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AVALIAÇÃO DA ATIVIDADE DA δ-AMINOLEVULINATO-DESIDRATASE E CONCENTRAÇÃO DE METALOTIONEÍNA EM FÍGADO E ENCÉFALO DE RATOS EXPOSTOS A CÁDMIO E ZINCO

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Apresentação

Conforme as recomendações do Programa de Pós Graduação em Ciências Biológicas: Bioquímica, esta dissertação está organizada em três partes principais:

Parte I – contêm os resumos, a lista de abreviaturas, a introdução e os objetivos do trabalho;

Parte II – é constituída por materiais e métodos e os resultados do trabalho, sendo este apresentado na forma de capítulos, onde constam dois artigos científicos em preparação;

Parte III – apresenta a discussão, as conclusões, as perspectivas, as referências citadas nas partes I e III e a lista de figuras.

Sumário

PARTE I

RESUMO

É bem conhecido que muitos efeitos tóxicos do cádmio (Cd) resultem da ação da interação com metais essenciais, incluindo zinco (Zn). Sendo um poluente ambiental, a exposição à Cd conduz a distúrbios no conteúdo e atividade de Zn no organismo, representando importante via para o desenvolvimento de sua toxicidade. Evidências suportam que Zn pode reduzir os efeitos do Cd, prevendo ou reduzindo a ação tóxica deste metal, enquanto que a deficiência de Zn pode intensificar a toxicidade de Cd. Com base nisto, este trabalho buscou investigar (1) o efeito da interação Zn-Cd sobre o tecido hepático de ratos adultos, por representar um importante alvo biológico à ação dos metais e (2) se estes efeitos são estendidos ao SNC mesmo protegido pela barreira hemato-encefálica. Através da avaliação de parâmetros bioquímicos, como δ-aminolevulinato-desidratase e metalotioneína, os resultados encontrados suportam a sobreposição do efeito tóxico de Cd sobre as funções essenciais de Zn, entretanto existe diferença entre os tecidos quanto ao mecanismo protetivo exercido sobre a toxicidade de Cd. Além disso, obtemos dados sobre os níveis de MT que contrapõem suas funções benéficas previamente descritas. Em resumo, essa dissertação reforça a ação tóxica de Cd por vias biológicas comuns ao Zn e expõe diferenças entre o tecido hepático e o SNC quanto ao mecanismo interativo.

Palavras-chave: cádmio, zinco, δ-aminolevulinato-desidratase, metalotioneína

ABSTRACT

It is well known that many toxic effects of cadmium (Cd) results from the action of the interaction with essential metals, for example zinc (Zn). Cd is an environmental pollutant, which exposure leads to disturbance in the content and atividade of Zn in the body. Evidences indicate that Zn can reduce the effects of Cd for provide or reduce the toxic action of this metal, whereas Zn deficiency can intensify the toxicity of Cd. On account of this, our study aimed investigate (1) the effect of Zn-Cd interaction on the liver tissue of adult rats, which represents important biological target for action of the metals and (2) if these effects are extended to the CNS even protected by the blood-brain barrier. Through the evaluation of biochemical parameters, as δ-aminolevulinate-dehydratase (δ-ALA-D) and metallothionein (MT), the results support the overlap of the toxic effect of Cd on the essential functions of Zn; however there is difference between the tissues on the protective mechanism exerted on the toxicity of Cd. Furthermore, we obtain data on the levels of MT that contrast to their beneficial functions previously described. In summary, this work reinforces the toxic effect of Cd by biological pathways common to the Zn and explains differences between the liver tissue and CNS in relation to the interactive mechanism.

Keywords: cadmium, zinc, δ-aminolevulinate-dehydratase, metallothionein

LISTA DE ABREVIATURAS

BHE – barreira hemato-encefálica

CAT – catalase

Cd – cádmio divalente; Cd^{2+}

DTT – ditiotreitol

δ-ALA-D – δ-aminolevulinato desidratase

GPx – glutationa peroxidase

LPO – peroxidação lipídica

MT – metalotioneína

PBG – porfobilinogênio

SOD – superóxido dismutase

TBARS – substâncias reativas ao ácido tio-barbitúrico

Zn – zinco divalente; Zn^{2+}

1. INTRODUÇÃO

A crescente emissão de resíduos tóxicos, por conta do desenvolvimento industrial e tecnológico das últimas décadas, tornou necessária uma maior compreensão dos riscos que tais poluentes poderiam causar ao ambiente e, principalmente, ao homem. Prevendo isto, muitos pesquisadores se voltaram para o estudo dos metais traço, por constituírem elevado impacto sobre sistemas biológicos, tornando sua investigação predominante na pesquisa toxicológica. Estes elementos são conhecidos pela sua elevada toxicidade biológica, porém se sabe que alguns são fundamentais para o desenvolvimento e funcionamento adequado dos diversos organismos. Assim, eles podem ser divididos em dois grupos distintos: tóxicos e essenciais (Goyer, 1997).

Os metais essenciais, como zinco (Zn), são designados assim, por participarem de atividades biológicas. Já metais tóxicos, como cádmio (Cd), não possuem essencialidade e, frequentemente, podem apresentar efeitos adversos ao organismo. É com base nesta distinção dos metais que, recentemente, surgem evidências sobre a interação de metais tóxicos com aqueles essenciais ao organismo. Isto se deve a observação de que alguns elementos traço são apenas parcialmente distinguíveis em sistemas biológicos devido a propriedades químicas semelhantes. Respectivamente, Cd e Zn enquadram-se nestas categorias; por exemplo, ambos apresentam configuração eletrônica e eletronegatividade muito semelhante (respectivamente, 1,69 e 1,65 pela escala de Pauling) o que conduz a ação dos metais por rotas metabólicas comuns. Contudo existe uma importante diferença entre os dois metais quanto ao raio atômico e por isto a mimetização de Zn por Cd não é perfeita, causando disfunções sobre o metabolismo de Zn. É por causa desta interação que Cd tem sido reconhecido como um anti-metabólito de Zn (Brzóska & Moniuszko-Jakoniuk, 2001)

1.1 Cádmio

Cd é um metal não-essencial amplamente encontrado no ambiente devido à utilização industrial em larga escala (World Health Organization, 1992). Além disso, Cd é constituído

por alguns produtos domésticos como baterias, pigmentos, plásticos e ligas metálicas (ATSDR, 1998). Cd também é observado em baixas quantidades nos alimentos, além de estar presente na fumaça de cigarros, sendo esta uma significante fonte de exposição ao metal (Saldivar et al., 1991; Stohs et al., 1997). Variadas formas de exposição ao metal, torna quase que inevitável o contato com os seres vivos e, uma vez que um sistema biológico se torna contaminado por Cd, passa a receber influência do metal por longa data. Isto se deve a alta taxa de retenção de Cd que apresenta meia-vida biológica de aproximadamente 30 anos (Goyer, 1991; Hideaki et al., 2008), fazendo com que se acumule durante a vida dos organismos. O impacto que possui este dado concentra-se em evidências de correlação positiva entre o envelhecimento e os níveis de Cd em humanos (Bin & Garfinkel, 1994).

A intoxicação pelo metal é prejudicial a muitos órgãos, incluindo o pulmão (Manca et al., 1991b), fígado, rim (Manca et al., 1991b; Casalino et al., 2002), testículos (Manca et al., 1991b; Morselt, 1991), cérebro (Méndez-Armenta et al., 2001, 2003) e placenta (Wazelhoff et al., 1985; Wier et al., 1990). Entre estas estruturas o tecido hepático representa um importante alvo para o acúmulo de Cd (Frazier & Puglese, 1978), porém devido à elevada meia-vida biológica, este elemento de transição pode apresentar ação sobre outros componentes do organismo, mesmo naqueles que possuem barreiras biológicas como, por exemplo, o sistema nervoso central (SNC).

Em relação à hepatotoxicidade de Cd, os principais efeitos associados ao metal são: produção de espécies reativas de oxigênio (Eaton et al., 1980; Goering et al., 1993), aumento da peroxidação lipídica (Manca et al. 1991a; Muller, 1986), indução ao dano do ADN (Nocentini, 1987), carcinogênese (Waalkes & Rehm, 1994) e também a modificação da expressão de proteínas (Beyersmann & Hechtenberg, 1997). Especificamente no tecido nervoso este elemento de transição é capaz de causar mudanças neuroquímicas (Carageorgiou et al., 2004; Hobson et al., 1986; Luchese et al., 2007; Minami et al., 2001) e influenciar sobre a formação da memória (Lukawski et al., 2005).

Os processos que desencadeiam a ação tóxica de Cd ainda não estão completamente elucidados, mas como observado até o momento, invariavelmente, os

mecanismos confluem na produção de radicais livres (Figura 1). Apesar de Cd não ter capacidade direta de gerar espécies reativas existem evidências do aumento na produção dos radicais superóxido, hidroxila e óxido nítrico por via indireta, uma vez que o elemento causa a inibição das enzimas responsáveis pela resposta antioxidante (Galan et al., 2001; Manca et al., 1991b). Cd também é capaz de gerar peróxido de hidrogênio, da qual representa uma significante fonte de radicais livres, já que o metal pode substituir o grupo prostético de proteínas contendo ferro ou cobre, liberando estes íons ao desencadeamento de stress oxidativo via reação de Fenton (Watjen & Beyersmann, 2004). Por conseqüência ao aumento de radicais livres, Cd provoca mobilização da resposta celular antioxidante, aumentando a atividade enzimática da catalase, superóxido dismutase e glutationa peroxidase (Gutteridge & Halliwell, 1990; Nakazawa et al., 1996; Fridovich, 1998). Além disso, antioxidantes inespecíficos como metalotioneína (MT) podem aumentar seus níveis em resposta ao stress oxidativo dependente de Cd (Maret, 2006; Sato & Bremner, 1993; Thornalley & Vasak, 1985). Mesmo com toda a maquinaria antioxidante, o organismo pode não conseguir neutralizar o status oxidativo obtido pela exposição ao Cd e os radicais livres em excesso podem reagir com moléculas importantes para o funcionamento celular, tais como, ADN (Littlefield & Hass, 1995), proteínas e lipídios (Méndez-Armenta & Rios, 2007). Assim, danos gerados pela oxidação destas moléculas podem conduzir a carcinogênese, proteínas com funções comprometidas e diminuição da fluidez de membranas biológicas. Neste último caso, havendo comprometimento da membrana mitocondrial interna, Cd pode disparar morte celular por apoptose (Wätjen et al., 2002).

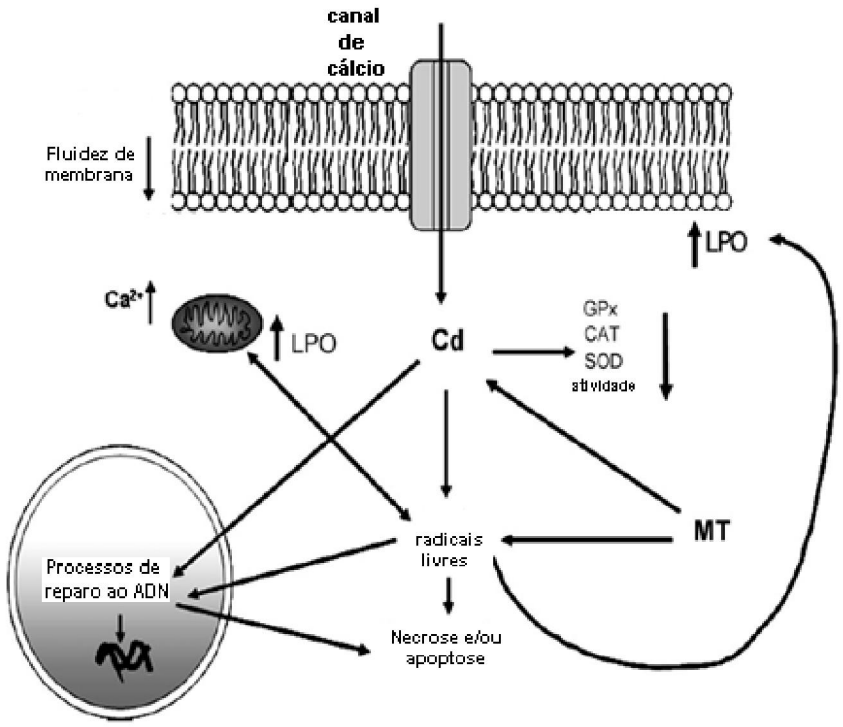

Figura 1. Ação de Cd por via direta e indireta. O processo de citotoxicidade de Cd centraliza-se na geração de radicais livres que irão, conseqüentemente, danificar importantes moléculas biológicas. *Adaptado de Méndez-Armenta & Rios, 2007.*

Entretanto, apesar da toxicologia do Cd centralizar-se sobre a produção indireta de radicais livres, Cd também pode apresentar efeito tóxico direto. Os mecanismos moleculares que caracterizam esta via de ação do metal ainda não são completamente entendidos, mas muitos trabalhos indicam que Cd pode agir por rotas comuns ao Zn (Brzóska & Moniusko-Jakoniuk, 2001), o que poderia acarretar em distúrbios celulares fundamentais. Uma importante observação disto é a mobilização de Cd em sítios moleculares onde Zn é o cofator natural. Por apresentarem propriedades químicas bastante similares, Cd compete e substitui Zn em sistemas biológicos, mimetizando deficientemente suas funções. Esta interação ocorre em fatores de transcrição dependente de Zn (*zinc-fingers*), metalotioneínas e metaloenzimas.

