) em trombócitos deste teleósteo.

Quatro subtipos de receptores P1 foram clonados e farmacologicamente caracterizados A_1, A_{2A}, A_{2B} e A_3, sendo todos metabotrópicos (RALEVIC & BURNSTOCK, 1998; FREDHOLM et al., 2001; ABBRACCCHIO et al., 2008). Os receptores A_1 e A_3 são acoplados a família das proteínas $G_{i/o}$ inibindo a formação de adenosina monofosfato cíclico (AMPc), enquanto os receptores A_{2A} e A_{2B} estimulam a produção de AMPc via proteínas da família G_s.

Os receptores de adenosina do subtipo A_1 possuem uma ampla distribuição filogenética, sendo encontrados no sistema nervoso central de muitos vertebrados (SIEBENALLER & MURRAY, 1986). Muitos estudos realizados em peixes demonstraram a caracterização farmacológica e a expressão destes receptores (LUCCHI ET al., 1992; LUCCHI et al., 1994; POLI et al., 1999; BERAUDI et al., 2003; BOEHMLER et al., 2009). Além disso, foi demonstrado que os receptores A_1 podem modular a liberação de neurotransmissores, como acetilcolina e glutamato em teleósteos (SATOH et al., 1997; ZHANG & SCHMDT, 1999; POLI et al., 2001). Os receptores A_2 também foram encontrados em peixes (REY & BURNSIDE, 1999; SATOH et al., 1997; SUNDIN & NILSSON, 1996). Entretanto, ROSATI e

colaboradores (1995), por meio de evidências farmacológicas, demonstraram a presença de receptores A_1, mas não de receptores A_2 no cérebro do peixe dourado "goldfish". Entretanto, em peixe zebra foram identificados dois genes para receptores A_{2A} e um gene para receptores A_{2B} (BOEHMLER et al., 2009).

A ação sinalizadora dos nucleotídeos é terminada por uma cascata de enzimas localizadas na superfície celular, denominadas ectonucleotidases. Esta degradação pode inativar a sinalização mediada pelo ATP nos receptores P2 e aumentar a sinalização mediada pela adenosina nos receptores P1 (KATO et al., 2004). A adenosina pode ser formada nos espaços intracelular e extracelular. Sua formação intracelular é devida à ação da enzima 5'-nucleotidase, que hidrolisa o AMP à adenosina e da hidrólise da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína hidrolase. A adenosina intracelular pode ser transportada ao espaço extracelular através de transportadores bidirecionais, por um mecanismo de difusão facilitada que regula os níveis deste nucleosídeo (CHAKRABARTI & FREEDMAN, 2009). No meio extracelular ela se comporta como uma molécula sinalizadora, influenciando a transmissão sináptica e a atividade do SNC (HARGUS et al., 2009; XIA et al., 2009). A sinalização adenosinérgica pode ser controlada pelo transporte bidirecional seguido por fosforilação até AMP pela adenosina cinase ou pela sua desaminação à inosina, promovida pela ADA nos meios intra e extracelular (FRANCO et al., 2001)

I.4.3.1. Ectonucleotidases

Ectonucleotidases são ectoenzimas que hidrolisam nucleotídeos extracelulares ao seu respectivo nucleosídeo. A sinalização mediada por

nucleotídeos extracelulares necessita de mecanismos eficientes para a inativação de seu sinal. Até o momento, não foram identificados transportadores responsáveis pela captação celular dos nucleotídeos e devido à alta densidade de sua carga, estes não permeiam as membranas celulares. Muitos trabalhos realizados evidenciaram a presença de uma variedade de enzimas localizadas na superfície celular denominadas ectonucleotidases, que são capazes de hidrolisar, e assim, inativar a sinalização mediada por nucleotídeos (ZIMMERMANN, 1994; 2001; ABBRACCCHIO et al., 2008) (Fig. 3). O produto final das reações de hidrólise é o nucleosídeo, que pode agir sobre seus próprios receptores ou ser captado pela célula e participar na rota de síntese das purinas.

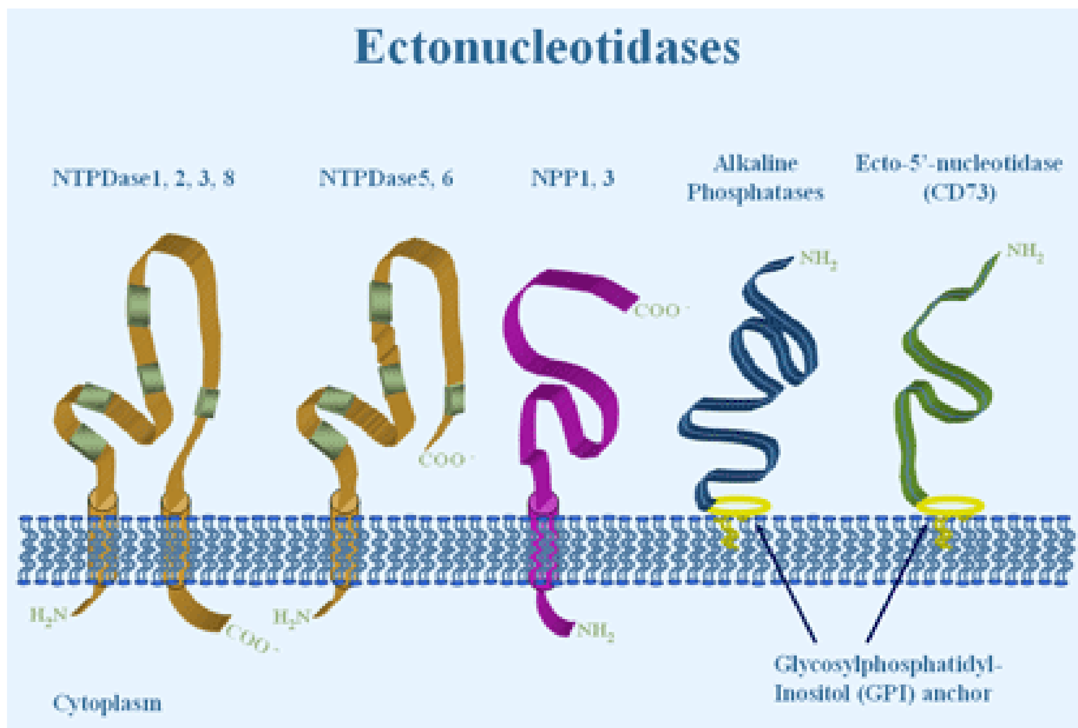

Figura 3: Estrutura das ectonucleotidases. Adaptado de www.crri.ca/sevigny.html

As ectonucleotidases desempenham um importante papel controlando a disponibilidade de ligantes para os purinoreceptores. Elas hidrolisam os

nucleotídeos, controlando a disponibilidade e a extensão da ativação dos receptores (ZIMMERMANN & BRAUN, 1999; YEGUTKIN, 2008). Além disso, estas enzimas possuem outros papéis relevantes como o envolvimento em interações célula-célula e célula-matriz, particularmente durante o desenvolvimento (ZIMMERMANN, 1996).

Estudos demonstraram que membros de muitas famílias de ectonucleotidases podem contribuir para a hidrólise de nucleotídeos extracelulares (Fig. 4). Estas enzimas não se sobrepõem apenas em sua distribuição tecidual, mas também em sua especificidade de substrato (KEGEL et al., 1997). Nucleosídeos 5'-tri e -difosfatados podem ser hidrolisados por membros da família das E-NTPDases (ectonucleosídeo trifosfato difosfoidrolase), família das E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase) e pelas fosfatases alcalinas (ZIMMERMANN, 1999; HENZ et al., 2009). Nucleosídeos 5'-monofosfatados podem sofrer hidrólise pela ação da ecto-5'-nucleotidase, fosfatases alcalinas e possivelmente por alguns membros da família das E-NPPs (ZIMMERMANN, 2001). Além disso, ectoenzimas capazes de fazer a interconversão de nucleotídeos, como a nucleosídeo difosfocinase e a miocinase já foram descritas (LU & INOUYE, 1996).

Figura 4: Degradação de nucleotídeos e nucleosídeos. Adaptado de YEGUTKIN, 2008.

Na família das NTPDases, já foram descritas até o momento, em mamíferos oito membros, que foram clonados e caracterizados (ZIMMERMANN, 2001; BIGONNESSE et al., 2004; ROBSON et al., 2006; SCHETINGER et al., 2007). Esta família de enzimas é encontrada em outras espécies de vertebrados, invertebrados, plantas, fungos, e protozoários (HANDA & GUIDOTTI, 1996; VASCONCELOS et al., 1996; SMITH et al., 1997; ZIMMERMANN, 1999; ZIMMERMANN & BRAUN, 1999).

Entre as NTPDases, os membros de 1, 2, 3 e 8 possuem seus sítio catalítico voltados para a superfície celular. Já os outros membros (NTPDase 4-7) possuem seu sítio catalítico voltado para o lúmen de organelas intracelulares. As NTPDases 5 e 6 também podem ser encontradas na membrana plasmática e possivelmente secretadas por clivagem proteolítica (MULERO et al., 1999).

Esta família de enzimas possui uma topologia de membrana comum com dois domínios transmembrana e uma alça extracelular, contendo cinco domínios denominados ACRs (Regiões conservadas da apirase) (Fig.3). Estas enzimas possuem uma ampla especificidade de substrato, hidrolisando nucleotídeos púricos e pirimídicos. Para a sua atividade catalítica máxima, estas enzimas necessitam de cátions divalentes, como cálcio e magnésio e um pH alcalino (ZIMMERMANN, 2000). Na maioria dos casos, os valores de K_M para ATP e ADP estão geralmente na ordem de micromolar (PLESNER, 1995).

A ecto-5'-nucleotidase, também conhecida como a proteína linfocitária CD73 hidrolisa nucleotídeos 5'-monofosfatatos púricos e pirimídicos ao respectivo nucleosídeo. Sua atividade enzimática é dependente de cátions divalentes, como cálcio e magnésio. A ecto-5'-nucleotidase é uma enzima ancorada a membrana plasmática por glicosil-fosfatidilinositol (GPI) (Fig.3), sendo que formas solúveis da enzima podem ser originadas mediante a ação de uma fosfolipase específica. Geralmente, o AMP é o nucleotídeo hidrolisado com maior eficiência, sendo que os valores de K_M para esta substância estão na faixa de micromolar (ZIMMERMANN, 1992). No sistema nervoso central, a ecto-5'-nucleotidase é encontrada principalmente em células gliais, mas estudos também demonstram esta atividade associada a neurônios (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998).

Estudos do nosso laboratório demonstraram a atividade da NTPDase e da ecto-5'-nucleotidase em membranas cerebrais de peixe zebra (RICO et al., 2003; SENGER et al., 2004). Estas enzimas possuem características cinéticas similares às ectonucleotidases já descritas em mamíferos como: 1) Dependência a cátions

divalentes; 2) pH ótimo para sua atividade na faixa de 7.0 a 8.0; 3) K_M na faixa de micromolar; 4) ampla especificidade a nucleotídeos.

I.4.3.2. Adenosina deaminase

Adenosina desaminase (ADA, E.C.3.5.4.4.) é uma enzima que catalisa a desaminação hidrolítica irreversível da adenosina em inosina e da 2'-desoxiadenosina em 2'-desoxinosina (FRANCO et al., 1998; ROMANOWSKA et al., 2007). Existem diferentes membros relacionados à família da ADA em células animais, os quais incluem ADA1, ADA2 e a ADA "mimética" (MAIER et al., 2005). Estas proteínas, junto com a adenina deaminase de leveduras e a AMP deaminase, são agrupadas na família das adenil-desaminase (MAIER et al., 2005).

Com relação às proteínas relacionadas a família da ADA, foi demonstrado que quase toda atividade da ADA humana é atribuída a ADA1 (ZAVIALOV & ENGSTROM, 2005). A ADA1 está localizada tanto no citosol quanto na membrana celular (FRANCO et al., 1997). Este membro da ADA tem um papel fundamental no sistema imunológico por sua atividade controlar a inibição mediada pela adenosina da proliferação das células T (GORRELL et al., 2001). A disfunção da ADA1 está relacionada a imunodeficiência severa combinada com danos tissulares (MORTELLARO et al., 2006). Estudos demonstraram que esta enzima está presente no SNC e co-localizada com receptores A_1 e A_{2B} em células neuronais e não neuronais, sugerindo um possível envolvimento da ecto-ADA em regular a sinalização mediada pela adenosina via receptores P1 (FRANCO et al., 1998; HERRERA et al., 2001).

As outras subfamílias da ADA compreendem a ADA2 e a ADAL. A ADA2 é abundante no plasma humano e possui diferentes características cinéticas quando comparadas à ADA1 (IWAKI-EGAWA et., 2006). Este membro da família da ADA já foi purificado e caracterizado em fígado de galinha (IWAKI-EGAWA et., 2004). Devido a sua habilidade em regular proliferação celular, a ADA2 foi considerada como parte de uma nova família de fatores de crescimento, denominada fatores de crescimento relacionados à adenosina desaminase (ADGFs) (ZAVIALOV & ENGSTROM, 2005; ZHANG & TAKEDA, 2007). Com relação a ADAL, não existem muitas informações sobre seus aspectos funcionais na literatura. Porém sabe-se que esta enzima apresenta domínios catalíticos, que podem ser importantes na desaminação da adenosina (MAIER et al., 2005; ROSEMBERG et al., 2007a).

Nosso laboratório realizou a identificação molecular e avaliou os padrões de expressão de diferentes genes relacionados a família da ADA em peixe zebra (ROSEMBERG et al., 2007a). Foi realizada uma procura dos diferentes genes da família da ADA em genoma de peixe-zebra e realizado um estudo filogenético, confirmando a presença de diferentes genes relacionados a ADA (ADA1, ADAL e duas isoformas de ADA2). Foi demonstrado que os diferentes membros da família da ADA são expressos em diferentes tecidos como: cérebro, brânquias, coração, fígado, músculo esquelético e rins.

Nosso grupo também realizou a caracterização cinética da atividade da adenosina desaminase, nas frações solúvel e de membrana, em cérebro de peixe zebra (ROSEMBERG et al., 2008). O pH ótimo da atividade da ADA foi na faixa de 6,0-7,0 para fração solúvel e 5,0 na fração membranosa. O K_M aparente ficou em

0,22±0,03 e 0,19±0,04 mM para as frações solúveis e de membrana, respectivamente. O valor do V_{max} aparente para a fração solúvel foi 12,3±0,73 nmol NH_3 min^{-1} mg^{-1} de proteína, enquanto o valor de V_{max} nas membranas cerebrais foi 17,5±0,51 nmol NH_3 min^{-1} mg^{-1} de proteína. Adenosina e 2'-deoxiadenosina foram desaminadas a taxas maiores quando comparados a nucleosídeos da guanina em ambas frações. Foi observado uma inibição significativa na presença de 0,1 mM de EHNA, um inibidor clássico da ADA.

