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PREVENÇÃO DOS EFEITOS TÓXICOS DO CLORETO DE MERCÚRIO EM RATOS JOVENS PELO CLORETO DE ZINCO: PAPEL DAS METALOTIONEÍNAS

TESE DE DOUTORADO

Nilce Coelho Peixoto

Santa Maria, RS, Brasil
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por

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Tese apresentada ao Programa de Pós-graduação em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**.

Orientadora: Prof[a]. Maria Ester Pereira

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Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-graduação em Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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elaborada por
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como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

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Santa Maria, 31 de outubro de 2006.

Dedico este trabalho aos meus primeiros educadores que,
embora não sejam professores,
doutrinam pela sabedoria proporcionada pela vida:
meus pais, Antão e Julieta.

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Àqueles que, embora aqui não referidos, tiveram algum tipo de participação direta ou indireta relacionada ao presente trabalho.

De como o que beneficia um prejudica outro

Dêmade, de Atenas, condenou um homem de sua cidade que comerciava com coisas necessárias aos enterros, acusando-o de tirar disso lucro excessivo, somente auferível da morte de muitas pessoas. Tal julgamento não me parece muito eqüitativo, pois não há benefício próprio que não resulte de algum prejuízo alheio e, de acordo com aquele ponto de vista, qualquer ganho fora condenável.

O mercador só faz bons negócios porque a mocidade ama o prazer; o lavrador lucra quando o trigo é caro; o arquiteto quando a casa cai em ruínas; os oficiais de justiça com os processos e disputas dos homens; os próprios ministros da religião tiram honra e proveito de nossa morte e das fraquezas de que nos devemos redimir; nenhum médico, como diz o cômico grego da antiguidade, se alegra em ver seus próprios amigos com saúde; nem o soldado seu país em paz com os povos vizinhos. Assim tudo. E o que é pior, quem se analise a si mesmo verá no fundo do coração que a maioria de seus desejos só nascem e se alimentam em detrimento de outrem. Em se meditando a propósito, percebe-se que a natureza não foge, nisso, a seu princípio essencial, pois admitem os físicos que toda coisa nasce, se desenvolve e cresce em conseqüência da alteração e corrupção de outra: “Logo que uma coisa qualquer muda de maneira de ser, disso resulta imediatamente a morte do que ela era antes”.

(Michel de Montaigne, 1533-1592)

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

PREVENÇÃO DOS EFEITOS TÓXICOS DO CLORETO DE MERCÚRIO EM RATOS JOVENS PELO CLORETO DE ZINCO: PAPEL DAS METALOTIONEÍNAS

Autor: Nilce Coelho Peixoto

Orientadora: Maria Ester Pereira

Data e local da defesa: Santa Maria, 31 de outubro de 2006.

O zinco, o cádmio e o mercúrio são metais divalentes pertencentes ao mesmo grupo da tabela periódica. O primeiro é um metal essencial e os demais são metais tóxicos. A característica comum mais notável entre eles é a capacidade de induzir à síntese de metalotioneínas (MT), que ocorre em dois órgãos vitais envolvidos na destoxificação, fígado e rins. A principal função das MT é a destoxificação de metais pesados e a regulação da homeostase de metais essenciais, como cobre e zinco. Há muitos estudos sobre a toxicidade do mercúrio e sobre o papel das MT em animais adultos. Entretanto, a sensibilidade de animais em desenvolvimento a vários compostos difere daquela observada em adultos e pode estar relacionada a diferentes intervalos pós-natais de desenvolvimento. O objetivo desta investigação foi verificar os efeitos dos pré-tratamentos com $CdCl_2$ e $ZnCl_2$ sobre os efeitos deletérios do $HgCl_2$ em ratos jovens e investigar se as MT estão envolvidas neste mecanismo de proteção. Ratos de três dias de idade foram injetados com uma dose diária (s.c.), nos cinco dias consecutivos, de salina, $CdCl_2$ (3,7 mg/kg) ou $ZnCl_2$ (27,0 mg/kg). Nos cinco dias subseqüentes os animais foram injetados com uma dose diária (s.c.) de salina ou $HgCl_2$ (5,0 mg/kg). Os animais foram sacrificados 24 h após a última dose e as amostras foram coletadas (sangue, figado e rins). Os pesos corporal e renal, a atividade da porfobilinogênio sintase (PBG-sintase) hepática e renal, a atividade da alanina aminotransferase, a creatinina, a uréia, a glicemia e a retenção do metal tóxico pelos tecidos foram significativamente alterados pelo $HgCl_2$. A exposição prévia ao $CdCl_2$ preveniu o efeito do mercúrio sobre a PBG-sintase renal, mas não alterou os níveis de mercúrio nos tecidos. Em geral, os efeitos do mercúrio foram prevenidos ou atenuados pelo zinco, exceto que o pré-tratamento com zinco aumentou o acúmulo de metal pesado nos rins e não modificou o aumento do peso renal induzidos pelo mercúrio. O conteúdo de MT foi

aumentado pelos tratamentos com mercúrio e zinco e a sua maior elevação foi induzida pelo zinco. A distribuição de metal nas frações subcelulares mostrou que em ambas, fração insolúvel (FI) e fração citosólica tratada a quente (CTQ), os conteúdos foram modificados pelos tratamentos. Embora a fração CTQ seja rica em MT, os maiores conteúdos de zinco e mercúrio foram verificados na FI de todos os tecidos analisados. As relações entre MT e metais na fração CTQ revelaram que nos tecidos hepático e renal sempre que há um aumento nos teores de metal, há um aumento no conteúdo de MT. A redução dos níveis de mercúrio hepático e sangüíneo e o aumento do conteúdo desse metal nos rins induzidos pelo zinco sugerem que o metal pesado contido no fígado é transportado para os rins pelo sangue. Esse processo também pode estar carreando MT do fígado para os rins. Além disso, é importante salientar que em células em proliferação, o que ocorre durante o crescimento acelerado, há MT nos núcleos e nas mitocôndrias. Desse modo, o alto conteúdo de mercúrio encontrado na FI, fração rica em núcleos e mitocôndrias, também estaria associado às MT. Considerando que o pré-tratamento com zinco induziu a um aumento de 80% no conteúdo de MT renal e o grupo tratado com zinco e mercúrio apresentou um conteúdo 25% maior de metal tóxico nessa proteína do que aquele verificado no grupo que foi tratado somente com mercúrio, esses resultados sugerem que as MT são, pelo menos em parte, responsáveis pela redução da toxicidade do mercúrio verificada em vários parâmetros analisados nesse trabalho.

Palavras-chave: zinco; mercúrio; cádmio; metalotioneínas; toxicidade renal; ratos em desenvolvimento.

ABSTRACT

Thesis of Doctor's Degree
Post-graduating Course in Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

PREVENTION OF THE TOXIC EFFECTS OF MERCURY CHLORIDE IN YOUNG RATS BY ZINC CHLORIDE: THE ROLE OF METALLOTHIONEINS

Author: Nilce Coelho Peixoto

Advisor: Maria Ester Pereira

Date and place of the defense: Santa Maria, October 31st, 2006.

Zinc, cadmium, and mercury are divalent metals and constitute same group of the periodic table. While zinc is an essential metal, the others are toxic metals. The most important common feature among these metals is the ability to induce the synthesis of metallothioneins (MT), which occurs in two vital organs involved in detoxification, the liver and kidney. The main role of MT is the detoxification of heavy metals and the regulation of homeostasis of essential trace metals, such as copper and zinc. There are several studies about mercury toxicity and the role of MT in adult animals. However, the sensitivity of developing animals to various compounds differs from that observed in adults and may to related to different pos-natal phases of the development. The aim of this investigation was to verify the effects of $CdCl_2$ and $ZnCl_2$ pretreatments on the deleterious effects of $HgCl_2$ in young rats and to investigate whether MT were involved in this protection mechanism. When pups were three days old, they received five consecutive injections (s.c.) of saline, $CdCl_2$ (3.7 mg/kg/day) or $ZnCl_2$ (27.0 mg/kg/day). On the five subsequent days, the animals were injected daily with one dose (s.c.) of saline or $HgCl_2$ (5.0 mg/kg). Pups were sacrificed 24 h after the last dose and samples were collected (blood, liver and kidneys). The body and renal weights, hepatic and renal porphobilinogen synthase (PBG-synthase) activity, alanine aminotransferase activity, creatinine, urea, glycemia, and the retention of heavy metal in tissues were significantly altered by $HgCl_2$. Prior exposure to $CdCl_2$ prevented the effect of mercury on renal PBG-synthase, but did not alter mercury levels in the tissues. In general, the effects of mercury were prevented or lessened by zinc, except that the zinc pre-treatment increased the retention of mercury in the kidneys and did not modify the increase of renal weight induced by mercury. MT contents were increased by treatments with mercury and zinc and the greatest

increase was induced by latter. The metal distribution in subcellular fractions showed that in both the insoluble fraction (IF) and heat treated cytosolic fraction (HTC), the contents were modified by the treatments. Although the HTC fraction is rich in MT, higher zinc and mercury contents were verified in the IF from all tissues analyzed. The relationships between MT and HTC metals showed that in the hepatic and renal tissues whenever there is an increase of metal levels there is increase of MT content. The reduction of hepatic and blood mercury levels and the increase of this metal in the kidneys induced by zinc suggests that the heavy metal contained in the liver is carried to the kidneys through the blood. This process also would transport MT from the liver to the kidneys. Moreover, it is important to emphasize that in cells in proliferation, which occur during rapid growth, there are nuclear and mitochondrial MT. Therefore, the high content of mercury found in the IF, enriched fraction in nucleus and mitochondria, would be bound to MT, as well. Considering that the zinc pre-treatment induced an increase of renal MT of around 80% and the group treated with zinc and mercury presented a content of mercury in this protein that was 25% higher than for the group treated only with mercury, these results suggest that MT are, at least in part, responsible for the reduction of the toxicity of mercury seen in the various parameters analyzed in this work.

Keywords: zinc; mercury; cadmium; metallothioneins; renal toxicity; developing rats.

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LISTA DE REDUÇÕES (ABREVIATURAS, PREFIXOS, SIGLAS E SÍMBOLOS)

AAS: espectrofotometria de absorção atômica (*atomic absorption spectrophotometry*);

ADN: ácido desoxirribonucléico (= DNA);

δ-ALA: ácido δ-aminolevulínico (δ-*aminolevulinic acid*);

δ-ALA-D: δ-aminolevulinato desidratase (δ-*aminolevulinic acid dehydratase*) (= PBG-sintase);

ALT: alanina aminotransferase;

ANOVA: análise de variância (*analysis of variance*);

ARN: ácido ribonucléico;

c (centi): prefixo que indica uma unidade de medida derivada igual a 100 vezes menor;

^{o}C: grau Celsius;

Cd: cádmio;

Cd^{2+}: íon cádmio;

$CdCl_2$: cloreto de cádmio;

CH_3Hg^+: íon metilmercúrio;

CTQ: citosol tratado a quente (= HTC);

d (deci): prefixo que indica uma unidade de medida derivada igual a 10 vezes menor;

Da: Dálton;

DNA: ácido desoxirribonucléico (*deoxyribonucleic acid*) (= ADN);

DTNB: ácido 5,5'-ditio*bis*-2-nitrobenzóico [*5,5'-dithio-bis(2-nitrobenzoic acid)*];

d. w.: peso seco (*dry weight*);

E. C.: Comissão de Enzimas *(Enzyme Comission)*;

EDTA: sal dissódico do ácido etilenodiaminotetraacético (*ethylenediaminetetraacetic acid*

disodium salt);

FI: fração insolúvel (= IF);

g: grama;

g: aceleração da gravidade (força centrífuga);

h: hora;

HCl: ácido clorídrico;

Hg: mercúrio;

Hg^{o}: mercúrio metálico; mercúrio elementar;

Hg^{2+}: íon mercúrico; íon mercúrio II;

$HgCl_2$: cloreto mercúrico; cloreto de mercúrio II;

HNO_3: ácido nítrico;

H_2SO_4: ácido sulfúrico;

HTC: citosol tratado a quente (***heat-treated cytosol)*** (= CTQ);

I_2: iodo metálico;

IF: fração insolúvel (***insoluble fraction)*** (= FI);

k (quilo): prefixo que indica uma unidade de medida derivada igual a 1.000 vezes maior;

K_2HPO_4: fosfato de potássio dibásico;

KH_2PO_4: fosfato de potássio monobásico;

KI: iodeto de potássio;

KOH: hidróxido de potássio;

l: litro;

L: litro;

LDH: lactato desidrogenase (*lactate dehydrogenase*);

m: metro;

m (mili): prefixo que indica uma unidade de medida derivada igual a 1.000 vezes menor;

M: molar;

μ (micro): prefixo que indica uma unidade de medida derivada igual a 1 milhão de vezes menor;

min: minuto;

mol: quantidade expressa pelo número de Avogadro ($6{,}02 \times 10^{23}$); massa, em g, de 1 mol de moléculas ou 1 mol de átomos;

MT: metalotioneína(s);

MT-I: isoforma I da metalotioneína;

n (nano): prefixo que indica uma unidade de medida derivada igual a 1 bilhão de vezes

menor;

n: número de repetições;

N: normal;

Na: sódio;

NaCl: cloreto de sódio;

NADH: dinucleotídeo de nicotinamida adenina (forma reduzida) [*nicotinamide adenine dinucleotide (reduced form)*];

NaOH: hidróxido de sódio;

N. D.: não-detectado;

$(NH_4)_2SO_4$: sulfato de amônio;

p: nível de significância;

P: nível de significância;

PBG: porfobilinogênio;

PBG-sintase: porfobilinogênio sintase (= δ-ALA-D);

pH: potencial hidrogeniônico;

PMSF: fluoreto de fenilmetano sulfonila (*phenylmethane sulphonyl fluoride*);

rpm: rotações por minuto;

Sal: salina;

SAL: salina;

s. c.: subcutânea; subcutaneamente;

S. E. M.: erro padrão da média (*standard error of mean*);

SH: grupamento(s) sulfidrílico(s);

SNC: sistema nervoso central;

u: prefixo que indica uma unidade de medida derivada igual a 1 milhão de vezes menor [= μ (micro)];

U: unidade;

w/v: peso/volume (*weight/volume)*;

Zn: zinco;

Zn^{2+}: íon zinco;

$ZnCl_2$: cloreto de zinco.

SUMÁRIO

CAPÍTULO 1
1 INTRODUÇÃO

Os efeitos provocados pela exposição ou intoxicação de organismos vivos ao mercúrio permanecem um problema sério, até a presente data, do ponto de vista toxicológico e terapêutico. A utilização desse metal pesado foi reduzida significativamente nas últimas décadas (CLARKSON, 1997; FURST & RADDING, 1998). Essa medida foi tomada por ocasião da Doença de Minamata, um acontecimento mundialmente divulgado (HARADA, 1978; WEISS, 1996; BISINOTI & JARDIM, 2004). A catástrofe ambiental, ocorrida na Baía de Minamata (sul do Japão), foi protagonizada pela fábrica Chisso Corporation, que utilizava o mercúrio metálico como catalisador na fabricação de plástico e, na década de 30, iniciou a deposição dos seus efluentes industriais nesse local. O metal pesado foi convertido à uma de suas formas orgânicas denominada metilmercúrio, que é mais tóxica que a forma metálica original, e incorporado à cadeia alimentar (KLAASSEN, 1996; BISINOTI & JARDIM, 2004; HARADA, 2005). Os humanos intoxicaram-se mediante o consumo de peixes contendo metilmercúrio, o que causou alterações no sistema nervoso central (SNC), as quais são congênitas (HARADA, 1978, 1995; KONDO, 2000). Até o final do século passado haviam sido reconhecidas oficialmente cerca de 2.500 pessoas como sendo vítimas da tragédia ambiental (HARADA *et al.*, 1998; WATTS, 1999; STRATTA *et al.*, 2001; BISINOTI & JARDIM, 2004; McCURRY, 2006), porém mais de 10.000 exigem o reconhecimento (HARADA *et al.*, 1998; BISINOTI & JARDIM, 2004; McCURRY, 2006).

A restrição global do uso do mercúrio, como recomendada por órgãos governamentais e organizações ambientais, nem sempre é respeitada (JUNGHANS, 1983; CLARKSON, 1997). Nos países subdesenvolvidos e em desenvolvimento há uma franca utilização da forma metálica, em particular na mineração do ouro. Nesses casos, assim como ocorreu em Minamata, ocorre contaminação ambiental e a exposição de humanos (BOISCHIO & HENSHEL, 1996; LEBEL *et al.*, 1996; RENZONI *et al.*, 1998; RISHER *et al.*, 2003; CASTILHOS *et al.*, 2006).

Embora o metilmercúrio seja a principal e a mais tóxica forma de mercúrio à qual os animais estão expostos (GOYER, 1996; BISINOTI & JARDIM, 2004), é importante salientar

a participação de outras duas, o mercúrio metálico (ou elementar) e o mercúrio inorgânico, uma vez que há interconversões entre essas três formas (BISINOTI & JARDIM, 2004; COUNTER & BUCHANAN, 2004).

A terapia medicamentosa adequada a ser instituída nos casos de exposições ou intoxicações pelo mercúrio ainda é um desafio para a ciência. É prescrita a administração de drogas quelantes com o fim de seqüestrar e aumentar a excreção do metal (HARTVIG, 1984; DOMINGO, 1995). Entretanto, do tratamento com esses antagonistas advêm efeitos colaterais adversos, como alterações nas concentrações de metais essenciais (CANTILENA & KLAASSEN, 1982; THOMAS & CHISOLM, 1986; BAPU *et al.*, 1994), além de, muitas vezes, provocar mais efeitos tóxicos que o próprio metal causador da intoxicação (JUNGHANS, 1983; DOMINGO, 1994; ROZA *et al.*, 2005).

Atualmente, a ciência tem salientado e divulgado, para inúmeras doenças e síndromes, a importância da utilização de terapias de reposição e de suplementação em uma variedade de especialidades da medicina (EBERLEIN-KÖNIG *et al.*, 1998; GOEL *et al.*, 2000, 2005, 2006; GOEL & DHAWAN, 2001; ZATTA *et al.*, 2003; DI DANIELI *et al.*, 2004; JOSHI *et al.*, 2004; LADD *et al.*, 2005; MOCCHEGIANI *et al.*, 2005; OZTURK & CILLIER, 2006; SOHRABRAND *et al.*, 2006). Essas terapias têm o fator conveniente de não envolverem substâncias estranhas ao organismo e, desse modo, a probabilidade do aparecimento de efeitos secundários indesejáveis é mínima. Os efeitos tóxicos provocados pela exposição ou intoxicação por metais pesados podem ser atenuados ou prevenidos pelo uso de metais essenciais, sobretudo zinco e cobre (LI *et al.*, 1995; BRZÓSKA & MONIUSZKO-JAKONIUK, 2001). Esses efeitos medicamentosos são conseqüências das interações entre esses metais (GOYER, 1995; BRZÓSKA & MONIUSZKO-JAKONIUK, 2001; IRATO & ALBERGONI, 2005; DOREA & DONANGELO, 2006), uma vez que pode haver competições, deslocamentos de metais não-essenciais com a posterior substituição por metais essenciais (IRATO & ALBERGONI, 2005) e formação de complexos em determinados sistemas biológicos (CHMIELNICKA *et al.*, 1983; GOYER, 1995).