Zinc fingers são domínios presentes em uma classe de fatores de transcrição de genes associados à detoxificação de metais tóxicos, cuja estrutura é fundamentalmente mantida por Zn, mas por vezes pode ser substituído por Cd de maneira imperfeita (Hartwig,

1994). Já MT é uma proteína com papel importante na homeostase de Zn (Cousins, 1985; Vasak, 2005), e alguns trabalhos têm encontrado que Cd pode ser quelado junto a sua estrutura substituindo Zn (Liu et al., 1995; Vallee, 1979), o que, conseqüentemente, poderia causar problemas sobre a distribuição de Zn intracelular. Por fim, Cd também pode substituir e mimetizar as funções de Zn como cofator de enzimas, como tem sido observado para a δ-ALA-D (Tsukamoto et al., 1979; Sommer & Beyersmann, 1984), onde o metal pode causar distúrbios sobre a sintonia fina da rota metabólica, uma vez que causa diminuição da sua eficiência cinética.

1.2 Zinco

No grupo dos metais traço considerados essenciais para o organismo Zn é o segundo mais abundante, sendo estimado um total de 2,3g em todo o corpo humano, que contém Fe em maior quantidade, com aproximadamente 4g (Coleman, 1992). Em sistemas biológicos, Zn possui papel fundamental em um grande número de proteínas estruturais, fatores de transcrição e enzimas, sendo dividido em três categorias por conta das suas funções: estrutural, regulatório e catalítico (Russel et al., 2002; Cousins, 1994). Quando Zn possui papel constitutivo ele coordena com domínios protéicos, provocando o dobramento observado nos *zinc-fingers* e assim torna a molécula biologicamente ativa. Em funções regulatórias, Zn apresenta papel crucial em muitos processos biológicos como, por exemplo, expressão do gene da metalotioneína (Cousins, 1994; Dalton et al., 1997). Por fim, como componente catalítico, o metal realiza funções como cofator em centenas de metaloenzimas, tais como, anidrase carbônica, RNA polimerases e δ-aminolevulinato desidratase (Cousins, 1994; Gibson et al., 1955).

Por representar um elemento essencial para o funcionamento de diversas biomoléculas, Zn é fisiologicamente importante para os organismos, com papel na manutenção da integridade celular e em funções biológicas, como a síntese protéica, o metabolismo de ácidos nucléicos e, conseqüentemente, o ciclo celular (Beyersmann &

Haase, 2001; Favier & Hininger-Favier, 2005). Como fator de crescimento Zn é importante imunoregulador, e possui ação citoprotetora com ação antioxidante, antiapoptótica e anti-inflamatória (Zalewski et al., 2005). Ainda, especificamente no SNC, Zn age como um fator na sinalização intracelular, assim como neuromodulador sináptico extracelular (Frederickson, 1989a; Xie & Smart, 1991; Valle & Falchuk, 1993), cujo distúrbio sobre o metabolismo pode conduzir a injúria cerebral excitotóxica (Frederickson et al., 1989b).

Quanto à interação Zn e Cd, existem muitos estudos que mostram Zn como um atenuador da toxicidade causada por Cd. Estes trabalhos têm indicado que Zn reverte os principais efeitos tóxicos de Cd como, stress oxidativo (Jemai et al., 2007), dano testicular (Webb, 1982), carcinogênese (Gunn et al., 1964), citotoxicidade (Stacey & Klaassen, 1981) e letalidade (Probst et al., 1977). O mecanismo de tolerância ao Cd após suplementação com Zn ainda é pouco conhecido, porém acredita-se que a indução da síntese de MT por Zn possa ser o processo envolvido na proteção pelo metal essencial (Probst et al., 1977). Entretanto, outras investigações observaram mecanismos alternativos de tolerância à Cd que levam em conta sistemas independentes de MT, tais como, redução de captação de Cd (Enger et al., 1986) e outras proteínas que se ligam ao elemento tóxico (Waalkes et al., 1988). Alternativamente, por apresentarem propriedades químicas semelhantes, Zn poderia diminuir danos celulares devido à competição direta por sítios onde Cd exibiria sua toxicidade (Gachot & Poujeol, 1992; Endo et al., 1996).

I.3 δ-aminolevulinato-desidratase (E.C. 4.2.1.24)

δ-ALA-D é uma enzima presente nos mais diversos organismos e estruturas biológicas (por exemplo, fígado e cérebro). Em mamíferos a proteína se apresenta no espaço citosólico como uma tiol-enzima, Zn-dependente, sendo responsável pela produção de porfobilinogênio (PBG), um precursor do grupo heme (Gibson et al., 1955). A atividade da δ-ALA-D é altamente sensível a metais, que interagem com os grupos tióis ou com o cofator, Zn (Emanuelli et al.; 1998; Jaffe et al., 2000; Nogueira et al., 2003). Assim, Cd pode inibir a

atividade da enzima por oxidar os grupos –SH ou mesmo causar leve ativação, uma vez que o metal é capaz de substituir Zn como cofator da enzima (Sommer & Beyersmann, 1984; Davis & Avram, 1978; Wilson et al., 1972).

Os metais traço divalentes, em geral, apresentam um alto grau de reatividade no metabolismo celular. Devido à natureza química devem ser rigidamente controlados, para que permaneçam biologicamente "inertes" e não se associem de forma inespecífica a biomoléculas e, portanto, apenas um pequeno intervalo deve ser mantido sob a forma "solúvel". Em sistemas biológicos, geralmente estes elementos encontram-se sob a forma "não-disponível", por estarem forte e especificamente ligados a biomoléculas. Entretanto, mesmo metais associados a substâncias orgânicas podem estar numa forma "biodisponível", onde a ligação elemento-molécula é tão fraca que permite deslocar este metal para uma faixa solúvel. Por conta disto que a δ-ALA-D representa um potencial bioindicador da carga tóxica de elementos traço. Sua atividade é altamente sensível a metais como Hg, Pb e Cd, sendo em geral inibida em concentrações que vão de 10^{-7} à 10^{-5}M (Bellinaso, 1985). A enzima parcialmente inibida apresenta-se desta forma com seus grupos tiólicos oxidados, podendo este processo ser revertido com a adição de DL-ditiotreitol (DTT), um composto sulfidrílico com capacidade redutora (Granick et al., 1973). Assim, utilizando uma técnica, *in vitro*, que mede a atividade da δ-ALA-D parcialmente inibida e sua atividade recuperada com DTT, se obtém a diferença entre as duas incubações e, com isso, a expressão do efeito tóxico causado por metais na forma solúvel ou biodisponível (Perottoni et al., 2005). A Figura 2 mostra, esquematicamente, a influência que os metais exercem sobre a atividade da enzima devido a ligação em sistemas biológicos.

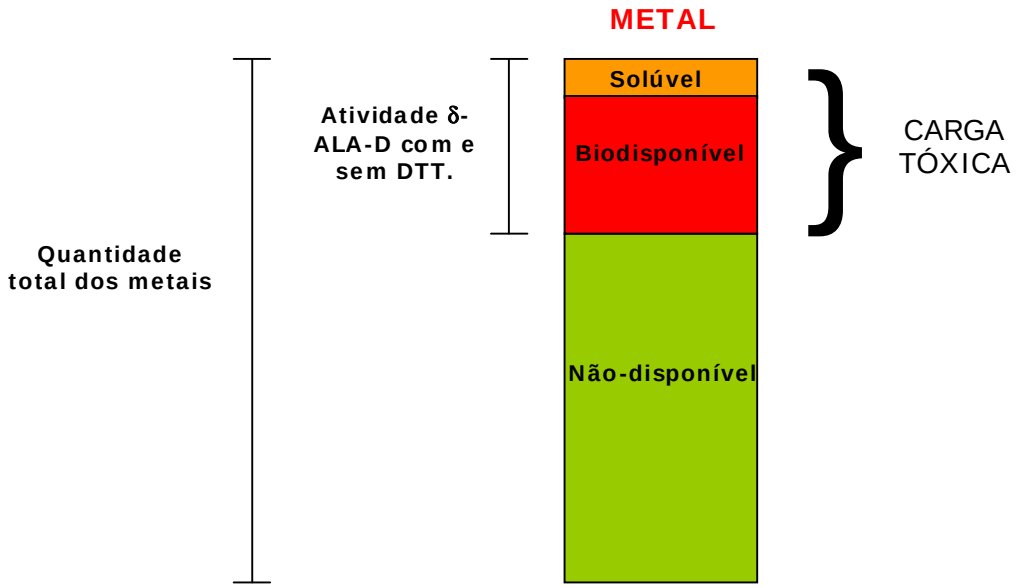

Figura 2. Formas químicas em que se apresentam os metais em sistemas biológicos e influência sobre a atividade da δ-aminolevulinato-desidratase.

I.4 Metalotioneínas

Em relação à importância que tem sobre o controle da viabilidade intracelular de metais, MT é apontada como importante alvo de ação na interação Zn-Cd. MT é uma proteína monomérica composta de dois domínios globulares com capacidade de se ligar a sete átomos de qualquer metal divalente (Romero-Isart & Vasak, 2002). O potencial de quelar metais é concedido pela presença do alto conteúdo de resíduos de cisteína em sua estrutura, possibilitando a formação de *clusters* tiol-metal.

MTs são constituídas por diversos tipos de seres vivos. Em mamíferos são encontradas em diferentes isoformas; tipo I e II presente em todos os órgãos, enquanto MT-III tem sido observada principalmente no cérebro e MT-IV é mais abundante em tecidos epiteliais estratificados (Moffatt & Denizeau, 1997). As diferenças entre os tipos de MTs não são meramente estruturais, e hoje se sabe que MT-I e MT-II podem ser induzidas por uma variedade de condições de stress e compostos, tais como, citocinas, espécies reativas de oxigênio e metais traço (Kägi, 1991), ao passo que, as isoformas III e IV não são responsivas por estes mesmos indutores. Sobre os metais, Zn e Cd são os mais fortes estimuladores da

síntese de MT-I e MT-II, com o Zn regulando a expressão através do fator de transcrição do tipo *zinc-finger* (MTF1 – *metal regulatory transcription factor 1*) (Auf der Maur et al., 1999; Heuchel et al., 1995), enquanto que Cd causa a indução por um outro tipo de fator nuclear (USF – *upstream stimulator factor*) (Li et al., 1998).

Ainda há muito para esclarecer sobre as funções da MT, mas devido à propriedade de quelar Zn, a proteína tem sido apontada como um importante controle na homeostase do metal essencial (Cousins, 1985; Vasak, 2005). Entre outras funções, MT é também capaz detoxificar Cd (Liu et al., 1995; Vallee, 1979), além de proteger contra stress oxidativo (Maret, 2006; Sato & Bremner, 1993; Thornalley & Vasak, 1985).

Quanto ao envolvimento da proteína em patologias, estudos mostram forte associação de MTs sobre a doença de Alzheimer. Neste distúrbio, as isoforma I e II apresentam níveis elevados (Duguid et al., 1989; Uchida, 1993; Adlard et al., 1998; Zambenedetti et al., 1998; Chuah & Getchell, 1999). Já MT-III (antigamente conhecida como fator de crescimento inibitório), as evidências apontam para uma deficiência desta isoforma em cérebro de indivíduos com Alzheimer (Uchida et al., 1991; Yu et al., 2001), o que reforça a hipótese de que a ausência deste subtipo da proteína poderia ser responsável por aberrações de neuritos, contudo a participação direta de MT-III na doença de Alzheimer tem sido bastante questionada (Erickson et al., 1994; Amoureux et al., 1997). Além disso, nos últimos anos tem se observado importante papel da proteína em alguns tipos de tumores. Aqui, é bastante evidente a participação de MT-I e MT-II por conta da modificação de seus níveis em células cancerígenas, incluindo hepatoma e glioma, havendo uma forte correlação negativa entre a mortalidade e os níveis da proteína presentes nestas células (Pedersen et al., 2009), o que colabora com a maior resistência ao tratamento radio- e quimioterápico, uma vez que possuem propriedades citoprotetivas e antioxidantes (Theocharis et al., 2003, 2004; Nielsen et al., 2007).

2. OBJETIVOS

Objetivo Geral: Investigar o mecanismo da ação tóxica de Cd sobre rotas metabólicas comuns ao Zn, em fígado e SNC de ratos adultos, através da avaliação de potenciais alvos moleculares (δ-ALA-D, MT), assim como o acúmulo destes metais, após exposição *in vivo*.

Objetivos Específicos:

Capítulo I – Reproduzir e aprofundar a investigação da interação Zn-Cd em fígado de ratos, uma vez que é um importante tecido para ação do Cd, servindo como comparação para posterior estudo em tecido nervoso (Capítulo II).

Capítulo II – Identificar possíveis danos sobre o metabolismo de Zn em cérebro de ratos por conta da exposição à Cd, levando em conta a proteção exercida pela barreira hemato-encefálica (BHE) sobre o transporte dos metais até o SNC.

PARTE II

CAPÍTULO I

Hepatic Cd-dependent Zn deposition and modulation of the δ-aminolevulinate-dehydratase activity and metallothionein levels

Artigo em preparação para ser submetido ao periódico *Toxicology and Applied Pharmacology*

Hepatic Cd-dependent Zn deposition and modulation of the δ-aminolevulinate-dehydratase activity and metallothionein levels

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Abstract

Cadmium (Cd) is an environmental pollutant that can be harmful to human and animals. Liver represents an important target for Cd accumulation in the body and, consequently, for its toxicity. Cd can exert its toxicity via disruption of normal Zn homeostasis. Here we examined the Zn-Cd interaction to determine how these two metals could affect δ-aminolevulinate-dehydratase (δ-ALA-D) and MT, two potential molecular endpoints for Cd hepatotoxicity. Cd exposure (0.25 and 1 mg/kg body weight, i.p., for 10 days) caused a marked increase in hepatic Zn deposition, as well as co-administration with Zn (2 mg/kg, i.p.) intensified this effect. Cd also caused a dose dependent increase in hepatic Cd content that was not modified by co-exposure to Zn. Zn treatment increased hepatic δ-ALA-D activity and Cd had the same effect, although the increase caused by Cd was less marked. Reactivation index of δ-ALA-D by DTT was decreased by Zn and Cd exposure, which indicates that Cd and mainly Zn protected enzyme from oxidation. Hepatic metallothionein (MT) was increased only after exposure to 1 mg/kg Cd and simultaneous exposure to Zn reduced the stimulation of MT synthesis. The results here presented clearly indicate that Cd can redistribute Zn from non-hepatic tissues to liver and this added to the modulation of the δ-ALA-D and MT indicate similar pathways in Cd hepatoxicity and Zn-homeostasis. Moreover the whole results indicate surprisingly negative action of MT in Cd-Zn interaction.