I.4.4. Sistema Colinérgico

A acetilcolina foi o primeiro neurotransmissor a ser identificado nas sinapses cerebrais (VAN DER ZEE & LUITEN, 1999). Esta molécula desempenha diversas funções no SNC, onde está relacionada a aspectos comportamentais, processos de aprendizagem e memória além de atuar na organização pelo córtex cerebral no movimento voluntário (PEPEU, 1972). No SNP, o sistema colinérgico inerva a musculatura voluntária. A acetilcolina é sintetizada no neurônio pré sináptico pela enzima colina-acetiltransferase a partir do Acetil-CoA e da colina (SOREQ & SEIDMAN, 2001). A etapa final da sua síntese ocorre no citoplasma onde a acetilcolina é transportada para o interior de vesículas pré sinápticas, onde permanece até que um potencial de ação ocorra e libere as vesículas com acetilcolina na fenda sináptica (SMITH, 1984; SOREQ & SEIDMAN, 2001). (Fig. 5)

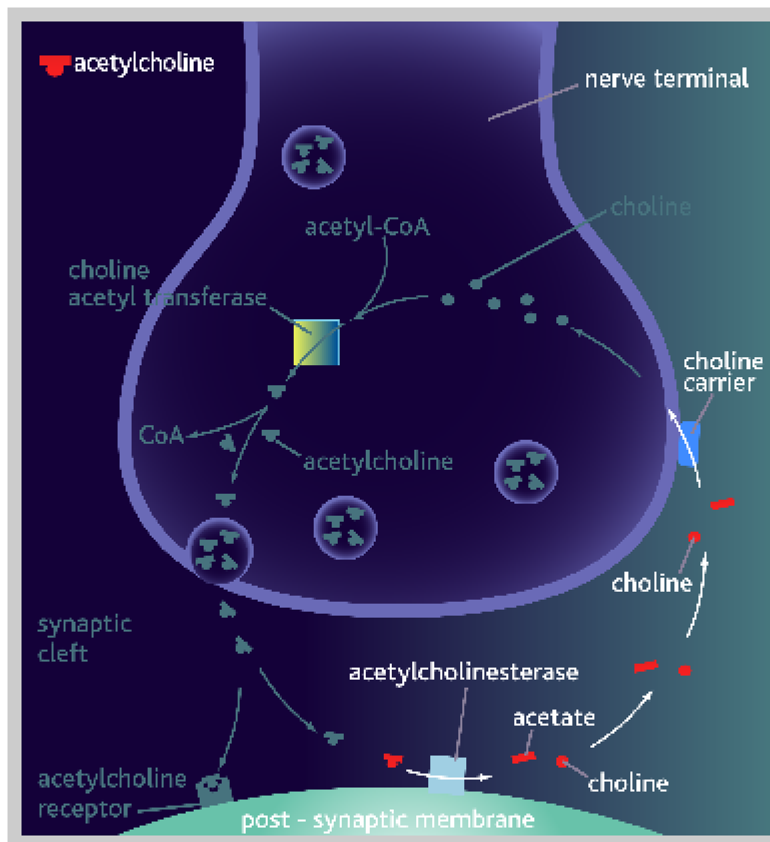

Figura 5: sinapse colinégica. Adaptado de http://www.chm.bris.ac.uk/webprojects 2006/Macgee/Web%20Project/ach_esterase_small_bord.png

De acordo com sua afinidade por agentes que mimetizam a ação da acetilcolina, os receptores colinérgicos foram divididos em duas classes: muscarínicos e nicotínicos (JONE & DUNLOP, 2007). Os receptores muscarínicos são metabotrópicos e possuem a acetilcolina e a muscarina, um alcalóide presente em certos cogumelos venenosos, como agonistas. Estes receptores são divididos em cinco subtipos (M1-M5), sendo que os receptores M1 e M2 estão presentes em neurônios do SNP e SNC (SARTER & PARIKH, 2005). Os receptores nicotínicos são ionotrópicos e possuem a acetilcolina e a nicotina como agonistas. Estes receptores são pentaméricos e formam combinações

homoméricas ou heteroméricas a partir de 12 subunidades diferentes (α2-α10, β2-β4) (OLIVERA-BRAVO et al., 2006)

I.4.4.1. Acetilcolinesterase

Uma vez liberada na sinapse, a acetilcolina é degradada pela enzima acetilcolinesterase (AChE, E.C. 3.1.1.7) em acetato e colina, sendo esta última recaptada pelo neurônio (SARTER & PARIKH, 2005). A AChE é uma enzima heterogênea encontrada em sinapses colinérgicas e nas junções neuromusculares (GRISARU et al., 1999).

O domínio catalítico da AChE é composto por uma tríade serina-histidina-glutamato que está localizada no fundo de uma estrutura estreita (gorge) que vai da superfície da proteína até o seu centro (SHAFFERMAN et al., 1992; ZIMMERMAN & SOREQ, 2006). Esta estrutura esta 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 & SUSSMAN, 2008).

A AChE varia no aspecto de sua estrutura nos diferentes tecidos (SILMAN & SUSSMAN, 2005). Estas formas são geradas por processamento alternativo seguido por modificações pós-traducionais (SILMAN & SUSSMAN, 2008). Existem três formas globulares (G1, G2 e G4) e três formas assimétricas (A4, A8 e A12). O SNC contém principalmente as formas globulares, enquanto as formas assimétricas são encontradas principalmente no SNP e músculo (RAKONCZAY et al., 2005). As formas globulares podem ser solúveis ou ancoradas a membrana

por seqüências de aminoácidos hidrofóbicos, enquanto as outras formas são ligadas à membrana por glicofosfolipídeos (TALESA, 2001). As formas assimétricas estão incluídas na matriz extracelular por uma cauda colágena e são abundantes nas junções neuromusculares (ALDUNATE et al., 2004).

O gene que codifica a AChE do peixe zebra foi clonado e seqüenciado por BERTRAND et al. (2001), revelando que esta enzima é codificada por somente um gene e que sua seqüência de 634 aminoácidos apresenta 62% de similaridade em relação aos mamíferos. Neste estudo não foi verificada a presença de um gene que codifique a butirilcolinesterase, indicando que possivelmente não há atividade desta enzima no peixe zebra.

I.5. OBJETIVOS

Considerando que: (1) metais tóxicos contaminam o ambiente aquático; (2) o peixe zebra é uma espécie suscetível a toxicidade de metais tóxicos e é um organismo consolidado em estudos toxicológicos; (3) os sistemas purinérgico e colinérgico exercem importantes papéis na sinalização do sistema nervoso central; (4) receptores e enzimas, envolvidas nestes importantes sistemas de neurotransmissão, já foram descritos nesta espécie, esta tese apresenta os seguintes objetivos específicos:

- Verificar o efeito *in vitro* do zinco e cádmio sobre a atividade da acetilcolinesterase e das ectonucleotidases, NTPDase e 5'-nucleotidase, em membranas cerebrais de peixe zebra.

- Verificar se o alumínio, em pH ácido ou neutro, altera parâmetros comportamentais e a atividade da AChE cerebral de peixe zebra.

- Avaliar a influência de metais tóxicos ($HgCl_2$, $Pb(CH_3COO)_2$, $Cd(CH_3COO)_2$, $CuSO_4$, $K_2Cr_2O_7$, $CoCl_2$, $ZnCl_2$, $MnCl_2$) na atividade da adenosina deaminase nas frações solúveis e membranosas do cérebro do peixe zebra.

PARTE II

II. 1. Capítulo 1- SENGER, M. R., ROSEMBERG, D. B., RICO, E. P., ARIZI, M. B., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2006. **In vitro effect of zinc and cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain.** Toxicology in vitro 20, 954-958

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In vitro effect of zinc and cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain

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Abstract

Zinc and cadmium are environmental contaminants that induce a wide range of effects on CNS. Here we tested the in vitro effect of these metals on acetylcholinesterase (AChE) and ectonucleotidase (NTPDase and ecto-5′-nucleotidase) activities in zebrafish brain. Both zinc and cadmium treatments did not alter significantly the zebrafish brain AChE activity. ATP hydrolysis presented a significant increase at 1 mM zinc (17%) and the AMPase activity had a dose-dependent increase at 0.5 and 1 mM zinc exposure (188% and 199%). After cadmium treatment, ATPase activity was significantly increased (53% and 48%) at 0.5 and 1 mM, respectively. Cadmium, in the range 0.25–1 mM, inhibited ADP hydrolysis in a dose-dependent manner (13.4–69%). Ecto-5′-nucleotidase activity was only inhibited (38%) in the presence of 1 mM cadmium. It is possible to suggest that changes on NTPDase and ecto-5′-nucleotidase activities can be an important mechanism involved in neurotoxic effects promoted by zinc and cadmium.
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Keywords: Zinc; Cadmium; Acetylcholinesterase; Ectonucleotidase; NTPDase; Ecto-5′-nucleotidase; Zebrafish

1. Introduction

Exposure to metals can develop a wide range of deleterious effects on exposed organisms. Zinc and cadmium are neurotoxic metals, known to be important environmental contaminants. Behavioral disorders and biochemical dysfunction have been observed in central nervous system (CNS) of zinc and cadmium-exposed animals (Carageorgiou et al., 2004).

Despite the toxicity observed at higher concentrations, zinc is the second most important trace metal in the body after iron. Total brain zinc concentrations, at micromolar range, are usually associated with zinc metalloenzymes and transcription factors (Barañano et al., 2001; Takeda, 2001). Smart et al. (2004) have shown that low concentrations of zinc are important in neurotransmission. After depolarization induced by electrical stimuli, K^+ or kainate, zinc is released at synaptic cleft in a Ca^{2+}-dependent manner (Barañano et al., 2001). The importance of zinc on excitatory glutamate receptors and on inhibitory GABA currents has already been described (Smart et al., 2004). Furthermore, the relevance of zinc in cognitive development and CNS homeostasis is well known (Takeda, 2001).

On the other hand, cadmium is a metal with many toxic properties at CNS (Carageorgiou et al., 2004). This metal affects the structure of nucleic acids, the activity of certain enzymes, the uptake of catecholamines and the levels of various neurotransmitters (Cooper and Manalis, 1984).

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Minami et al. (2001) demonstrated the toxicity of this metal in rat brain, relating a possible action of cadmium in the balance of excitatory and inhibitory synaptic neurotransmission.

Acetylcholine is a classical neurotransmitter that plays several roles at CNS. After released, acetylcholine is rapidly removed from the synaptic cleft by acetylcholinesterase (AChE, EC 3.1.1.7), which belongs to the family of type B carboxylesterases and cleaves acetylcholine into choline and acetate. Moreover, studies have been performed in order to elucidate the mechanisms involving the cholinergic and non-cholinergic activities of AChE in both central and peripheral nervous systems (Cousin et al., 2005).

Acetylcholine and ATP are co-released together at nerve endings (Burnstock, 2004). Besides the energetic function, ATP can be an important signaling molecule, acting in metabotropic P2Y receptors and ionotropic P2X receptors (Cunha and Ribeiro, 2000). Studies demonstrated that ATP can control the acetylcholine release through a dual opposite modulation, acting on facilitatory P2X or inhibitory P2Y receptors (Cunha and Ribeiro, 2000).

This nucleotide is converted to adenosine by cell-surface enzymes called ectonucleotidases. These enzymes constitute the pathway responsible for inactivation of the ATP signal controlling the purinergic neurotransmission. ATP is hydrolyzed to AMP by a family of enzymes named NTPDases (nucleoside triphosphate diphosphohydrolases) and an ecto-5′-nucleotidase (EC 3.1.3.5) promotes the AMP hydrolysis to adenosine, an important neuromodulatory messenger (Zimmermann, 2001). Adenosine is able to modulate acetylcholine release through inhibitory adenosine A_1 or facilitatory adenosine A_{2A} receptors (Magalhães-Cardoso et al., 2003).

Zebrafish (*Danio rerio*) is a small freshwater teleost widely used as a vertebrate model of developmental, neurobiological and toxicological studies (Hill et al., 2005; Senger et al., 2005). Zebrafish presents a unique situation among vertebrates, because AChE is the only ACh-hydrolyzing enzyme in this organism (Behra et al., 2003). The genome of this teleost does not encode a functional butyrylcholinesterase, another enzyme that can also hydrolyze ACh. AChE gene is already cloned and sequenced and this enzyme activity was detected in zebrafish brain (Bertrand et al., 2001). Furthermore, cholinergic receptors are also expressed in neuronal tissues of this teleost (Zirger et al., 2003).

Recently, the characterization of NTPDase and ecto-5′-nucleotidase activities in zebrafish brain membranes have been described (Rico et al., 2003; Senger et al., 2004). In the literature, both P2X and P2Y receptors have already been identified in this specie (Kucenas et al., 2003).

Considering that both cholinergic and purinergic systems are present in zebrafish brain and that zinc and cadmium are important toxic substances, the aim of this study was to test the in vitro effect of zinc and cadmium on AChE and ectonucleotidase activities in zebrafish brain.

2. Materials and methods

2.1. Animals

Adult male and female zebrafish were obtained from commercial supplier and housed for 2 weeks in a 50-L aquarium containing continuously aerated distilled water. The fish were kept between 23 and 26 °C under a natural light–dark photoperiod. All procedures for the use of animals were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2. Chemicals

Zinc chloride ($ZnCl_2$; CAS number 7648-85-7) and cadmium acetate [$Cd(CH_3COO)_2$; CAS number 543-90-8] were purchased from QM (Brazil) and Nuclear (Brazil), respectively. Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium and magnesium chloride, acetylthiocholine, 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.3. In vitro treatments

The metals were added to reaction medium before the pre-incubation with the enzyme and maintained throughout the enzyme assays. Zinc and cadmium were tested in a final concentration of 0.05–1 mM.

2.4. Biochemical measurement of AChE activity

Zebrafish brains were gently homogenized on ice in 60 vol (v/w) of 0.05 M Tris–HCl, pH 8.0, using a Teflon–glass homogenizer. AChE activity was measured according to the method of Ellman et al. (1961). AChE activity in the homogenate was measured by determining the rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Before starting the reaction with addition of substrate, samples containing 10 µg of protein and the reaction medium described previously were pre-incubated at 25 °C for 10 min. The hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Protein concentration and linearity of absorbance towards time were also investigated. Controls without zebrafish brain homogenate were performed in order to determinate the non-enzymatic hydrolysis of ACSCh. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. We performed at least three different replicate experiments.