Organismos jovens são especialmente vulneráveis a fatores externos (FRANKOVÁ & BARNES, 1968; CHASE *et al.*, 1969; WALSH, 1982; MOSER, 2000; SINGH & RISHI, 2005). Conseqüentemente, animais em fases precoces da vida, período em que encontram-se em desenvolvimento e crescimento, são os sujeitos experimentais de investigações visando à avaliação do grau de toxicidade desses agentes e à proteção desses organismos (TRAUTH *et al.*, 2000; SINGH & RISHI, 2005).

A presente tese versa sobre a capacidade do zinco e do cádmio em atenuar ou prevenir

os efeitos deletérios conseqüentes da exposição de ratos em desenvolvimento ao mercúrio.

1.1 Objetivos

O presente trabalho tem como objetivo geral investigar a capacidade do zinco e do cádmio em prevenir ou reduzir os efeitos tóxicos induzidos pelo mercúrio em ratos jovens.

Tomando-se por base o objetivo geral, os objetivos específicos foram definidos. As conseqüências da exposição ao mercúrio são avaliadas por meio das seguintes variáveis: determinação do peso corporal, determinação dos pesos úmidos dos rins e do fígado, avaliação da atividade da enzima porfobilinogênio sintase (PBG-sintase) de fonte renal e hepática, quantificação dos níveis dos metais mercúrio e zinco retidos nos tecidos renal, hepático e sangüíneo, avaliação das funções renal e hepática, quantificação do teor de glicogênio hepático, determinação do nível de glicose sérica e quantificação do nível de metalotioneína (MT) renal, hepática e sangüínea. A capacidade do tratamento com zinco ou cádmio em evitar ou atenuar os efeitos provocados pelo mercúrio é avaliada mediante a modificação desses parâmetros alterados pelo mercúrio.

1.2 Justificativa

O mercúrio figura como um dos mais perniciosos entre os metais classificados como pesados, seus efeitos deletérios são investigados e conhecidos desde algumas décadas. Porém, as conseqüências da exposição ao mercúrio em organismos em desenvolvimento, cuja sensibilidade é relatada maior, são pouco estudadas. Além disso, o tratamento farmacológico prescrito para as intoxicações causadas por esse agente é, até o momento, inadequado, uma vez que é paliativo e tóxico. O zinco e o cádmio são metais dotados da capacidade de induzir à síntese de proteínas ligantes de metal. De acordo com isso, o zinco e o cádmio poderiam ser tratamentos alternativos nesses casos de intoxicações por mercúrio em animais jovens.

A presente tese é apresentada conforme as seguintes partes constituintes seqüenciais:

- **CAPÍTULO 2: REVISÃO BIBLIOGRÁFICA**;
- **CAPÍTULO 3: ARTIGO 1**

  Efeitos do zinco e do cádmio sobre a inibição da d-**ALA-D induzida pelo $HgCl_2$ e os níveis de Hg nos tecidos de ratos jovens**

  (Effects of zinc and cadmium on $HgCl_2$-d-ALA-D inhibition and Hg levels in tissues of suckling rats)
- **CAPÍTULO 4: ARTIGO 2**

  Efetividade do $ZnCl_2$ em proteger contra a nefrotoxicidade induzida pelo $HgCl_2$ em ratos recém-nascidos

  (Effectiveness of $ZnCl_2$ in protecting against nephrotoxicity induced by $HgCl_2$ in newborn rats)
- **CAPÍTULO 5: MANUSCRITO**

  Níveis de metalotioneína, zinco e mercúrio nos tecidos de ratos jovens expostos ao zinco e subseqüentemente ao mercúrio

  (Metallothionein, zinc and mercury levels in tissues of young rats exposed to zinc and subsequently to mercury)
- **CAPÍTULO 6: DISCUSSÃO GERAL;**
- **CAPÍTULO 7: CONCLUSÃO GERAL.**

CAPÍTULO 2
2 REVISÃO BIBLIOGRÁFICA

2.1 Mercúrio

O mercúrio é um elemento químico que não possui função biológica e causa efeitos deletérios e, por essas razões, arrolado como metal tóxico (GOYER, 1996; KLAASSEN, 1996). É ubíqüo no meio ambiente, deriva de fontes naturais e de atividades humanas. Por tratar-se de um agente potencialmente tóxico, é indicado que deve-se minimizar ao máximo seu emprego em qualquer tipo de atividade (CLARKSON, 1997; FURST & RADDING, 1998). As diversas formas, inorgânicas e orgânicas, nas quais pode apresentar-se, refletem a diversidade de efeitos que pode provocar. Dentre as principais, destacam-se as formas inorgânicas mercúrica (Hg^{2+}) e o mercúrio elementar [ou mercúrio metálico (Hg^{o})] e a forma orgânica metilmercúrio (CH_3Hg^{+}). O mercúrio metálico tem predileção por afetar o SNC e os rins (FREDRIKSSON *et al.*, 1992; RISHER *et al.*, 2003; COUNTER & BUCHANAN, 2004; YOSHIDA *et al.*, 2005), enquanto que a forma mercúrica, cujo principal representante é o cloreto de mercúrio ($HgCl_2$), é um característico nefrotóxico (CLARKSON, 1997; HERAK-KRAMBERGER & SABOLIC, 2001). Na atualidade, o mercúrio ainda é usado na extração do ouro, assim a exposição ocupacional ocorre nos garimpeiros e na população alocada nas imediações do garimpo (LEBEL *et al.*, 1996; BISINOTI & JARDIM, 2004; CASTILHOS *et al.*, 2006). Populações ribeirinhas que habitam locais não tão próximos das regiões de garimpagem, mas que cujas águas e fauna aquática estão contaminadas pelos rejeitos derivados da atividade de extração, também são alvos potenciais do mercúrio. A exposição dessas populações é magnificada em virtude da baixa renda, razão pela qual sua principal fonte protéica alimentar é por meio do consumo de peixes originários do ambiente contaminado (LEBEL *et al.*, 1996; RENZONI *et al.*, 1998; CHAN *et al.*, 2003; CASTILHOS *et al.*, 2006).

A forma mercúrica é alvo de inúmeras investigações porque, além de sua toxicidade intrínseca, é atribuída à ela a toxicidade do mercúrio elementar e, parcialmente, a do

metilmercúrio, uma vez que, por oxidação e desmetilação, respectivamente, ambas as formas são convertidas em Hg^{2+} (COUNTER & BUCHANAN, 2004). As alterações provocadas pela exposição de organismos vivos ao mercúrio são inúmeras. Dados da literatura relatam modificações no desempenho de tarefas comportamentais (ROCHA *et al.*, 1993, 2001; SAKAMOTO *et al.*, 1993), no peso corporal (SAKAMOTO *et al.*, 1993; ROCHA *et al.*, 1995), no peso, na histologia e na fisiologia de alguns órgãos (MAGOS *et al.*, 1982; ROCHA *et al.*, 1995; EMANUELLI *et al.*, 1996; HOMMA-TAKEDA *et al.*, 1999; SHIGEMATSU *et al.*, 2000; ROZA *et al.*, 2005). Além disso, são comuns alterações na atividade de enzimas como PBG-sintase proveniente de várias fontes (ROCHA *et al.*, 1995; EMANUELLI *et al.*, 1996; ROZA *et al.*, 2005), alanina aminotransferase (ALT) (GILL *et al.*, 1990; BUCIO *et al.*, 1995) e acetilcolinesterase de nervo periférico (OMATA *et al.*, 1982) e cerebral (MORETTO *et al.*, 2004), na uréia (HOLT & WEB, 1986; EMANUELLI *et al.*, 1996; EWALD & CALABRESE, 2001), na concentração tecidual de metais endógenos (BAPU *et al.*, 1994; FENG *et al.*, 2004), entre outras.

Nos casos de intoxicações por mercúrio é recomendado o afastamento do indivíduo da fonte de contaminação e a terapia medicamentosa é por intermédio da administração de agentes quelantes, que são antagonistas de metais por reagirem com eles e assim formarem complexos estáveis que são posteriormente excretados nesta forma complexada (JUNGHANS, 1983; HARTVIG, 1984; DOMINGO, 1995).

2.2 Cádmio

O cádmio, assim como o mercúrio, é considerado um metal tóxico. Quanto ao seu grau de toxicidade, pode ser equiparado ao chumbo e ao mercúrio, ou seja, é potencialmente tóxico (GOYER, 1996; KLAASSEN, 1996). Suas aplicações obtiveram grande êxito partindo dos achados de suas propriedades anticorrosivas. Industrialmente é utilizado na fabricação de baterias de cádmio e níquel, de células fotovoltaicas, de lâmpadas a vapor e na obtenção de pigmentos. Ademais, ele é um subproduto da mineração do zinco e do cobre e está presente no tabaco (WHO, 1992; GOYER, 1996; KLAASSEN, 1996). O cádmio gera prejuízos especialmente nos sistemas renal (TANG *et al.*, 1998; HERAK-KRAMBERGER & SABOLIC, 2001) e esquelético (OGOSHI *et al.*, 1992; BRZÓSKA *et al.*, 2001). A exposição ocupacional ocorre em trabalhadores de fábricas que utilizam o cádmio e em mineiros e na

população geral dá-se pelo hábito de fumar e pelo consumo de fígado, rins e frutos do mar, os quais concentram o metal (WHO, 1992; GOYER, 1996; KLAASSEN, 1996).

Apesar de ser classificado como um metal tóxico, dependendo da dose e da idade em que os animais são expostos, não são verificadas alterações no desenvolvimento e na atividade da enzima PBG-sintase (PEIXOTO, 2000), efeitos comumente induzidos pela exposição a metais pesados como o mercúrio e o chumbo (ROCHA *et al.*, 1995; PEIXOTO, 2000). Além disso, a exposição induz à síntese de proteínas ligantes de metal e MT (OTVOS & ARMITAGE, 1980; ONOSAKA & GEORGE CHERIAN, 1981; RITA MISRA *et al.*, 1997). Embora essas proteínas tenham a propriedade de quelar o metal e assim torná-lo indisponível para exercer seus efeitos tóxicos (HIDALGO *et al.*, 2001; CHAN *et al.*, 2002; DABRIO *et al.*, 2002), há relatos de nefrotoxicidade mais acentuada quando o cádmio encontra-se nessa forma do que quando na forma de sal (DORIAN *et al.*, 1995).

2.3 Zinco

O zinco é classificado como metal essencial e tem várias funções biológicas comprovadas. Primariamente, destacam-se suas funções catalítica, estrutural e regulatória (CHAN *et al.*, 2002; MARET, 2005; MATHIE *et al.*, 2006). A replicação e a transcrição do ácido desoxirribonucléico (ADN) e a síntese protéica são processos que representam o papel essencial do zinco na regulação da proliferação e diferenciação celulares (ECKHERT & HURLEY, 1977; GEORGE CHERIAN *et al.*, 2003). Sua essencialidade também é evidente considerando-se que ele é imprescindível à uma variedade de funções bioquímicas e fisiológicas, nas quais estão envolvidas várias enzimas e proteínas que o requerem (VALLEE, 1995; ROFE *et al.*, 2000; ZATTA *et al.*, 2003; TAKEDA *et al.*, 2004a, 2004b, 2005). A manutenção das concentrações adequadas de zinco é de suma importância de modo a manter todos esses eventos sob um controle rígido. A homeostase do metal é realizada por meio de proteínas sensoras e transportadoras de membranas, as quais regulam o influxo e o efluxo de zinco celular e vesicular, e por outras proteínas como tioneínas e MT, que estão envolvidas no tráfego do metal entre organelas e na manutenção da concentração de zinco disponível dentro de uma faixa de variação específica (MASON *et al.*, 1981; CUAJUNGCO & LEES, 1997; ROFE *et al.*, 2000; MARET, 2005).

O zinco possui a capacidade de induzir à síntese de proteínas ligantes de metal e MT

(EATON *et al.*, 1980; GOERING & FOWLER, 1987; PEDERSEN *et al.*, 1998). Entre os metais, é classificado como o melhor indutor (EATON *et al.*, 1980; BRACKEN & KLAASSEN, 1987).

Há um considerável número de publicações relatando os efeitos benéficos do zinco em relação às várias alterações indesejáveis provocadas por alguns agentes como o mercúrio (GALE, 1984; ZALUPS & CHERIAN, 1992), o cádmio (TANG *et al.*, 1998; BRZÓSKA *et al.*, 2001), o cobre (BREWER *et al.*, 1998), organofosforados (GOEL *et al.*, 2005, 2006), e por algumas patologias, como o diabetes (TOBIA *et al.*, 1998; QURAISHI *et al.*, 2005), a aterosclerose (REN *et al.*, 2006), a gastrite (TRAN *et al.*, 2005) e a cirrose (BIANCHI *et al.*, 2000). Atualmente, o zinco é considerado um possível antagonista do mercúrio (COUNTER & BUCHANAN, 2004).

2.4 Animais em desenvolvimento

Os roedores apresentam fases de desenvolvimento rápido pós-natal. Essas fases estão compreendidas desde o momento do nascimento e estendem-se até o final do período de desmame (3 semanas de idade, aproximadamente) (GOTTLIEB *et al.*, 1977). Os estágios de desenvolvimento acelerado são os seguintes:

- primeira fase: situa-se desde o dia do nascimento e estende-se até o sexto dia de vida;
- segunda fase: compreendida entre o oitavo e o décimo terceiro dia de idade;
- terceira fase: ocorre do décimo sétimo ao vigésimo terceiro dia de vida.

Durante esses intervalos de desenvolvimento rápido há um aumento mais pronunciado do peso da maioria dos órgãos quando comparado com as taxas de desenvolvimentos que verifica-se imediatamente antes e depois desses intervalos (KOBAYASHI, 1963; WINICK & NOBLE, 1965; GOTTLIEB *et al.*, 1977). Esse aumento mais significativo do desenvolvimento é atribuído à maior quantidade de mielina, de proteína, de ADN e de ácido ribonucléico (ARN) presente nos órgãos em virtude da intensa síntese dos mesmos (WINICK & NOBLE, 1965; GOTTLIEB *et al.*, 1977; MORGANE *et al.*, 2002).

Vários trabalhos determinaram que animais não completamente desenvolvidos têm uma marcada sensibilidade quando aplica-se insultos externos (BARONE *et al.*, 2000; RICE

& BARONE, 2000; MENDOLA *et al.*, 2002; MORGANE *et al.*, 2002). Esses insultos tanto podem ser sutis, como estresse, manipulação, subnutrição e privação do contato materno (COWLEY & WIDDOWSON, 1965; GOLDMAN, 1965; ADLARD & DOBBING, 1971; DOBBING & SANDS, 1971; FRANKOVÁ & BLATNÍKOVÁ, 1979; ROCHA & VENDITE, 1990), ou podem ser agressões propriamente ditas, como exposições a metais pesados, praguicidas ou outros agentes tóxicos (RIBEIRO-DA-SILVA *et al.*, 1994; ASTON *et al.*, 1996; MOSER, 2000; FERRI *et al.*, 2003; ROZA *et al.*, 2005; SINGH & RISHI, 2005; DOREA & DONANGELO, 2006). As deficiências provocadas por esses insultos impostos nesta idade precoce da vida podem ser irreversíveis, ainda que cessada sua aplicação e recompostas as condições adequadas dos animais (FREDRIKSSON *et al.*, 1992; RICE & GILBERT, 1995; TRAUTH *et al.*, 2000). Há relatos da presença de certas deficiências em animais adultos expostos a insultos sutis durante a vida precoce (FRANKOVÁ & BARNES, 1968; VENDITE *et al.*, 1985). A grande vulnerabilidade desses organismos quanto a agentes químicos está relacionada à imaturidade dos órgãos e membranas e à incapacidade de processar adequadamente os mesmos (NIES & SPIELBERG, 1996; HODGSON, 1997; TYL, 1998).

2.5 PBG-sintase (E.C. 4.2.1.24)

A enzima citosólica PBG-sintase, também denominada δ-aminolevulinato desidratase (δ-ALA-D), é classificada, segundo a *Enzyme Comission*, como E.C. 4.2.1.24, sendo uma liase que catalisa a condensação de duas moléculas do substrato ácido δ-aminolevulínico (δ-ALA) para a síntese de uma molécula de porfobilinogênio (PBG) com a liberação de duas moléculas de água (BERNARD & LAUWERYS, 1987; JAFFE, 1995). Está amplamente distribuída nos organismos vivos e é altamente conservada (JAFFE, 1995). Seu alto conteúdo de grupamentos contendo enxofre (SHEMIN, 1976; TSUKAMOTO *et al.*, 1979) determina sua grande afinidade por metais, preponderantemente, os divalentes (BORDER *et al.*, 1976; SCHEUHAMMER, 1987). A ligação dos metais a esses grupamentos químicos altera sua capacidade catalítica (BORDER *et al.*, 1976; DESPAUX *et al.*, 1977; DAVIS & AVRAM, 1980; SCHEUHAMMER, 1987).

Dados da literatura relatam que alguns metais, como zinco, são ativadores da PBG-

sintase (DESPAUX *et al.*, 1977; BERNARD & LAUWERYS, 1987), enquanto que outros, como mercúrio e chumbo, são inibidores da sua atividade (TSUKAMOTO *et al.*, 1980; GOERING & FOWLER, 1987; NOGUEIRA *et al.*, 2003). Além disso, alguns metais foram classificados como ativadores da enzima em baixas concentrações e inibidores da PBG-sintase quando presente em altas concentrações (DESPAUX *et al.*, 1977; BERNARD & LAUWERYS, 1987). As características de ubiqüidade (GIBSON *et al.*, 1955; BERNARD & LAUWERYS, 1987; JAFFE, 1995) e sua natureza sulfidrílica (SHEMIN, 1976; TSUKAMOTO *et al.*, 1979) são as responsáveis pela sua utilização como biomarcador da exposição a metais pesados.

2.6 MT

As MT pertencem à uma superfamília de proteínas ligantes de metal descritas por Margoshes e Vallee, em 1957, por ocasião de sua descoberta em rim de cavalo (CAI *et al.*, 1999; NATH *et al.*, 2000; DABRIO *et al.*, 2002). São proteínas ubíqüas, já foi descrita sua presença em fungos, leveduras, bactérias, mamíferos, crustáceos, vegetais, moluscos e em outros organismos (STILLMAN, 1995; PEDERSEN *et al.*, 1998; KLAASSEN *et al.*, 1999; ROMERO-ISART & VASÁK, 2002). Estão alocadas numa classe especial porque detêm características peculiares (DUNN *et al.*, 1987; STILLMAN, 1995; NATH *et al.*, 2000; CHAN *et al.*, 2002; DABRIO *et al.*, 2002; ROMERO-ISART & VASÁK, 2002):

- localização preponderantemente citosólica;
- peso molecular baixo (6.000-7.000 Da, em mamíferos, corresponde a uma única cadeia polipeptídica com 61-68 resíduos de aminoácidos);
- ricas em cisteína (25-30%);
- ausência de aminoácidos aromáticos e de histidina;
- ausência de pontes dissulfeto;
- distribuição dos resíduos cisteinil altamente conservada;
- resistência ao calor;
- alto conteúdo de metais.