Keywords: cadmium, zinc, metallothionein and δ-aminolevulinate-dehydratase

Introduction

On account of its large scale industrial use (Page et al. 1986), cadmium (Cd) is a pollutant widespread in the environment (World Health Organization, 1992). Cd poisoning is harmful to many human and animals tissues and the liver represents an important target for Cd accumulation in the body (Frazier and Puglese, 1978). Cd hepatotoxicity can be associated with an over production of reactive oxygen species (ROS) (Eaton et al., 1980; Goering et al. 1993), lipid peroxidation (Manca et al. 1991; Muller, 1986), carcinogenesis (Waalkes and Rehm, 1994), and modification of the expression of proteins (Beyersmann and Hechtenberg, 1997).

Currently, the exact mechanism(s) involved in Cd toxicity is(are) not yet clear, but chronic Cd exposure causes disturbances on metabolism of zinc (Zn) (Bonner et al., 1980b; Brzóska et al., 2001, 2005; Brzóska and Moniuszko-Jakoniuk, 2004a, 2005c; Kalużyński et al., 2003; Mahaffey et al., 1981; Noel et al., 2004; Ogoshi et al., 1992). Consequently, negative interactions between these metals can cause disturbances in cell biology, because Zn is an essential element for over three hundred metalloenzymes (Vallee and Auld, 1990), and has important role in the cell cycle and in the expression of many proteins (Beyersmann and Haase, 2001).

Mammalian δ-aminolevulinate dehydratase (δ-ALA-D) is cytoplasmatic thiol- and Zn-dependent enzyme that is responsible for the production of porphobilinogen (PBG), a heme precursor (Gibson et al., 1955). δ-ALA-D activity is highly sensitive to metals that interact with thiols or Zn (Emanuelli et al., 1998; Jaffe et al., 2000, Nogueira et al. 2003). Accordingly, δ-ALA-D can be inhibited by Cd either via oxidation of -SH groups (Emanuelli et al., 1998; Jaffe et al., 2000) or competition with Zn (Tsukamoto et al., 1979; Sommer and Beyersmann, 1984). However, at lower concentrations this metal can replace Zn as a cofactor in mammalian enzyme though the catalytic efficiency is considerably reduced (Sommer and Beyersmann, 1984).

Metallothionein (MT) is a protein rich in cysteine residues, with is thought to be involved in detoxication of Cd (Liu et al., 1995; Vallee, 1979), homeostasis of Zn (Cousins, 1985; Vasak, 2005) and protection against oxidative stress (Maret, 2006; Sato and Bremner, 1993; Thornalley and Vasak, 1985). In addition to chelate Cd and Zn, MT I and II, the two isoforms found in hepatic tissue, they may have expression induced by the two metals (Tandon et al., 2001).

This work seek for further understanding about the interaction of Zn and Cd, particularly, we were interested in determining on how these two metals could affect δ-ALA-D and MT, two important molecular targets for Cd hepatotoxicity. Here we have investigated the potential hepatoxic effects that could result from subchronic Cd intoxication associated or not with Zn supplementation. The study of Cd and Zn interaction are of particular importance, in view of the fact that literature have provided some points of evidence indicating that Cd can compete with Zn and impairs its function (Bauer et al., 1980; Coogan et al., 1992; Gachot and Poujeol, 1992); however, to the best of our knowledge, literature have not investigated simultaneously the participation of hepatic MT and δ-ALA-D on the break of Zn-homeostasis caused by Cd. For this purpose, content of Zn and Cd were measured after *in vivo* exposure to these metals, and δ-ALA-D activity and MT levels were used as molecular endpoints of Cd hepatotoxicity.

Material and Methods

Animals

Male Wistar rats (± 240 g and ± 80 days) were used. The animals were housed 3 for cage at a constant room temperature (22 ºC) under a 12 hr light: 12 hr dark cycle. Food and water were provided *ad libitum*. Animals were cared in accordance with the “National Institutes of Health Guide for Care and Use of Laboratory Animals” and all experiments were approved by our ethics committee for animal use at the Federal University of Rio Grande do Sul.

Chemicals

Cadmium acetate dihydrate was obtained from Baker Analysed (Deventer, Netherlands) and zinc acetate dihydrate from Riedel-de Haën (Seelze, Germany); mercury chloride, trichloroacetic acid, *p*-dimethylamino benzaldehyde, glacial acetic acid and perchloric acid from Merck (Darmstadt, Germany); δ-aminolevulinic acid, DL-dithiothreitol, metallothionein-I from rabbit liver, *o*-phenylendiamine, thiobarbituric acid and malonaldehyde bis- (dimethyl acetal) were purchased from Sigma (St. Louis, MO, USA); monoclonal mouse anti-metallothionein, which recognizes rat MT-I and MT-II, and peroxidase-conjugated to goat anti-mouse IgG as secondary antibody were purchased from Dako Corporation (Carpinteria, CA, USA.). The other reagents used were of analytical grade and were obtained from commercial suppliers.

Treatment and Tissue preparation

Rats were randomly divided into 6 groups. Based in literature (Hopf et al., 1990; Kown et al., 2000) the rodents were treated for 10 days with intraperitoneal injections of: 1) NaCl 0.9 % (control group); 2) 2 mg/kg of Zn (equivalent to 6.71 mg/kg of zinc acetate); 3) 0.25 mg/kg of Cd (equivalent to 0.59 mg/kg of cadmium acetate); 4) 1 mg/kg of Cd (equivalent to 2.37 mg/kg of cadmium acetate); 5) 0.25 mg/kg of Cd plus 2 mg/kg of Zn; 6) 1mg /kg of Cd plus 2 mg/kg of Zn. At 24 hrs after the last injection animals were sacrificed and the whole liver was rapidly removed separating a part for biochemical analyses and other part for investigate Zn and Cd content. The hepatic tissue was placed on ice and homogenized in saline (1:7) and centrifuged at 4000 g for 10 min. The supernatant was used for quantification of δ-ALA-D activity and MT immunocontent.

Cd and Zn Quantification

Determination of total Zn and Cd in hepatic samples was performed following US EPA method 3052 (USEPA, 1996). The samples were digested with nitric acid and hydrogen peroxide in boiling water. Extreme care was taken to avoid samples contamination and only reagents with low background impurities were used. Zn concentrations were determined using a Perkin-Elmer 3300 flame atomic absorption spectrometer and Cd levels were estimated with a Perkin-Elmer SIMAA-6000 graphite furnace atomic absorption spectrometer. The minimum

detection limits achieved were 0.495 and 0.008 μg/g for Zn and Cd. All analyses were realized at Ecology Center of Federal University of Rio Grande do Sul.

δ-ALA-D activity

Liver δ-ALA-D activity was determined based on the method previously described by Sassa (1982) with few modifications. The rate of porphobilinogen (PBG) formation was measured in a medium containing 3 mM δ-ALA and 80 mM sodium phosphate buffer, pH 6.4, at 37ºC. Study on the reactivation of the enzyme was also performed in the presence of 2 mM of DTT (Perottoni et al., 2005). After 30min of incubation, the product PBG was determined using Ehrlich's reagent at 555 ηm, with a molar absorption coefficient of 6.1×10^4 M^{-1} for the Ehrlich-PBG salt.

Metallothionein Enzyme Linked Immunoassay

Metallothionein was determined with the method described by Cousins (1991) with some modifications. The homogenate of liver was kept at -20 °C until use. The whole experiment was conducted at room temperature with 96-well microtiter plates and MT-I was used as standard. Wells with standard and samples were incubated for 1 h and washed three times with phosphate buffered saline (PBS) plus 0.05 % Tween 20 (washing solution). Then 1 % bovine serum albumin (BSA) was applied in wells to block non-binding sites for 30 min and washed three times. Primary antibody was added to each well and incubated for 30 min, then washed three times. Secondary antibody conjugated with horseradish peroxidase was incubated for 30 min and after washed three times. Finally, solution of *o*-phenylendiamine was added to each well for reaction with the peroxidase. After of 30 min, HCl 3 M was added to stop the reaction, and the plates were read at 490 ηm. The values were expressed as μg MT/mg protein.

Protein Assay

Protein was measured by the method of Lowry et al. (1951), which BSA was used as standard.

Statistical analysis

Statistics data were obtained for SPSS 15.0 software and reported as mean ± SEM. Changes body weight due to treatment were analyzed with a three-way ANOVA. Two-way ANOVA was applied to measure differences in biochemical parameters. Further group differences were investigated by Duncan's multiple range test with $p < 0.05$ considered as statistically significant.

Results

Development of the rats was not modified by Zn, whereas 1 mg of Cd reduced body weight and caused the death of two rats in this group (**Table 1**). Zn co-treatment decreased the body weight loss caused by Cd and no other overt signs of toxicity were observed. The extension of metals effects on body weight of the rats is indicated by three-way ANOVA analysis. Cd showed main effect on animals body weight ($F = 192.18; p < 0.001$), although all variables isolated or together presented effect significant on clinical board (except *Zn x day*).

Hepatic content of Zn was increased about two times after treatment with 2 mg of Zn. Interestingly, isolated exposure with Cd caused an increase in hepatic content of Zn and this was significantly higher than that provided by Zn-treatment as observed in 1 mg of Cd. In relation to comparison between similar Cd groups (with or without Zn), the metals together did significantly increase the deposition of Zn (**Figure 1**). The evaluation of the variables force revealed significant effect to Zn and Cd on Zn content (respectively, $F = 50.97; p < 0.001; F = 126.95; p < 0.001$).

Cd was not detected in the liver of control group or in animals exposed to Zn (**Figure 2**); however, hepatic Cd content increased after cadmium acetate treatment and the deposition was dose dependent. Co-administration of Zn did not modify the profile and the level of Cd accumulation. Analysis of effects on dependent variable indicated just significant effect of Cd ($F = 182.99; p < 0.001$)

In order to obtain information about the possible intracellular effect of Cd and Zn, hepatic δ-ALA-D was determined (**Figure 3**). All groups exhibit a significant increase in δ-ALA-

D activity in comparison to the control; however groups treated with Zn showed the highest effects. Also there was not difference between similar Cd groups. Moreover, enzyme reactivation by DTT (**Figure 4**) decreased significantly in all groups which Zn treated group had this effect more pronounced. The two-way ANOVA to δ-ALA-D activity showed significant main effect of Zn ($F = 23.36$; $p < 0.001$); however interaction between metals also represented important action on dependent variable ($F = 3.22$; $p = 0.051$). Similarly, the assessment to reactivation index indicated both Zn and metals interaction as main effects (respectively, $F = 5.55$; $p < 0.05$; $F = 5.36$; $p < 0.01$).

Zn did not change MT content. Similarly, 0.25mg of Cd alone or together with Zn did not modify MT levels (**Figure 5**). However, there was a significant increase hepatic of MT in rats exposed to 1 mg of Cd or to 1mg of Cd plus Zn; however, the MT levels in Cd plus Zn were about 1/3 of that in the group exposed only to 1mg of Cd. Statistical analysis of variable effects on MT pointed main effect to Cd ($F = 450.37$; $p < 0.001$); however Zn and Cd-Zn interactions had significant action (respectively, $F = 170.29$; $p < 0.001$; $F = 124.98$; $p < 0.001$).

Discussion

Cadmium can cause severe toxicity in rodents, such as changes in development and even mortality (Li and Lim, 2007; Waalkes and Rehm, 1994). Similar results were obtained here, since highest dose of Cd caused mortality and a reduction in body weight. Furthermore, Zn co-administration attenuated the Cd toxicity, which has also been reported in literature (Jemai et al., 2007). Thus, this supports the idea that there is overlap between the toxic and physiological mechanisms designed for each element.

In literature there are evidences that Cd causes accumulation of Zn in the liver (Brzóska et al., 2000; Eybl et al., 1998; Oishi et al., 2000) and thus we quantified both metals. Interestingly, our results show that Cd induced a strong increase of Zn in the tissue. In view of this, we can suppose that Zn had reduced excretion or even was mobilized from other sources into the liver, such as food or peripheral tissues. In regard to increased mobilization of Zn from

diet, this seems unlikely, since no change on the consumption of food was observed by us (data not shown) and there is no study that has evaluated alteration on intestinal Zn absorption due to Cd. Here, the source of Zn retained in the liver seems to be from peripheral tissues, because no change in Zn excretion has been reported after of Cd exposure, while transport from other tissues into liver has been observed (Brzóska et al., 2000; Oishi et al., 2000). In respect to the protective effect performed by Zn treatment (associated with increase of its levels), it could provide a consequent decrease in the retention of Cd, but this was the opposite of the observed and the element accumulated in liver independent of the presence of Zn. In previous studies both metals have been suggested compete in many biological sites (Asmuss et al., 2000; Gachot and Poujeol, 1992) and such specific interaction would be explained due to similar chemical properties (Bin and Garfinkel, 1994). Then, increased Zn levels on account of Cd treatment could represent a competitive effect between the metals, which the essential element would be transferred from non-hepatic tissues into the liver to counterbalance with the higher accumulation of Cd and thus partly would neutralize the toxic effects.