2.5. Determination of ectonucleotidase activities

Brain membranes were prepared as described previously (Barnes et al., 1993). Whole zebrafish brains were

homogenized in 60 vol (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. This preparation was centrifuged at 1000*g* for 10 min and the pellet was discarded in order to remove the nuclear and cell debris. The supernatant was centrifuged for 25 min at 40,000*g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and recentrifuged for 20 min at 40,000*g*. The final pellet was resuspended and used in the enzyme assays. The material was maintained at 2–4 °C throughout preparation.

NTPDase and 5′-nucleotidase assays were performed according to Rico et al. (2003) and Senger et al. (2004), respectively. Brain membranes of zebrafish (3–10 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the ecto-5′-nucleotidase activity) in a final volume of 200 μL. The samples were pre-incubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The assays were stopped by the addition of 200 μL 10% trichloroacetic acid and the samples were chilled on ice for 10 min before the measurement of inorganic phosphate (Pi) (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity is expressed as nanomole of Pi released per minute per milligram of protein. We performed at least three different replicate experiments.

2.6. Protein determination

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

2.7. Statistical analysis

Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA). A Duncan multiple range test considering $P \leqslant 0.05$ as significant followed the analysis.

3. Results

The effect of zinc and cadmium (varying from 0.05 to 1 mM) was demonstrated on AChE, NTPDase and ecto-5′-nucleotidase activities in zebrafish brain. Both zinc (Fig. 1A) and cadmium (Fig. 1B) treatments were not able to promote any significant changes on zebrafish brain AChE activity.

Zinc, when added to the reaction medium, affected ATP and AMP hydrolysis. Whereas ATP hydrolysis presented a significant increase only at 1 mM zinc (17%), the AMP hydrolysis promoted by 5′-nucleotidase significantly increased at 0.5 and 1 mM zinc exposure (188% and 199%) (Fig. 2A). However, there were no significant changes on ADP hydrolysis in all zinc concentrations tested (Fig. 2A).

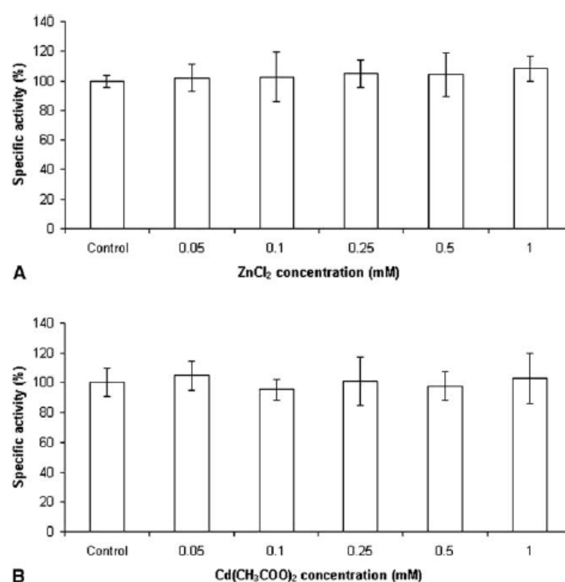

Fig. 1. In vitro effects of varying concentrations of zinc (A) and cadmium (B) on AChE activity in zebrafish brain. Bars represent the mean ± of at least three different experiments. The AChE control activity (without metal) was 37.97 ± 3.59 and 35.21 ± 1.45 μmol of thiocholine released per hour per milligram of protein for cadmium and zinc, respectively (ANOVA followed by a Duncan multiple range test, considering $P \leqslant 0.05$ as significant).

Cadmium, at 0.5 and 1 mM, promoted a significant increase in ATPase activity (53% and 48%, respectively) (Fig. 2B). ADP hydrolysis was inhibited in a dose-dependent manner ranging from 0.25 to 1 mM cadmium (13.4–69%). The ecto-5′-nucleotidase activity was only inhibited (38%) in the presence of 1 mM cadmium (Fig. 2B).

4. Discussion

Here, we have tested the in vitro effect of zinc and cadmium on AChE and ectonucleotidase activities in zebrafish brain. The AChE activity was not altered, but nucleotide hydrolysis were affected in the presence of zinc and cadmium. Zinc increased ATPase and AMPase activities, but there were no changes on ADPase activity. However, cadmium strongly increased ATP hydrolysis, but promoted a significant inhibition of ADP and AMP hydrolysis in zebrafish brain membranes.

Several studies have shown the influence of zinc on many cellular mechanisms. This divalent cation is an important molecule involved at neurotransmission (Barañano et al., 2001; Smart et al., 2004) and in activation of different metalloenzymes, such as zinc hydrolases (Hernick and Fierke, 2005). In addition, zinc is also necessary to

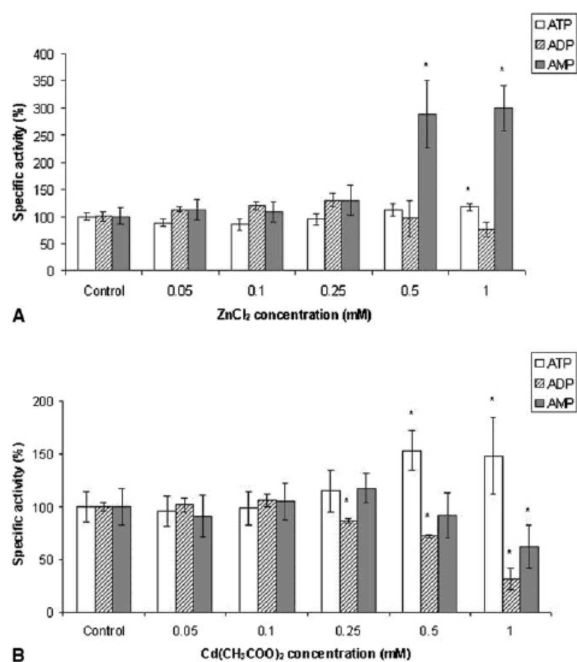

Fig. 2. Effects of varying concentrations of zinc (A) and cadmium (B) on nucleotide hydrolysis in zebrafish brain membranes. Bars represent the mean ± of at least three different experiments. The control ATPase, ADPase and AMPase activities for cadmium were 617.1 ± 88.3; 147.9 ± 5.5 and 30.5 ± 5.4 nmol Pi min^{-1} mg^{-1} of protein, respectively. The control ATPase, ADPase and AMPase activities for zinc were 564.4 ± 58.2; 147.9 ± 28.21 and 24.2 ± 5.2 nmol Pi min^{-1} mg^{-1} of protein, respectively. *Significantly different from control group ($P \leqslant 0.05$) using ANOVA followed by a Duncan multiple range test.

mobilize defense against reactive oxygen species (ROS) and H_2O_2-induced apoptosis (Chung et al., 2005).

Considering the toxicological effects, zinc is a metal tested in many distinct organisms with variable biochemical and physiological responses. For example, Brocardo et al. (2005) showed an inhibitory effect of zinc on AChE activity from cerebral cortex and hippocampus of rats. Another report demonstrated different results: the ChE activity of the *Adamussium colbecki* scallop is not altered by the in vivo zinc exposure (Corsi et al., 2004). The results presented herein demonstrated that zebrafish brain AChE is not affected by in vitro zinc treatment.

There was no evidence about a possible toxicity of zinc on NTPDase activity at the present moment. On the other hand, studies demonstrate that zinc can modulate ATPase activity in brain. This metal significantly increases the copper-transporting ATPase (DiDonato et al., 2002), Na^+–K^+–ATPase (Lovell et al., 1999) and Mg^{2+}-independent Ca^{2+} activity (Gandhi and Ross, 1988). The results obtained are consistent with these studies, demonstrating that the ATP hydrolysis in zebrafish brain membranes is also modulated by zinc. Furthermore, ecto-5′-nucleotidase contains catalytically important zinc at active site (Senger et al., 2004). Based on these findings, it is possible to suggest the importance of this zinc ligand-site on the activation of AMPase activity in zebrafish brain.

The effect of cadmium exposure on AChE was also tested in another animal species. Carageorgiou et al. (2004) verified the in vitro and in vivo effect of cadmium on this enzyme activity on adult rat brain. After in vitro treatment, concentrations higher than 0.1 mM decreased the acetylcholine hydrolysis. However, after in vivo short (8 h) and long-term (4 months) exposure, cadmium promoted different effects on rat brain AChE. The short-term exposure caused a dose-dependent reduction, while a long-term cadmium administration activated the brain AChE activity. Studies also have shown the in vivo effect of cadmium on brain AChE of different fishes. In *Barbus conchonious*, an exposure to cadmium concentration of 12.6 mg/L during 48 h stimulated the brain AChE activity (Gill et al., 1991). Nevertheless, De La Torre et al. (2000) demonstrated that *Cyprinus carpio* AChE presented no changes after transferred to water contaminated cadmium solution (1.6 mg/L). The similar response to cadmium found to *D. rerio* and *C. carpio* could be attributed to the relatively low phylogenetic distance showed between these species. An inhibition of the NTPDase activity by cadmium acetate has been already described on rat cerebral cortex synaptosomes (Barcellos et al., 1994). This metal inhibited both ATP and ADP hydrolysis in a concentration-dependent manner. The concentration tested ranged to 0.05–1 mM, the same used in our experiments.

A plausible explanation to the contrasting cadmium effect on ATP and ADP hydrolysis is that it causes activation of one member of the NTPDase family and a concomitant inhibition on another member in brain membranes of zebrafish. From the eight-well characterized enzymes of mammals NTPDase family, four members (NTPDase 1–3 and 8) are tightly bound to plasma membrane with active site facing the extracellular milieu. According to the literature, mammalian NTPDase1 hydrolyzes both ATP and ADP at the same rate, whereas NTPDase2 substantially prefers triphosphonucleosides as substrates (Zimmermann, 2001). Orthologous NTPDase1 (GenBank accession number AAH78240) and NTPDase2 (GenBank accession numbers CAE49096, NP_001004643 and XP_697600) genes are present in zebrafish genome. Hence, the decreased ADP hydrolysis and increased ATP hydrolysis observed in the presence of cadmium may be due to NTPDase1 inhibition and NTPDase2 activation.

This study has shown that the purinergic, but not cholinergic system, is affected by in vitro zinc and cadmium treatments in zebrafish brain. Our findings are important to better understand the toxic effect of both metals on CNS. E-NTPDases that are being expressed and contributing for these metals responses are still unknown. Based on the complexity of zinc and cadmium effect in this teleost, further molecular analysis and in vivo assays will be important to elucidate the influence of both contaminants in neurotransmission.

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II. 2. Capítulo 2- SENGER, M. R., SEIBT, K. J., GHISLENI, G., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2009. **Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish *(Danio rerio)* brain.** Manuscrito submetido ao periódico Aquatic Toxicology

Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish *(Danio rerio)* brain

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Abstract

Aluminum is a toxic metal that is known to impact fish biota. Zebrafish has been used as an attractive model for toxicology and behavioral studies. Here we have investigated the effect of aluminum exposure on brain acetylcholinesterase activity and behavior parameters in zebrafish. *In vivo* exposure of zebrafish to 50 µg/L $AlCl_3$ during 96 h at pH 5.8 significantly increased (36 %) acetylthiocholine hydrolysis in zebrafish brain. There were no changes on acetylcholinesterase activity when fish were exposed to the same concentration of $AlCl_3$ at pH 6.8. Semi-quantitative RT-PCR has not shown significant alterations on the expression levels of *ache* mRNA in zebrafish brain. *In vitro* concentrations of $AlCl_3$ varying from 50 to 250 µM increased AChE activity (28 to 33 %, respectively). Moreover, we observed that, animals exposed to $AlCl_3$ at pH 5.8 presented a significant decrease on locomotor activity, as evaluated by the number of line crossings (25 %), distance traveled (14.1 %) and maximum speed (24 %), besides an increase on the absolute turn angle (12.7 %). These results indicate that sublethal levels of aluminum can modify behavioral parameters and acetylcholinesterase activity in zebrafish brain.

Keywords: acetylcholinesterase; AChE; aluminum; zebrafish; behavior; locomotion

1. Introduction

Aluminum (Al) is a non-essential toxic metal extremely common throughout the world, being the third most abundant element in the earth crust. Al is innocuous under alkaline or circumneutral conditions whereas in acidic environments induce severe risks to the aquatic biota, including fish (Waring et al., 1996). Studies have postulated that those chronic water acidifications associated with Al are involved in the Atlantic salmon population decline (Monette and McCormick, 2008). Furthermore, Al is known to cause toxic effects to a variety of organs systems including brain (Oteiza et al., 1993). The precise molecular mechanism by which Al exerts its neurotoxic effects is still not completely understood. Evidence that Al accumulation contributes to Alzheimer disease remains contradictory, although epidemiological studies have indicated a relation between the concentration of Al in potable water and Alzheimer disease (Shcherbatykh and Carpenter, 2007; Rondeau et al., 2009).

Acetylcholine is a classical neurotransmitter secreted from the presynaptic nerve terminals. After released, acetylcholine is rapidly removed from the synaptic cleft by acetylcholinesterase (AChE, EC 3.1.1.7), which belongs to the family of type B carboxylesterases that cleaves acetylcholine into choline and acetate (Soreq and Seidman, 2001). Studies have demonstrated that AChE is a well-known biomarker for several environmental contaminants (Senger et al., 2006, Naravaneni and Jamil, 2007). Studies suggest that Al interacts with the cholinergic system in both *in vitro* and *in vivo* systems. The results of these studies are paradoxials, because some authors report decreases on AChE activity (Hetnarski, et al., 1980; Kumar al., 1998) whereas others report an activation of AChE in the

presence of aluminum (Peng et al., 1992; Sarkarati et al., 1999; Zatta, et al., 1994; 2002).

Zebrafish is a consolidated model system in neuroscience, toxicological and behavioral studies (Gerlai et al., 2000; Senger et al., 2005; 2006). Zebrafish have recently become a focus of neurobehavioral studies since larvae display learning, sleep, drug addiction, and other neurobehavioral phenotypes that are quantifiable and relate to those seen in man (Guo, 2004; Best and Alderton, 2008). Furthermore, the organization of the zebrafish genome and genetic pathways controlling signal transduction and development are highly conserved between zebrafish and human (Postlethwai et al., 2000). This species also holds a great potential for our understanding of the genetic basis of behavior and associated behavioral disorders (Amsterdam and Hopkins, 2006; Krens et al., 2006).

This teleost presents a unique situation among vertebrates, because AChE is the only ACh-hydrolysing enzyme in this organism (Behra et al., 2003). It has been demonstrated that butyrylcholinesterase is not found in zebrafish genome and AChE is encoded by a single gene that was already cloned, sequenced, and functionally detected in zebrafish brain (Bertrand et al., 2001). Furthermore, cholinergic receptors are also expressed in neuronal tissue of this teleost (Zirger et al., 2003; Williams and Messer, 2004).