O alto conteúdo de resíduos de cisteína proporciona-lhes alto conteúdo de metais, uma vez que a afinidade pelos metais é conferida pela presença dos grupamentos sulfidrílicos

(CHAN *et al.*, 2002; ROMERO-ISART & VASÁK, 2002). Os átomos de metais situam-se em regiões específicas da cadeia polipeptídica em agrupamentos denominados *clusters* (OTVOS & ARMITAGE, 1980; STILLMAN, 1995). Cada molécula protéica é capaz de ligar 7, 12 ou mesmo 18 átomos de metal dos Grupos 11 e 12 (STILLMAN, 1995; CHAN *et al.*, 2002). A composição metálica da proteína depende do tecido e da exposição prévia ao metal (ROMERO-ISART & VASÁK, 2002).

A síntese das MT pode ser induzida pela exposição a qualquer um dos seguintes fatores: estresse (HIDALGO *et al.*, 1990; KONDOH *et al.*, 2003), íons metálicos (essenciais ou tóxicos) (EATON *et al.*, 1980; GOERING & FOWLER, 1987), hormônios (DUNN *et al.*, 1987; NATH *et al.*, 2000), agentes farmacológicos (BRACKEN & KLAASSEN, 1987; NATH *et al.*, 2000), radiações (CAI *et al.*, 1999), praguicidas (NATH *et al.*, 2000; KONDOH *et al.*, 2003), compostos químicos em geral (BRACKEN & KLAASSEN, 1987; THEOCHARIS *et al.*, 2001). Os tecidos que mais expressam as MT são o fígado, os rins e os intestinos, mas estão presentes em vários outros tecidos (CAI *et al.*, 1999; ROMERO-ISART & VASÁK, 2002).

As funções biológicas dessas moléculas ainda não estão completamente elucidadas. Entretanto, algumas investigações revelaram que estão envolvidas na homeostase de metais essenciais, como o zinco e o cobre, e no mecanismo de destoxificação de metais e radicais livres (HIDALGO *et al.*, 2001; CHAN *et al.*, 2002; DABRIO *et al.*, 2002).

CAPÍTULO 3

ARTIGO 1:

Título: **Efeitos do zinco e do cádmio sobre a inibição da** d**-ALA-D induzida pelo $HgCl_2$ e os níveis de Hg nos tecidos de ratos jovens**

(Effects of zinc and cadmium on $HgCl_2$-d-ALA-D inhibition and Hg levels in tissues of suckling rats)

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Effects of zinc and cadmium on $HgCl_2$-δ-ALA-D inhibition and Hg levels in tissues of suckling rats

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Abstract

The effects of $CdCl_2$ and $ZnCl_2$ pretreatments on the inhibition of δ-ALA-D (δ-aminolevulinic acid dehydratase) activity and Hg contents in liver and kidneys of suckling rats intoxicated with $HgCl_2$ were investigated. Zn-pretreatment prevented the effects of mercury at a higher magnitude than $CdCl_2$. Hepatic and renal δ-ALA-D activities were significantly inhibited by $HgCl_2$ and prior exposure to $CdCl_2$ partially prevented the renal effect of mercury but not altered the mercury levels in both tissues. Pretreatment with $ZnCl_2$ abolished mercury-induced δ-ALA-D-inhibition in kidneys and liver and induced an increase in renal (about three times) and a decrease in hepatic (to one-third) Hg contents when compared to the group injected only with mercury. In face of zinc effects to prevent Hg-δ-ALA-D inhibition and to alter Hg-deposition levels in kidney and liver, these results suggest that these effects may be partially due to the synthesis of metallothioneins (MT). In fact, liver MT content presented by animals pretreated with zinc was significantly greater than control and Hg-treated groups, but the increase showed by renal tissue (about 60%) was not significant. Although the MT is rich in cysteine (-SH) and consequently can form a great number of MT–Hg complex, other mechanisms should be also involved in zinc protection on mercury toxicity.

Keywords: Cadmium and zinc chloride; Mercuric chloride; δ-aminolevulinic acid dehydratase; Suckling rats; Tissues metal levels; Metallothionein

1. Introduction

Mercury, cadmium, and lead are potent toxic agents that are released into the environment mainly through anthropogenic actions. Numerous studies have been conducted to determine the molecular mechanism(s) underlying their toxic actions. Although various molecular and cellular targets have been identified, the precise involvement of such targets in the toxicity of these metals has not been definitely established yet (Clarkson, 1983; Annau and Cuomo, 1988; Bressler and Goldstein, 1991; Rossi et al., 1991; Rocha et al., 1993, 1995). The majority of experiments with mercury have been conducted in adult animals (Friberg, 1956; Mengel and Karlog, 1980; Nielsen and Andersen, 1989, 1990). The sensitivity of developing animals to various compounds (including metals) may differ from that observed in adults (Jugo, 1976;

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Kostial et al., 1978; Walsh, 1982; Webb and Holt, 1982; Pereira et al., 1999), thus making important to examine the effects of metals on developing organisms.

In experiments conducted with young rats the administration of mercuric chloride (Rocha et al., 1995, 2001) and methylmercury chloride (Rocha et al., 1993) inhibit the rat δ-aminolevulinic acid dehydratase (δ-ALA-D) activity. This is a Zn-dependent, sulfhydryl-containing enzyme, that catalyses the condensation of two δ-aminolevulinic acid (δ-ALA) molecules to form porphobilinogen, which is a heme precursor (Gibson et al., 1955). Considering its high sensitivity to metals, the activity of this enzyme can be used as useful biomarker of heavy metals exposition and/or intoxication.

Zinc, cadmium, and mercury are divalent metals that constitute group II B of the periodic table of elements. Although zinc is an essential trace metal while the remaining two are toxic metals, they share biological responses such as affinity towards thiol groups, antagonism to biological cations, participation in redox reaction, modulation of essential elements homeostasis, and preferential excretion through feces via bile. However, the most important common feature among these metals is the ability to induce synthesis of metallothioneins (MT). These proteins are characterized by low molecular mass (6000–7000 Da), a high content of cysteine (SH) (about 30% in vertebrates), a lack of aromatic amino acid residues, and heat stability (Dunn et al., 1987; Romero-Isart and Vasák, 2002). Beyond metals, the MT are induced by hormones, stress, irradiation, and other factors (Dunn et al., 1987; Park et al., 2001; Tandon et al., 2001). The biosynthesis of MT happens in two vital organs involved in detoxification of toxins, named liver and kidney (Dunn et al., 1987; Romero-Isart and Vasák, 2002; Kondoh et al., 2003). In newborn rats, ontogenic changes in the hepatic MT happen after birth until about 16th day post-partum and appear to be related to the increase in liver weight (Mason et al., 1981). The main role of MT is detoxification of heavy metals and in regulation of the homeostasis of essential trace metals (such as copper and zinc) (Dunn et al., 1987; Romero-Isart and Vasák, 2002).

Considering that the sensitivity of target organs and, most specifically, the sensitivity of molecular targets (δ-ALA-D and MT) to metals can change considerably as a function of development (Mason et al., 1981), the present study investigated the effect of $CdCl_2$ and $ZnCl_2$ pretreatments on the inhibition of δ-ALA-D and mercury accumulation in tissues induced by mercury exposure during the early post-natal periods. The MT contents were assessed with the aim of verifying the protection capacity of this protein on mercury intoxication effects.

2. Materials and methods

2.1. Chemicals

Glacial acetic acid, *ortho*-phosphoric acid, perchloric acid, absolute ethanol, $HgCl_2$, $CdCl_2$, $ZnCl_2$, NaCl, K_2HPO_4, KH_2PO_4, H_2SO_4, HNO_3, NaOH, perhydrol® 30%, sodium tetrahydroborate, β-mercaptoethanol, sucrose, HCl, phenylmethylsulphonylfluoride (PMSF), chloroform, calcium disodium ethylenediaminetetraacetate (EDTA), 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), L-cysteine hydrochloride monohydrate, and tris(hydroxymethyl) aminomethane (Tris) were obtained from Merck (Rio de Janeiro, RJ, Brazil); δ-aminolevulinic acid, bovine albumin, and Coomassie brilliant blue G from Sigma (St. Louis, MO). Trichloroacetic acid was obtained from Reagen and *para*-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han., Germany).

2.2. Animals

Wistar rats obtained from the General Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12 h light/dark cycle. The breeding regimen consisted of grouping three virgin females (90- to 120-day-old) with one male for 20 days. After this period, pregnant rats were selected and housed individually in opaque plastic cages (50 cm × 25 cm × 18 cm). Pregnant rats were checked once daily between 3:00 and 6:00 p.m. for the presence of pups.

2.3. Treatment with metals

When pups were 3 days old, they received five subcutaneous (s.c.) injections on consecutive days of saline (NaCl 90 mg/kg), $CdCl_2$ (3.7 mg/kg) or $ZnCl_2$

(27.0 mg/kg). On five subsequent days the animals were injected daily with one dose (s.c.) of saline or $HgCl_2$ (5.0 mg/kg). The pups were separated from their dams, weighted, injected, and placed in individual cages for about 10 min. The metals were dissolved in saline solution and all the solutions were injected at a volume of 10 ml/kg body weight.

2.4. Tissue preparation

Rats were weighted, anesthetized with ether and killed by decapitation about 24 h after the last dose of saline or mercury. The kidneys and liver were removed and weighted. Samples of these tissues were collected for enzymatic activity assays, to determine mercury, and metallothionein levels.

For δ-ALA-D activity assays, the liver and kidneys were quickly removed and placed on ice. Kidneys were homogenized in 5 and 7 volumes of 150 mM NaCl with 10 up-and-down strokes at ~1200 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged at 8000 × *g* for 30 min using a Janetisky K-24 refrigerated centrifuge. The supernatant fraction was used in the enzyme assays.

To determine mercury levels in the tissues, liver and kidney were removed, placed in a vial, and frozen (−20 °C).

For metallothionein level assays, the liver and kidneys were homogenized in 4 volumes of 20 mM Tris–HCl buffer, pH 8.6, containing 0.5 M PMSF as agent antiproteolytic and 0.01% β-mercaptoethanol as a reducing agent. The homogenate was then centrifuged at 17,000 × *g* for 30 min to obtain a supernatant containing metallothioneins (Viarengo et al., 1997).

2.5. Enzyme assay

δ-ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen) formation, except that 76 mM sodium phosphate buffer (pH 6.8) and 2.2 mM δ-ALA were used. The reaction product was determined using a modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 for the Ehrlich-porphobilinogen salt. The incubation was initiated by adding 100 μl of tissue preparation (0.5–1.0 mg of protein) (final volume, 275 μl) and was carried out for 40 and 90 min for liver and kidneys, respectively, at 39 °C. The specific activity was expressed as nmol of PBG formed per hour per mg protein. Protein concentrations were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard. All samples were run in triplicate.

2.6. Mercury content determination

The determination of mercury was made according to the method described by Emanuelli et al. (1996). Prior to mercury analysis, samples were allowed to thaw and then received 10 ml of HNO_3 (65%) and 1 ml of perhydrol® (30%). Samples were maintained at room temperature for 24 h and heated at 45 °C on a sand bath for 6 h. Samples were filtered and the volume was adjusted to 25 ml with 0.5% HNO_3 (w/v). This solution was used for the determination of mercury by atomic absorption spectrometry (AAS) with the aid of the cold vapor technique using a Perkin Elmer 3030 Spectrometer and a Perkin Elmer MHS-10 Hydride Generation System. The detection limit for this method is 0.02 μg of Hg per gram of tissue. All samples were run in triplicate.

2.7. Metallothionein content determination

Metallothionein content determination was assayed according to the method of Viarengo et al. (1997) as modified by Petrovic et al. (2001). Aliquots of 1 ml of supernatant were added with 1.05 ml of cold (−20 °C) absolute ethanol and 80 μl of chloroform; the samples were then centrifuged at 6000 × *g* for 10 min. The collected supernatant was combined with three volumes of cold ethanol (−20 °C), maintained at −20 °C for 1 h and centrifuged at 6000 × *g* for 10 min. The metallothionein-containing pellets were then rinsed with 87% ethanol and 1% chloroform and centrifuged at 6000 × *g* for 10 min. The metallothionein content in the pellet was evaluated using the colorimetric method with Ellman's reagent. The pellet was resuspended in 150 μl 0.25 M NaCl and subsequently 150 μl 1 N HCl containing EDTA 4 mM were added to the sample. A volume of 4.2 ml 2 M NaCl containing 0.43 mM DTNB buffered with 0.2 M Na–phosphate, pH 8.0 (Ellman, 1958) was then added to the sample at room temperature. The sample was finally centrifuged at

3000 × g for 5 min; the supernatant absorbance was evaluated at 412 nm and metallothionein concentration was estimated utilizing cysteine as a reference standard and expressed as micrograms of SH per gram of wet weight. All samples were run in duplicate.

2.8. Statistical analysis

Body and organ weights, δ-ALA-D activity, mercury levels of tissues and relative percentage of MT were analyzed by one-way ANOVA followed by Duncan's multiple range test when appropriate. Each litter contributed only one rat for each experimental group in order to avoid a litter effect (Abbey and Howard, 1973).

3. Results

3.1. Body, liver, and kidney weights

The effects of the metal treatments on corporal, renal and hepatic weights are illustrated in Fig. 1. One-way ANOVA revealed that the body weight was significantly affected by the administration of mercury (Cd-pretreatment, $F(3,24) = 21.64$, $P < 0.0001$;

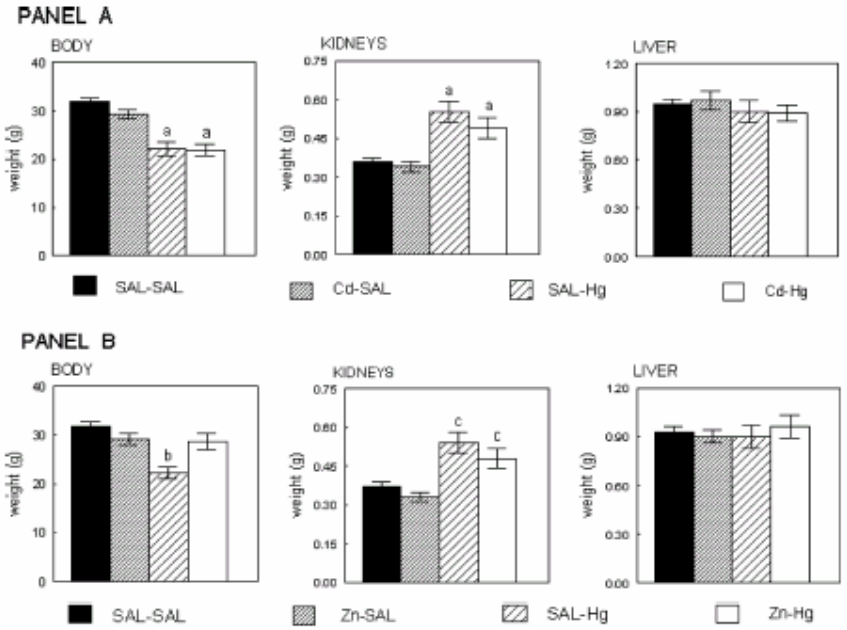

Fig. 1. Body, kidneys, and liver weights of 13-day-old rats intoxicated with $HgCl_2$ pre-exposed to $CdCl_2$ or $ZnCl_2$. Data shown as mean ± *S.E.M.* Rats were treated (s.c.) for five consecutive days from post-natal days 3–7 with saline (90 mg/kg) or $CdCl_2$ (3.7 mg/kg) (panel A); or saline or $ZnCl_2$ (27 mg/kg) (panel B). Subsequently the animals received (s.c.) from day 8–12 five doses of saline or $HgCl_2$ (5.0 mg/kg). Duncan's multiple range test: (a) Significantly different from groups treated with saline–saline and cadmium–saline ($P < 0.01$); (b) significantly different from the others groups from the same panel ($P < 0.01$); (c) Significantly different from groups treated with saline–saline and zinc–saline ($P < 0.05$).

Zn-pretreatment, $F(3, 24) = 10.32$, $P < 0.001$). $HgCl_2$ treated groups presented a decrease in weight gains of about 30% when pretreated with saline or cadmium ($P < 0.01$, Duncan's multiple range test) and 10% when pretreated with zinc. Previous exposure to zinc prevented the mercury-induced loss of weight since there was no significant difference between zinc–mercury group and saline–saline group.

Liver weight was not altered, whereas renal weight was significantly increased by mercury treatment. One-way ANOVA revealed a significant effect of $HgCl_2$ treatment on renal weights in both pretreatment models ($CdCl_2$, $F(3, 20) = 12.61$, $P < 0.0001$; $ZnCl_2$, $F(3, 12) = 6.32$, $P < 0.008$). $HgCl_2$ treated animals presented an increase in renal weights of about 50% ($P < 0.05$, Duncan's multiple range test). Previous exposure to zinc or cadmium has not prevented the increasing of renal weight induced by mercury.

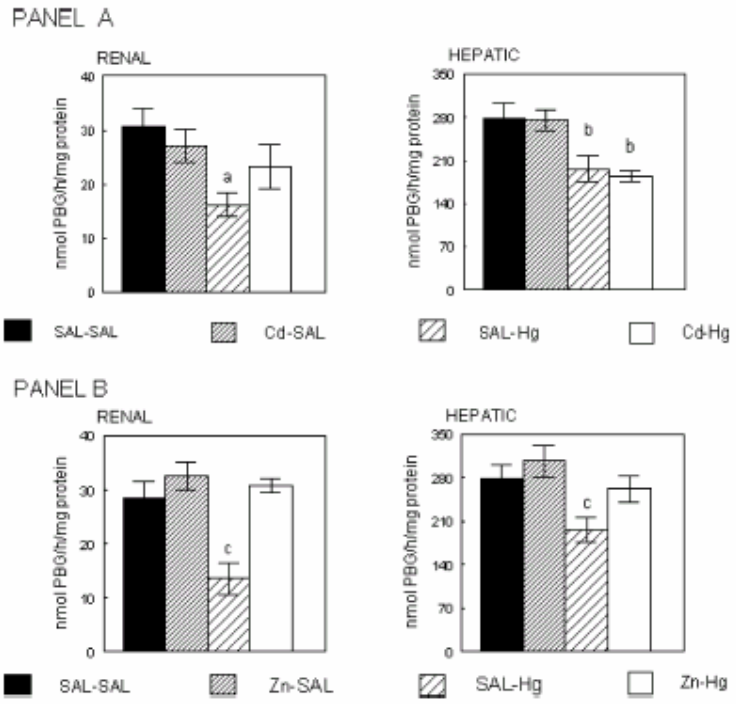

Fig. 2. Renal and hepatic δ-ALA-D specific activities of 13-day-old rats intoxicated with $HgCl_2$ pre-exposed to $CdCl_2$ or $ZnCl_2$. Data shown as mean ± *S.E.M.* Rats were treated as described in Fig. 1. δ-ALA-D specific activity is expressed as nanomol of porphobilinogen formed per hour per milligram of protein. Duncan's multiple range test: (a) significantly different from groups treated with saline–saline and cadmium–saline ($P < 0.05$); (b) significantly different from groups treated with saline–saline and cadmium–saline ($P < 0.01$); (c) significantly different from the others groups from same panel ($P < 0.05$).