Although hepatotoxic effect induced by Cd has been extensively investigated, the mechanism of accumulation of Zn caused by it remain unclear and, therefore, can not be ensured certainly what biological functions are affected and what the main cellular compartment that the metals act in these context. δ-ALA-D has brought us some answers about it. The activity of the enzyme showed modifications associated with all treatments, which corresponded to the entry and the triggering of responses by metals in intracellular space. Disturbance performed by Cd usually cause inhibition of δ-ALA-D activity (Nogueira et al., 2003; Santos et al., 2005), despite this we found outcome opposite to these. Nevertheless, this controversial result has been shown by other study (Folmer et al., 2004), of which attributed to a protection against oxidation generated by Cd or an induction of synthesis of the enzyme. Furthermore, reported data show also the metal to be able to mimetize and replace Zn as cofactor of the enzyme (Sommer and Beyersmann, 1984). This does not allow us to say with certain what of these processes predominated. However, lower enzyme reactivation by

DTT associated to the treatments gives greater security to affirm that there was important metal-induction in the kinetic velocity caused for Cd and mainly Zn, whereas addition in the number of enzymatic units would not be consistent to explain these results.

MT is a protein with features that cross as Cd detoxification as Zn-homeostasis and, hence, its expression is expected to be strongly induced by both metals (Tandon et al., 2001). Zn is a classical inducer of MT, even so, our experimental conditions did not observe this and only with higher dose of Cd there was increase in the expression of MT. Many authors explain Cd induction due to its chelation to the protein (Din and Frazier, 1985; Friberg et al., 1974; Onosaka and Cherian, 1981). Then, for this reason, would be expected to obtain a greater amount of MT in the group that received this same dose of Cd with Zn to adapt this result to our observations. However, unexpectedly, we found in these animals MT levels almost three times lower when compared to the group that received only Cd. Here, the partial reversal of the toxic effect of Cd by essential metal seems obvious, since Zn content was significantly different between these groups and treatment with Zn alone not altered the levels of MT. These results show that MT may have performed a negative role in those animals exposure to 1 mg of Cd which this is surprising because few works has observed similar effect (Liu et al., 1995; Webb and Etienne, 1977) and this makes necessary investigate further its relevance as a molecule not only protective, but also with toxic action. However, due to MT be responsible for intracellular Zn distribution (Brady, 1982; Ou and Ebadi, 1992), we can suppose that the toxic role played by elevated levels of the protein was to become more difficult the access to the cellular Zn; the presence of higher amount of MT was achieved to detoxify the Cd, but this increased the probability of Zn be bonded to it and, consequently, cause disturbs on metal-homeostasis due to false depletion of the element. Also the process above described, on accumulation of Zn hepatic triggered by Cd, could demonstrate a physiological response in favor of this assumption.

In summary, the results presented indicate that hepatic deposition of Zn is markedly modified by Cd co-administration. This added to the modulation of the two molecular targets used here (δ-ALA-D and MT) indicate similar pathways to Cd hepatoxicity and Zn-

homeostasis. Moreover, this work contributed with surprisingly negative role played by the MT, which this makes necessary many investigations to point the size of the impact that MT has on the uptake and cellular distribution of Zn. Finally this would become further clarified a critical point in Zn-Cd interaction, since the protein has functions in Cd detoxification and intracellular Zn regulation.

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Table

Table 1

Body weight in 1st and 11th day of treatment with Zn and Cd.

	1st day	11th day
control	230.8 ± 3.9	257.7 ± 6.0 # *
2 mg Zn	243.4 ± 2.8	243.8 ± 4.5
0.25mg Cd	239.0 ± 2.4	239.8 ± 4.1
0.25mg Cd + 2mg Zn	243.3 ± 2.6	244.3 ± 3.6
1mg Cd	237.4 ± 1.8	202.9 ± 4.0 # ‡
1mg Cd + 2mg Zn	235.2 ± 2.3	218.3 ± 2.5

Values are indicated as mean ± S.E.M. in gram (n = 9). 1 mg of Cd group presented two deaths (n = 7 in 11th day). # Significantly different of respective group in first day. $^{*}p < 0.05$ *and* $^{\ddagger}p < 0.001$.

Figure Legends

Figure 1. Hepatic concentration of zinc after exposure to Zn and/or Cd. Adult rats were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 6. Columns not sharing the same letter were different at $p < 0.05$ (Duncan's multiple range test).

Figure 2. Hepatic concentration of cadmium after exposure to Zn and/or Cd. Adult rats were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 5-6. Columns not sharing the same letter were different at $p < 0.05$ (Duncan's multiple range test).

Figure 3. Hepatic δ-ALA-D activity of rats after exposure to Zn and/or Cd. Adult rats were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 7-9. Columns not sharing the same letter were different at $p < 0.05$ (Duncan's multiple range test).

Figure 4. Reactivation index of hepatic δ-ALA-D by DTT after exposure to Zn and/or Cd. Adult rats were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 7-9. Columns not sharing the same letter were different at $p < 0.05$ (Duncan's multiple range test).

Figure 5. Hepatic concentration of MT of rats after exposure to Zn and/or Cd. Adult rats were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 5-9. Columns not sharing the same letter were different at $p<0.05$ (Duncan's multiple range test).

Fig. 1

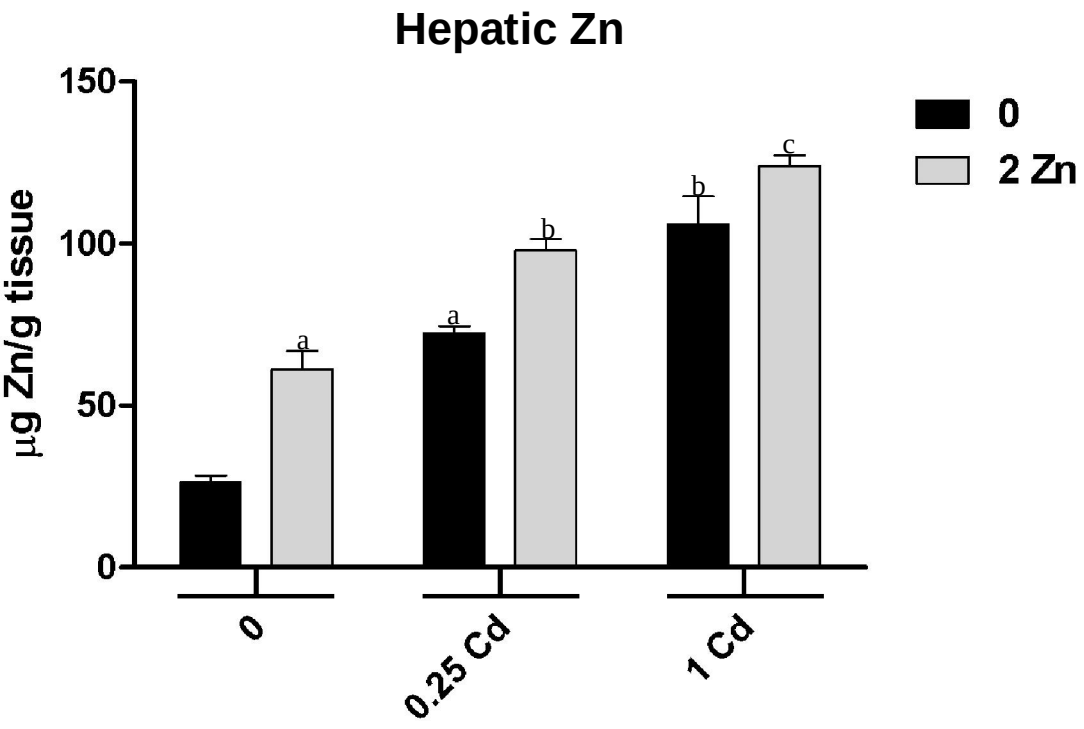


Fig. 2

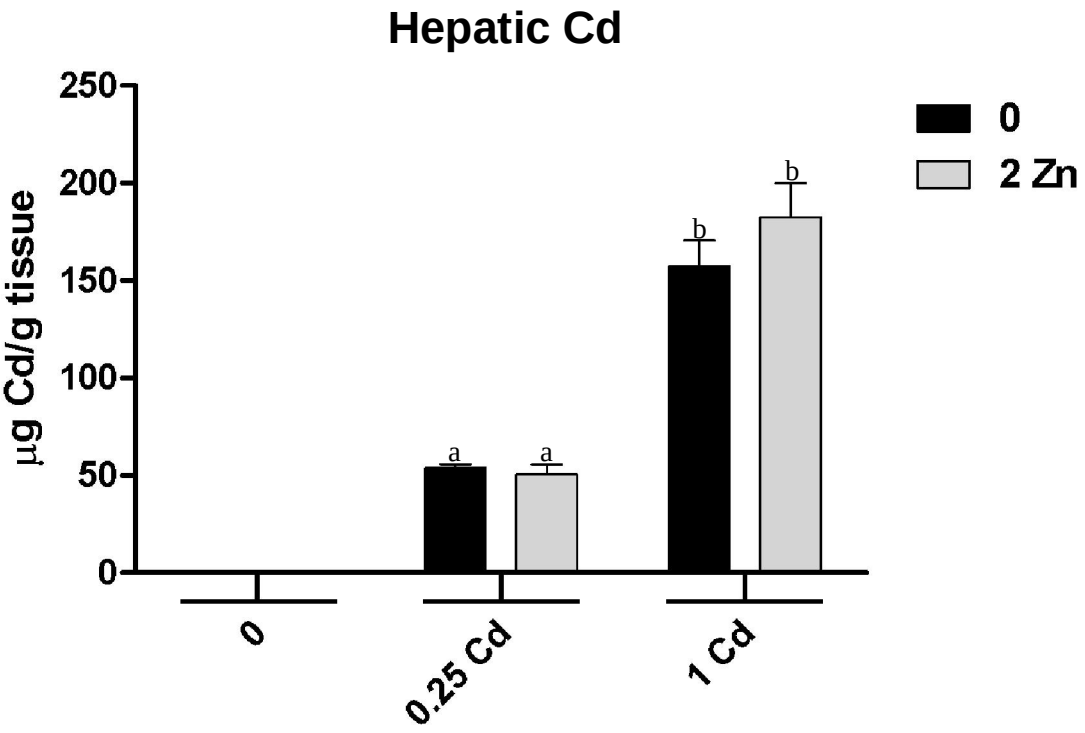

Hepatic Cd
µg Cd/g tissue
250
200
150
100
50
0
0
2 Zn
a
a
b
b
0
0.25 Cd
1 Cd

Fig. 3

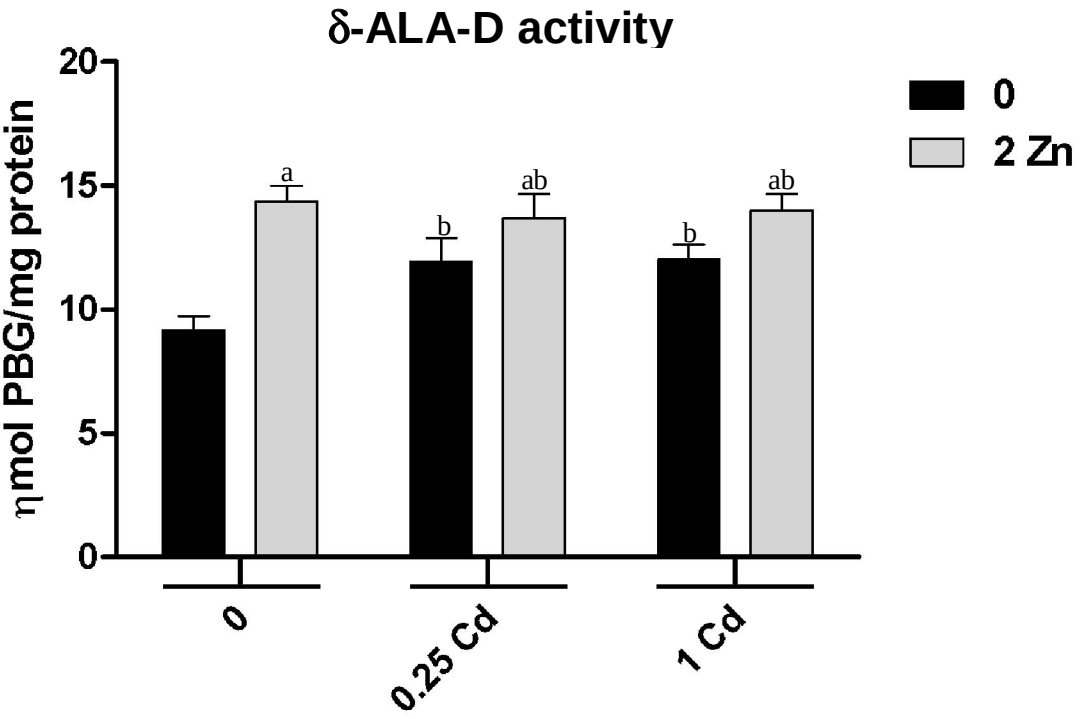

δ-ALA-D activity
ηmol PBG/mg protein
20
15
10
5
0
a
b
ab
b
ab
0
0.25 Cd
1 Cd
0
2 Zn