Considering that aluminum is an important pollutant that has been correlated with fish population decline and neurodegenerative disorders and the fact that zebrafish may be an ideal vertebrate model system for behavioral, neurological diseases and toxicology studies, the aim of this study is to evaluate the effects of

Al exposure on brain acetylcholinesterase activity and gene expression pattern as well as on the behavior of this specie.

2. Methods

2.1. Animals

A total of 105 adult zebrafish were obtained from commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50-L aquarium. The fish were kept on a 14 h light/dark cycle (lights on at 7:00 am) at a temperature of 25±2°C. Animals feeding and maintenance of fish were done according to Westerfield (2000). All procedures for the use of animals were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2. Chemicals

Aluminum ($AlCl_3$, CAS number 7784-13-6) were purchased from Quimibrás Indústrias Químicas (Brazil). Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium citrate, Coomasie Blue G, bovine serum albumin, acetylthiocholine, 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (USA). All other reagents used were from analytical grade.

2.3. In vivo treatments

For *in vivo* treatments, animals were divided into four groups: control group (pH 6.8), $AlCl_3$-treated group (pH 6.8), control group (pH 5.8) and $AlCl_3$-treated

group (pH 5.8). The control groups were maintained in the 5-L test aquarium water at pH 6.8 or acidified with HCl to reach pH 5.8. The treated fish were maintained in the 5-L test aquarium containing 50 μg/L $AlCl_3$ at pH 5.8 or pH 6.8 during 24 h (acute treatment) or 96 h (subchronic treatment) (Brodeur et al., 2001). Immediately after the exposure, the fish were euthanized.

2.4. In vitro treatments

For *in vitro* assays, $AlCl_3$ at final concentrations of 50, 100, and 250 μM were directly added to reaction medium, pre-incubated for 10 min with the homogenate and maintained throughout the enzyme assay. For the control group, the enzyme assay was performed in the absence of $AlCl_3$.

2.5. Determination of AChE activity

Zebrafish were euthanized by decapitation, their brains were removed from the cranial skull by the dissection technique. A pool of five total brains of zebrafish was used for each experiment. The brains were homogenized on ice in 60 volumes (v/w) of 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 rate of acetylthiocholine hydrolysis (0.8 mM) was determined in a final volume of 2 ml with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB using a method previously described (Ellman et al., 1961). Before the addition of substrate, samples containing protein (10 μg) and the reaction medium described above were preincubated for 10 min at 25°C. The acetylthiocholine hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (30-second

intervals). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of acetylthiocholine. The linearity of absorbance related to time and protein concentration was previously determined. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. Four different experiments were performed and the assays were run in triplicate.

2.6. Protein determination

Protein was measured using Coomassie Blue as color reagent and bovine serum albumin as standard (Bradford, 1976).

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

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

Total RNA was isolated from zebrafish brain using TRIzol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometer and all samples were adjusted to 160 ng/μL. cDNA species were synthesized using SuperScript III ™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit following the supplier instructions. One microliter of RT reaction mix was used as a template for each PCR. PCR for *ache* 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 1U Taq DNA polymerase (Invitrogen). PCR for β-actin gene was performed in 20 µL using 0.1 µM of each primer, 0.2 µM dNTP, 2 mM $MgCl_2$ and 0.5U Taq DNA polymerase (Invitrogen). PCR were conducted at 1 min at 94°C, 1 min at 60°C (AChE) and at 54°C (β-actin), and 1 min at 72°C for 35 cycles. A post-extension cycle was performed for 10 min at 72°C. For each PCR set, a negative control was included. PCR products were analyzed on 1% agarose gel, containing GelRed® and visualized with ultraviolet light. The low DNA Mass Ladder (Invitrogen) was used as a molecular marker and normalization was performed 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 Kodak 1D Image Analysis Software.

2.8. Behavioral analysis

The behavior of fish was recorded between 10:00 h and 12:00 h and all animals were maintained at ph 5.8. In the behavioral assessment, control and $AlCl_3$-treated group (50 µg/L of $AlCl_3$ to 96 hours) were placed individually into the experimental tank (30 x 15 x 10 cm, length x height x width) and were first habituated to test tank for 30 seconds, as previously described (Gerlai et al., 2000). Their locomotor activity was videorecorded for 5 minutes after the habituation period and simultaneously analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, Illinois). The tank was divided into equal sections with four vertical and three horizontal lines and the follow locomotion patterns were

measured: distance travelled, maximum speed, number of line crossing (vertical and horizontal lines) and absolute turn angle.

2.9. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and expressed as means ± S.D. A Tukey multiple test range as post-hoc was performed considering $P \leq 0.05$ as significant. For behavioral studies, data were analysed using using an unpaired, two-tailed Student´s t test, considering $P \leq 0.05$ as significant.

3. Results

The aluminum exposure were evaluated on brain AChE activity and behavioral parameters of zebrafish. The *in vivo* experiments were performed after 24 and 96 h of exposure to 50 µg/L of $AlCl_3$ at pH 5.8 and pH 6.8. We evaluated the control and $AlCl_3$-treated group at pH 6.8 in order to confirm influence of pH on toxic effects of aluminum. The exposure to $AlCl_3$ at pH 6.8 did not promote a significant difference on AChE activity in zebrafish brain in both schedules of treatment. There were no significant changes after $AlCl_3$ exposure during 24 h at pH 5.8. However, this enzyme activity was significantly increased (36 %) after 96 h of $AlCl_3$ exposure (pH 5.8) when compared to control (pH 5.8) (Fig. 1).

In order to verify if the increase on acetylthiocholine hydrolysis could be a consequence of transcriptional control, RT-PCR analysis was performed when alterations on AChE activity were observed. There were no significant changes on

the *ache* mRNA transcript levels after 96 h of exposure at pH 5.8 in zebrafish brain (Fig.2).

To evaluate if aluminum could promote a direct effect on the enzyme activity, we tested the *in vitro* effect of $AlCl_3$ on AChE activity in zebrafish brain. $AlCl_3$ concentrations tested (50 - 250 μM) promote a significant increase on AChE activity ranging from 28 to 33 % (Fig.3).

Swimming activity of control group (pH 5.8) and $AlCl_3$-treated group (pH 5.8) were evaluated in the open field. $AlCl_3$-treated group presented impairment a decrease (25%) on locomotor activity, as evidenced by a decrease on the number of crossings, when compared to control group (Fig.4A). In figure 4B, we have shown that $AlCl_3$-treated group significantly decreased the distance traveled (14.1 %) during the 5 min of the task. When maximum speed was analyzed, the $AlCl_3$-treated group showed a decrease of 24 % when compared to its respective control group (Fig.4C). The measurement of absolute turn angle behavior reflects changes in the zebrafish swimming direction and this parameter showed a significant increase in degrees direction changes for $AlCl_3$-treated group (12.7 %) (Fig.4D).

4. Discussion

In the present study, we observed significant changes on brain AChE activity and behavior parameters in zebrafish exposed to sublethal Al exposure. The precise mechanism by which Al exerts its neurotoxic effects is still not completely understood. However, literature suggests that Al interacts with the cholinergic system, acting as a cholinotoxin (Gulya et al., 1990).

In vivo experiment carried out after exposure for 96 h to 50 µg/L $AlCl_3$ at pH 5.8 showed a significant increase of AChE activity on zebrafish brain. However, this metal at the same concentration and exposure time did not promote any effect on zebrafish brain AChE on circumneutral pH 6.8. Studies reported that Al chloride exposure at low pH values induce more severe physiological consequences in fish when compared to circumneutral exposure. Brodeur et al. (2001) has shown that Atlantic salmon (*Salmo salar*) exposed for 36 days to acidified water (pH 5.2) with 50 µg/L $AlCl_3$ have altered the bioenergetics of the fish when compared to circumneutral Al exposed fish. Furthermore, Monette and McCormick (2008) demonstrated that gill ionoregulatory responses of the same fish specie were more prominent in a 6 day-exposure time with 43-68 µg/L of Al at acid pH. In addition, the solubility of Al increases as a direct result of decreased pH leading to the increased presence of inorganic Al, the most toxic form of Al that to fish (Gensemer and Playle, 1999; Finn, 2007). Therefore, our results are in agreement with the before mentioned studies since we observed significant alterations of AChE only in $AlCl_3$-treated group at pH 5.8.

There are mechanisms that could regulate AChE after *in vivo* experiments, which include modifications at transcriptional level or direct effect of metal on protein. In order to verify whether the *ache* gene expression pattern could be modulated when zebrafish were exposed to 50 µg/L $AlCl_3$ for 96 h at pH 5.8, we performed semi-quantitative RT-PCR experiments. Our results showed that $AlCl_3$-treated group did not demonstrate alterations in the *ache* RNA transcript levels, suggesting that the increase of AChE activity observed in this exposure was not directly related to higher *ache* gene expression pattern.

Our *in vitro* study demonstrates that $AlCl_3$ was able to increase AChE activity. These results could be related to the fact that in vitro experiments evaluate the direct effect of the metal on the enzyme, without the influence of others biological systems, such as cell signaling pathways. Alterations of AChE by divalent cations mediated through allosteric anionic sites are well-known (Roufogalis et al., 1973). Furthermore Gulya et al. (1990) suggested that increase on AChE activity after Al exposure may be due to allosteric interaction between the cation and the peripheric anionic site of the enzyme. Furthermore, previous studies have suggested that modifications on lipid membrane could be responsible for a change in the conformational state of the AChE molecule, which could induce the activation of AChE activity observed after long-term exposure of Al (Kaizer et al., 2005).

The involvement of cholinergic systems on locomotor activity, operant tasks, exposure to novel stimuli and the performance of spatial memory tasks is well established (Pepeu and Giovannini, 2004). Our results evaluating zebrafish behavior after $AlCl_3$ exposure are in accordance with Allin and Wilson (1999), which observed reduced swimming activity in juvenile rainbow trout when exposed to Al in acidic water. However, Brodeur et al (2001) using Atlantic salmon observed a contradictory result, describing that salmon presents an increased swimming activity when exposed to acidic water and Al. These contradictory results indicate that fish can react differently to sublethal levels of Al on acidic waters. Many factors can interfere for these differences, such as the relative level of toxicity of the acidic water, or some species-specific variations. Therefore, alterations in fish behavior can limit the fish survival in the wild (Brodeur et al., 2001). Reduced swimming

activity can affect the ability of the fish to forage, avoid predation, migrate and successfully reproduce (Allin and Wilson, 1999).

Reliable animal models are required to facilitate the understanding of neurodegenerative pathways in AD. Models should allow for the testing of compounds at various points of the pathogeneses cascade in order to search for disease modifying drugs. Furthermore, they remain a valuable tool for identifying molecular, cellular and pathological changes that trigger the onset of cognitive decline in AD. The zebrafish is an effective and simple model organism for studies of development and disease processes in the nervous system (Newman et al., 2007). Considering that studies have linked Al-induced neurotoxicity with neurodegenerative disorders such as (AD) (Flaten, 2001), the evaluation of neurochemical and behavioral changes induced by aluminum of zebrafish can contribute for a better understanding about the mechanisms related to the neurodegeneration induced by this metal.

This study showed that Al treatment, at acid pH, cause alterations on brain AChE activity and behavioral parameters in zebrafish. The activation of brain AChE activity could be involved in the behavior and neurotoxic effect induced by aluminum on central nervous system.

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Figure legends

Figure 1: *In vivo* effect of $AlCl_3$ on acetylcholinesterase activity in zebrafish brain. Data represent means ± S.D. of four different experiments, performed in triplicate. * indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

Figure 2: Acetylcholinesterase and β-actin mRNA expression in zebrafish brain. Fish were exposed to Aluminum after 96 h of exposure at pH 5.8 and the RT-PCR experiments were conducted. The PCR products were subjected to electrophoresis on a 1 % agarose gel, using β-actin as 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. The data were expressed as means ± S.D. and analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

Figure 3: *In vitro* effect of different concentrations of aluminum on acetylcholinesterase activity in zebrafish brain. Data represent means ± S.D. of four different experiments, each one performed in duplicate. * indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

Figure 4: Effect of aluminum acid exposure (50 μg/L, during 96 h at pH 5.8) on the swimming behavior during 5 minutes of videorecording. Figure (A) show the number o line crossing, (B) total distance traveled, (C) maximum speed and (D) show the absolute turn angle. Data are representative of 10 animals per group, presented as mean ± S.D. considering $P<0.05$ significant.