3.2. Renal and hepatic δ-ALA-D

The effects of cadmium and zinc pretreatments on mercury δ-ALA-D inhibition are shown in Fig. 2.

One-way ANOVA revealed significant effects of $HgCl_2$ treatment on renal δ-ALA-D activity in cadmium and zinc pretreatments ($CdCl_2$ (panel A), $F(3, 20) = 3.58$, $P < 0.03$; $ZnCl_2$ (panel B), $F(3, 12) = 11.20$, $P < 0.009$). Posthoc comparisons by Duncan's multiple range test showed that $HgCl_2$ treated rats pretreated with saline presented a decrease in enzyme activity of about 50% ($P < 0.05$). Previous exposition to cadmium partially prevented the δ-ALA-D inhibition induced by mercury (24% inhibition), whereas the previous exposition to zinc totally prevented the inhibition of the enzyme induced by mercury.

One-way ANOVA for liver δ-ALA-D activities revealed significant effects of $HgCl_2$ (Cd-pretreatment, $F(3, 24) = 7.71$, $P < 0.0009$); Zn-pretreatment, $F(3, 24) = 4.36$, $P < 0.01$). Posthoc comparisons (Duncan's multiple range test) showed that

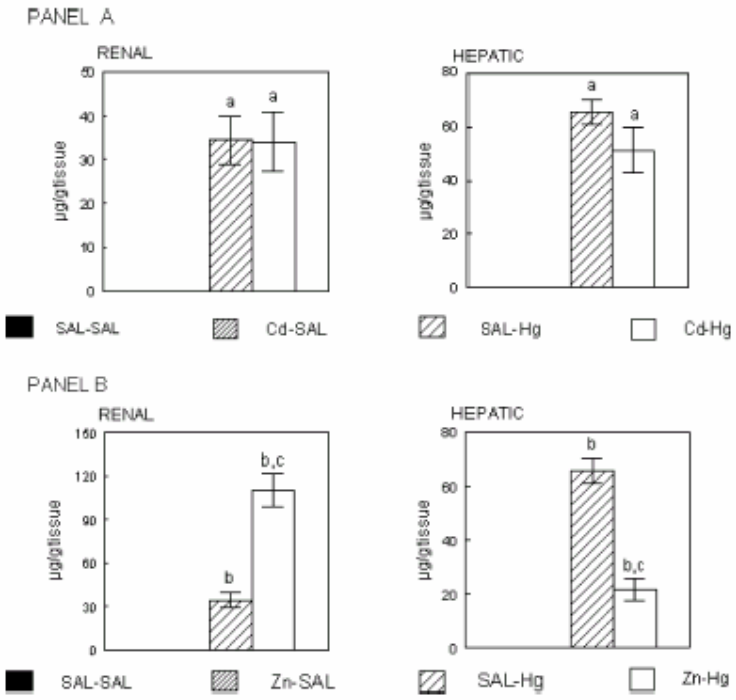

Fig. 3. Renal and hepatic mercury content of 13-day-old rats intoxicated with $HgCl_2$ pre-exposed to $CdCl_2$ or $ZnCl_2$. Data shown as mean ± *S.E.M.* Rats were treated as described in Fig. 1. The mercury level is expressed as micrograms of mercury per gram of wet tissue. Duncan's multiple range test ($P < 0.01$): (a) significantly different from groups treated with saline–saline and cadmium–saline; (b) significantly different from groups treated with saline–saline and zinc–saline; (c) significantly different from group treated with saline–mercury.

saline–mercury and cadmium–mercury groups presented significant inhibition of the δ-ALA-D activities in the range of 30% ($P < 0.01$). Previous exposure to zinc prevented δ-ALA-D inhibition induced by mercury. The liver enzyme activities were similar for the two groups, zinc–mercury and saline–saline.

3.3. Mercury quantification

The mercury content of kidneys and liver is shown in Fig. 3. The both tissues of Hg-untreated animals presented Hg levels lower than the detection limit of the method used in this work (0.02 μg). The one-way ANOVA revealed significant effects of the $HgCl_2$ treatment on mercury levels (Cd-pretreatment, kidney $F(3, 8) = 20.01$, $P < 0.0004$, liver $F(3, 8) = 49.36$, $P < 0.001$; Zn-pretreatment, kidney $F(3, 8) = 69.18$, $P < 0.0001$, liver $F(3, 8) = 108.83$, $P < 0.0001$). Posthoc comparisons by Duncan's multiple range test showed that all groups treated with $HgCl_2$ presented greater levels of this metal than saline-treated groups in both tissues analyzed ($P < 0.01$). Whereas the Cd-pretreatment did not alter the mercury content of tissues, the Zn-pretreatment induced alterations in mercury levels in both tissues of rats exposed to $HgCl_2$. The animals injected with zinc prior to mercury presented, in renal tissues, mercury levels about three folds greater than those from animals exposed only to mercury ($P < 0.05$). In the liver, the pre-exposure to zinc reduced the mercury levels to one-third of the group exposed only to mercury ($P < 0.05$).

3.4. Metallothionein content

The MT assay was conducted only in animals pretreated with zinc because only this pretreatment modified the δ-ALA-D activity inhibition and Hg-deposition levels induced by $HgCl_2$ treatment. The MT content was quantified as tissues SH content and the results were analyzed as relative percentage of control group. The Table 1 shows the effects of zinc pretreatment on MT levels in rats intoxicated with mercury.

One-way ANOVA revealed a significant effect of treatment on %MT hepatic [$F(3, 8) = 24.537$, $P < 0.002$] but not renal [$F(3, 8) = 1.018$, $P = 0.434$]. Posthoc comparisons by Duncan's multiple range test of hepatic MT showed that both groups treated with $ZnCl_2$ ($P < 0.01$) and the group treated with $HgCl_2$ ($P < 0.05$) presented greater levels of MT than saline-treated group. Zn-pretreatment induced an increase of 200% in the hepatic MT and of 60% in the renal MT. The animals treated only with mercury presented an increase in hepatic MT of 80%. The renal MT level was not altered by treatment with Hg.

Table 1
Metallothionein content in kidneys and liver of young rats injected for five consecutive days with one daily injection (s.c.) of saline (90 mg/kg) or $ZnCl_2$ (27.0 mg/kg) (treatment 1) and subsequently for five consecutive days with one daily injection (s.c.) of saline (90 mg/kg) or $HgCl_2$ (5.0 mg/kg) (treatment 2)

Treatment 1	Treatment 2	MT levels (% of control group)	
($n = 3$)		Renal	Hepatic
Saline	Saline	100 ± 0	100 ± 0
Zinc	Saline	153.3 ± 31.0	329.5 ± 31.5[a,b]
Saline	Mercury	109.6 ± 34.1	186.2 ± 11.4[a]
Zinc	Mercury	160.2 ± 38.7	369.1 ± 37.9[a,b]

Data are presented as mean ± S.E.M of relative percentage of saline–saline group (hepatic MT: 0.273±0.043, renal MT: 0.094± 0.021, expressed as mg of SH/g fresh tissue). Duncan's multiple range test (at least $P < 0.05$).
[a] Significantly different from group treated with saline–saline.
[b] Significantly different from group treated with saline–mercury.

4. Discussion

The present work was conducted with the objective of investigating the sensitivity of young rats to cadmium and zinc as MT synthesis inductors as well as a preventive treatment of Hg intoxication using δ-ALA-D as biomarker.

$HgCl_2$ reduced body weight gain and increased the renal weight. The effect observed on body weight gain was less accentuated than that reported by Rocha et al. (1995), who used the same schedule of treatments. However, the increase in renal weight was more pronounced than that observed by authors mentioned above. Adult rodents also exhibit an increase of renal weight after exposition to mercurials (Rocha et al., 1995, Emanuelli et al., 1996). The present study indicates that the decrease in body weight gain induced by mercury intoxication was partially prevented by pretreatment with five doses of $ZnCl_2$, but not $CdCl_2$.

Inhibition of δ-ALA-D induced by organic or inorganic forms of mercury has been extensively reported in the literature (Rocha et al., 1993, 1995, 2001; Emanuelli et al., 1996). Now we show that renal but not hepatic δ-ALA-D-inhibition caused by $HgCl_2$ is partially prevented by previous administration of $CdCl_2$. Cd^{2+} prevention of renal Hg^{2+}-induced inhibition of δ-ALA-D, may be a consequence of MT synthesis, since these proteins can be induced by exposure to metals such as copper, cadmium and zinc (Dunn et al., 1987; Goering and Fowler, 1987; Park et al., 2001) and functioning as a detoxification mechanism as well as storage of heavy metals (Dunn et al., 1987; Cai and Cherian, 2003). However, the Cd-pretreatment was not effective at preventing the hepatic-δ-ALA-D inhibition. The lack of effects on this organ suggests that the levels of MT synthesized were not enough to bind significant amounts of free metal (Gad and Ash, 1998), considering the high levels of metal that accumulated in the hepatic tissue.

Zn-pretreatment modified the majority of mercury effects in every studied parameters. For instance, Zn^{2+} prevented the decrease in body weight gain (Fig. 1), prevented renal and hepatic δ-ALA-D activity inhibition (Fig. 2), and induced a decrease in liver and an increase in kidney of mercury contents (Fig. 3). Besides, Zn-pretreatment induced an increase in hepatic and renal MT of about 200 and 60%, respectively (Table 1). The formation of MT–Hg complex in high quantities in liver could overflow to the plasma and then be taken up by renal cells and degraded by lysosomal proteases increasing the pool of renal metal (Goering and Klaassen, 1983). Assuming the high cysteine content in the MT molecule, it is possible that this protein can sequester a high percentage of this toxic metal in an inert complex, making them less available to interact with sensitive organelles or enzyme systems. Another aspect is the high content of free zinc as consequence of zinc released of Zn-MT (complex formed after Zn-treatment and before exposure to mercury, when begin the metals change in the MT) plus zinc administrated (Goering and Fowler, 1985). It is very important when considering the ability of Zn to reverse the δ-ALA-D inhibition induced by heavy metals (Chiba and Kikuchi, 1984; Goering and Fowler, 1985) and by oxidative agents (Emanuelli et al., 1998).

In conclusion, considering the effects of zinc on prevention δ-ALA-D inhibition and modification of tissues Hg levels, $ZnCl_2$ can be considered as potential preventive therapeutic agent against Hg toxicity. However, based on these results it is not possible to know whether Zn^{2+} treatments could ameliorate the toxicity caused by Hg^{2+}.

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CAPÍTULO 4

ARTIGO 2:

Título: **Efetividade do $ZnCl_2$ em proteger contra a nefrotoxicidade induzida pelo $HgCl_2$ em ratos recém-nascidos**

(Effectiveness of $ZnCl_2$ in protecting against nephrotoxicity induced by $HgCl_2$ in newborn rats)

Autores: Nilce Coelho Peixoto e Maria Ester Pereira

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Effectiveness of $ZnCl_2$ in protecting against nephrotoxicity induced by $HgCl_2$ in newborn rats

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Abstract

This work investigated the preventive effects of $ZnCl_2$ on renal and hepatic alterations induced by $HgCl_2$ in young rats. Wistar rats of 3 days old were treated (s.c.) on consecutive days with saline or $ZnCl_2$ 27 mg/kg/day from the 3rd to the 7th and with saline or $HgCl_2$ 5.0 mg/kg/day from the 8th to the 12th day of life. Pups were sacrificed 24 h after the last dose and samples were collected. The creatinine and urea dosages, used as renal parameters, presented increases of 35% and 500%, respectively. The alanine aminotransferase and lactic dehydrogenase activities, used as hepatic parameters, presented a decrease (40%) and no alteration, respectively, by mercury exposure. The glycemia was diminished and the hepatic glycogen was not modified by mercury. All the mercury effects were prevented by zinc. These results suggest that mercury intoxication of young rats alters the renal function but does not modify the hepatic parameters, and previous exposure to zinc is able to avoid the renal damage.

Keywords: Young rats; Mercury; Zinc; Renal function; Hepatic function; Glycemia; Glycogen

1. Introduction

Mercury is a divalent metal without biological function (nonessential). In the environment, it is constantly transformed and found in both organic and inorganic forms (Clarkson, 1997; Counter and Buchanan, 2004). Consequently, its adverse effects are countless and, frequently, very serious. Methylmercury chloride is noteworthy among the organic forms and metallic mercury (elemental) and mercury chloride among the inorganic forms. The last which is the object of this investigation, may be originated from the oxidation of elemental mercury or the demethylation of methylmercury and can originate either metallic mercury through reduction or methylmercury through methylation (Counter and Buchanan, 2004; Lodenius and Malm, 1998; WHO, 1991). Mercury chloride has been recognized as a nephrotoxic agent (Fowler, 1992; Magos et al., 1982). Exposure to this form of metal causes many effects on kidneys related to both biochemical and structural functions (Ewald and Calabrese, 2001; Girardi and Elias, 1991, 1995; Homma-Takeda et al., 1999). As consequence, porphyrinuria and proteinuria are very common (Fowler, 1992). Although the hepatic organ is not defined as a target for mercury, alterations in some enzymatic activities (Akhilender Naidu et al., 1984; Ji et al., in press; Oliveira et al., 2004; Peixoto et al., 2003; Roza et al., 2005), in lipid peroxidation (Girardi and Elias, 1991; Ji et al., in press) and in processes related to primary metabolism, such as glucose and glycogen levels, have been described in the literature (Akhilender Naidu et al., 1984; Bleau et al., 1996; Srivastava, 1982). Furthermore, mercury causes alteration in regulation of appetite (Counter and Buchanan, 2004; Freundt and Ibrahim, 1990) and reduction of amino acids and sugars intestinal absorption (Farmanfarmaian et al., 1989).

Zinc also is a divalent metal and it is classified as an essential metal. Biologically it is an important enzymatic cofactor, but among its features, the most interesting is undoubtedly the ability to induce the synthesis of detoxificant proteins (Dunn et al., 1987; Kondoh et al., 2003), such as metallothioneins or metal binding proteins.

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These molecules would act by sequestering the xenobiotic and, consequently, inhibiting or diminishing its effect (Klaassen et al., 1999; Nath et al., 2000). Thus, the zinc exposure would avoid the deleterious effects of mercury (Peixoto et al., 2003; Zalups and Cherian, 1992).

Investigations utilizing young rats in association with tissue damage and the possible preventive character of zinc on this noxious effect are uncommon (Peixoto et al., 2003). This communication was designed to investigate the possible deleterious effects of $HgCl_2$ in developing rats. Creatinine and urea were utilized as renal function markers, and alanine aminotransferease (ALT) and lactic dehydrogenase (LDH) activities as hepatic function markers. The blood glucose level was also monitored. Moreover, this paper aimed at investigating whether $ZnCl_2$ prevents these alterations.

2. Materials and methods

2.1. Chemicals

Mercury chloride, zinc chloride, NaCl, KOH, ethanol, HCl, ammonium sulfate, iodine, and KI were obtained from Labsynth (Diadema/SP/Brazil). Glycogen was purchased from Merck (Darmstadt/Germany). The kits for determination of creatinine, urea, ALT and LDH were acquired from Labtest (Lagoa Santa/MG/Brazil). The kit for dosage of glucose was purchased from Bioclin (Belo Horizonte/MG/Brazil).

2.2. Animals

Wistar rats obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12-h light/dark cycle and at a controlled temperature (23 ± 2 °C). The animals had free access to water and commercial food.

2.3. Breeding

The breeding regimen consisted of grouping three females (90–120 days old) and one male for 20 days. After this period, pregnant rats were selected and housed individually in opaque plastic cages (50 × 25 × 18 cm). Pregnant rats were checked once a day between 3:00 and 6:00 p.m. to verify the possible presence of pups.

2.4. Newborn animals

The day of birth was defined as 0 days old. At 1 day old, the number of pups of each litter was reduced to 9. The number of litters used was 17. Males and females were used without distinction.

2.5. Pretreatment

Three-day-old Wistar rats were treated with NaCl 90 mg/kg/day (saline) or $ZnCl_2$ 27 mg/kg/day (s.c.) during 5 consecutive days (from 3 to 7 days old).

2.6. Intoxication

From the 8th to the 12th day of life the rats received one daily dose of saline or $HgCl_2$ 5.0 mg/kg (s.c.). The animals were weighed daily to adjust to the dose. Of the 153 animals used, 83 were injected with mercury (43 pretreated with saline and 40 pretreated with zinc) and only one pretreated with zinc died after receiving 4 doses of the heavy metal.

2.7. Samples preparation

At 24 h after the last dose of saline or mercury the pups were euthanized. Blood samples and livers were collected. Blood sera were obtained by total blood centrifugation at 3,000*g* and the sera were frozen until analysis (until 5 days). Liver samples were processed to glycogen analysis.

2.8. Biochemical determinations

2.8.1. Creatinine

The estimation of creatinine was carried out by measuring the quantity of product formed, creatinine picrate, and by utilizing creatinine as standard. The reaction was conduced in a medium containing picric acid 20.2 mmol/L and NaOH 145.4 mmol/L in a thermostatized cuvette at 37 °C with 100 μL of sample added. The absorbance was recorded at 510 nm.

2.8.2. Urea

The urea was determined by the quantity of the product formed, indophenol blue, and it was used as standard. The incubation, at 37°C for 5 min, was started by adding 10 μL of sample to a medium containing phosphate buffer 19.34 mmol/L pH 6.9, sodium salicylate 58.84 mmol/L, sodium nitroprusside 3.17 mmol/L, and urease (⩾12.63 UK/L). The reaction was stopped by adding oxidant solution (final concentrations: NaOH 0.07 mol/L and sodium hypochlorite 3.01 mmol/L), and the mixture was incubated for 5 min to achieve color development. The absorbance was measured at 600 nm.

2.8.3. Alanine aminotransferase (ALT)

The enzymatic activity was determined by the Reitman and Frankel (1957) method in a medium containing buffer 55.8 mmol/L pH 7.4, α-ketoglutaric acid 1.67 mmol/L, L-alanine 83.3 mmol/L, and sodium azide 12.8 mmol/L. After 2 min of thermoequilibration at 37 °C, the incubation was started by adding 25 μL of sample for 30 min at 37 °C. Subsequently, HCl 0.45 mol/L (to stopping the reaction) and 2,4-dinitrophenylhydrazine 0.45 mmol/L (as color reactive) were mixed and the tubes were placed at ambient temperature for 20 min. Finally, NaOH 0.33 mol/L was added to produce intense color. The absorbance was determined at 505 nm and the activity (in U/mL) was calculated by comparison with a calibration curve utilizing sodium pyruvate as standard.