Fig. 4

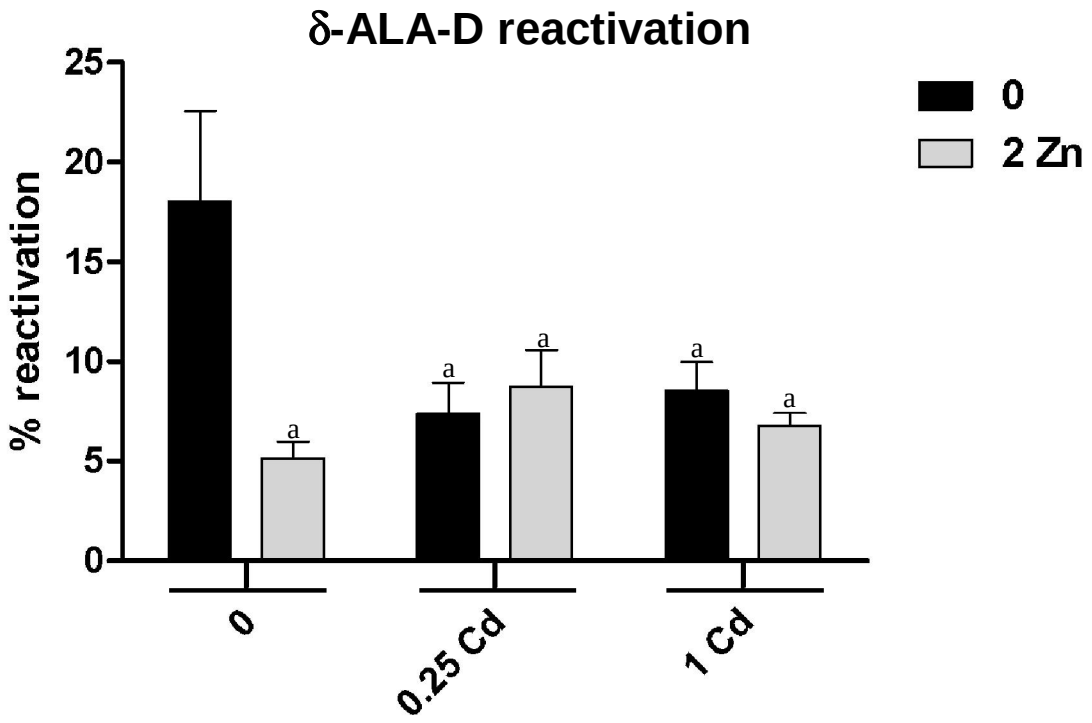
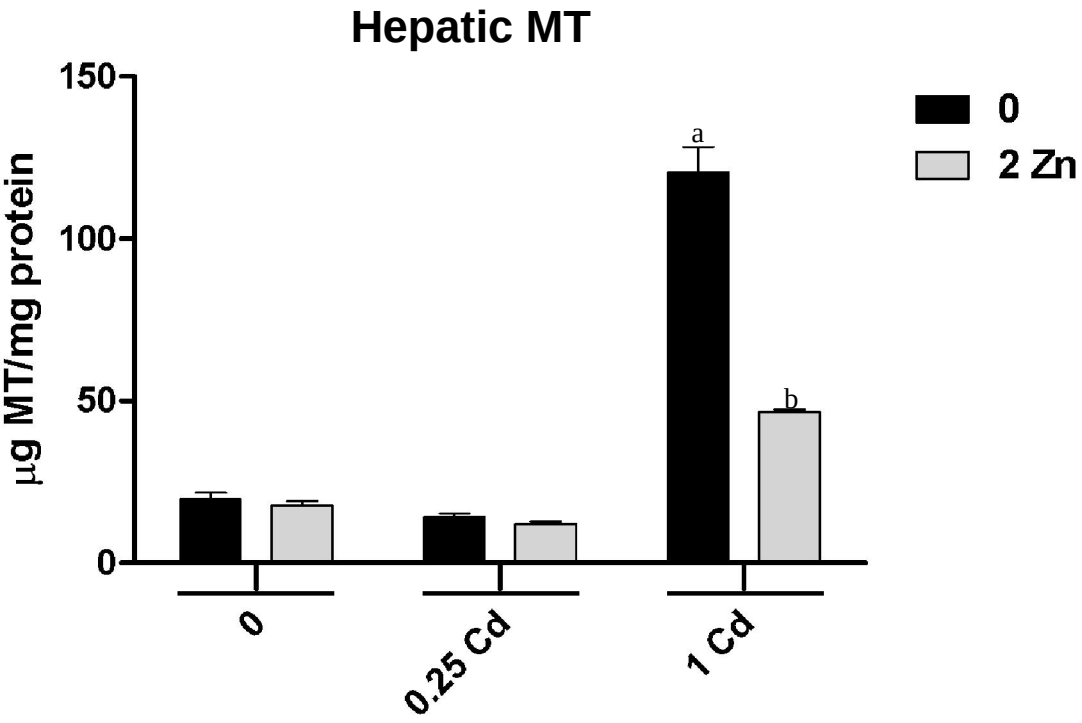

δ-ALA-D reactivation
% reactivation
25
20
15
10
5
0
a
a
a
a
0
2 Zn
0
0.25 Cd
1 Cd

Fig. 5



CAPÍTULO II

Effect of cadmium on metallothionein levels and δ-aminolevulinate-dehydratase activity in the nervous tissue

Artigo em preparação para ser submetido ao periódico *Toxicology and Applied Pharmacology*

Effect of cadmium on metallothionein levels and δ-aminolevulinate-dehydratase activity in the nervous tissue

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Abstract

Cadmium (Cd) is a pollutant widespread in environmental which it has neurotoxic effects. Recently, the mechanism of Cd action has been appointed by similar zinc (Zn) pathway indicating an interaction between the metals on biological systems; however this association was more observed in peripheral tissues than in central nervous system. Thus, the present study was designed to investigate Zn-Cd interaction in nervous system. Adult rats were exposed to Zn (2 mg/kg/day) and/or to different doses of Cd (0.25 and 1 mg/kg/day). After ten days of treatments, animals were killed and the whole brain was used for assess Zn and Cd content, as well as parameters biochemical, such as δ-aminolevulinate-dehydratase activity (δ-ALA-D), metallothionein levels (MT) and the rate of lipoperoxidation. The amount of Zn in the brain did not modify between treatments, while Cd was slightly increased in Cd-exposure animals. δ-ALA-D had activity induced mainly for Zn-treatments and this corresponded to an elevation in expression of the enzyme. The levels of MT showed no interference on Zn-treatments, but Cd was surprisingly able of reduce its levels in association with 0.25 mg of Cd, while 1 mg of Cd caused an increase. These results related with TBARS levels indicated that the nervous tissue was protected of excessive oxidative-stress performed by Cd, when its levels were higher. The overall results indicated be remote the Zn-Cd interaction in brain because Zn did not accumulate in the tissue. However this study become more clarified the biochemical effects in the brain of adult rats after exposure to Cd, as well as it is the first to show a dual effect of Cd on MT.

Keywords: cadmium, zinc, metallothionein and δ-aminolevulinate-dehydratase

Introduction

Cadmium (Cd) is non-essential metal widespread in environmental due to industrial utilization in large scale (World Health Organization, 1992). This element is extremely toxic to human heath and it presents long biological half-life (Goyer, 1991). Cd has been shown harmful in the central nervous system (CNS) and neurological effects performed by Cd include changes in neurochemistry (Carageorgiou et al., 2004; Hobson et al., 1986; Luchese et al., 2007; Minami et al., 2001) and influence on memory formation (Lukawski et al., 2005). Nevertheless, the molecular mechanisms involved in neurotoxic effects of Cd are not completely understood. In peripheral tissues Cd has been suggested as antimetabolite of zinc (Zn), because it acts in common pathways to the essential metal (Brzóska and Moniusko-Jakoniuk, 2001). Consequently, the extent of this interaction to the nervous tissue could cause serious problems to the functioning of the CNS, since Zn has important role as an intracellular signal factor as well as an extracellular synaptic modulator (Frederickson, 1989a; Xie and Smart, 1991; Valle and Falchuk, 1993) and disturbs on its metabolism may lead to excitotoxic brain injury (Frederickson et al., 1989b).

δ-aminolevulinate dehydratase (δ-ALA-D) is an important enzyme for evaluate exposure to divalent metals such as Zn and Cd. It is a cytoplasmic thiol-enzyme, Zn-dependent, with role in the porphobilinogen production (PBG), a heme precursor (Gibson et al., 1955). The δ-ALA-D is widely found in the body and its activity is sensible to Cd due to oxidation of structural –SH groups (Emanuelli et al., 1998; Jaffe et al., 2000) or even because Cd is able to replace Zn as cofactor of the enzyme (Sommer and Beyersmann, 1984).

The control on intracellular metals availability is appointed as the main characteristic of metallothionein (MT). This protein has high content of cysteine residues and thus it has capacity of detoxify Cd (Liu et al., 1995; Vallee, 1979), participate of the metabolism of Zn and Cu (Cousins, 1985; Vasak, 2005) and protect against oxidative stress (Maret, 2006; Sato and Bremner, 1993; Thornalley and Vasak, 1985). MTs are presents in mammalian cells in different isoforms; in the CNS, type I and II are not present in neurons but abundant in

astrocytes (Aschner, 1996; Hidalgo et al., 2001), while MT-III has been observed both cells (Maters et al., 1994; Uchida et al., 1994).

In this paper, we studied whether even with protection of blood-brain barrier (BBB) Cd is able of cause toxic effects on brain. An initial study conducted by us in liver tissue has noted the action of Cd on metabolism of Zn (Braga et al., in preparation) and thus we try to extend these findings to the CNS. In order to evaluate this mechanism was measured in adult rats the content of Zn and Cd after exposure *in vivo*. Also was tested the activity of the δ-ALA-D, the content of MT and rate of lipid peroxidation by levels of thiobarbituric acid-reactive substances (TBARS).

Material and Methods

Chemicals

δ-aminolevulinic acid (δ-ALA), DL-dithiothreitol, metallothionein-I from rabbit liver, *o*-phenylendiamine, thiobarbituric acid (TBA) and malonaldehyde bis- (dimethyl acetal) were obtained from Sigma (St. Louis, MO, USA); monoclonal mouse anti-metallothionein-I/II, and peroxidase-conjugated to goat anti-mouse IgG as secondary antibody were purchased from Dako Corporation (Carpinteria, CA, USA.).

Animals and treatments

Male Wistar rats with approximately 80 days (± 240 g) were maintained at a constant room temperature (22 ºC) under natural lighting conditions with water and food provided *ad libitum*. The care of animals was followed in accordance with the "National Institutes of Health Guide for Care and Use of Laboratory Animals".

According to the literature (Hopf et al., 1990; Kown et al., 2000), rats were randomly separated into six groups and injected daily via i.p. with: 1) NaCl 0.9 % (control group); 2) 2 mg/kg of Zn (equivalent to 6.71 mg/kg of zinc acetate); 3) 0.25 mg/kg of Cd (equivalent to 0.59 mg/kg of cadmium acetate); 4) 0.25 mg/kg of Cd plus 2 mg/kg of Zn; 5) 1 mg/kg of Cd (equivalent to 2.37 mg/kg of cadmium acetate); 6) 1mg /kg of Cd plus 2 mg/kg of Zn. The

treatment was extended by 10 days and all groups exposed to cadmium and zinc received the metals in the form of acetate together with control vehicle saline. Twenty-four hours after the last injection animals were weighted and killed, and then whole brain was removed and separated in two parts to trace elements analyses and evaluation of biochemical parameters. This last fraction was placed on ice and homogenized in saline (1:5) and centrifuged at 4000 x g at 4 ºC for 10 min to yield the supernatant that was used in the evaluation of the δ-ALA-D activity and content of MT and TBARS.

Zn and Cd determination

The total Zn and Cd present in brain was determined by atomic absorption spectrometry. Zn was determined by a Perkin-Elmer 3300 flame atomic absorption spectrometer and Cd levels were estimated with a Perkin-Elmer SIMAA-6000 graphite furnace atomic absorption spectrometer. The whole process of analysis was based on US EPA method 3052 (USEPA, 1996) with few modifications and all care was taken to avoid samples contamination. The minimum detection limit achieved was 0.495 and 0.008 μg/g for Zn and Cd. All analyses were realized at Ecology Center of Federal University of Rio Grande do Sul.

δ-ALA-D activity

Enzyme activity was evaluated as previously described (Sassa, 1982). The rate of product porphobilinogen (PBG) formation was obtained with a medium containing 3 mM δ-ALA mixed with 80 mM sodium phosphate buffer (pH 6.4) and then incubated at 37 ºC for 2 hrs. Reactivation of the δ-ALA-D was performed with addition of 2 mM DTT to verify the percentage of inhibition of the enzyme caused by treatments (Perottoni et al., 2005). The product PBG was determined using Ehrlich's reagent at 555 ηm, with a molar absorption coefficient of 6.1×10^4 M^{-1} for the Ehrlich-PBG salt.

Metallothionein content

MT levels were determined through an ELISA described by Cousins (1991), which we extend to the study of brain. The tissue fraction was kept at -20 °C until use. The whole experiment was conducted at room temperature in 96-well microtiter plates and MT-I was

used as standard. Wells were coated for 1 h with standard and samples and then washed three times with phosphate buffered saline plus 0.05 % Tween 20 (washing solution). Blocking solution (1 % BSA) was applied for 30 min and plate was washed three times. Primary antibody (1:5000) was added to each well and incubated for 30 min and then washed three times. Polyclonal anti-MT-I/II antibody conjugated with horseradish peroxidase (1:5000) was added for 30 min and after washed three times. Finally, *o*-phenylendiamine was incubated in the dark for 30 min for reaction with the peroxidase. After this, 3 M HCl was added and the plate was measured in 490 ηm. The values were expressed as μg MT/mg protein.