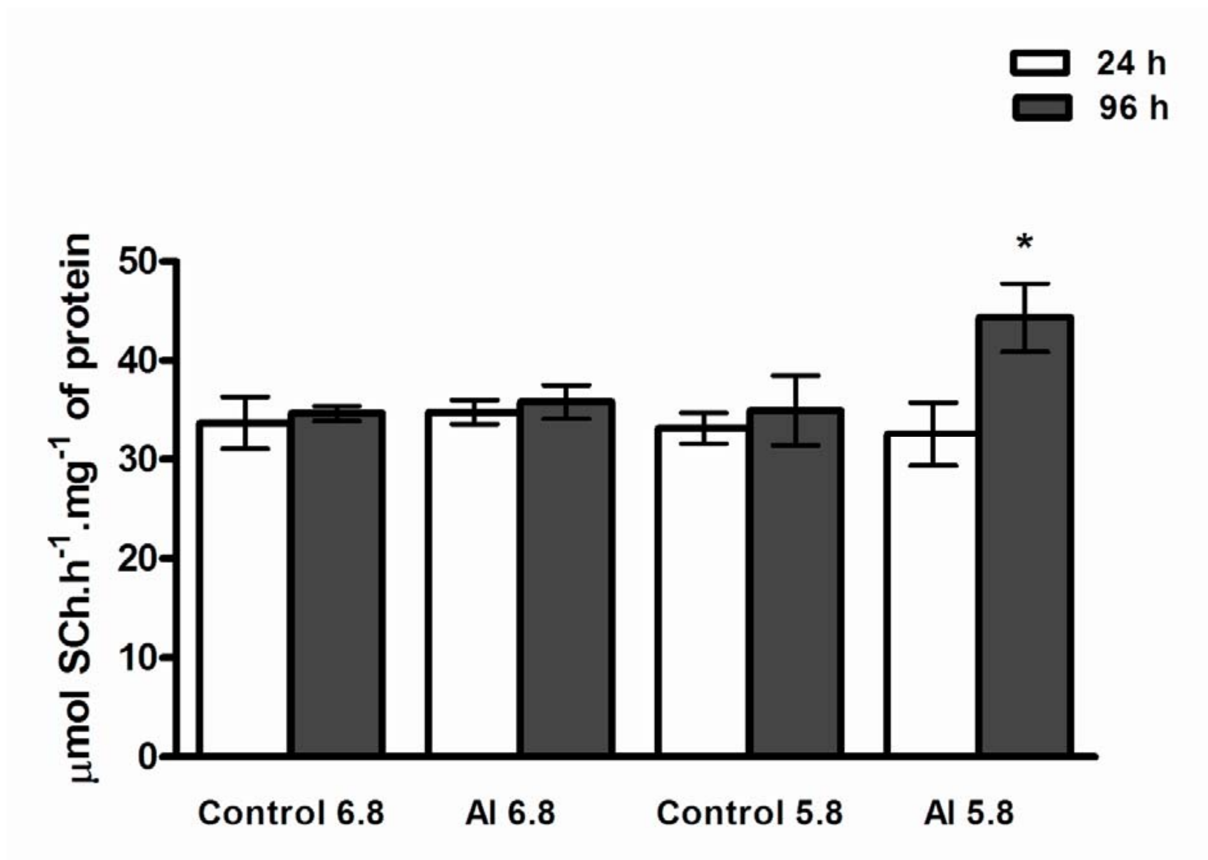

Fig. 1

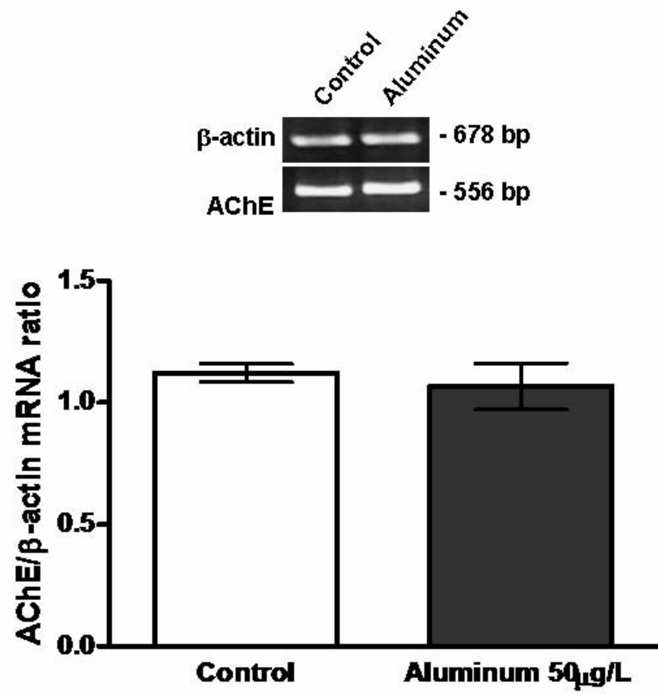

Fig. 2

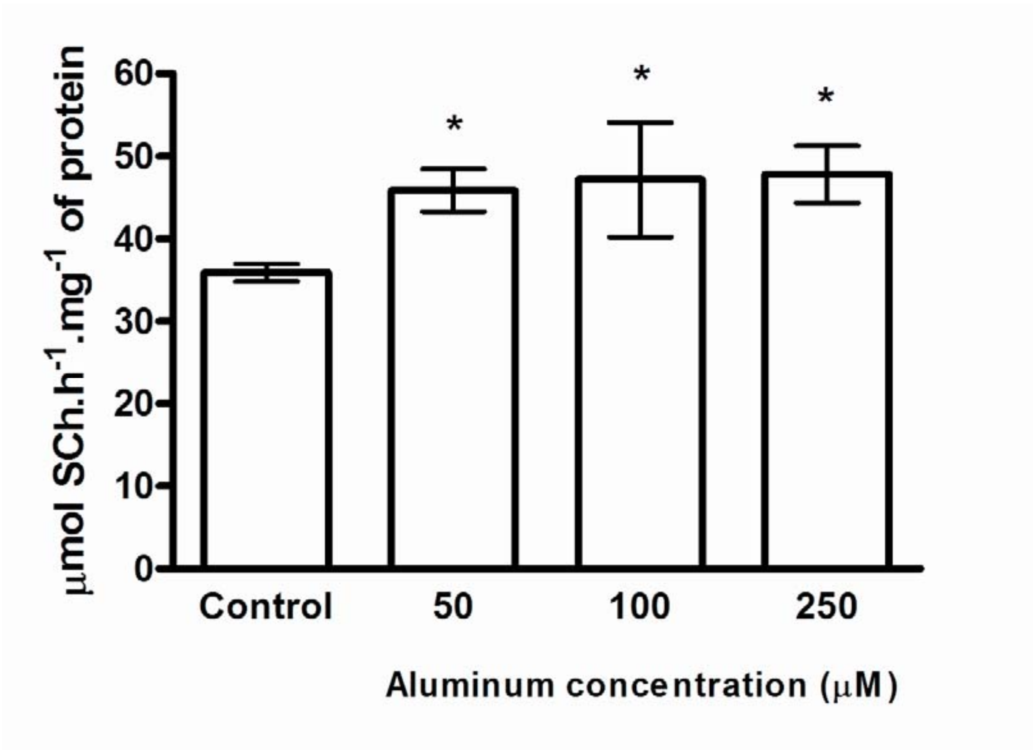

Fig. 3

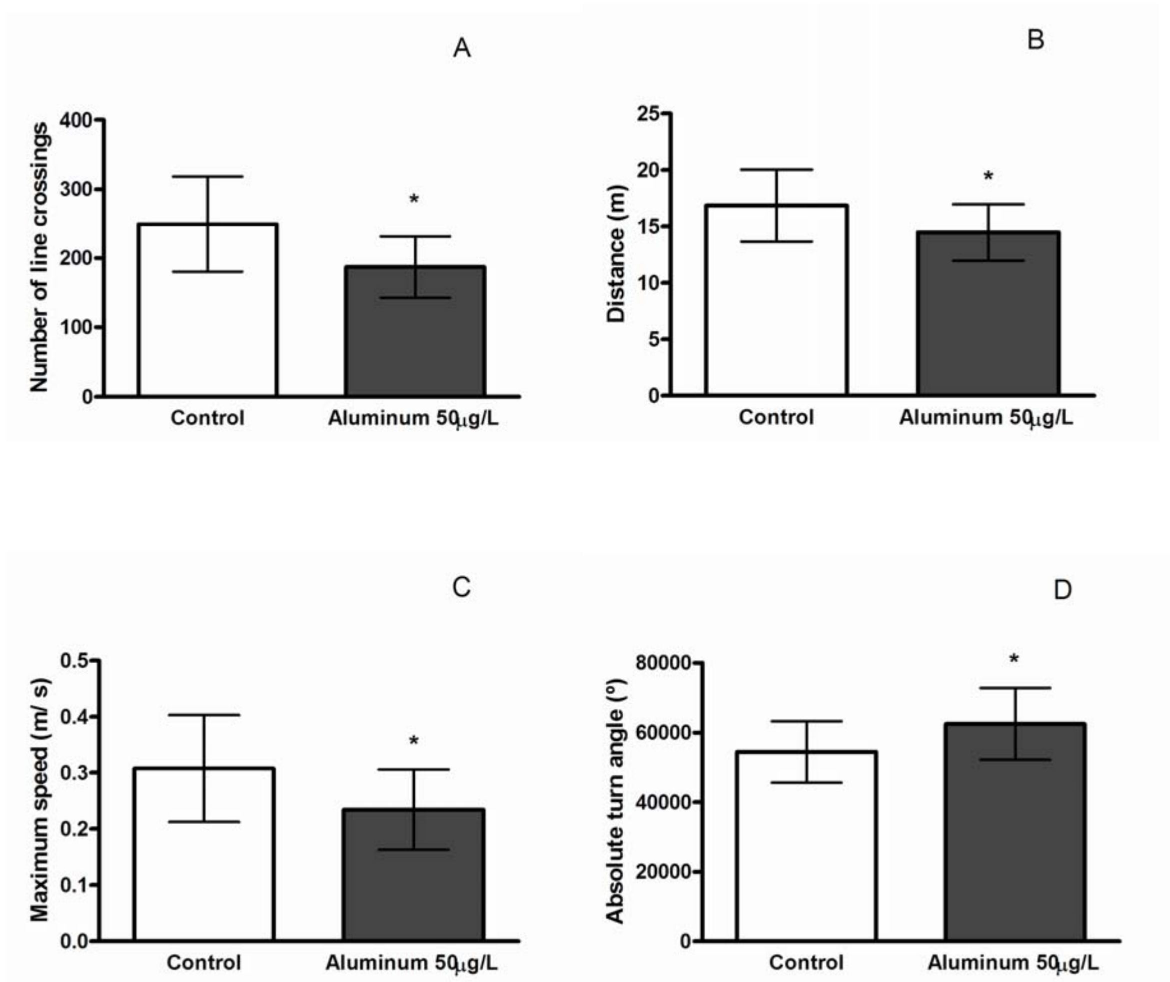

Fig. 4

II. 3. Capítulo 3- SENGER, M. R., ROSEMBERG, D. B., SEIBT, K. J., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2009. **Influence of trace metals on adenosine deaminase activity in zebrafish brain.** Manuscrito submetido ao periódico Toxicology

Influence of toxic metals on adenosine deaminase activity and gene expression in zebrafish (*Danio rerio*) brain

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Abstract

Toxic metals are widespread environmental contaminants that induce neurotoxicity even at very low concentrations. In this study, we investigated the effects of mercury chloride, cadmium acetate, potassium dichromate, lead acetate, zinc chloride, copper sulphate, cobalt chloride, and manganese chloride on soluble and membrane adenosine deaminase (ADA) activity and gene expression in zebrafish brain. Among the metals tested, only mercury was able to inhibit the adenosine deamination in vitro. The inhibition was observed from 5 to 250 μM $HgCL_2$ (84.6 – 92.6%) in soluble fraction while in membrane fractions the inhibition varied from 50 to 250 μM (20.9 – 26 %). In order to verify if mercury chloride could bind to ADA, we performed in vitro experiments with chelants to test if these compounds prevent or reverse the inhibition promoted by $HgCl_2$. The inhibition promoted by $HgCl_2$ was partially or fully abolished by the chelants. The in vivo exposure of zebrafish to 20 μg/L of $HgCl_2$ was evaluated after acute (24h) and subchronic (96h) treatments on ADA activity in soluble and membrane fractions. The ADA activity from soluble fraction was inhibited after both acute (24.5 %) and subchronic (40.8 %) exposures while the ADA activity from brain membranes was inhibited only after subchronic exposure (21.9 %). Semi-quantitative RT-PCR analysis showed that $HgCl_2$ did not alter the ADA gene expression. This study has shown that ADA activity was inhibited by mercury and this inhibition can be one of the neurotoxic targets of this heavy metal.

Key words: adenosine, adenosine deaminase, zebrafish, brain, toxic metals, mercury.

1. Introduction

Toxic metals are ubiquitous pollutants that are introduced in the aquatic environment by anthropogenic sources, mainly by industrial effluents (Kostial, 1978; Rai, 2009). Exposure to these substances promotes a wide range of deleterious effects to the aquatic and terrestrial biota even at sublethal concentrations. The high toxic effect of heavy metals, their persistence in the environment and their accumulation in the food chain, make them highly hazardous for animal and humans, mainly acting at central nervous system (CNS) (Jarup, 2003).

Nucleotides and nucleosides have important roles in energetic and signaling systems in the CNS in both physiological and pathological conditions. Adenosine can be rapidly obtained by ATP breakdown via ectonucleotidase activity (NTPDase and 5'-nucleotidase) or be released from any cell when the intracellular concentration goes up (Fredholm, 2002, Yegutkin, 2008). Extracellularly, adenosine acts as a neuromodulator, controlling both inhibitory and excitatory synapses acting on G-protein coupled receptors (A_1, A_{2A}, A_{2B}, A_3) (Burnstock, 2007). Extracellular adenosine concentrations can be regulated by neural cell uptake through bi-directional nucleoside transporters followed by phosphorylation to AMP by adenosine kinase or deamination to inosine by adenosine deaminase (ADA) (Fredholm et al., 2005, Rosemberg et al., 2007). Furthermore, studies have shown that the extracellular concentrations of adenosine may also be regulated by ecto-ADA activity (Franco et al., 1998; Romanowska et al., 2007). Adenosine deaminase (ADA, E.C.3.5.4.4.) is an enzyme witch catalyzes the hydrolytic

deamination of adenosine to inosine in both cytosol and on the cell membrane (Franco et al., 1997; Rosemberg et al., 2008).

Zebrafish (*Danio rerio*) is a small teleost widely used in toxicological and biochemical studies (Rubinstein, 2006; Senger et al., 2006b). This fish combines the relevance of a vertebrate with the scalability of an invertebrate (Lieschke and Currie, 2007). Studies performed in our laboratory demonstrated the presence of NTPDases, 5'-nucleotidase, and adenosine deaminase activities in zebrafish brain (Rico et al., 2003; Senger et al., 2004, Rosemberg et al., 2008). A recent data from our group has also reported the differential expression pattern of ADA-related genes in zebrafish tissues, including brain, demonstrating that distinct ADA related genes (*ada1, ada2-1, ada2-2,* and *adal*) are present in this specie (Rosemberg et al., 2007a). In addition, Western blot analysis revealed the presence of a band of 35 kDa for A(1)R in membrane preparations and a band of 43 kDa for ADA in both cytosol and membranes in goldfish (Beraudi et al., 2003).

Considering that toxic metals are important environmental pollutants and parameters of purinergic signaling and adenosine deaminase activity have been reported in zebrafish brain, the aim of this work is verify the effect of toxic metals on ADA activity and gene expression in soluble and membrane fractions of zebrafish brain.

2. Materials and methods

2.1. Chemicals

Adenosine, EDTA, EGTA, Coomassie Blue G, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenol and sodium

nitroprusside were purchased from Merck (Darmstadt, Germany). Mercury chloride ($HgCl_2$, CAS number 7487-94-7) and lead acetate [$Pb(CH_3COO)_2$, CAS number 301-04-2] were purchased from Quimibrás Indústrias Químicas (Brasil). Cadmium acetate [$Cd(CH_3COO)_2$, CAS number 543-90-8], copper sulphate ($CuSO_4$, CAS number 7758-99-8), potassium dichromate ($K_2Cr_2O_7$, CAS number 7778-50-9), and cobalt chloride ($CoCl_2$, CAS number 7646-79-9) were purchased from Nuclear (Brazil). Zinc chloride (ZnCl2, CAS number 7648-85-7) was purchased from QM (Brasil). Manganese chloride ($MnCl_2$, CAS number 7791-18-6) was purchased from Vetec Química Fina LTDA (Brasil). All other reagents used were from high analytical grade.