2.8.4. Lactic dehydrogenase (LDH)

The measurement of enzymatic activity was carried out through the reduction of absorbance due to the oxidation of NADH and calculated using its molar absorption coefficient (6.30×10^3). The reaction was conduced in a medium containing NADH 282 μmol/L, sodium azide 14.7 mmol/L, sodium pyruvate 1.18 mmol/L, and buffer 49 mmol/L pH 7.5 in a thermostatized cuvette at 37 °C with 20 μL of sample added. The absorbance was recorded at 340 nm.

2.8.5. Glucose

The quantification of glucose was carried out by measuring the product formed in a reaction medium containing phosphate buffer 9.9 mmol/L pH 7.4, phenol 9.9 mmol/L, glucose oxidase ⩾12,000 U/L, peroxidase ⩾1,000 U/L and 4-amino-antipyrine 0.4 mmol/L. The incubation was started by adding 10 μL of sample and realized at 37 °C for 10 min. The absorbances were measured at 500 nm and the sample glucose concentrations were compared to the glucose standard curve.

2.8.6. Glycogen

The sample of liver, around 200 mg, was added to 2.0 mL of KOH 30% and heated at 100 °C until full dissolution of tissue and, then cooled in cool

water. Ethanol (2.0 mL) was added and the mixture was heated at 70 °C for 10 min. Next, the tubes were cooled in ice for 3 min and centrifuged at 3,000 rpm for 5 min. Supernatant was discarded and 0.2 mL of HCl 5N and 3.8 mL of distilled water were added to the pellet. The tubes containing extracted glycogen were frozen until quantification analyses (until 5 days).

The glycogen content was determined by the Krisman method (1962). The sample (50 μL) was mixed with iodine reactive [I_2 0.01% and KI 0.1% in a saturated solution of $(NH_4)_2SO_4$] and the resultant absorbance was determined at 460 nm and compared with a glycogen standard.

2.9. Statistical analysis

All assays were carried out in triplicate. The results were analyzed by one-way ANOVA followed by Duncan's multiple range test when appropriate. Each litter contributed only with one experimental sample (n) for each one of the determinations (Abbey and Howard, 1973).

2.10. Ethics

The experiments of this work involved the utilization of experimental rats. However, aiming for the protection and welfare of animals, the studies were conduced in accordance with the national and institutional guidelines (University Ethics Committee Guidelines—Process No. 23081.013915/2004-06) for experiments with animals.

3. Results

The effects of mercury on renal function were assessed by creatinine and urea contents and are illustrated in Figs. 1 and 2, respectively. One-way ANOVA revealed that both parameters were significantly altered by mercury exposure: creatinine, $F(3,12) = 6.981$, $P<0.01$; urea, $F(3,12) = 3.961$, $P<0.05$. The group treated with mercury presented increases in creatinine [Duncan's multiple range test: $P<0.05$ (Fig. 1)] and in urea levels [$P<0.05$ (Fig. 2)] when compared with the control group. These results also demonstrate that the previous exposure to zinc was able to avoid these increases.

In regard to hepatic toxicity, two enzymes were investigated, ALT and LDH, whose activities are shown in Figs. 3 and 4, respectively. Both activities were significantly altered by treatments: ALT, $F(3,12) = 5.339$, $P<0.02$; LDH, $F(3,12) = 4.130$, $P<0.05$. The mercury exposure significantly decreased the ALT activity ($P<0.05$) and the previous treatment with $ZnCl_2$ impeded the mercury effect. In relation to LDH, the Duncan's multiple range test showed that this enzyme was not significantly altered by metals, although the Hg-intoxicated rats presented the LDH activity around 80% of control, effect also prevented by $ZnCl_2$ exposure.

The blood glucose and hepatic glycogen levels are shown in Table 1. One-way ANOVA revealed that the blood glucose level was altered by treatment [$F(3,16) = 3.180$, $P = 0.05$]. The post hoc analyses showed that the administration of mercury caused a decrease in glycemia ($P<0.05$) and that pretreatment with zinc partially prevented this effect. The effect on glucose level seems to be independent of glycogen content, since this hepatic parameter was not modified by mercury exposure [one-way ANOVA: $F(3,20) = 1.068$, $P = 0.385$].

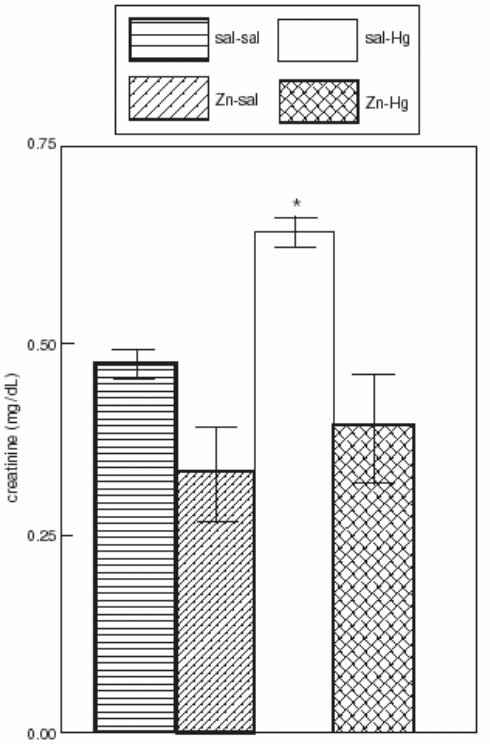

Fig. 1. Serum creatinine level of young rats treated with $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3rd–7th day old) and intoxicated with $HgCl_2$ (5 mg/kg/day; s.c.) for 5 subsequent days (8th–12th day old). Duncan's multiple range test: (*) significantly different from other groups ($P<0.05$; $n = 4$).

4. Discussion and conclusion

The purpose of this work was to investigate the renal and hepatic toxic effects of mercury chloride in neonate rats and the possible prevention of these by zinc chloride. Creatinine and urea levels and ALT and LDH activities indirectly assessed renal and hepatic alterations, respectively (Devlin, 1997). Mammals present a high sensitivity to extern insults when these are applied in early phases of life. In rodents, this phase happens from birth to weaning (Gottlieb et al., 1977) and is associated with intense development, the reason that rats in this age were chosen as experimental animals.

These findings confirm, now for young rats, the renal insufficiency caused by heavy metal. The Hg-intoxicated

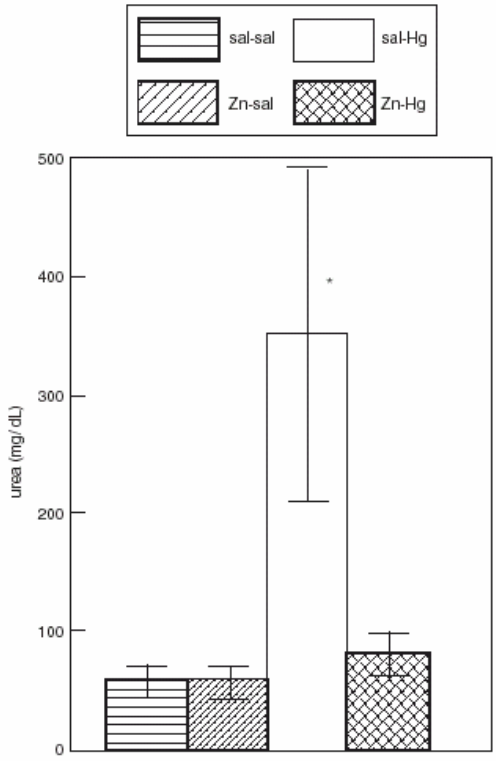

Fig. 2. Serum urea level of young rats treated with $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3rd–7th day old) and intoxicated with $HgCl_2$ (5 mg/kg/day; s.c.) for 5 subsequent days (8th–12th day old). Duncan's multiple range test: (*) significantly different from other groups ($P<0.05$; $n=4$).

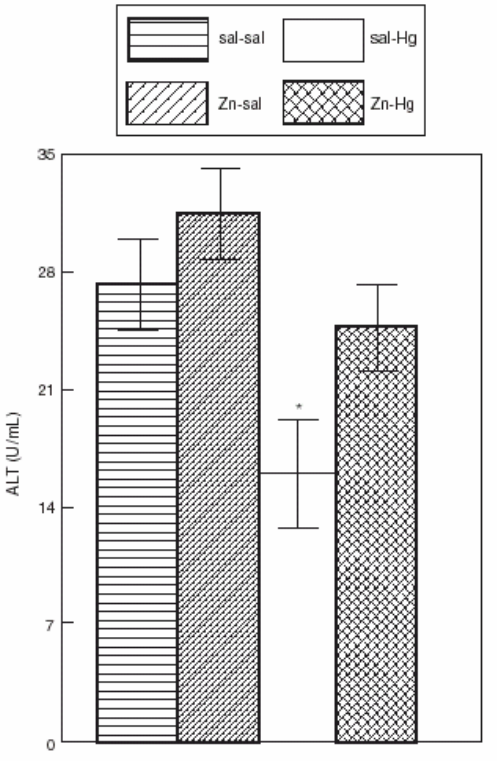

Fig. 3. Serum ALT activity of young rats treated with $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3rd–7th day old) and intoxicated with $HgCl_2$ (5 mg/kg/day; s.c.) for 5 subsequent days (8th–12th day old). Duncan's multiple range test: (*) significantly different from other groups ($P<0.05$; $n=4$).

rats showed an increase of 35% in creatinine and of 500% in urea levels. The previous exposure to zinc prevented this toxic effect. The preventive zinc effects on mercury intoxication have been associated with the ability of zinc to induce the synthesis of detoxificant proteins, such as metallothioneins (Dunn et al., 1987; Kondoh et al., 2003; Peixoto et al., 2003). In fact, a previous study of this laboratory demonstrated that zinc administrated in the same dose used in this work was able to prevent the inhibitory effect on δ-ALA-D activity and the decrease in the body weight gain caused by mercury in parallel to the increase of metallothionein levels (Peixoto et al., 2003). Although the precise mechanism involved in this effect is not clear yet, these detoxificant proteins would sequester the toxic metal turning it unavailable to cause its damage effects (Klaassen et al., 1999; Nath et al., 2000; Romero-Isart and Vasak, 2002). The ALT and LDH hepatic parameters were subtly modified by mercury exposure. The Hg-intoxicated rats presented a reduction of 40% and 20% (not significant) of these activities, respectively. However, this effect does not characterize hepatic toxicity, since it is verified as an increase of these activities as consequence of cellular lesion (Devlin, 1997). Still, the $ZnCl_2$ impeded the Hg inhibitory effect.

The mercury effect on glycemia was interestingly observed in the experiment. The Hg-intoxicated rats, although not hypoglycemic, presented a decrease of 25% in this parameter, and the zinc partially prevented this reduction. Considering that blood glucose level is closely related to glycogen metabolism, we evaluated the liver content of this polymer and we verified that the glycogen content was not modified by the treatment. Thus, the decrement in the glucose level seems not to be associated with an alteration in glycogen metabolism. Literature data relate to damage to nutrients absorption, including glucose, caused by mercury in aquatic organisms (Farmanfarmaian

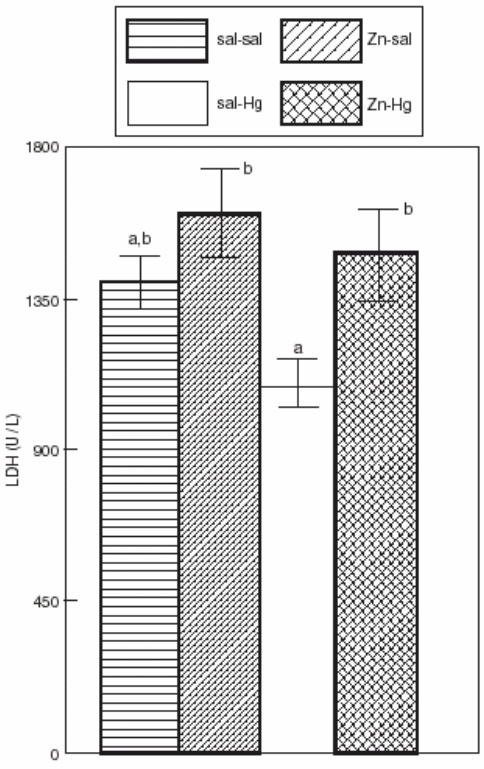

Fig. 4. Serum LDH activity of young rats treated with $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3rd–7th day old) and intoxicated with $HgCl_2$ (5 mg/kg/day; s.c.) for 5 subsequent days (8th–12th day old). Duncan's multiple range test: groups that share the same letter are statistically similar. (a) Significantly different from Zn–sal and Zn–Hg groups ($P<0.05$; $n=4$) (b) Significantly different from sal–Hg group ($P<0.05$, $n=4$).

Table 1
Serum glucose and liver glycogen of young rats treated with $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3rd–7th day old) and intoxicated with $HgCl_2$ (5 mg/kg/day; s.c.) for 5 subsequent days (8th–12th day old)

Treatment	Serum glucose (mg/dL) $n=5$	Hepatic glycogen (g/100 g tissue) $n=6$
Saline–saline	136 ± 8.9[a]	5.67 ± 0.38
Zn–saline	130 ± 10.3[a]	4.72 ± 0.59
Saline–Hg	101 ± 6.4[b]	5.86 ± 1.16
Zn–Hg	126 ± 8.6[a,b]	4.35 ± 0.34

[a]Duncan's multiple range test: groups that share the same letter are statistically similar.
[b]Significantly different from saline–saline and Zn–saline groups ($P=0.05$).

et al., 1989; Sastry and Rao, 1984; Sastry et al., 1982). This metal seems to act on the active transport and facilitated diffusion components of uptake (Farmanfarmaian, 1985; Farmanfarmaian and Socci, 1985). If this effect also is related to subcutaneous exposure is not clear. However, it is known that mercury anorexigenic effects on appetite mechanism are regulated by the central nervous system (Counter and Buchanan, 2004; Freundt and Ibrahim, 1990). If this is the case, in some way, the zinc would prevent the effect of Hg on this central mechanism. More specific investigations need to be done to confirm this effect. However, our present results suggest an important protective effect of zinc on several metabolic and toxic effects of mercury, and these are important in order to open a new perspective of prevention and/or therapy for mercury exposure.

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CAPÍTULO 5

MANUSCRITO:

Título: **Níveis de metalotioneína, zinco e mercúrio nos tecidos de ratos jovens expostos ao zinco e subseqüentemente ao mercúrio**

(Metallothionein, zinc and mercury levels in tissues of young rats exposed to zinc and subsequently to mercury)

Autores: Nilce Coelho Peixoto, Maria Ângela Serafim, Érico Marlon de Moraes Flores, Maria João Bebianno e Maria Ester Pereira

Situação: em fase final de preparação.

Metallothionein, zinc and mercury levels in tissues of young rats exposed to zinc and subsequently to mercury

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Abstract:

Several studies have described about mercury toxicity and about role of metallothioneins (MT) in the detoxification of heavy metals and in the regulation of trace metals homeostasis. However, there are few data obtained from young animals, mainly when considered the specific pos-natal developmental phase; and this is important since young animals seem to be more sensitive to toxicant agents than adults. The objective of this work was to investigate whether the MT participate of protector mechanism conferred by zinc pre-treatment on toxic effects induced by mercury in neonate rats. Pups received $ZnCl_2$ (5 doses of 27 mg/kg/day, s.c.) and subsequently $HgCl_2$ (5 doses of 5 mg/kg/day, s.c.). MT contents were increased by treatments and the greatest increase was induced by zinc. Although the heat treated cytosolic (HTC) fraction is rich in MT, higher zinc and mercury contents were verified in the insoluble fraction (IF) from all tissues analyzed. The relationships between MT and HTC metals showed for both hepatic and renal tissues that the increase in metal levels occurs in parallel to increase in MT content. It is important to emphasize that cells in proliferation have nuclear and mitochondrial MT. Therefore, the high content of mercury found in the IF would be bound to MT, as well. These results suggest that MT are, at least in part, responsible for the reduction of the toxicity of mercury, mainly for avoid or soften renal toxicity.

Keywords: metallothionein, metals, zinc, mercury, mammals, rats.

Acknowledgements: N. C. P. was recipient of fellowships from CAPES (PDEE).

1. Introduction

Metallothioneins (MT) are ubiquitous proteins of low molecular weight, rich in cysteine. This high content of sulfhydrilic amino acids (around 30%) confers unique metal binding properties to them (Chan et al., 2002; Dabrio et al., 2002). Factors such as exposure to toxic or essential metals (Goering and Fowler, 1987; Pedersen et al., 1998; Bebianno and Langston, 1999; Peixoto et al., 2003), stress (Kondoh et al., 2003), radiations (Cai et al., 1999), and other agents (Rojas et al., 1996; Theocharis et al., 2001) promote induction of the synthesis of these molecules (Dunn et al., 1987). In relation to their biological functions, it is attributed that they have antioxidant properties (Cai and Cherian, 2003; Kondoh et al., 2003), are involved in the homeostasis of essential metals, such as zinc and copper (Dunn et al., 1987; Dabrio et al., 2002), and can act as detoxifying agent (Stillman, 1995; Dabrio et al., 2002) of metal ions, due to the affinity with sulfhydrilic groups.

As homeostatic agent, MT has properties to maintain the essential metal concentrations at physiologic levels with the function to release, or even, to sequester these metals in order to maintain their physiological function (Dunn et al., 1987; Dabrio et al., 2002). From these essential metals, the biological role of zinc, as an excellent inductor of MT synthesis is widely recognized beyond its role as enzymatic cofactor and structural in other metalloproteins (Eaton et al., 1980; Cosson, 1989). Hepatic and brain dysfunctions in mammals may be associated with low levels of zinc due to the specific role of this metal in normal neurological function (Zatta et al., 2003; Takeda et al., 2005) and in regulation of carbohydrate hepatic metabolism (Rofe et al., 2000). DNA replication and transcription and protein synthesis are biological processes that also require zinc. Thus, the regulation of zinc endogenous levels is necessary for proliferation and differentiation of the cells (Mutch and Hurley, 1974; Eckhert and

Hurley, 1977).

From toxic metals, mercury, a divalent metal without any biological function, is known to cause several deleterious effects in adults (Emanuelli et al., 1996; Shigematsu et al., 2000) as in developing organisms (Peixoto et al., 2003, 2004; Roza et al., 2005) affecting mainly the central nervous (Pereira et al., 1999; Rocha et al., 2001) and renal systems (Magos et al., 1974; Emanuelli et al., 1996; Peixoto and Pereira, in press). Young animals, mainly during the first days after birth, seem to be more sensitive to toxicant agents than adults (Jugo, 1976; Nielsen and Andersen, 1996). This vulnerability can be related to organ immaturity to process these agents (Schulz et al., 1962; Winick and Noble, 1965).