Thiobarbituric acid-reactive substances

Index of lipid peroxidation by formation of TBARS was measured in according to Draper and Hadley (1990) with some adaptations. Supernatant of samples were precipitated with 15 % TCA and centrifuged at 4000 x g for 10 min. In 96-well microtiter plates were placed samples deproteinized and the standard malondialdehyde (MDA). Then 0.67 % TBA was added to microplate and after heated in boiling water bath for 30 min. TBARS were determined at 532 ηm of absorbance and the values were expressed as μmol MDA equivalents/mg protein.

Protein concentration

The protein content was estimated by the method of Lowry et al. (1951) using BSA as standard.

Statistical analysis

Experimental results are reported as mean ± S.E.M. Statistical analysis for STATISTICA 5.1 software (Statsoft, Tulsa, OK, USA) was performed on changes of body weight of the animals with an ANOVA for repeated measures. Two-way ANOVA was applied to measure differences in biochemical parameters. Differences in groups were investigated with the Tukey Post Hoc test and $p < 0.05$ was considered statistically significant.

Results

Figure 1 reports data on body weight of the rats before and after exposition to the metals. On it can be seen that control had significantly increased the initial body weight on eleventh day. Unlike this, high dose of Cd negatively interfered in the development of the animals after of the treatment ($p < 0.001$), while the same effect was not observed when co-administered similar dose of Cd with Zn ($p = 0.88$). The protective effect of Zn on clinical board delivered by Cd was reinforced by observed mortality (two rats in nine) in the group that received 1 mg of Cd, whereas the Cd plus Zn group showed no death in addition to increased consumption of food (data not shown). Also, the size of the effect of Cd on body weight of the rats is indicated by ANOVA/MANOVA analysis. Metals and day as well as its interactions (*except Zn x day*) had effect on dependent variable; however Cd predominated as the main effect ($F = 192.18; p < 0.001$).

The content of Zn reported in **table 1** had no difference between groups and even treatment with Zn did not modify the levels of the metal in the brain. In respect to the quantity of Cd, Zn-treatments caused no change in the levels of Cd and it was only detected when administered to the animals (**Table 1**). Moreover, groups that received 1 mg of Cd significantly had more accumulation than groups exposed with 0.25 mg Cd ($p = 0.03$ to *0.25 Cd x 1 Cd* and $p = 0.009$ to *0.25 Cd plus 2 Zn x 1 Cd plus 2 Zn*). Analysis of variance showed significant main effect of Cd ($F = 52.17; p < 0.001$) for brain Cd levels.

δ-ALA-D activity of Zn and Cd plus Zn-treated animals significantly was higher than control animals ($p = 0.004$ for 2 Zn, p = 0,011 for 0.25 Cd plus 2 Zn and $p < 0.001$ for 1 Cd plus 2 Zn), whereas single exposure to Cd caused no effect (**Figure 2A**), though 1 mg of Cd induced a substantial increase in enzyme activity ($p = 0.11$). Also, there was no difference between treatments with similar dose of Cd, but co-administration of the metals showed the highest activities. In addition to this, DTT did not alter δ-ALA-D activity and thus the reactivation index was similar in all groups (**Figure 2B**). The two-way ANOVA for δ-ALA-D activity yielded a significant main effect of Zn ($F = 25.10; p < 0.001$), in spite of Cd has

showed an important effect (*F = 3.13; p = 0.055*). Statistical analysis for reactivation index indicated no significant result, however Zn caused moderate effect (*F = 3.08; p = 0.089*).

The brain levels of MT present in the groups are depicted in **Figure 3**. In relation to the control, treatment with Zn showed no alteration (p = 0.94), as well as 1 mg of Cd with or without Zn (p = 0.14 and p = 0.62, respectively). Interestingly, groups that received 0.25 mg of Cd had significant decrease in the amount of MT in comparison to the control (p = 0.002 for *control x 0.25* Cd and p = 0.007 for *control x 0.25 Cd plus 2 Zn*) and in relation to the groups administered with 1 mg Cd (p < 0.001 for all comparisons). In general Zn did not alter the effects of Cd on the content of MT (p = 1 and p = 0.92, respectively for *0.25 Cd x 0.25 Cd plus Zn* and *1 Cd x 1 Cd plus 2 Zn*). Indeed, the analysis of variance showed no significant interference of Zn and changes on the amount of MT are only assigned for Cd (*F = 38.71; p < 0.001*).

The effect of Cd on lipid peroxidation in brain was determined by evaluation of TBARS levels (**Figure 4**). Compared to the control, the content of TBARS was significantly enhanced in the groups exposed to 0.25 mg of Cd (p = 0.02) and 0.25 mg of Cd plus 2 mg of Zn (p = 0.010). Surprisingly, 1 mg of Cd and 1mg of Cd plus Zn caused no modification on the content. The rate of lipid peroxidation did not differ between 0.25 mg to 1 mg Cd, but there was significance between 0.25 mg of Cd plus Zn and 1 mg of Cd plus Zn (p = 0.003). Although Zn has performed an increase in TBARS levels, this result was not significant, as well as it caused no interference on effect of Cd. This is reflected in two-way ANOVA test for variable dependent, where Cd and its interaction with Zn were indicated as the main effects on TBARS levels (respectively*, F = 11.26; p < 0.001; and F = 4.60; p = 0.017*).

Discussion

In previous report (Braga et al., in preparation) we discussed the toxic effects of Cd on hepatic tissue together with the protective action of Zn. Here, we extend the investigation to

the nervous tissue. Hence, when appropriate, some results will are compared to this study and the reader can obtain more details through of the source.

The evaluation of effects on the clinical board of the animals shows lethality and damage in association with 1 mg of Cd. Impairment on development of rats performed by Cd has been observed by researchers (Li and Lim, 2007; Waalkes and Rehm, 1994). Moreover, our results show that Zn protected the animals of such effects. In support of this result some works have found an improvement on the body weight gain in young rats due to the co-administration with Zn (Jacquillet et al., 2006; Jemai et al., 2007). Overall these observations preliminary reinforce the evidence of interaction between Cd and Zn in biological systems (Brzóska and Moniuszko-Jakoniuk, 2001).

Investigations on CNS have detected Cd in brain of rodents exposed *in vivo* to Cd (Arvidson and Tjälve, 1986; Eybl et al., 1998). However, the levels of metal found are in the range of microgram which is much lower than those usually obtained in peripheral tissues because of the protection exerted by BBB. Here, we report accumulation of Cd in brain near this value and, furthermore, there was an association between dose and amount of metal present in the tissue. In respect to the Zn content, since it is an essential metal could be expected an elevation in the levels after treatment by itself, but this has not happened and nor the increase of Zn dependent of Cd occurred as in previous study about liver tissue (Braga et al.,in preparation). Takeda et al. (2002) observed that transport of ^{65}Zn in the brain is a slow and tightly controlled process whose peak of metal uptake occurs six days after intravenous injection. Thus, unchanged levels of Zn may not indicate lack of Zn transport to the brain, because the methodology used by us does not reflect the qualitative content of the element.

The activity Zn-dependent of δ-ALA-D is important in study of the effects of Cd on Zn-homeostasis. In agreement with previous studies (Folmer et al., 2004; Luchese et al., 2007; Santos et al., 2005) Cd alone caused no significant effect on brain δ-ALA-D, although 1mg of Cd has substantially increased the activity. However, Zn modulating δ-ALA-D is a sustainable result. The modification of enzyme activity by Zn indicates that the brain has also suffered

intracellular action performed by metal, in spite of it has non-accumulated in the tissue. This ensures the hypothesis that the BBB was able to control the levels of Zn, but not inhibit its transport into brain; otherwise it would prevent an increase in availability of metal to the δ-ALA-D in nervous cells. Moreover, the resulting activation of δ-ALA-D by Zn (and perhaps Cd) could represent a protection as cofactor, or an induction of expression of the enzyme. Reactivation by DTT revealed no alteration on activity in treatments, thus we can conclude that Zn increased enzyme synthesis, such as has been previously reported in intoxication by Pb (Fujita et al., 1981; Rocha et al., 1995).

Another important target for interaction Zn-Cd is directed to the MT. Initially, in respect to the methodology applied, we have the certain of which the levels of MT are not total, since the primary antibody recognizes only isoforms I and II, but Palmiter et al. (1992) has shown that MT-III is unresponsive to Zn and Cd, so the content of MT found is consistent with effect achieved by the two metals. In fact, in whole animals, Cd is the strongest inducer of MT -I and -II followed by Zn (Beyersmann and Hechtenberg, 1997) and in spite of this we find no induction of MT in brain by exposure to both metals. In contrast, treatments with 0.25 mg of Cd produced intensive reduction in the amount of MT. In regard to this unusual result, MT levels have also been reported decrease in the hippocampus after Cd exposure (Ushakova and Kruchinenko, 2008). Interestingly, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn showed no modification in comparison to the control or, from another point of view, these treatments were able of maintain higher concentration of MT in relation to the groups with 0.25 mg. In view of these results Cd performed a dual effect in the expression of MT, although it is difficult to know which mechanisms may be involved in both situations. In this sense, induction of the expression of MT by the highest dose of Cd seems obvious, but this would be a premature conclusion, since the levels of the protein could be maintained due to increased half-life of MT. Many works have observed decline in turnover of MT with Cd associated to molecule (Bremner et al., 1978; Feldman et al., 1978; Feldman and Cousins, 1976; Chen et al., 1975), thus increased amounts of the metal could also govern the levels of MT. Meanwhile, no alteration in Zn treatment is consistent with absence of its accumulation in the tissue. This is

an interesting result, because here was not possible to extend the competitive effect between Zn and Cd on metallothionein expression observed in hepatic tissue (Braga et al., in preparation).

Lipid peroxidation has been reported as important process in Cd neurotoxicity (Manca et al., 1991a, 1991b; Pal et al., 1993). TBARS levels were remarkably enhanced in the brain of rats administered with 0.25 mg of Cd (with or without 2 mg of Zn), while 1 mg of Cd showed no difference in relation to the control. These results have support in the literature, which has been found both increase (Nemmiche et al., 2007; Santos et al., 2005) as no change of the content of TBARS (Folmer et al., 2004) after treatment with Cd. These data corroborate the dual effect ascribed to Cd such as it was seen to MT. Synthesis of MT in response to the cadmium-induced ROS is not novelty (Méndez-Armenta et al., 2003; Thomas et al., 1986) and our results for combination of the two factors showed that while the levels of MT were high, the content of TBARS was lower. Furthermore, co-administration of Zn did not perform effects on TBARS levels and thus supports the absence of competition between metals in the brain.

The intersection of all the results shows clear conclusions in this work. Although Zn increased synthesis of δ-ALA-D it performed no action on MT. Consequently, the protective effect of Zn on the clinical conditions seems to be more associated with peripheral tissues (as observed by us in liver tissue) than with CNS. Exposure to Cd became the metal detectable in the brain and even in low amounts caused effects on lipid peroxidation associated with modifications in the content of MT. On account of MT act as ROS scavenger and Cd chelation, we can not affirm that effects predominated in this study. Importantly, was also observed a dual effect of Cd on expression of MT and this is probably connected to different doses of exposure to the element. However, further investigations are necessary to clarify which the mechanisms modulate the different levels of MT. Finally, transport of Cd into nervous tissue shows a limited protection by BBB and this added to long biological half-life of the metal (about 30 years) makes important more investigations on impact that it can cause in CNS.

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Table

Table 1

Concentrations of Zn and Cd in brain after exposure Zn and/or Cd.

	Zn (μg/g tissue)	Cd (ηg/g tissue)
control	14.0 ± 0.3 (n = 6)	ND (n = 5)
2 mg Zn	13.7 ± 0.2 (n = 5)	ND (n = 3)
0.25mg Cd	14.1 ± 0.6 (n = 6)	122.2 ± 22.1 $a^{\dagger}b^{*}$ (n = 6)
0.25mg Cd + 2mg Zn	13.2 ± 0.4 (n = 6)	89.0 ± 21.0 a^{*} (n = 5)
1mg Cd	13.0 ± 0.3 (n = 6)	209.8 ± 20.8 $a^{\dagger}b^{\dagger}c^{*}d^{\dagger}$ (n = 5)
1mg Cd + 2mg Zn	13.2 ± 0.3 (n = 6)	198.8 ± 17.7 $a^{\dagger}b^{\dagger}d^{\dagger}$ (n = 4)

Values are indicated as mean ± S.E.M. Statistically significant differences (ANOVA, Tukey's test) are indicated by: [a]vs. control, [b]vs. 2 mg Zn, [c]vs. 0.25 mg Cd, [d]vs. 0.25 mg Cd plus 2 mg Zn and [e]vs. 1 mg Cd. $^{*}p < 0.05$, $^{\dagger}p < 0.01$ *and* $^{\dagger}p < 0.001$.

Figure Legends

Figure 1. Body weight of rats before and after exposure to Zn and/or Cd. The animals were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. Groups were compared between 1^{st} and 11^{th} day and statistically significant differences (ANOVA, Tukey's test) were indicated (#). $^{*}p < 0.05$ *and* $^{‡}p < 0.001$.

Figure 2. A) Brain Specific activity (n = 6-9) and B) reactivation with DTT (n = 5-8) of the δ-aminolevulinate dehydratase after exposure to Zn and/or Cd. The animals were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. Statistically significant differences (ANOVA, Tukey's test) are indicated by: ^{a}vs. control and ^{c}vs. 0.25 mg Cd. $^{*}p < 0.05$, $^{†}p < 0.01$ *and* $^{‡}p < 0.001$.