2.2. Experimental Animals

Adult zebrafish (*Danio rerio*) were obtained from commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50-L aquarium filled with continuously aerated unchlorinated water. The temperature was kept between 25 $\pm$ 1 ºC under a 14-h light-dark photoperiod. Animals feeding and maintenance were done according to Westerfield, (2000). The use and maintenance of zebrafish were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.3. Preparation of soluble and membrane fractions

Zebrafish were sacrificed and whole brains were initially homogenized in 20 volumes (v/w) of chilled phosphate buffer saline (PBS) 2 mM EDTA, 2 mM EGTA, pH 7.4 in a glass-Teflon homogenizer in order to obtain both cellular fractions. The

preparation of brain membranes was according to the method described previously (Barnes et al., 2003) with minor modifications. The homogenate was centrifuged at 800 *g* for 10 min and the pellet was discarded. After removing the nuclear and cell debris, the supernatant was centrifuged for 25 min at 40 000 *g*. The resultant supernatant and the pellet corresponded to the soluble and membrane fractions, respectively. The supernatant was collected and kept on ice for enzyme assays. The pellet was frozen in liquid nitrogen, thawed, resuspended in PBS once 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 for biochemical assays. The material was maintained at 2-4^{0}C throughout preparation.

2.4. Determination of adenosine deaminase activity

Adenosine deaminase activity was determined using a Berthelot reaction as previously reported (Weisman et al., 1988). The brain fractions prepared (5-10 µg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for the experiments with soluble and membrane fractions assays, respectively, in a final volume of 200 µl. The samples were preincubated for 10 min at 37ºC and the reaction was initiated by the addition of adenosine to a final concentration of 1.5 mM. After incubated for 75 min (soluble fraction) and 120 min (membranes), the reaction was stopped by the addition of 500 µl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct

non-enzymatic hydrolysis of substrates. The reaction mixtures were immediately mixed to 500 µl of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37ºC for 15 min and the ammonia produced were quantified by a colorimetric assay at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nmol of NH_3. min^{-1}. mg $protein^{-1}$.

2.5. *In vitro* assays

The metals were added to reaction medium before preincubation with the enzyme and maintained throughout the enzyme assays. Metals were tested at final concentration of 50-250 µM (Fig. 1). For $HgCl_2$ experiments, we tested this metal in a final concentration of 1-250 µM (Fig. 2).

To verify if $HgCl_2$ could bind directly to the enzyme, we performed *in vitro* experiments with DTT and EDTA. The adenosine deamination was measured in the presence of 250 µM $HgCl_2$ for membrane fractions or 5 µM and 250 $HgCl_2$ for soluble fractions. The control group was performed in the absence of $HgCl_2$. The fractions were preincubated 5 min in the reaction mixture and DTT or EDTA (500 µM) were added and preincubated during more 5 min (Groups E + DTT and E + EDTA); or preincubated 5 min with DTT or EDTA (500 µM) and after 5 min with the fractions (E) (Groups DTT + E and EDTA + E). Then, the determination of adenosine deaminase activity was performed as described above.

2.6. *In vivo* assays

The animals were introduced to the test aquariums (10 L) containing solution of $HgCl_2$ in a final concentration of 20 µg/L, which was been reported in the aquatic environment (Berzas Nevado et al., 2003; Senger et al., 2006). The animals were maintained in the test aquarium for acute (24 h) and subchronic (96 h) exposures.

2.7. Protein determination

Protein was measured using Coomassie blue as color reagent (Bradford, 1976) and bovine serum albumin as a standard.

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

The analysis of the expression of ADA related genes *ada1, ada2-1, ada2-2,* and *adal* was carried out by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA from zebrafish brain was isolated using Trizol reagent (Invitrogen) in accordance with manufacturer's instructions. 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 instructions. The *β-actin* primers were used as described previously (Chen et al., 2004). Primer sequences of ADA-related genes were designed and RT-PCR conditions were performed according Rosemberg et al. (2007a).

2.9. Statistical analysis

All experiments were carried out in replicate and means ± S.D. of at least three independent experiments were presented. Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey test as post-hoc. *P* values ≤ 0.05 were considered as significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer.

3. Results

The effect of mercury chloride, cadmium acetate, potassium dichromate, lead acetate, zinc chloride, copper sulphate, cobalt chloride, and manganese chloride (varying from 100 to 250 µM) were tested on soluble (Fig. 1A) and membrane fraction (Fig. 1B) ADA activity. Among the metals tested, only $HgCl_2$ inhibited the adenosine deamination in zebrafish brain.

In order to verify if lower mercury concentrations could be able to affect the ADA activity, we have tested concentrations ranging from 1 to 250 µM on soluble (Fig. 2A) and membrane (Fig. 2B) fractions. In soluble fractions, the inhibition has been observed at 5 µM and has been maintained until 250 µM (84.6 – 92.6%). In membrane fractions, the inhibition varied from 20.9 – 26 % at 50 to 250 µM $HgCl_2$, respectively. Our experiments demonstrated that the inhibition promoted by 5 µM $HgCl_2$ were partially and totally recovery by DTT when added after and before the soluble fraction, respectively. EDTA recovered partially the ADA activity only when added before the enzyme on the soluble fraction (Fig. 3A). Both DTT and EDTA did not prevent the inhibition promoted by 250 µM $HgCl_2$ in soluble fraction. For

membrane fractions, only DTT reverted partially the $HgCl_2$ inhibition when added before the enzyme preparation.

The in vivo exposure of zebrafish to 20 µg/L of $HgCl_2$ was evaluated after acute (24 h) and subchronic (96 h) treatments on ADA activity in soluble (Fig. 4A) and membrane fractions (Fig. 4B). Significant mortality was not observed in control and treated groups in the different exposure times and no changes in the swimming behavior were observed between control and treated groups (Senger et al., 2006a). The ADA activity of soluble fraction was inhibited after both acute (24.5 %) and subchronic (40.8 %) exposure times. The membrane ADA activity was inhibited only after subchronic exposure (21.9 %). Semi-quantitative RT-PCR experiments were conducted to verify whether $HgCl_2$ treatment could alter the expression of zebrafish brain ADA-related genes and the band densitometry using enzyme/β-actin ratios were determined for each treatment. The results have shown that $HgCl_2$ was not able to modulate ADA gene expression in zebrafish brain (Fig. 5).

4. Discussion

The results presented herein demonstrate the influence of toxic metals on ADA activity and expression in zebrafish brain. Among the eight metals tested in vitro, only $HgCl_2$ was able to inhibit ADA activity in soluble and membrane fractions. Our experiment with DTT and EDTA demonstrated that the inhibition was partially or fully abolished by these chelating agents, suggesting that $HgCl_2$ can bind on the enzyme structure and thus alter adenosine deamination on zebrafish brain.

There are few studies on the literature about the effect of metals on ADA activity. The role of divalent cations in structure and function of murine ADA has been previously demonstrated (Cooper et al., 1997). The authors showed that a single zinc or cobalt cofactor are bound in a high affinity site deep within the substrate binding cleft and it is required for catalytic function. Furthermore, metal ions bound at additional sites inhibited the enzyme and zinc, cobalt, cooper, manganese, and cadmium were able to inhibit the ADA activity at micromolar range. The decrease on ADA activity promoted by these added metals was immediately reversed by addition of EDTA to the reaction solution, indicating that they might be associated at sites readily accessible on the enzyme. The same reversion result of the inhibitory effect promoted by mercury on soluble and membrane bound ADA activity was observed in the present report, which lead us to suggest that these same enzyme sites are involved with the inhibitory effect of $HgCl_2$ on ADA activity from zebrafish brain.

The ADA activity of soluble fraction was significantly decreased after acute and subchronic exposures while the membrane ADA activity was inhibited only after subchronic exposure. The RT-PCR results showed that $HgCl_2$ did not interfere on ADA-related genes expression, indicating that this metal was not able to modulate the mRNA synthesis of enzymes able to deaminate adenosine. In this regard, it is possible to suggest that the significant decrease on adenosine deamination promoted by $HgCl_2$ *in vivo* might be due to a direct effect on enzymatic activities, as verified in the in vitro experiments.

Previous studies from our laboratory performed a phylogenetic analysis and have found the presence of ADA1, ADA2, and ADAL in zebrafish brain

(Rosemberg et al., 2007a). It has been suggested that the presence of these enzymes could be contributing in a different manner for the regulation of adenosine/inosine levels in distinct cellular fractions of porcine and zebrafish brains (Kukulski et al., 2004; Rosemberg et al., 2007a). Traditionally, ADA1 has been considered a cytosolic enzyme, constituting 90–100% of the total intracellular ADA activity (Franco et al., 2007; Iwaki-Egawa and Watanabe, 2002). Evidence demonstrated that cell-surface ADA1 are colocalized with A_1, A_{2B} receptors and concentrative nucleoside transporters, suggesting that this enzyme might be also involved in a fine tuning modulation of purinergic signaling (Saura et al., 1998; Herrera et al., 2001; Hirsch et al., 2007). No differences on catalytically activity or molecular characteristics between intracellular ADA1 and ecto-ADA1 have been found so far (Franco et al., 2007). It has been shown that subcellular fractions from rat cultured brain cells and rat brain synaptosomes reveal the coexistence between ADA1 and the membrane bound enzyme 5'-nucleotidase (Trams and Lauter, 1975; Franco et al., 1986). Previous study demonstrated that, together with ADA1, ADA2 also contributes to adenosine deamination on extracellular milieu, belonging to a new family of growth factors with ADA activity (Zavialov and Engström, 2005). In fact, zebrafish ADA2 sequences shared a signal peptide suggesting their potential role to cleave adenosine at the extracellular space (Rosemberg et al., 2007a). Although it has no evidence related to ADAL functionality and location so far, the presence of conserved amino acids involved on adenosine deamination, its similarity with ADA1 and the presence of *adal* mRNA transcripts on zebrafish brain (Rosemberg et al. 2007a) might indicate a putative role of this enzyme member to cleave adenosine. Considering that the inhibition induced by $HgCl_2$ on adenosine

deamination of soluble fraction was more pronounced that the observed on the membrane ADA activity, it is possible to suggest a differential cellular location of these ADA-related enzymes in zebrafish brain and a distinct effect of $HgCl_2$ on these enzyme family members. Moreover, these aspects might be also important to explain the absence of effect after cadmium acetate, potassium dichromate, lead acetate, zinc chloride, copper sulphate, cobalt chloride, and manganese chloride treatments on total adenosine deamination on soluble and membrane fractions.

Extracellular nucleotides and nucleosides are important signaling molecules that require effective mechanisms for their signal regulation (Yegutkin, 2008). This regulation is exerted by a broad range of nucleotide-degrading and interconverting extracelular enzymes (Zimmerman, 2006; Abbracchio et al., 2009). Our laboratory has already characterized the presence of NTPDase, 5'-nucleotidase and ADA activity in brain membranes of zebrafish, enzymes that are required for the extracellular degradation of ATP to adenosine (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008). In addition, we have already demonstrated that NTPDase and 5`nucleotidase activities are inhibited by toxic elements like mercury, lead, zinc, cadmium, and copper (Rosemberg et al., 2007b; Senger et al., 2006a; 2006b). Adenosine deamination is an important mechanism able to promote tissue homeostasis and adenosine signaling in brain. (Franco et al., 1997; Sun et al., 2005). Furthermore, the product of this reaction, inosine, has signaling properties on brain by act on A_3 adenosine receptors (Shen et al., 2005). Although the inhibitory effect promoted by $HgCl_2$ on ADA activity indicates a possible decrease on inosine synthesis, it might be an important mechanism to increase the levels of the neuromodulator adenosine since previous data have shown that

different exposure times to this metal decrease nucleotide hydrolysis in zebrafish brain membranes (Senger et al., 2006a). Further experiments involving other purinergic signaling parameters are still required to elucidate this hypothesis.

Altogether, this study demonstrated that mercury inhibit the ADA activity, an important enzyme of the purinergic metabolism, suggesting that the adenosine/inosine levels could be altered. These results suggest that both extracellular and intracellular purine metabolism are modulated by mercury chloride and could be utilized as a tool to the bioindication of these hazardous substances on the environment utilizing zebrafish as a model.

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Figure legends

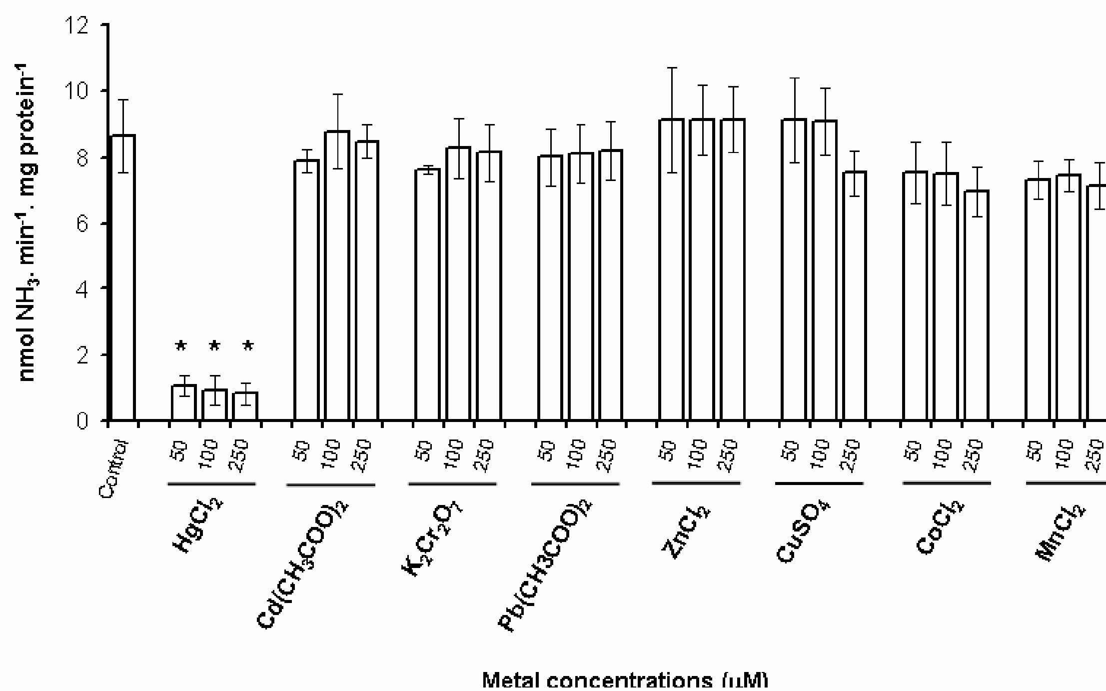
Fig. 1: *In vitro* effect of toxic metals on soluble (A) and membrane-bound (B) ADA activity from zebrafish brain. Data represent means ± S.D. of at least three different replicate experiments. * represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 2: Effect of mercury chloride concentrations on soluble (A) and membrane (B) preparations from zebrafish brain. Data represent means ± S.D. of three different replicate experiments. * represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 3: Effect of DTT and EDTA on the inhibition promoted by mercury chloride on ADA activity from soluble (A) and membrane (B) fractions of zebrafish brain. The adenosine deamination was measured in the absence (control group) or in the presence of 250 μM $HgCl_2$; preincubated 5 min with DTT or EDTA (500 μM) and after 5 min with the enzyme preparations (E) (Groups DTT + E and EDTA + E); or preincubated 5 min with the enzyme preparations and after more 5 min with DTT or EDTA (500 μM) (Groups E + DTT and E + EDTA). Data represents means ± S.D. of four different replicate experiments. * represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$). # represents a significant difference from control and $HgCl_2$ group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 4 Effect of acute (24 h) and subchronic (96 h) mercury chloride (20 μg/L) exposure on soluble (A) and membrane-bound (B) ADA activity from zebrafish brain. Data represent means ± S.D. of at least four different replicate experiments. The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 5: ADA gene expression patterns after subchronic (96 h) mercury exposure (20 μg/L). The figure shows β-actin, ADA1, ADAL, ADA2-1, and ADA2-2 expression in brain of zebrafish. The results were expressed as optical densitometry (O.D.) of ADA related genes versus β-actin expression. Data represent means ± S.D. of four different replicate RT-PCR experiments.