Using young rats as models, results from literature and from this laboratory have demonstrated that exposure to mercury chloride (25 mg/kg) for 5 consecutive days causes cerebral (Rocha et al., 1995; Roza et al., 2005) and corporal weight decrease (Rocha et al., 1995; Peixoto et al., 2003), renal weight increase (Rocha et al., 1995; Peixoto et al., 2003; Roza et al., 2005), mercury accumulation in tissues (Peixoto et al., 2003; Roza et al., 2005), inhibition of porphobilinogen synthase activity (Rocha et al., 1995; Peixoto et al., 2003; Roza et al., 2005), alterations in renal and hepatic functions and in glycemia (Peixoto and Pereira, in press). The previous administration of zinc avoids or softens some of these toxic effects (Peixoto et al., 2003; Peixoto and Pereira, in press). This is an interesting aspect of zinc, since the intoxication by metals usually are treated with chelating agents, which have a disadvantage of being not specific and sometimes more toxic than the metal itself (Bapu et al., 1994; Roza et al., 2005). On account of this and of the preventive therapeutic properties attributed to zinc and of its advantage to be an endogenous metal make it a promising alternative as preventive agent in poisoning cases by metals. In this context, the objective of this study was to

investigate whether MT are produced as consequence of exposure to zinc and whether this metalloprotein participates on the mechanism conferred by zinc pre-treatment of neonate rats exposed to mercury.

2. Materials and Methods

2.1. Experimental animals

The studies were conduced in accordance with the national and institutional guidelines (University Ethics Committee Guidelines – Process number 23081.013915/2004-06) for experiments with animals.

Wistar rats obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12-h light/dark cycle at a controlled temperature (23 ± 2^{o}C). The animals had free access to water and commercial food. The breeding consisted of grouping three females (90-120 days old) and one adult male for 20 days. After this period, pregnant rats were selected and housed individually in opaque plastic cages (50x25x18 cm). Pregnant rats were checked once a day between 3:00 and 6:00 p.m. to verify the possible presence of pups.

2.2. Young rats

The day of birth was defined as 0 days old. At 1 day old, the number of pups of each litter was reduced to 9. The number of litters used was 5. Males and females were used without distinction.

2.3. Treatments

Three-day-old Wistar rats were treated with NaCl 90 mg/kg/day (saline) or $ZnCl_2$ 27 mg/kg/day (s.c.) during 5 consecutive days (from 3 to 7 days old). From the 8th to the 12th day of life the rats received one daily dose of saline or $HgCl_2$ 5.0 mg/kg (s.c.). The animals were weighed daily to adjust to the dose. Each litter contributed with two animals to the saline-saline, Zn-saline and Zn-Hg treatments. For the saline-Hg

treatment, each litter contributed with three animals because of the deleterious effects caused on their development (Peixoto et al., 2003), except for one litter, where one rat of this treatment died. The litter was considered as experimental *n*, always.

2.4. Samples

At 24 h after the last dose of saline or mercury, the pups were euthanized. Blood, liver and kidneys were excised. The blood samples were collected in tubes with heparin. For each litter, the tissues from animals of the same treatment were pooled, weighed and frozen at –20^{o}C. After, they were lyophilized, weighed again and stocked until analysis.

2.5. Sample preparation

The lyophilized tissues were homogenised in 5.0 mL of Tris-HCl buffer 0.02 M (pH 8.6) at 4^{o}C. A subsample (2 mL) of the homogenate was used for the determination of metal concentrations. Another aliquot (3 mL) of homogenate was centrifuged at 30,000 *g* for 45 min at 4^{o}C. The supernatant, after being separated from the pellet (insoluble fraction - IF), was denatured at 80^{o}C for 10 min to precipitate the high molecular weight proteins and re-centrifuged at 30,000 *g* for 45 min at 4^{o}C. The heat-treated cytosol (HTC) was used for the determination of MT concentrations as described below.

2.6. Metal determinations

Metal quantification was performed on dried subsamples of the homogenate, insoluble and heat-treated cytosolic fractions after wet digestion with nitric acid. Zinc was analyzed using flame atomic absorption spectrophotometry and mercury by cold vapour atomic spectrophotometry. Metal concentrations were expressed as µg/g dry

weight of tissue and, for the correlations between MT and metals, as nmol/g dry weight of tissue. Recovery of Hg and Zn from the tissues was 85.70 ± 5.87% and 96.67 ± 7.83%, respectively.

The mercury content in tissues of rats treated with zinc alone was not determined since this metal was not detected in these tissues of rats submitted to same schedule o treatment (Peixoto et al., 2003).

2.7. MT determination

Aliquots (7.5-25.0 µL) of the heat denatured cytosol were used to quantify the MT by differential pulse polarography, using a method described by Bebianno and Langston (1989). The standard addition method was used for calibration of MT concentrations with rabbit liver MT standard (MT-I from Sigma) 10 mg/L in distilled water. The MT concentrations are expressed as µg/g dry weight of tissue and, for the correlations between MT and metals, as nmol/g dry weight of tissue.

In order to express MT on a molecular weight basis, a molecular weight of 6,494 Da was used (Wong and Klaassen, 1979).

2.8. Statistical analysis

All statistical analyses were performed using the program Statistica for Windows (Statistical Software, Tulsa, OK, USA). The one-way analysis of variance (ANOVA) was performed in order to determine if the treatments alter the parameters investigated. When the analyses were significant (at level of 0.05), the Duncan´s multiple range test ($p < 0.05$) was used to determine the significant differences among the means. Comparisons between groups were performed by Student *t* test. Linear regression analysis ($p < 0.05$) was used to analyse the possible correlation between two

parameters.

3. Results

3.1. Metal concentrations in tissues

Zinc concentrations in the different tissues of groups of animals treated with zinc are higher than those animals from the other groups (Figure 1), with the exception of the blood, that although with the same pattern the differences between treatments were not significant (Figure 1C). Furthermore, Zn levels in the liver of rats treated with zinc (Figure 1A) were 3- and 5- fold higher than in the kidney (Figure 1B) and blood (Figure 1C), respectively. Interestingly, the mercury administration also induced a significant increase of this essential metal in the liver in relation to the control group (Figure 1A).

When rats were treated with mercury, the three tissues presented significantly higher levels of this metal when compared to rats treated only with saline solution (Figure 2). However in this case, a similar pattern was observed for liver and blood, while in the zinc pre-treated rats this metal seems to prevent the increase of Hg accumulation. Interestingly, the Hg concentrations accumulated in the liver and kidney were similar (Figure 2A-B) and around 10-fold lower in the blood (Figure 2C). But when rats were treated with Hg this metal was more accumulated in the kidney of zinc treated rats (3-fold and 50-fold when compared to the liver and blood respectively) and significantly higher than that of kidney Sal-Hg treated rats (Figure 2B).

3.2. Subcellular distribution of zinc and mercury in tissues

The zinc levels in the subcellular fractions are illustrated in Table 1. The highest percentage of Zn in the liver was in the IF. Zinc in the zinc-pre-treated rats accumulated significant higher levels of this metal in both hepatic subcellular fractions than the saline-pre-treated groups. However, the percentage of Zn was not modified in the

insoluble fraction, but presented significantly increased in the HTC fraction from Zn-sal and Zn-Hg treated rats when compared to the Sal-sal treated rats (Table 1).

In the renal tissue also the insoluble fraction presented higher Zn levels than the HTC fraction but the percentage of both fractions were higher than those in the liver. Besides, in Zn treated rats, the increase of zinc content was only significant in the HTC fraction. The same pattern exits in the blood: highest Zn percentage in the IF fraction. Similarly to total tissue, zinc levels in the subcellular fractions in the blood did not increase although some changes in the percentage of Zn distribution occurred in Zn and Hg treated rats (Table 1).

The subcellular distribution of mercury in the tissues is presented in Table 2. The percentage of Hg in the liver was similar between IF and HTC fractions and these two subcellular fractions represent only 40% of total Hg distribution which means that the high molecular weight protein fraction represent around 60% of total Hg in this tissue. The Hg content in both liver fractions of intoxicated rats was higher that untreated ones (Sal-sal) but in the IF fraction this increase was higher in Sal-Hg treated rats than in Zn-Hg treated ones. In the HTC although Hg content were similar, the percentage (in relation to total Hg - Figure 2) of mercury content present in Zn-Hg treated rats was significantly higher than in the Sal-Hg group. Similarly, in the kidney the IF fraction the mercury content was higher than in the HTC fraction and the percentage of these two fractions represent in this tissue around 50% of the total Hg accumulated. The Hg levels in these two subcellular fractions were significantly higher in zinc pre-exposure rats than in the rats treated only to Hg, although the percentage of Hg contained in each fraction was not modified; and, both Hg groups presented higher Hg contents in both fractions than in saline ones. In the blood, however, Hg was only detected in the IF fraction and represent only about 20% of total Hg, and higher metal

level were verified in Sal-Hg intoxicated rats. In the HTC fraction Hg was not detected in the presence of the mercury, whereas in the blood pellet the higher metal levels were in Sal-Hg rats.

3.3. MT concentrations in tissues

The MT levels in the different tissues are presented in Figure 3. In rats treated with saline solution, MT levels in the blood were 2 to 3-fold higher than in the other tissues. In the liver of rats exposed to Zn or to Hg presented a small increase of MT content that were significant when these comparisons were done between two groups (Figure 3A). In the kidney, the zinc pre-treatment induced a significant increase of MT around 80%, and no changes occurred in MT levels after Hg exposure (Figure 3B). MT in the blood was not modified by treatments.

3.4. Relationships between MT and metals in the HTC

The relationships between MT and zinc levels in the HTC are shown in Figure 4. In liver and kidney MT concentrations increased linearly with the increase of zinc concentrations in this subcellular fraction (Figure 4A and 4B). Nevertheless, in the blood there was no relationship between these two parameters (data not shown).

The relationships between MT and mercury in the HTC fraction in the liver and kidney are presented in Figure 5. MT concentrations increased significantly with the increase of mercury concentrations in both tissues (Figure 5A and 5B). For the blood this relationship does not exist because the presence of mercury in HTC fraction was not detected.

In Table 3, the MT/metal molar ratios in the tissues are presented. The results reveal that MT was undersaturated with both metals (Zn and Hg), independently of the

treatment administered to the animals. Still, it is possible to observe that highest binding of Zn to MT was in the liver and this binding increase with Zn treatment. Nevertheless, the ratio does not change in the other tissues (kidney and blood) of both zinc treated groups. In relation to Hg treated rats although the binding increase with Hg treatment slightly higher in the kidney the MT/Hg molar ratios were not significantly different between both Hg treated groups.

Table 1. Subcellular distribution of zinc in the liver, kidney and blood of young rats treated with $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3^{rd}-7^{th} day old) and exposed to $HgCl_2$ (5 mg/kg/day; s.c.) for 5 consecutive days (8^{th}-12^{th} day old)

	Zn (μg/g d.w.)			
	Treatment			
Fraction	Sal-sal	Zn-sal	Sal-Hg	Zn-Hg
Liver				
IF	74.3 ± 16.9[b]	256.2 ± 89.3[a]	133.9 ± 26.7[b]	269.9 ± 83.8[a]
	(34.0)	(30.7)	(36.3)	(31.8)
HTC	11.6 ± 1.8[b]	131.8 ± 59.5[a]	41.9 ± 25.1[b]	120.6 ± 50.5[a]
	(5.1)	(15.3)[*]	(11.8)	(14.9)*
Kidney				
IF	96.6 ± 29.3[a]	140.5 ± 34.5[a]	107.8 ± 40.2[a]	145.0 ± 39.2[a]
	(62.7)	(51.6)	(59.7)	(59.3)
HTC	57.7 ± 10.7[b]	98.7 ± 22.6[a]	42.1 ± 7.1[b]	83.8 ± 12.6[a]
	(37.2)	(35.1)	(24.9)	(33.7)
Blood				
IF	20.5 ± 4.9[a]	26.6 ± 13.1[a]	28.7 ± 7.4[a]	32.3 ± 14.3[a]
	(38.3)	(53.8)	(71.1)	(60.0)
HTC	11.7 ± 3.2[a]	13.9 ± 7.8[a]	8.6 ± 1.7[a]	14.4 ± 1.9[a]
	(21.3)	(27.5)	(22.1)	(24.9)

IF: insoluble fraction; HTC: heat-treated cytosolic fraction

The percentage of zinc in each fraction in relation to total zinc content in each tissue (Figure 1) is shown in parentheses.

Data represent mean ± standard deviation (n = 5) and the values followed by different letters in the same line are statistically different ($p < 0.05$). ***** Statistically different from Sal-sal group ($p < 0.02$).

Table 2. Subcellular distribution of mercury in the liver, kidney and blood of young rats treated as described in Table 1

	Hg (μg/g d.w.)		
	Treatment		
Fraction	Sal-sal	Sal-Hg	Zn-Hg
Liver			
IF	N.D.[c]	36.8 ± 3.1[a]	17.0 ± 4.5[b]
		(18.2)	(15.4)
HTC	N.D.[b]	29.5 ± 14.6[a]	29.8 ± 10.7[a]
		(14.9)	(25.9)*****
Kidney			
IF	N.D.[c]	41.6 ± 14.3[b]	159.8 ± 39.1[a]
		(30.6)	(33.5)
HTC	N.D.[c]	28.1 ± 7.6[b]	74.7 ± 7.9[a]
		(21.1)	(16.4)
Blood			
IF	N.D.[c]	3.5 ± 0.6[a]	1.5 ± 0.7[b]
		(24.3)	(16.3)
HTC	N.D.	N.D.	N.D.

IF: insoluble fraction; HTC: heat-treated cytosolic fraction

N. D.: not detected

The percentage of contained mercury in each fraction in relation to total mercury content in tissue (Figure 2) is shown in parentheses.

Data represent mean ± standard deviation (n = 5) and the values followed by different letters in the same line are statistically different ($p < 0.05$). *Statistically different from Sal-Hg group ($p < 0.05$).

Table 3. MT/Zn and MT/Hg molar (μmol/g d.w.) ratios from heat-treated cytosolic fraction in the liver, kidney and blood of young rats treated as described in Table 1

	Treatment			
Tissue	Sal-sal	Zn-sal	Sal-Hg	Zn-Hg
MT/Zn				
Liver	1/0.7	1/3	1/1	1/3
Kidney	1/2	1/2	1/2	1/2
Blood	1/0.2	1/0.2	1/0.2	1/0.2
MT/Hg				
Liver	1/0	_	1/0.3	1/0.2
Kidney	1/0	_	1/0.4	1/0.5
Blood	1/0	_	1/0	1/0

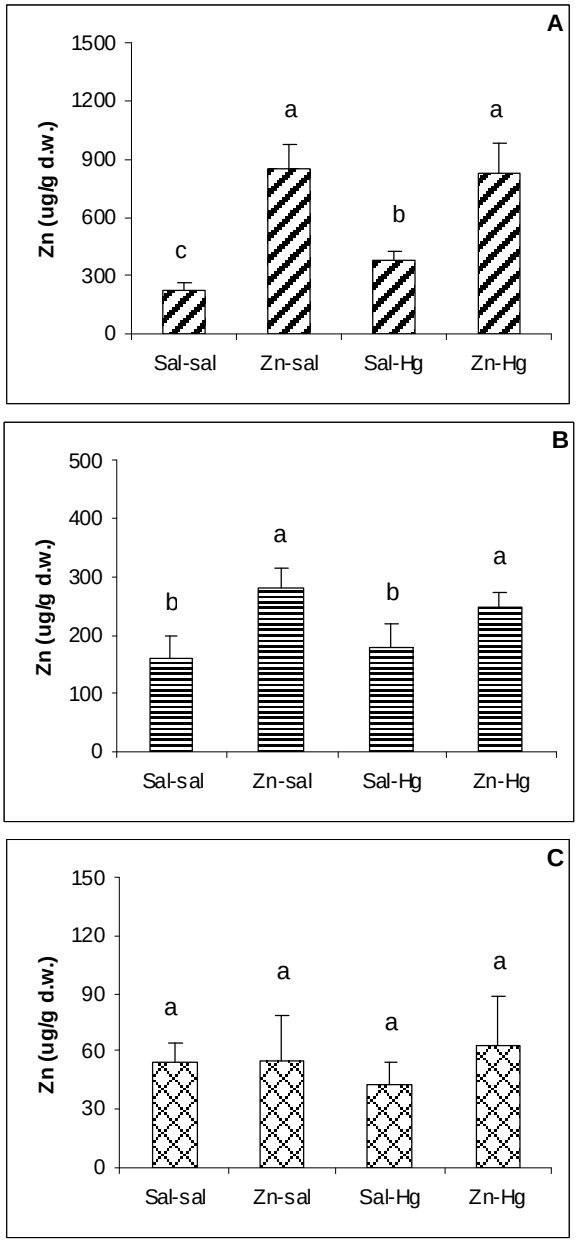

Figure 1. Zinc concentrations in the liver **(A)**, kidney **(B)** and blood **(C)** of young rats treated with saline (Sal-sal) and $ZnCl_2$ (27 mg/kg/day; s.c.) for 5 consecutive days (3^{rd}-7^{th} day old) (Zn-sal) solutions and exposed to $HgCl_2$ (5 mg/kg/day; s.c.) for 5 consecutive days (8^{th}-12^{th} day old) (Sal-Hg and Zn-Hg).

Data represent mean ± standard deviation (n = 5) and the bars followed by different letters are statistically different ($p < 0.05$).

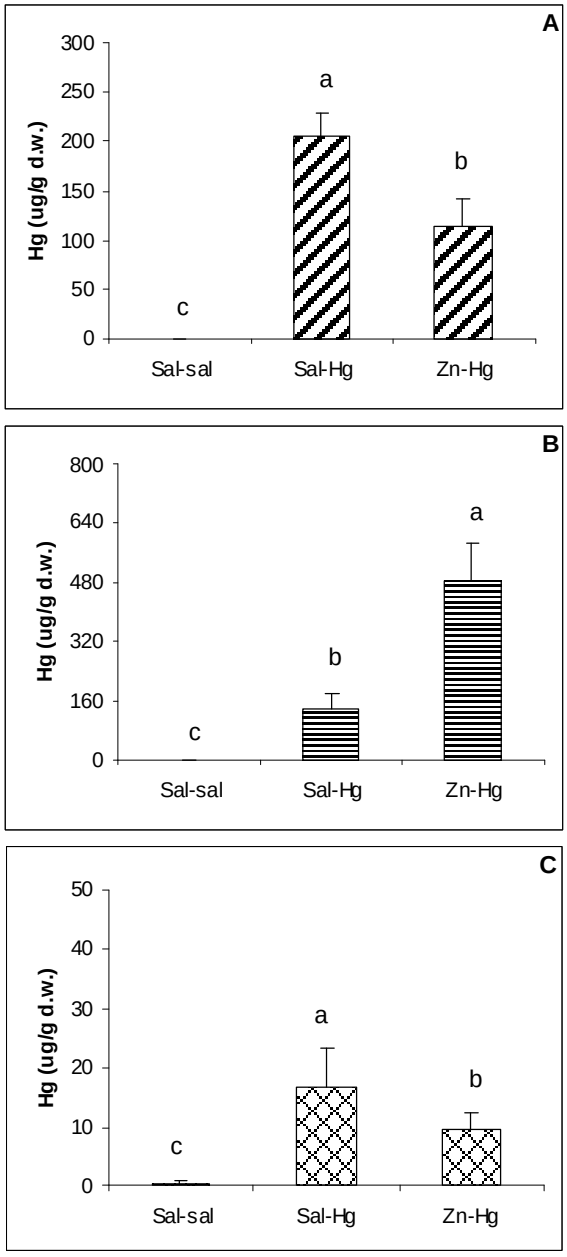

Figure 2. Mercury concentrations in the liver **(A)**, kidney **(B)** and blood **(C)** of young rats treated as described in Figure 1.