Figure 3. Brain concentration of metallothionein after exposure to Zn and/or Cd. The animals were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 6-9. Statistically significant differences (ANOVA, Tukey's test) are indicated by: ^{a}vs. control, ^{b}vs. 2 mg Zn, ^{c}vs. 0.25 mg Cd and ^{d}vs. 0.25 mg Cd plus 2 mg Zn. $^{*}p < 0.05$, $^{†}p < 0.01$ *and* $^{‡}p < 0.001$.

Figure 4. Brain concentration of TBARS after exposure to Zn and/or Cd. The animals were daily administered with i.p. injections of 2 mg of Zn, 0.25 mg of Cd, 0.25 mg of Cd plus 2 mg of Zn, 1 mg of Cd and 1 mg of Cd plus 2 mg of Zn. 24 hrs after the last dose the animals were killed. Data are represented as mean ± S.E.M. for n = 5-8. Statistically significant differences (ANOVA, Tukey's test) are indicated by: ^{a}vs. control, ^{c}vs. 0.25 mg Cd and ^{d}vs. 0.25 mg Cd plus 2 mg Zn. $^{*}p < 0.05$ *and* $^{†}p < 0.01$.

Fig. 1

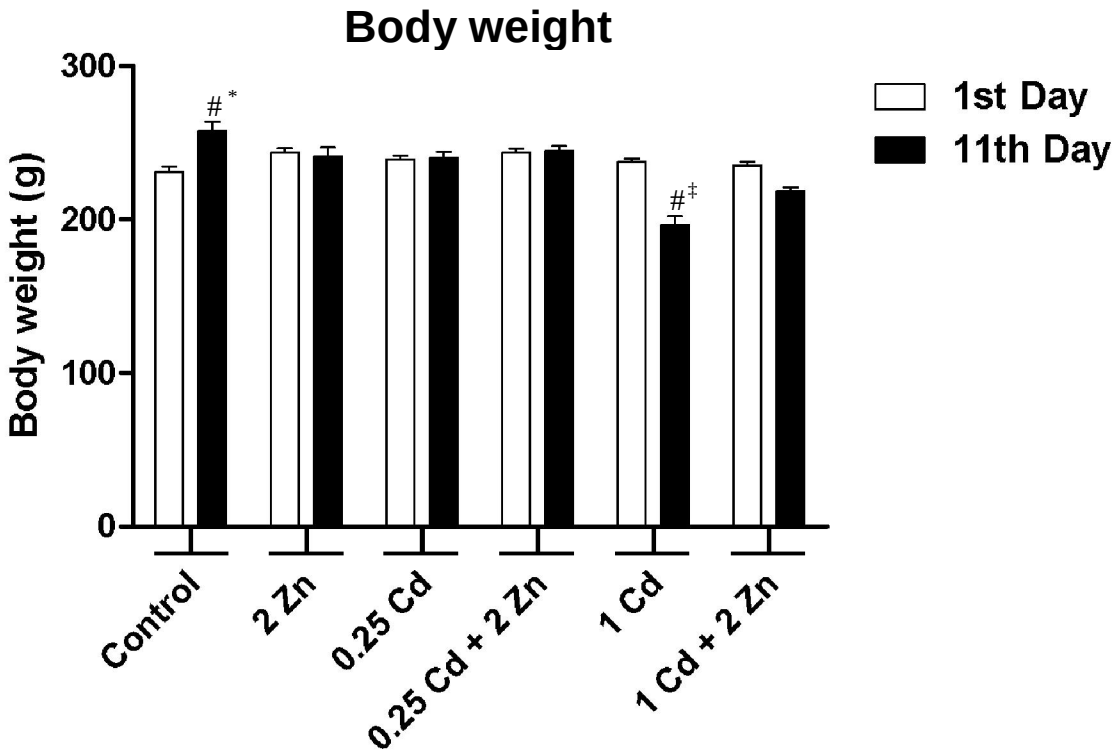

Body weight
300
200
100
0
Body weight (g)
#*
#‡
1st Day
11th Day
Control
2 Zn
0.25 Cd
0.25 Cd + 2 Zn
1 Cd
1 Cd + 2 Zn

Fig. 2

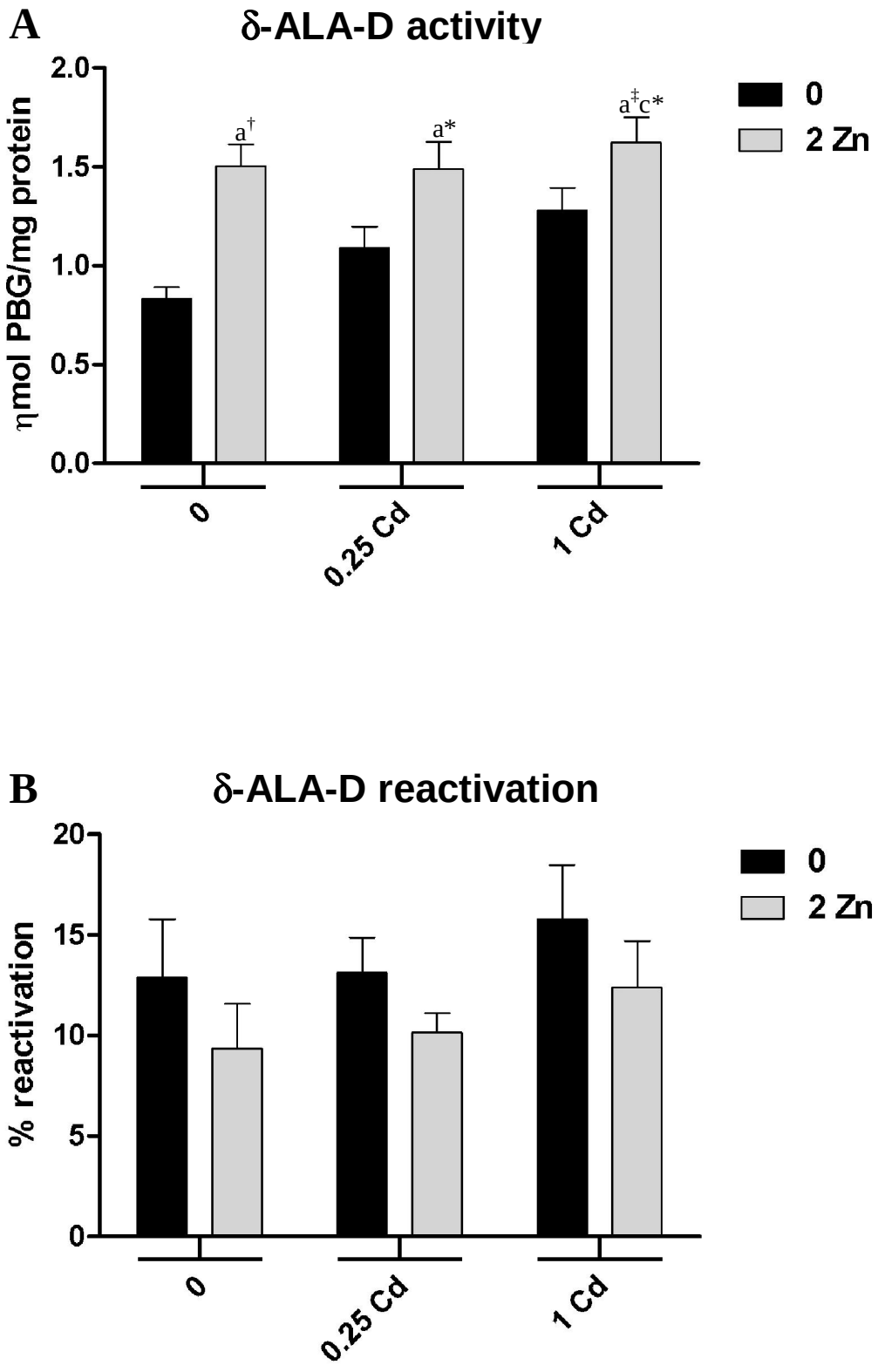

A
δ-ALA-D activity
ηmol PBG/mg protein
2.0
1.5
1.0
0.5
0.0
a†
a*
a‡c*
0
2 Zn
0
0.25 Cd
1 Cd
B
δ-ALA-D reactivation
% reactivation
20
15
10
5
0
0
2 Zn
0
0.25 Cd
1 Cd

Fig. 3

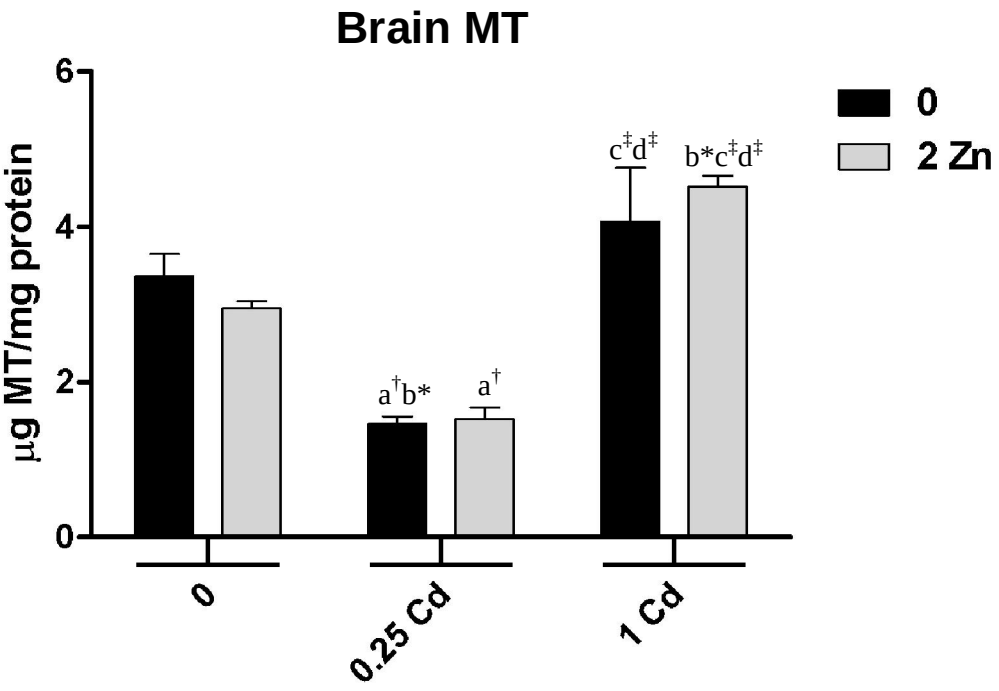
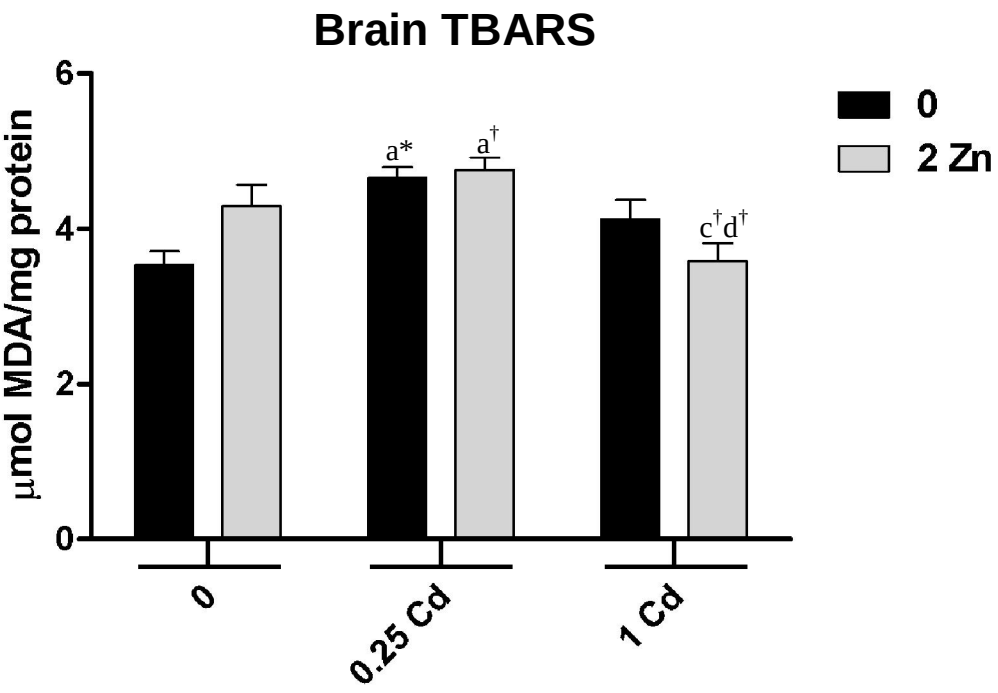


Fig. 4



PARTE III

1. DISCUSSÃO

A avaliação dos efeitos sobre o quadro clínico dos animais mostrou letalidade e crescimento negativo em associação com 1mg de Cd. Ação prejudicial de Cd sobre o desenvolvimento de ratos também tem sido observada por outros pesquisadores (Li & Lim, 2007; Waalkes & Rehm, 1994). Além disso, nossos resultados mostram que Zn protegeu os animais de tais efeitos e, em apoio a este resultado, alguns trabalhos têm encontrado benefícios sobre o ganho de peso corporal devido à co-administração com Zn (Jacquillet et al., 2006; Jemai et al., 2007). Assim, estas observações preliminares reforçam as evidências sobre a interação Zn-Cd em sistemas biológicos (Brzóska & Moniuszko-Jakoniuk, 2001).