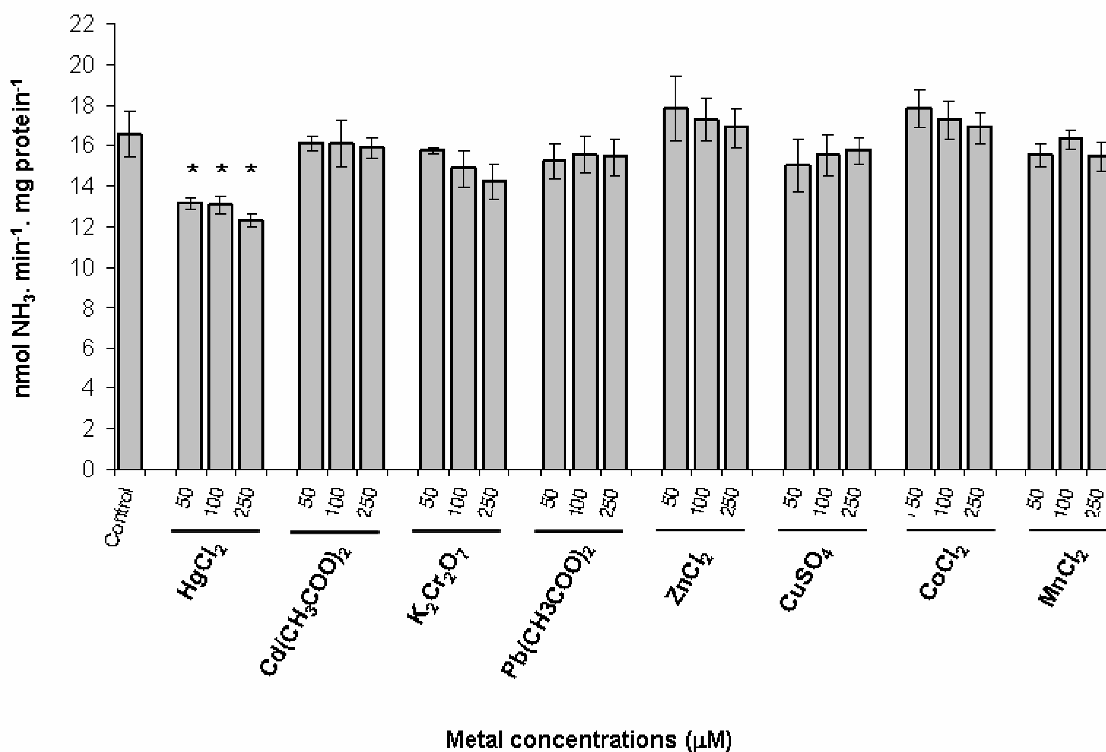
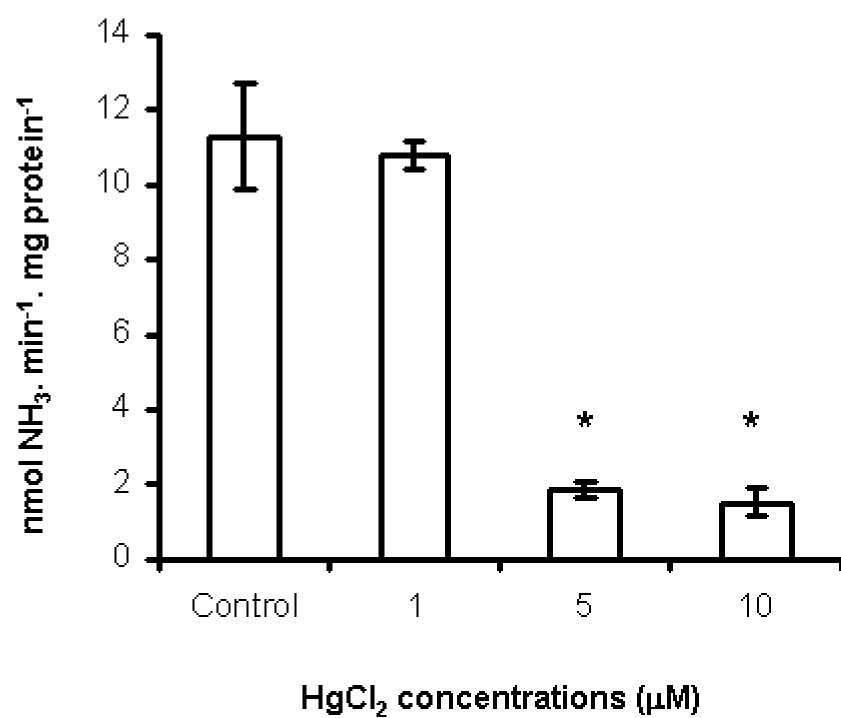


Fig. 1

A



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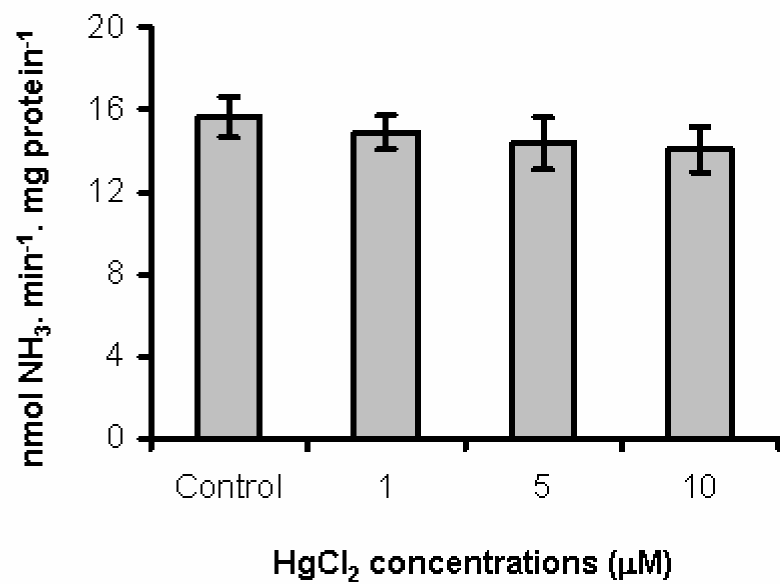
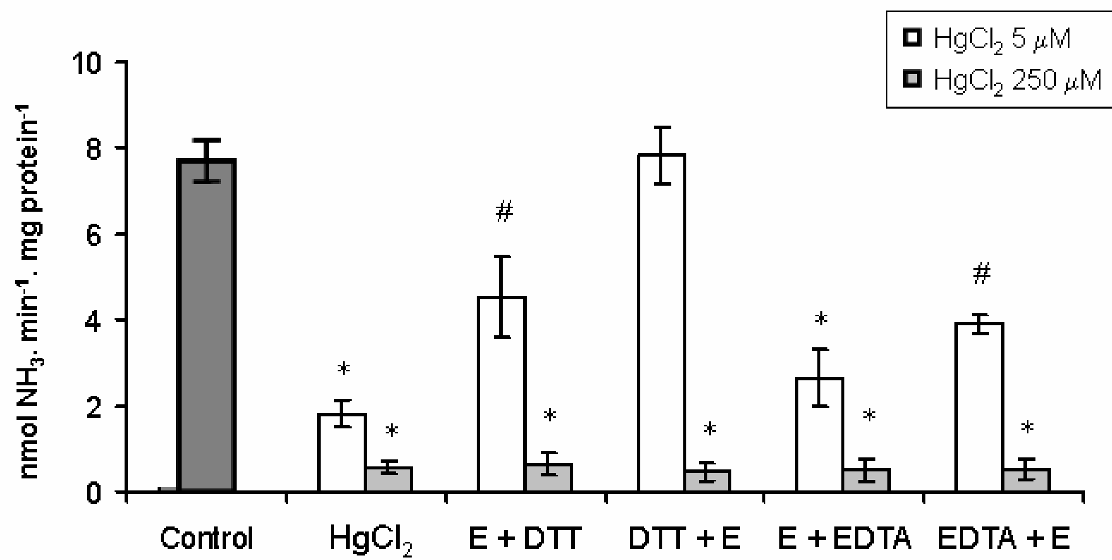


Fig. 2



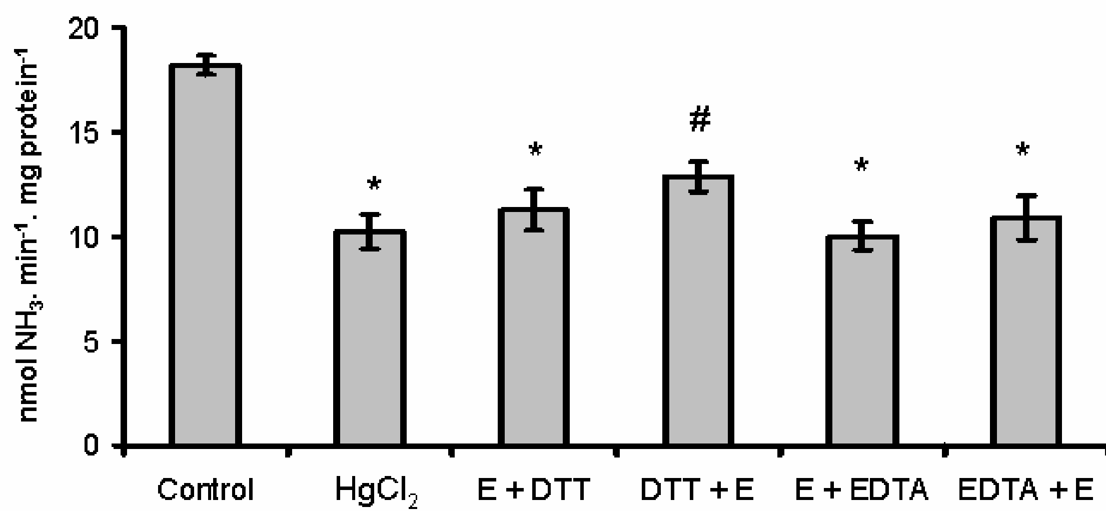
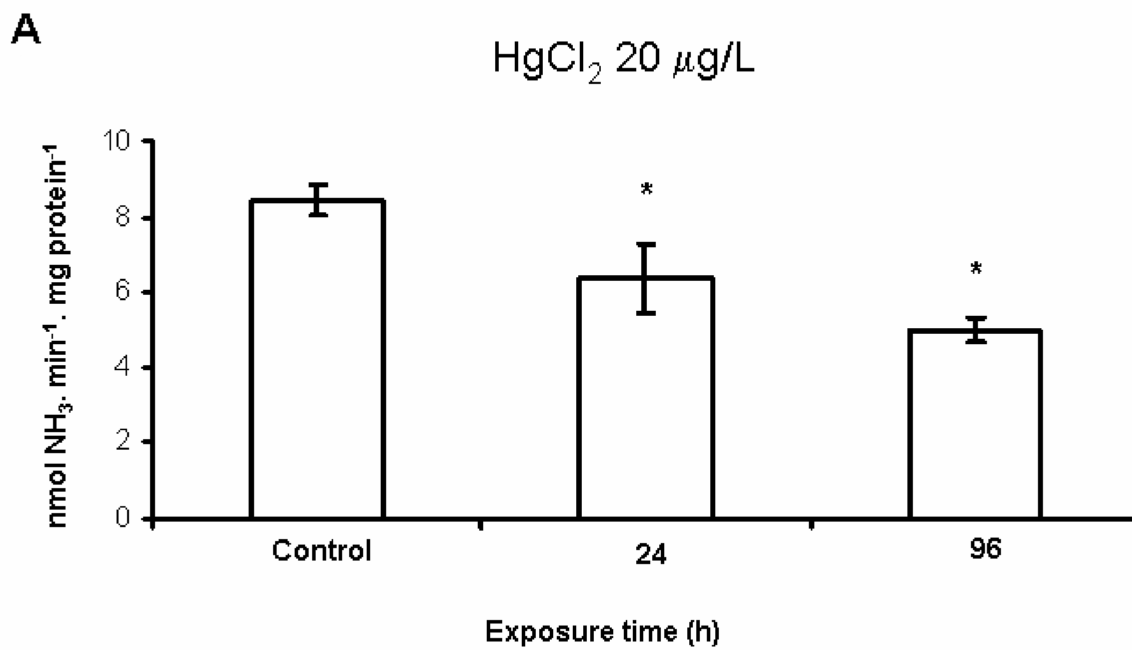