NOTE: the mercury in the liver (**A**) and kidneys (**B**) of the rats of the saline-saline (Sal-sal) group was not detected.

Data represent mean ± standard deviation (n = 5) and the bars followed by different letters are statistically different ($p < 0.05$).

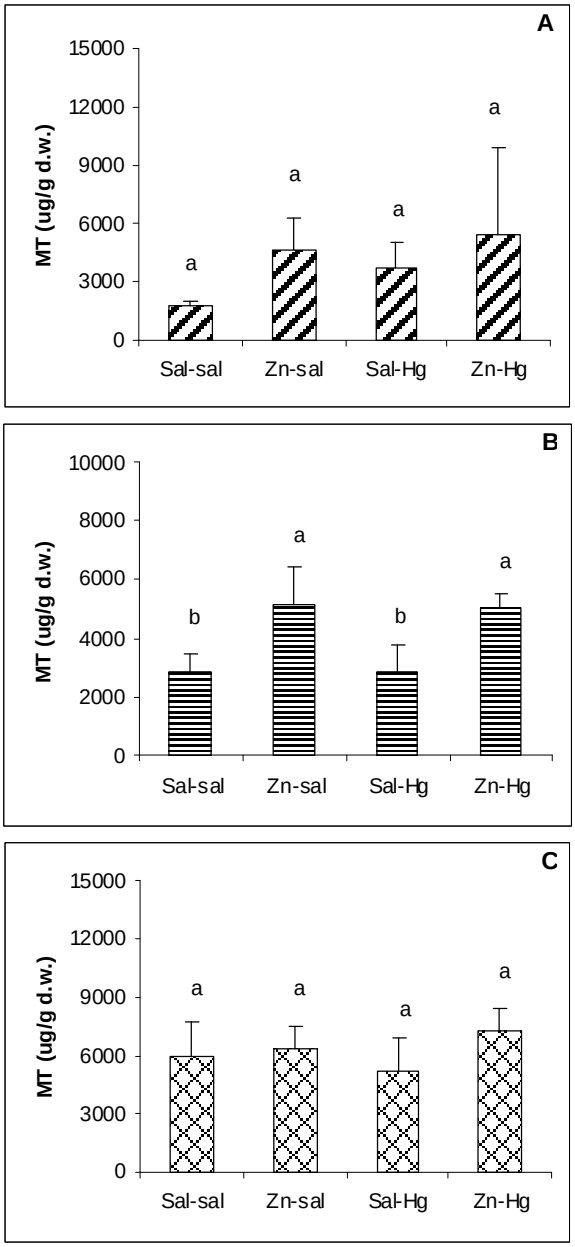

Figure 3. MT concentrations in the liver **(A)**, kidney **(B)** and blood **(C)** of young rats treated as described in Figure 1.

Data represent mean ± standard deviation (n = 5) and the bars followed by different letters are statistically different ($p < 0.05$).

The Zn-sal and Sal-Hg groups are statistically different from Sal-sal group ($p < 0.005$).

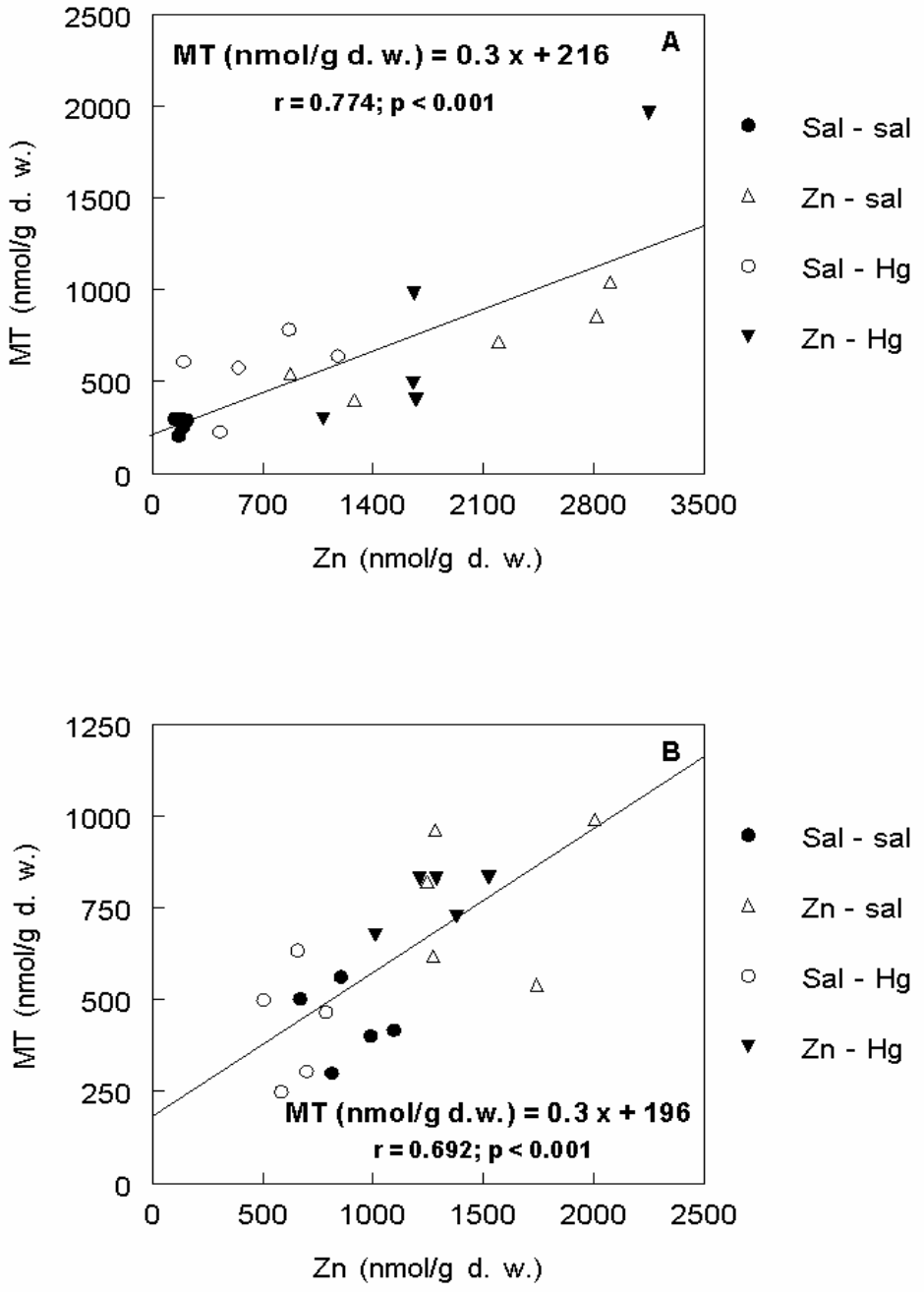

Figure 4. Relationship between MT concentrations (nmol/g d.w.) and zinc concentrations (nmol/g d. w.) in the heat-treated cytosolic fraction in the liver **(A)** and kidney **(B)** of young rats treated as described in Figure 1.

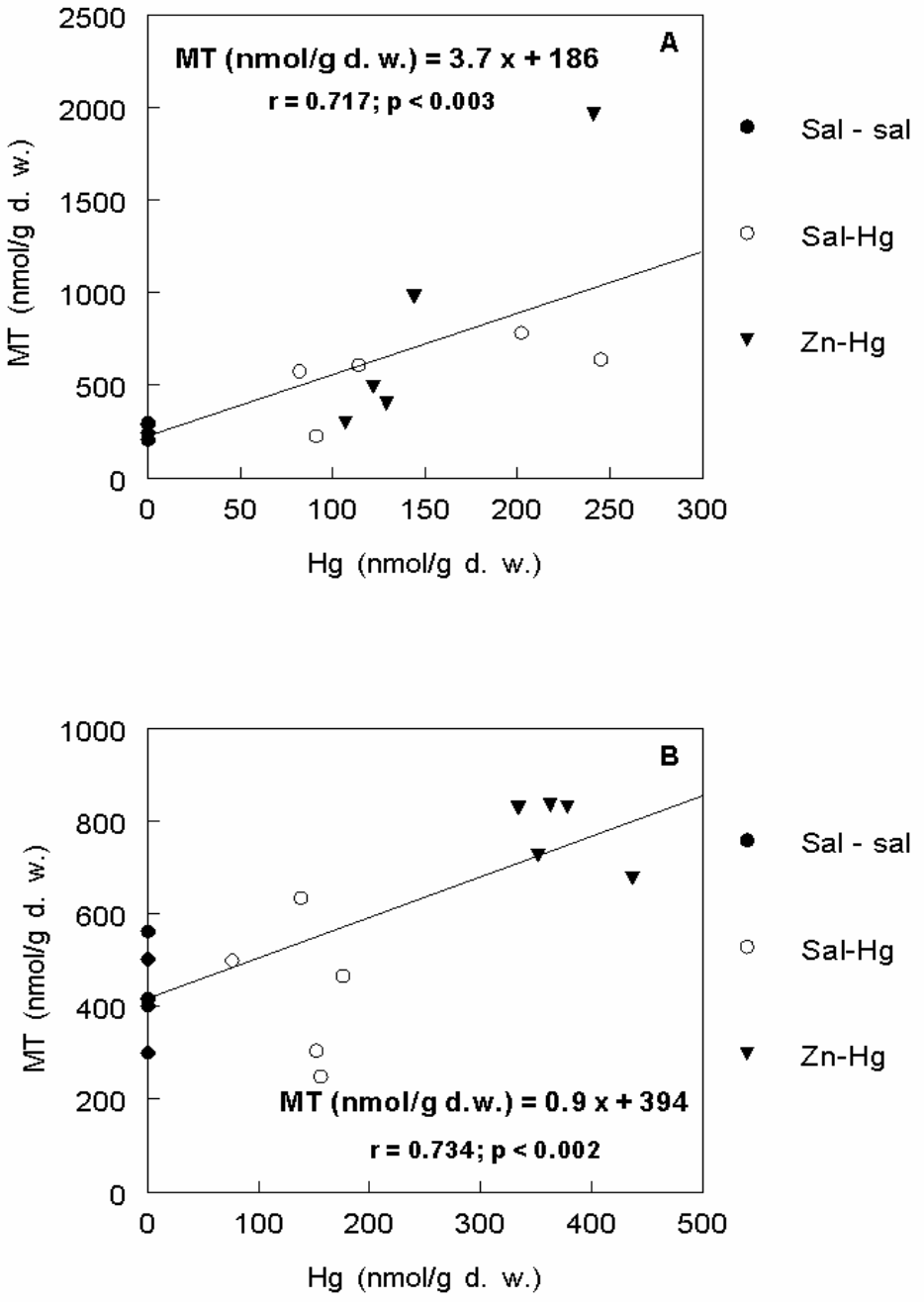

Figure 5. Relationship between MT concentrations (nmol/g d.w.) and mercury concentrations (nmol/g d.w.) in the heat-treated cytosolic fraction in the liver **(A)** and kidney **(B)** of young rats treated as described in Figure 1.

4. Discussion

The purpose of this study was to assess the involvement of MT in the protector mechanism of zinc in animals submitted to intoxication by mercury in a precocious age such as related in our previous studies (Peixoto et al., 2003; Peixoto and Pereira, in press).

In general, the MT contents were increased by metal treatments and the higher increase was induced by zinc exposure. These results demonstrated that although the increase of zinc contents after zinc pre-treatment were around 3 times to liver and 50% to kidneys, this metal was more effective in induce MT synthesis than mercury, whose increase of metal accumulation after mercury intoxication was much bigger in all tissues. These results agree with those of other authors that verified the effectiveness of zinc on MT synthesis (Cosson, 1989; Pedersen et al., 1998; Kondoh et al., 2003; Irato and Albergoni, 2005). However, the metal distribution in subcellular fraction can better explain these metal effects since the both IF and HTC fractions metal contents were modified by treatments. This is interesting once the HTC fraction is rich in MT; although higher content of zinc and mercury (except liver HTC mercury from Zn-Hg group) were verified in pellet fraction (IF) to all tissues analyzed.

Zinc contents in HTC and IF fractions presented similar behaviour than those verified for total tissues levels. Mainly to liver, similarly is remarkable, since both fractions of zinc-groups presented high zinc content. In the animals exposed only to mercury this additional quantity was distributed in both liver fraction, though the increase was smaller than those verified in zinc pre-treated groups. However, the higher HTC zinc percentages verified in zinc groups in relation to saline group are in accord with the light increase of MT in liver of these animals.

In kidneys, the treatment with zinc induced to a rise in level of this metal mainly

in the HTC fraction and the posterior exposure to mercury did not modify this parameter. This increase in the HTC zinc level agrees with the increase in the MT levels found in this tissue, since the high levels of zinc and MT were detected in the HTC. In blood the treatments did not alter the zinc and MT contents.

Except to liver HTC fraction, the effects of treatments on Hg content in subcellular fractions seem to those on total tissue Hg levels, once that the zinc pre-treatment decreased the Hg level in the liver IF and increased in both fractions from kidney, in relation to group exposed only to Hg. In relation to liver HTC mercury level, zinc did not impede the mercury retention, and still it induced an increase in percentage of Hg retained in this fraction in relation to tissue total Hg. Considering the small total percentage (IF plus HTC) of mercury in these fractions and the reduction of total tissue mercury content induced by zinc pre-treatment (Figure 2), these results suggested that the principal alteration of mercury content in liver may be consequence of change of Hg level contained in other fractions not rich in MT.

In regard to renal mercury content, the rise induced by zinc pre-treatment in the IF fraction was more prominent (3.8 times) than in the HTC fraction (2.7 times), although these alterations did not modify the percentage of Hg retained in each fraction in relation to total tissue level.

Similarly to liver, the effects of zinc in to reduce Hg content in IF fraction occurred in parallel to decline in total blood, suggesting that this reduction was consequence of the reduction displayed in the IF fraction and in rest of the cellular material not evaluated (fraction not resistant to heat), once that HTC fraction did not present Hg. The absence of metal in the HTC fraction is in agreement with similar levels of blood MT presented by all groups.

The relationships between MT and HTC metal showed that in the hepatic and

renal tissues whenever there is increase of metal level there is increase of MT content. Nevertheless, the molar ratios between MT and metal did not demonstrate the saturation of these proteins by metals, but it is possible to observe higher zinc concentration bound to MT than Hg. This was waited, once zinc is an essential metal just bound in MT of control groups. Moreover, the high zinc level in liver of rats treated only with mercury may be related to light increase of MT in this organ, once mercury also induces MT synthesis (Clarkson, 1997; Chapman and Chan, 1999; Tandon et al., 2001; Counter and Buchanan, 2004). If this is true, this protein could be bound to zinc.

The hepatic and blood mercury levels reductions and the increase of the content of this metal in the kidneys induced by zinc suggest that the heavy metal contained in the liver was carried to renal tissue through blood. This process also would be transporting the MT from liver to kidney, since hepatic tissue is the main organ in synthesis of MT. Moreover, it is important to emphasize that in cells in proliferation, as occur during the rapid growth, have been observed the presence of nuclear and mitochondrial MT (Nartey et al., 1987; Cherian and Apostolova, 2000; Ye et al., 2001). Therefore, the high content of mercury found in the IF of the tissues, enriched fraction in nucleus and mitochondria, would be bound to MT, too.

Considering that the zinc pre-treatment induces an increase of renal MT of around 80% and the group treated with zinc and mercury presents a content of mercury of 25% bigger in this protein than group treated only with mercury, these results suggest that the MT are, at least in part, responsible by reduction of toxicity of mercury as verified previously (Peixoto et al., 2003; Peixoto and Pereira, in press).

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CAPÍTULO 6
DISCUSSÃO GERAL

O propósito deste trabalho foi avaliar a efetividade do tratamento prévio com cloreto de zinco e cloreto de cádmio em evitar ou amenizar os efeitos deletérios provocados pela exposição de ratos neonatos ao cloreto de mercúrio.

A exposição à uma dose (s.c.) diária de 5,0 mg/kg de $HgCl_2$ (equivalente a 3,7 mg/kg de Hg^{2+}) durante cinco dias em ratos jovens (PEIXOTO, 2000) causou várias alterações verificadas após 24 h da última dose administrada. Os pesos corporal e renal apresentaram-se diminuído e aumentado, respectivamente. Entretanto, o metal tóxico não causou alteração no peso hepático. Houve inibição na atividade da enzima PBG-sintase proveniente de fonte renal e hepática. O nível de mercúrio retido no tecido renal, hepático e sangüíneo apresentou-se elevado e as quantidades desse metal acumuladas nas frações insolúvel (FI) e citosólica tratada a quente (CTQ) refletem os níveis demonstrados pelos tecidos, exceção à fração CTQ sangüínea, na qual não foi detectada a presença do metal pesado. As concentrações de zinco encontradas no tecido renal e sangüíneo não foram alteradas, porém, no fígado houve aumento do nível do metal essencial (70% em relação ao grupo controle). Essa alteração no teor de zinco hepático, causada pelo tratamento com mercúrio, mostra que há uma redistribuição do metal essencial nesse caso. Os resultados relativos aos teores de zinco contido nas frações subcelulares demonstraram uma concordância com aqueles verificados nos tecidos, salvo para o tecido hepático, no qual as frações não apresentaram altos níveis do metal endógeno. A função renal, avaliada pelo aumento da creatinina e da uréia séricas, mostrou-se prejudicada, o que demonstra que os animais expostos ao mercúrio desenvolveram nefrotoxicidade, mais especificamente têm um prejuízo na filtração glomerular (HOLT & WEBB, 1986; GIRARDI & ELIAS, 1991; CLARKSON, 1997). Quanto à função hepática, verificada pela dosagem da atividade das enzimas séricas ALT e lactato desidrogenase (LDH), foi demonstrado que houve diminuição na atividade da primeira e nenhuma modificação na atividade da segunda. Entretanto, a limitação de 40% na atividade da ALT não tem expressão clínica, desde que a hepatoxicidade é caracterizada pela elevação da atividade da proteína e não pela sua diminuição (LEHNINGER *et al.*, 1995; CHAMPE &

HARVEY, 1996). A quantidade de glicogênio contida no tecido hepático não foi alterada, ao passo que o nível de glicose sérica apresentou-se diminuído como conseqüência da exposição. As concentrações de MT renal e sangüínea não foram alteradas pelo metal. Por outro lado, houve um aumento no nível hepático dessa proteína. As correlações entre MT e metais (zinco e mercúrio) mostraram que essas foram significativamente positivas entre essas duas variáveis para os tecidos hepático e renal, mas não para o sangue. As quantidades de zinco ligadas às MT em qualquer uma das fontes pesquisadas não foram alteradas quando os animais receberam o tratamento com mercúrio, entretanto, a exposição provocou aumento nas razões molares MT/Hg dos tecidos hepático e renal.