1.1 Acúmulo de Zn e Cd

Na literatura existem trabalhos indicando que Cd causa acúmulo de Zn no fígado (Brzóska et al., 2000; Eybl et al., 1998; Oishi et al., 2000) e por isso nós quantificamos os dois metais. Nossos resultados mostram que Cd induziu um forte aumento de Zn no tecido. Tendo em vista isto, poderíamos supor que Zn teve a excreção reduzida ou mesmo o elemento foi mobilizado de outras fontes para o fígado, tais como comida ou a partir de outros tecidos. No que diz respeito ao aumento da mobilização de Zn da dieta, isto parece improvável, uma vez que nenhuma mudança foi observada sobre o consumo de comida (dados não mostrados) e não há registro de estudos avaliando a alteração na absorção de Zn intestinal devido à Cd. Aqui, a fonte do Zn retido no órgão parece advir de tecidos periféricos, pois nenhuma mudança em sua excreção tem sido reportada depois da exposição à Cd, ao passo que o transporte de outros tecidos para o fígado tem sido observado (Brzóska et al., 2000; Oishi et al., 2000). Em respeito ao efeito protetor desempenhado pelo tratamento com Zn, poderia se pensar que o aumento de seus níveis estaria associado a uma diminuição na retenção hepática de Cd, mas isto foi o oposto do observado e o metal tóxico acumulou independente da presença de Zn. Em estudos prévios ambos os metais são sugeridos competirem por diversos sítios biológicos (Asmuss et al., 2000; Gachot & Poujeol, 1992) e tal interação

específica seria explicada devido a propriedades químicas semelhantes (Bin & Garfinkel, 1994). Assim, os níveis aumentados de Zn por conta do tratamento com Cd poderiam representar um efeito competitivo entre os metais, do qual Zn seria transferido de tecidos não-hepáticos em direção ao fígado para contrabalançar com o maior acúmulo de Cd, neutralizando parcialmente seus efeitos tóxicos.

Em relação ao transporte dos metais no SNC, Cd tem sido detectado em encéfalo de roedores após exposição *in vivo* (Arvidson & Tjälve, 1986; Eybl et al., 1998). Entretanto, devido à proteção exercida pela BHE, os níveis encontrados para o elemento estão na faixa do micrograma, os quais são muito menores do que aqueles obtidos em tecidos periféricos. Nós reportamos acúmulo encefálico de Cd próximo a este valor e, além disso, houve associação entre dose e quantidade de metal presente no tecido. Com relação ao conteúdo de Zn, por ser um elemento essencial, poderia se esperar uma elevação no conteúdo do metal após seu tratamento, porém isto não ocorreu e nem mesmo foi observado o aumento de Zn dependente de Cd como foi obtido no tecido hepático. Com relação a este resultado, Takeda et al. (2002) observaram que o transporte de Zn no cérebro é um processo lento e rigidamente controlado, cujo pico de captação do metal ocorre seis dias depois de injeção intravenosa. Desta maneira, níveis inalterados de Zn podem não indicar ausência de seu transporte ao tecido nervoso, visto que a metodologia utilizada por nós não reflete, qualitativamente, o conteúdo do elemento.

1.2 Ação de Zn e Cd sobre a δ-ALA-D

Embora o efeito hepatotóxico de Cd tem sido extensivamente investigado, o mecanismo de acúmulo de Zn provocado por ele ainda não é completamente entendido e, portanto, não se podem garantir quais funções biológicas são afetadas e, muito menos, qual o principal compartimento celular onde os metais agem neste contexto. O estudo da δ-ALA-D trouxe-nos algumas respostas em torno destes questionamentos. A atividade da enzima hepática mostrou modificações associadas com todos os tratamentos, correspondendo à

entrada e o desencadeamento de respostas pelos metais no espaço intracelular. Distúrbios causados por Cd, geralmente estão associados com a inibição da atividade da δ-ALA-D (Nogueira et al., 2003; Santos et al., 2005), apesar disto, nós encontramos resultado oposto. Todavia, este dado controverso tem sido mostrado por outro trabalho (Folmer et al., 2004), sendo atribuído, ou a uma proteção contra oxidação gerada por Cd, ou a uma indução de síntese da enzima. Além disso, dados reportados na literatura indicam que Cd é capaz de mimetizar e substituir Zn como cofator da δ-ALA-D (Sommer & Beyersmann, 1984). Por conta disto, não podemos saber ao certo qual destes processos predominaram no presente estudo. Entretanto, menor reativação da enzima por DTT indica que houve indução na sua velocidade cinética causada por Cd e, principalmente Zn, enquanto que a adição no número de unidades enzimáticas não seria consistente para explicar estes resultados.

A extensão do estudo da δ-ALA-D para o tecido nervoso teve como objetivo avaliar possíveis similaridades e diferenças na interação Zn-Cd em relação ao tecido hepático. De acordo com estudos anteriores (Folmer et al., 2004; Luchese et al., 2007; Santos et al., 2005) Cd sozinho não causou efeitos significantes sobre a δ-ALA-D presente no encéfalo, embora 1mg de Cd tenha aumentado substancialmente sua atividade. Entretanto, a modulação da enzima por Zn é um resultado sustentável, indicando que o encéfalo tem também sofrido ação intracelular devido a este metal, apesar da ausência de seu acúmulo no tecido. Isto assegura a hipótese de que a BHE foi capaz de controlar os níveis de Zn, mas não inibir seu transporte ao SNC, pois, caso contrário, preveniria o aumento na viabilidade do metal à δ-ALA-D presente nas células nervosas. Como complemento, a ativação da δ-ALA-D por Zn (e talvez Cd) poderia representar uma proteção como cofator, ou uma indução da expressão da enzima. Assim, reativação por DTT revelou nenhuma alteração dos tratamentos sobre a atividade e desta maneira podemos concluir que Zn aumentou a síntese enzimática, tal como tem sido relatado para intoxicação com Pb (Fujita et al., 1981; Rocha et al., 1995).

1.3 Ação de Zn e Cd sobre MT

Com base na força da interação entre os metais e o eminente risco que Cd pode representar sobre a quebra da homeostase de Zn, nosso grupo buscou investigar o conteúdo de MT hepático e encefálico. MT é uma proteína com características que cruzam tanto a desintoxicação de Cd como a homeostase de Zn e daí sua expressão ser fortemente induzida pelos dois metais (Tandon et al., 2001). Zn é um clássico indutor de MT, contudo, sob o tecido hepático, nossas condições experimentais não permitiram observar isto e somente na maior dose de Cd houve aumento na expressão da proteína. Muitos autores explicam a indução por Cd através de seu "sequestro" pela própria MT (Din & Frazier, 1985; Friberg et al., 1974; Onosaka & Cherian, 1981). Por esta razão, seria esperado obter maior quantidade de MT no grupo que recebeu 1mg de Cd junto com Zn, uma vez que nossas observações indicam um melhor status clínico sobre o desenvolvimento destes animais em comparação com aqueles que receberam somente 1mg de Cd. Contudo, inesperadamente, o grupo com co-administração dos metais apresentou níveis de MT quase três vezes menores. A reversão do efeito tóxico de Cd pelo metal essencial parece óbvia, pois, além do conteúdo de Zn ter sido significativamente diferente entre estes grupos, o tratamento somente com Zn não alterou os níveis de MT. Estes resultados mostram que MT pode ter realizado um papel negativo naqueles animais expostos a 1mg de Cd, o que é surpreendente, pois poucos trabalhos têm observado efeito similar (Liu et al., 1995; Webb and Etienne, 1977) e isto torna necessário investigar mais sua relevância como uma molécula não somente protetora, mas também com ação tóxica. Entretanto, devido a MT ser responsável pela distribuição intracelular de Zn (Brady, 1982; Ou and Ebadi, 1992), nós podemos supor que o papel tóxico realizado pelos altos níveis da proteína foi de tornar mais difícil o acesso ao Zn intracelular; a presença de maior quantidade de MT foi atingida para detoxificar Cd, mas isto pode ter também aumentado a probabilidade de Zn estar ligado a ela e, conseqüentemente, causar perturbação sobre a homeostase do metal devido ao falso empobrecimento do elemento no tecido. Também o processo descrito acima, sobre acúmulo de Zn hepático desencadeado por Cd, poderia demonstrar uma resposta fisiológica em favor desta suposição.

O estudo da MT no tecido nervoso apresentou resultados divergentes daqueles observados no tecido hepático. Para início de discussão devemos esclarecer que, em relação à metodologia aplicada, nós temos a certeza de que os níveis obtidos para a MT não são totais, uma vez que o anticorpo primário reconhece somente as isoformas I e II, porém Palmiter et al. (1992) têm mostrado que MT-III não é responsiva à Zn e Cd e, portanto, o conteúdo de MT encontrado coincide com os efeito adquirido através dos dois metais. De fato, Cd é o mais forte indutor de MT-I e –II seguido por Zn (Beyersmann & Hechtenberg, 1997) e, apesar disto, nós encontramos nenhuma indução de MT no encéfalo após a exposição aos metais. Em contraste, tratamentos com 0,25mg de Cd produziram redução intensiva na quantidade de MT. No que diz respeito a este resultado incomum, níveis de MT tem também apresentado diminuição em hipocampo após intoxicação por Cd (Ushakova & Kruchinenko, 2008). Intrigantemente, 1mg de Cd sozinho ou co-administrado com 2mg de Zn mostraram nenhuma modificação em comparação ao controle ou, de outro ponto de vista, estes tratamentos foram capazes de manter maior concentração de MT em relação aos grupos com 0,25mg de Cd. Por estes resultados, Cd produziu um efeito dual na expressão da MT, contudo é difícil saber quais mecanismos podem estar envolvidos nestas duas situações. Neste sentido, indução da expressão da MT associada à dose mais alta de Cd parece evidente, mas isto seria uma conclusão prematura, pois os níveis da proteína poderiam ser mantidos devido ao aumento na meia-vida da MT. Muitos trabalhos têm observado um declínio no “turnover” da MT com Cd associado a sua molécula (Bremner et al., 1978; Feldman et al., 1978; Feldman & Cousins, 1976; Chen et al., 1975) e, com isso, aumento na quantidade do metal poderia também determinar os níveis de MT observados. Enquanto isso, nenhuma alteração no tratamento com Zn é consistente com a ausência de seu acúmulo no tecido. Este é um dado interessante, porque no encéfalo não foi possível estender o efeito competitivo entre Zn e Cd sobre a expressão de MT, como ocorreu no tecido hepático.

1.4 Stress oxidativo induzido por Cd em tecido nervoso

Indiferentemente, peroxidação lipídica também tem sido reportada como um importante processo na neurotoxicidade de Cd (Manca et al., 1991a, 1991b; Pal et al., 1993). Níveis encefálicos de TBARS foram notavelmente elevados naqueles ratos administrados com 0,25mg de Cd (com ou sem 2mg de Zn), enquanto 1mg de Cd mostrou nenhuma diferença em relação ao controle. Estes resultados são suportados pela literatura, onde, após tratamento com Cd, é encontrado tanto um aumento (Nemmiche et al., 2007; Santos et al., 2005) como nenhuma mudança no conteúdo de TBARS (Folmer et al., 2004). Estes dados corroboram com o efeito dual descrito para Cd tal como foi visto para MT neste mesmo tecido. Síntese de MT em resposta ao aumento de radicais livres não é novidade (Méndez-Armenta et al., 2003; Thomas et al., 1986) e enquanto os níveis encefálicos de MT foram altos, o conteúdo de TBARS esteve mais baixo. Além disso, co-administração de Zn também não interferiu na ação do Cd sobre os níveis de TBARS, suportando a ausência de competição entre os metais.

2. CONCLUSÕES GERAIS

Os resultados sugerem que a ação tóxica de Cd tanto em tecido hepático como nervoso ocorre por vias onde Zn exerce suas funções de essencialidade. Entretanto, a BHE torna o encéfalo menos vulnerável a interação Zn-Cd, uma vez que Zn teve os níveis basais inalterados e Cd apresentou quantidade em torno de 10^3 vezes menor do que aqueles encontrados no fígado. Por conta disto, a proteção exercida por Zn sobre a exposição à Cd, mostra ser mais efetiva sobre órgãos periféricos do que sobre o SNC.

A avaliação dos efeitos sobre a δ-ALA-D revela o espaço intracelular como sítio de ação dos metais. Zn e Cd aumentaram a atividade da δ-ALA-D, porém o metal essencial apresentou maior eficiência sobre a modulação da enzima. Especificamente, este aumento foi traduzido por diferentes formas entre o tecido hepático e nervoso: no fígado a maior atividade indica proteção da enzima, enquanto que no encéfalo os metais induziram sua expressão.

A análise dos níveis encefálicos de MT juntamente com o conteúdo de TBARS indica um importante efeito antioxidante realizado pela proteína contra o stress oxidativo gerado por Cd. Entretanto, apesar dos benefícios sugeridos à proteína, nossos dados em tecido hepático indicaram que a elevada quantidade de MT pode ser prejudicial para o desenvolvimento dos animais. No encéfalo, Cd apresentou um efeito dual sobre a regulação dos níveis de MT, sendo ou modulada negativamente (em 0,25mg de Cd) ou positivamente (1mg de Cd).

Por fim, este trabalho indica ação benéfica de Zn sobre os efeitos de Cd. Este mecanismo de ação esteve bastante associado à regulação periférica da MT, que pelas nossas investigações ora esteve em um contexto positivo ora negativo à célula.

3. PERSPECTIVAS

1) Examinar com maior detalhe a ação negativa da MT, pois existem poucos trabalhos que indicam ação prejudicial a proteína.

2) Avaliar o mecanismo dual desempenhado por Cd quanto a expressão de MT no tecido nervoso.

3) Verificar outros efeitos de Cd no tecido nervoso, visto que a BHE exerce limitada proteção ao metal, além de ser um elemento com elevada meia-vida biológica.

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