Fig. 3



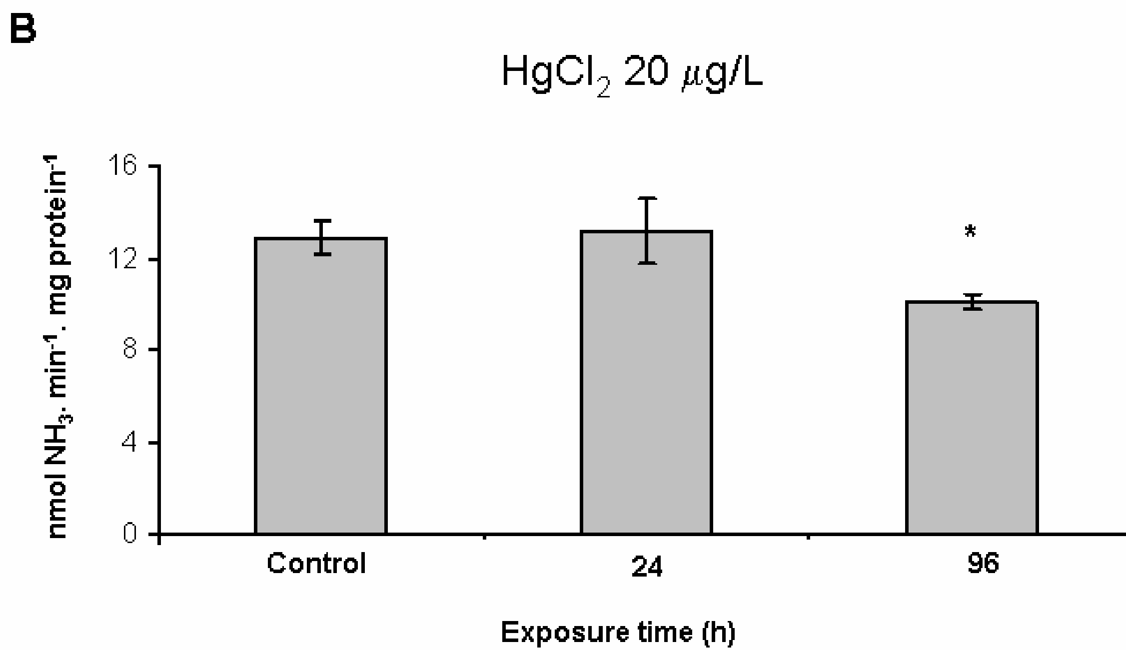


Fig. 4

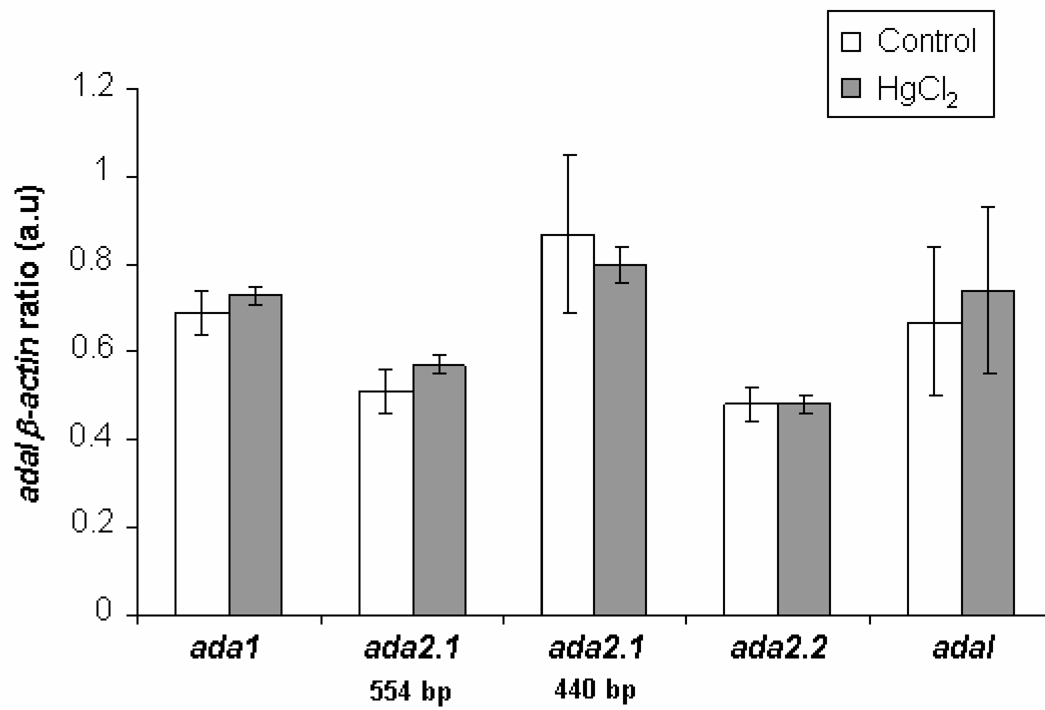


Fig. 5

PARTE III

III.1. Discussão

No capítulo 1, foi testado o efeito *in vitro* do zinco e do cádmio sobre a atividade da acetilcolinesterase e ectonucleotidases em cérebro de peixe zebra. A atividade da acetilcolinesterase não foi alterada, mas a hidrólise dos nucleotídeos foi afetada na presença destes metais tóxicos. O zinco aumentou a hidrólise de ATP e AMP, mas não teve efeito sobre a hidrólise de ADP. O cádmio aumentou fortemente a hidrólise do ATP, mas promoveu uma inibição significativa na hidrólise do ADP e AMP em membranas cerebrais do peixe zebra. Este estudo demonstrou que as enzimas do sistema purinérgico, mas não a AChE, são afetados pela exposição *in vitro* ao zinco e cádmio no cérebro do peixe zebra. As diferenças observadas com relação aos efeitos promovidos por cádmio e zinco sugerem que a atividade enzimática dos membros da família das NTPDases podem ser diferentemente afetados por estes metais, sendo que nossos resultados sugerem uma inibição da NTPDase1 e um ativação da NTPDase2. Nossos resultados contribuem para uma maior compreensão dos efeitos tóxicos de cádmio e zinco na sinalização purinérgica e colinérgica. Baseado na complexidade dos efeitos do zinco e cádmio neste teleósteo, futuros estudos in vivo serão importantes para elucidar a influência destes contaminantes na neurotransmissão purinérgica.

O capítulo 2 foi realizado com o intuito de verificar o efeito da exposição ao alumínio (Al) na atividade da AChE cerebral e em parâmetros comportamentais de peixes zebra. O experimento *in vivo* realizado após 96 horas de exposição a 50 µg/L de $AlCl_3$ em pH 5,8 demonstrou um aumento significativo na atividade da

AChE cerebral do peixe zebra. Entretanto, este metal na mesma concentração e tempo de exposição não promoveu nenhum efeito na AChE cerebral do peixe zebra em pH neutro 6,8. O aumento da atividade da AChE em pH 5.8 e a ausência de mudanças na atividade da AChE em pH 6.8 reforçam a hipótese de que os efeitos tóxicos do $AlCl_3$ são mais intensos em pH ácidos. Existem mecanismos que podem regular a atividade da AChE após experimentos *in vivo*, dentre os quais podemos incluir modificações em nível transcricional, ou efeito direto do metal na proteína. A fim de verificar se o padrão de expressão do gene da AChE pode ser modulado quando o peixe zebra foi exposto a 50 µg/L de $AlCl_3$ em pH 5,8 por 96 horas, nós realizamos experimentos de RT-PCR semiquantitativo. Nossos resultados demonstraram que o grupo tratado com alumínio nessas condições não apresentou alterações nos níveis de transcritos de RNA da AChE, sugerindo que o aumento da atividade da AChE observado nesta exposição não foi diretamente relacionado a uma maior expressão desta enzima. Os experimentos *in vitro* demonstraram que o $AlCl_3$ foi capaz de aumentar a atividade da AChE. Talvez este aumento possa estar relacionado ao fato que os experimentos *in vitro* avaliam o efeito direto do metal sobre a enzima, sem a influência de outros sistemas biológicos, como vias de sinalização celular.

Nossos resultados avaliando o comportamento do peixe zebra após o tratamento com $AlCl_3$ estão de acordo com ALLIN & WILSON (1999), que observaram uma atividade natatória reduzida em trutas arco-íris quando expostas ao Al em pH ácido. Entretanto, BRODEUR et al. (2001), usando o salmão atlântico como modelo experimental, observou um resultado contraditório, descrevendo que o salmão apresenta uma atividade natatória aumentada quando exposto ao Al em

pH ácido. Variações no comportamento dos peixes podem limitar sua sobrevivência em seu habitat natural (BRODEUR et al., 2001). Uma redução na atividade natatória pode afetar a habilidade do peixe foragear, evitar predação, migrar e se reproduzir com sucesso (ALLIN & WILSON, 1999). Muitos fatores podem interferir para estas diferenças, como o nível relativo de toxicidade da água acidificada ou algumas variações específicas de cada espécie.

Os resultados do capítulo 3 mostram a influência de metais tóxicos na atividade e expressão da ADA em cérebro de peixe zebra. Entre os oito metais testados *in vitro*, apenas o cloreto de mercúrio foi capaz de inibir a atividade da ADA em frações solúveis e de membrana. Nossos experimentos com DTT e EDTA demonstraram que a inibição foi parcialmente ou totalmente abolida por estes agentes quelantes, sugerindo que o cloreto de mercúrio pode se ligar na estrutura protéica, e assim alterar a desaminação da adenosina. A atividade da ADA foi significativamente diminuída na fração solúvel após as exposições aguda e sub crônica, enquanto que na fração de membrana este efeito inibitório só foi observado após a exposição sub-crônica. Os resultados de RT-PCR mostraram que o mercúrio não interferiu na expressão dos genes relacionados à ADA, indicando que este metal tóxico não foi capaz de modular a síntese de RNAm das enzimas capazes de desaminar a adenosina. Nesse sentido, é possível sugerir que a diminuição significativa da desaminação da adenosina promovida pelo mercúrio, após exposição *in vivo* pode ser devido a um efeito direto na atividade enzimática, como verificada nos experimentos *in vitro*. Estes resultados sugerem que o metabolismo das purinas no espaço extracelular e intracelular é afetado pelo mercúrio e pode ser utilizado como uma ferramenta toxicológica para a

bioindicação deste metal deletério ao meio ambiente utilizando o peixe zebra como modelo experimental.

III.2. Conclusão Final

Com os resultados apresentados nesta tese de doutorado, pode-se concluir que as enzimas envolvidas na degradação de nucleotídeos e nucleosídeos (NTPDase, 5'-nucleotidase e ADA) e acetilcolina (Acetilcolinesterase) são moduladas por metais tóxicos em cérebro de peixe zebra. Além de serem possíveis alvos da neurotoxicidade dos metais, a alteração destas atividades enzimáticas pode ser utilizada como bioindicador da presença ambiental destes poluentes.

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III.4. Anexos

III.4.1. Índice de Figuras

Parte I

Figura 1 Peixe zebra (*Danio rerio*)

Figura 2 Vias da sinalização purinérgica. Adaptado de YEGUTKIN, 2008

Figura 3 Estrutura das ectonucleotidases. Adaptado de www.crri.ca/sevigny.html

Figura 4 Degradação de nucleotídeos e nucleosídeos. Adaptado de Yegutkin, 2008.

Figura 5 sinapse colinérgica. Adaptado de http://www.chm.bris.ac.uk/webprojects2006/Macgee/Web%20Project/ach_esterase_small_bord.png

Parte II

II.1. Capítulo 1

Fig. 1: *In vitro* effect of varying concentrations of cadmium (A) and zinc (B) on AChE activity in zebrafish brain. Bars represent the mean ± of at least three different experiments. The AChE control activity (without metal) was 37.97 ± 3.59 and 35.21 ± 1.45 μmol of ASCh released.h^{-1}.mg^{-1} of protein for cadmium and zinc, respectively. (ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant).

Fig. 2: *In vitro* effect of varying concentrations of cadmium (A) and zinc (B) on nucleotide hydrolysis in zebrafish brain membranes. Bars represent the mean ± of at least three different experiments. The control ATPase, ADPase and AMPase activities for cadmium were 617.1 ± 88.3; 147.9 ± 5.5 and 30.5 ± 5.4 nmol Pi.min^{-1}.mg^{-1} of protein, respectively. The control ATPase, ADPase and AMPase activities

for zinc were 564.4 ± 58.2; 147.9 ± 28.21 and 24.2 ± 5.2 nmol $Pi.min^{-1}.mg^{-1}$ of protein, respectively. * Significantly different from control group ($P \leq 0.05$) using ANOVA followed by a Duncan multiple range test.

II. 2. Capítulo 2

Figure 1: *In vivo* effect of $AlCl_3$ on acetylcholinesterase activity in zebrafish brain. Data represent means ± S.D. of four different experiments, performed in triplicate. * indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

Figure 2: Acetylcholinesterase and β-actin mRNA expression in zebrafish brain. Fish were exposed to Aluminum after 96 h of exposure at pH 5.8 and the RT-PCR experiments were conducted. The PCR products were subjected to electrophoresis on a 1 % agarose gel, using β-actin as 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. The data were expressed as means ± S.D. and analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

Figure 3: *In vitro* effect of different concentrations of aluminum on acetylcholinesterase activity in zebrafish brain. Data represent means ± S.D. of four different experiments, each one performed in duplicate. * indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test, considering a $P<0.05$ significant.

Figure 4: Effect of aluminum acid exposure (50 μg/L, during 96 h at pH 5.8) on the swimming behavior during 5 minutes of videorecording. Figure (A) show the number o line crossing, (B) total distance traveled, (C) maximum speed and (D) show the absolute turn angle. Data are representative of 10 animals per group, presented as mean ± S.D. considering $P<0.05$ significant.

II.3. Capítulo 3

Fig. 1: *In vitro* effect of toxic metals on soluble (A) and membrane-bound (B) ADA activity from zebrafish brain. Data represent means ± S.D. of at least three different replicate experiments.The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 2: Effect of mercury chloride concentrations on soluble (A) and membrane (B) preparations from zebrafish brain. Data represent means ± S.D. of three different replicate experiments. The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 3: Effect of DTT and EDTA on the inhibition promoted by mercury chloride on ADA activity from soluble (A) and membrane (B) fractions of zebrafish brain. The adenosine deamination was measured in the absence (control group) or in the presence of 250 μM $HgCl_2$; preincubated 5 min with DTT or EDTA (500 μM) and after 5 min with the enzyme preparations (E) (Groups DTT + E and EDTA + E); or preincubated 5 min with the enzyme preparations and after more 5 min with DTT or EDTA (500 μM) (Groups E + DTT and E + EDTA). Data represents means ± S.D. of four different replicate experiments. The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 4 Effect of acute (24 h) and subchronic (96 h) mercury chloride (20 μg/L) exposure on soluble (A) and membrane-bound (B) ADA activity from zebrafish brain. Data represent means ± S.D. of at least four different replicate experiments. The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p \leq 0.05$).

Fig. 5: ADA gene expression patterns after subchronic (96 h) mercury exposure (20 μg/L). The figure shows β-actin, ADA1, ADAL, ADA2-1, and ADA2-2 expression in brain of zebrafish. The results were expressed as optical

densitometry (O.D.) of ADA related genes versus β-actin expression. Data represent means + S.D. of four different replicate RT-PCR experiments.

III.4.2. Cartas de submissão

III.4.2.1. Cópia eletrônica do resumo do manuscrito submetido ao periódico Aquatic Toxicology

Dear cbonan,

Your submission entitled "Aluminum exposure alters behavioral parameters and increase acetylcholinesterase activity in zebrafish (Danio rerio) brain" has been received by Aquatic Toxicology

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III.4.2.2. Cópia eletrônica do resumo do manuscrito submetido ao periódico Toxicology

Ms. No.: TOX-09-481
Title: Influence of toxic metals on adenosine deaminase activity and gene expression in zebrafish (Danio rerio) brain
Authors: Mario R Senger; Denis B Rosemberg; Kelly J Seibt; Renato D Dias; Mauricio R Bogo;

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