A administração de cinco doses (s.c.) de 3,7 mg/kg/dia de $CdCl_2$ (correspondente a 2,1 mg/kg de Cd^{2+}) (GOERING & FOWLER, 1987) do terceiro ao sétimo dia de vida pós-natal dos ratos não alterou nenhuma das variáveis estudadas. Entretanto, a exposição dos ratos neonatos sob esse mesmo protocolo ao $ZnCl_2$, com doses diárias de 27 mg/kg (equivalente a 13 mg/kg de Zn^{2+}) (GOERING & FOWLER, 1987) causou elevação nos níveis desse metal nos tecidos hepático e renal e o aumento no teor renal foi causado por um aumento desse metal apenas na fração CTQ. Os conteúdos de MT hepática e renal e a razão molar MT/Zn hepática também foram aumentados pela exposição a esse metal essencial.

O tratamento com uma dose (s.c.) diária de 3,7 mg/kg de $CdCl_2$ durante os cinco dias que antecederam a exposição ao mercúrio foi capaz de evitar apenas a inibição da atividade da enzima PBG-sintase de fonte renal, enquanto que os efeitos do mercúrio sobre o peso corporal, o peso renal, a atividade da enzima PBG-sintase hepática e as concentrações de mercúrio renal e hepática permaneceram inalterados.

A exposição ao zinco, anteriormente ao mercúrio, por cinco dias, com uma dose (s.c.) diária de 27 mg/kg de $ZnCl_2$ preveniu diversos efeitos causados pelo metal tóxico. O zinco impediu a diminuição de peso corporal, a inibição da atividade enzimática da PBG-sintase de ambas as fontes estudadas, o acúmulo tão acentuado de mercúrio no tecido hepático e sangüíneo, os aumentos na creatinina e uréia séricas e a diminuição da atividade da ALT sérica. Além disso, evitou parcialmente a alteração na concentração de glicose sérica. Por outro lado, quanto ao teor de mercúrio encontrado nos rins, o zinco provocou uma elevação do nível desse metal e também não impediu o aumento do peso desse órgão. Os níveis de zinco presentes nos tecidos renal e hepático mostraram-se aumentados quando houve a pré-exposição e o acréscimo para o fígado foi superior àquele verificado nesse mesmo tecido dos animais que receberam apenas o metal pesado. Os níveis de mercúrio encontrados nas frações subcelulares dos três tecidos acompanharam os perfis demonstrados pelos tecidos, salvo para

a fração CTQ hepática, em que o nível foi similar àquele apresentado pelos animais que receberam somente mercúrio. O tratamento com zinco não alterou as quantidades desse metal contidas nas duas frações subcelulares sangüíneas. O incremento de zinco nos rins dos animais representou o que ocorreu na fração CTQ. Considerando-se o fígado, ambas as frações apresentaram elevados níveis de zinco, acompanhando o aumento já citado no tecido. As quantificações das MT mostraram que a exposição prévia ao zinco aumentou ainda mais o seu conteúdo hepático em relação ao encontrado nesse órgão dos ratos que receberam apenas mercúrio. No tecido renal, o tratamento simultâneo provocou aumento no nível das proteínas, enquanto que no sangue essa conseqüência não foi vista. As correlações entre MT e os dois metais nos tecidos renal e hepático foram positivas, o que não ocorreu para o sangue. As razões molares entre a proteína e os dois metais estudados não foram alteradas, exceção feita à razão molar hepática entre MT e zinco, a qual foi aumentada.

Alterações marcantes, como no peso corporal e de órgãos, são achados comuns nos casos de exposição de animais em fase de desenvolvimento ou adultos ao mercúrio (ROCHA *et al.*, 1995; EMANUELLI *et al.*, 1996; PEIXOTO, 2000; ROZA *et al.*, 2005). O retardo no ganho de peso corporal e, conseqüentemente, no crescimento dos animais neonatos submetidos a esse tipo de insulto podem ser atribuídos ao fato de que o mercúrio é anorexigênico (FREUNDT & IBRAHIM, 1990; COUNTER & BUCHANAN, 2004) e o zinco, de alguma forma, impede esses efeitos.

A medida da atividade da enzima sulfidrílica serve como um marcador da exposição a metais pesados, uma vez que metais divalentes possuem afinidade pelos grupamentos contendo enxofre e essa ligação causa um prejuízo na atividade enzimática (DESPAUX *et al.*, 1977; DAVIS & AVRAM, 1980). A pré-exposição dos animais ao zinco preveniu completamente a inibição da atividade da enzima proveniente de ambas as fontes analisadas, isso pode ser explicado tendo-se como referência que o zinco tem um papel crítico nessa enzima, ele é requerido para a manutenção dos grupamentos SH no estado reduzido (TSUKAMOTO *et al.*, 1979; BEBER *et al.*, 1998; EMANUELLI *et al.*, 1998). Em relação ao efeito protetor do cádmio sobre a enzima, pode ser postulado que o metal, nessa dose utilizada, ligaria à proteína produzindo efeito alostérico positivo (DESPAUX *et al.*, 1977; DAVIS & AVRAM, 1980; BERNARD & LAUWERYS, 1987).

As altas concentrações de mercúrio detectadas no fígado e nos rins estão de acordo com dados que apontam esses tecidos como os que mais retêm o metal (MENGEL & KARLOG, 1980; NIELSEN & ANDERSEN, 1989; SAKAMOTO *et al.*, 1993; CLARKSON, 1997; COUNTER & BUCHANAN, 2004). Num rato intoxicado, a quantidade de mercúrio

presente no rim, por g de tecido fresco, é a metade daquela presente no fígado (33 e 65 μg, respectivamente), porém quando calculado quanto de mercúrio o órgão todo é capaz de reter, essa proporção diminui para um terço (18 e 55 μg, respectivamente). Para um rato pré-tratado com zinco, o teor de mercúrio retido no rim, por g de tecido fresco, é cinco vezes maior que aquele apresentado pelo fígado (110 e 22 μg, respectivamente), entretanto, o cálculo feito para o órgão todo mostra que o rim contém o triplo da quantidade de metal retida pelo fígado (54 e 20 μg, respectivamente). Esses cálculos demonstram que há uma inversão das proporções.

O sangue, embora tenha apresentado um teor elevado de metal pesado, suas concentrações correspondem a 10% daquelas encontradas no tecido hepático, porém o perfil de retenção entre os grupos de tratamento é semelhante, o tratamento com zinco provocou um decréscimo de, aproximadamente, 50% no conteúdo do metal contido nesses dois tecidos. Essas observações evidenciam que, nos animais em que houve exposição ao zinco, o sangue transporta o metal pesado do fígado para os rins (CHAN *et al.*, 1993), desde que a soma da quantidade de mercúrio retido por ambos tecidos é muito similar (cerca de 74 μg), independente do tratamento aplicado.

Quanto às quantificações dos glicídios, pode-se inferir que, embora haja uma estreita relação entre o nível de glicose sangüínea e o metabolismo do glicogênio (LEHNINGER *et al.*, 1995; HARRIS, 1997), o decréscimo na glicemia [ainda que os níveis não possam ser considerados hipoglicemia (BATTELINO *et al.*, 1999; GAVETE *et al.*, 2005; THYSSEN *et al.*, 2006)], provocado pelo mercúrio, parece não estar relacionado a um desequilíbrio no teor do polímero hepático, uma vez que esse não apresentou-se alterado. O zinco preveniu parcialmente o decréscimo da glicemia. Assim, parece que, de alguma maneira, o metal essencial interfere nesse mecanismo, embora esse não esteja esclarecido com os presentes experimentos. Porém, sugere-se que pode haver alguma relação com o fato do mercúrio alterar o mecanismo do apetite dos animais (FREUNDT & IBRAHIM, 1990; COUNTER & BUCHANAN, 2004).

O conteúdo de MT presente nos tecidos mostrou que o tecido renal apresenta quantidades significativamente aumentadas sempre que os animais foram pré-expostos ao zinco (EATON *et al.*, 1980; ZALUPS & CHERIAN, 1992). Para os demais tecidos analisados, fígado e sangue, não houve diferença entre os grupos de tratamento. Entretanto, para o tecido hepático, quando foram feitas comparações entre dois grupos, foram verificadas diferenças entre o grupo tratado somente com salina e os grupos tratados somente com zinco e somente com mercúrio.

Os dados pertinentes às relações entre metais na fração CTQ e MT demonstraram que há correlação positiva para os tecidos hepático e renal, tanto para zinco quanto para mercúrio. Por outro lado, para o tecido sangüíneo não há correlação entre o conteúdo de zinco na fração CTQ e MT, e a relação, considerando o mercúrio, não foi calculada, porque a fração CTQ proveniente desse tecido apresentou quantidades não-detectáveis do metal pesado.

As concentrações de mercúrio contidas nas frações subcelulares refletem os teores do metal contidos no tecido, uma vez que sempre que houve alteração na quantidade de metal, paralelamente, a alteração também ocorreu nas frações. Exceção feita à fração CTQ de fígado, na qual a quantidade absoluta não foi modificada pelo pré-tratamento com zinco e, ademais, a quantidade relativa apresentou-se aumentada quando comparada com o grupo tratado somente com o metal tóxico. Além disso, considerando-se os percentuais, é possível concluir que a maior percentagem desse metal está presente na fração não-analisada (precipitado proveniente da segunda centrifugação do material tecidual).

De uma maneira geral, a exposição aos metais causa uma elevação nas quantidades de MT presentes nos tecidos (ONOSAKA & GEORGE CHERIAN, 1981; BEBIANNO & LANGSTON, 1992, 1995). Deste modo, o conteúdo de MT aumenta quando aumenta o conteúdo de metal presente no tecido (BEBIANNO & LANGSTON, 1992, 1995; CHAN *et al.*, 1993). Essa evidência é mais notável para o zinco, já que as alterações no conteúdo desse metal nos tecidos são menores proporcionalmente às alterações causadas pelo metal pesado. Isso demonstra a propriedade peculiar do zinco como um dos melhores indutores da síntese de MT (EATON *et al.*, 1980; BRACKEN & KLAASSEN, 1987). O acréscimo de cerca de 75% na concentração de zinco nos rins foi capaz de induzir aumentos significativos no conteúdo da proteína ligante de metal, ao passo que aumentos bem mais pronunciados na concentração de mercúrio presente nesse tecido não provocaram esse efeito. Os aumentos no teor de zinco hepático, provocados pelos tratamentos com os metais, embora tenham sido percentualmente maiores que aqueles observados nos rins, inferem que, assim como o mercúrio contido no fígado é transportado por intermédio do sangue para os rins, isso também poderia estar ocorrendo em relação às MT (CHAN *et al.*, 1993), já que os aumentos na quantidade dessas proteínas nem sempre foram significativos nesse tecido.

A avaliação dos cálculos das razões molares entre MT e metal na fração CTQ demonstrou que há sempre mais zinco associado à proteína do que mercúrio. Entretanto, houve aumento apenas no conteúdo de MT/zinco quando houve exposição a esse metal e apenas no tecido hepático desses animais. O aumento no conteúdo de MT hepática dos animais que receberam apenas mercúrio pode ser explicado pelo fato de que o metal tóxico

quando nesta forma inorgânica (cloreto de mercúrio) também induz à síntese de MT (CLARKSON, 1997; CHAPMAN & CHAN, 1999; TANDON *et al.*, 2001; COUNTER & BUCHANAN, 2004), mas ainda assim há mais zinco ligado à essas proteínas do que mercúrio, o que pode ser justificado pelo aumento no conteúdo do metal endógeno provocado pelo metal tóxico nesse tecido especificamente.

Considerando-se que nesse trabalho as MT foram quantificadas por meio de dois métodos diferentes, colorimétrico, com a utilização do ácido 5,5'-ditio-*bis*-2-nitrobenzóico (VIARENGO *et al.*, 1997; PETROVIC *et al.*, 2001), e polarográfico (BEBIANNO & LANGSTON, 1989), cabem aqui algumas ponderações. O método colorimétrico baseia-se na quantificação dos grupamentos SH (ELLMAN, 1958,1959), pelos quais os metais divalentes têm afinidade (ELLMAN, 1958). Sabe-se que o mercúrio é o metal que tem a maior afinidade por esses grupos, portanto, trata-se de uma ligação muito estável (ELLMAN, 1958; CLARKSON, 1997; DABRIO *et al.*, 2002; ROMERO-ISART & VASÁK, 2002). No modelo experimental utilizado, onde os animais são expostos ao mercúrio, provavelmente esses grupamentos ligados ao mercúrio não são quantificados, o que levaria a resultados falsamente diminuídos. Entretanto, comparando-se os resultados obtidos pelos dois métodos usados, observa-se que, para esse modelo experimental, ambos se prestam. No tecido renal quando houve exposição prévia ao zinco houve aumentos de mais de 50% quando utilizou-se o método colorimétrico e de cerca de 80% quando o método utilizado foi o de polarografia, embora a análise estatística não tenha revelado diferenças para o primeiro método, provavelmente em virtude da grande dispersão dos resultados. Para o fígado dos animais tratados somente com mercúrio os aumentos foram de 86% e 120%, respectivamente para os métodos colorimétrico e polarográfico. Enquanto que os acréscimos relativos na quantidade de MT presente no fígado dos animais que receberam somente zinco ou os dois metais simultaneamente foram superiores a 230% e cerca de 200%, respectivamente para a colorimetria e a polarografia. Sugere-se que os resultados obtidos por ambas as metodologias tenham sido similares também porque a quantidade de mercúrio contida na fração CTQ é relativamente pequena, o que não causaria uma interferência significativa na determinação das MT contidas nessa fração subcelular pelo método colorimétrico. Assim, embora as quantidades de MT possam estar subestimadas pelo método, o erro inserido nesses resultados é pequeno.

Em relação às quantidades elevadas de metais detectadas na FI celular é possível que esses também estejam associados às MT. Embora essas proteínas sejam consideradas tipicamente de localização citosólica (CHERIAN *et al.*, 1987; DUNN *et al.*, 1987; NARTEY

et al., 1987; CHERIAN & APOSTOLOVA, 2000; GEORGE CHERIAN *et al.*, 2003), alguns autores identificaram a presença dessas nas mitocôndrias e no núcleo celular em determinadas situações (CHERIAN *et al.*, 1987; NARTEY *et al.*, 1987; CHERIAN & APOSTOLOVA, 2000; YE *et al.*, 2001). As MT de localização atípica foram detectadas quando há alta proliferação celular, mais especificamente, durante a fase de desenvolvimento e em células tumorais (CHERIAN *et al.*, 1987; NARTEY *et al.*, 1987; CHERIAN & APOSTOLOVA, 2000). Estudos mostraram que a localização nuclear das MT é transitória, ocorre do vigésimo dia de idade pré-natal até, aproximadamente, duas semanas de vida pós-natal de ratos (CHERIAN *et al.*, 1987). Deste modo, sabendo-se que a idade dos animais empregados no presente trabalho, no momento da coleta do material biológico, era de 13 dias, que a FI é enriquecida em mitocôndrias e núcleos e que as MT não foram quantificadas nesse material, sugere-se que pode haver altas concentrações das proteínas nessa fração.

Tomando-se os presentes resultados em conjunto, considera-se o zinco como uma alternativa mais adequada do que o cádmio quanto às suas propriedades preventivas aos efeitos tóxicos causados pelo mercúrio em ratos jovens, porque esse metal foi mais eficiente em evitar o aparecimento desses efeitos e de amenizá-los quando surgiram.

Quanto ao mecanismo envolvido neste processo é possível sugerir que as MT têm um papel fundamental. A nefrotoxicidade, principal conseqüência da exposição ao mercúrio detectada nos ratos neonatos, foi completamente prevenida pelo metal essencial e a presença dessas proteínas foi marcante nos rins, embora a quantidade de mercúrio nesse tecido tenha aumentado quando houve pré-exposição. Entretanto, a quantidade de metal tóxico presente na MT renal dos animais neonatos pré-tratados com zinco e posteriormente tratados com mercúrio foi cerca de 25% maior que aquela presente no tecido renal dos animais tratados apenas com mercúrio. Assim, propõe-se que, embora o conteúdo de mercúrio renal esteja mais elevado no grupo exposto aos dois metais, esse estaria quelado às MT e, desta forma, indisponível para causar toxicidade.

CAPÍTULO 7
CONCLUSÃO GERAL

Mediante a análise dos resultados obtidos através do presente trabalho é possível concluir que:

1) a exposição s.c. de ratos Wistar jovens (do oitavo ao décimo segundo dia de vida pós-natal) ao cloreto de mercúrio na dose total de 25,0 mg/kg (5,0 mg/kg/dia):
    a) afeta o desenvolvimento e crescimento normal dos animais, como verificado pelo prejuízo no ganho de peso corporal e aumento no peso renal;
    b) reduz a atividade da enzima PBG-sintase proveniente das fontes renal e hepática, cuja quantificação serve para o biomonitoramento da exposição;
    c) causa uma elevação nos teores de mercúrio presente nos tecidos renal, hepático e sangüíneo;
    d) prejudica a função renal, uma vez que o nível sérico de creatinina e de uréia encontra-se elevado;
    e) diminui a concentração de glicose sangüínea;
    f) provoca uma redistribuição do metal endógeno zinco para o fígado;
    g) induz a um aumento nos níveis de MT no fígado;
2) o tratamento s.c. (do terceiro ao sétimo dia de vida), aplicado aos animais durante os cinco dias que antecedem a exposição ao mercúrio, com cloreto de cádmio na dose total de 18,5 mg/kg (3,7 mg/kg/dia) previne apenas a alteração na atividade da enzima PBG-sintase renal;
3) o tratamento s.c. (do terceiro ao sétimo dia de vida), administrado aos ratos durante os cinco dias que precedem a exposição ao mercúrio, com cloreto de zinco na dose total de 135,0 mg/kg (27,0 mg/kg/dia):
    a) previne a alteração de peso corporal, mas não de peso renal, dos animais jovens;
    b) evita a inibição na atividade da enzima PBG-sintase de ambas as fontes utilizadas, fígado e rim;
    c) previne a diminuição da concentração de glicose no sangue;

d) diminui a retenção do metal tóxico no fígado e no sangue, porém aumenta a quantidade desse no tecido renal;

e) de uma maneira geral, as concentrações do metal essencial e do metal tóxico contidas nas frações subcelulares, correspondem com as alterações causadas no tecido;

f) provoca aumento nos níveis de MT renal e hepático, mas não nos níveis sangüíneos;

4) o cloreto de zinco é mais eficiente como agente preventivo aos efeitos deletérios do cloreto de mercúrio do que o cloreto de cádmio nas doses empregadas;

5) existe uma correlação positiva entre as quantidades de metal, zinco e mercúrio, e MT contidas na fração CTQ dos tecidos renal e hepático, porém isso não ocorre com o tecido sangüíneo;

6) a quantidade de zinco associada às MT é maior que a de mercúrio em todos os três tecidos analisados;

7) a razão molar ente mercúrio e MT no tecido renal é mais pronunciada quando há pré-exposição ao cloreto de zinco;

8) as proteínas ligantes de metal MT estão envolvidas no mecanismo de prevenção dos efeitos tóxicos renais do mercúrio exercido pelo cloreto de zinco.

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