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EFEITOS PROTETORES DO ZINCO SOBRE ALTERAÇÕES COMPORTAMENTAIS E BIOQUÍMICAS INDUZIDAS PELO MERCÚRIO EM RATOS JOVENS

TESE DE DOUTORADO

CARINA FRANCISCATO

Santa Maria, RS, Brasil

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EFEITOS PROTETORES DO ZINCO SOBRE ALTERAÇÕES COMPORTAMENTAIS E BIOQUÍMICAS INDUZIDAS PELO MERCÚRIO EM RATOS JOVENS

por

CARINA FRANCISCATO

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
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Orientadora: Maria Ester Pereira

SANTA MARIA, RS, BRASIL

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

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Elaborada por **Carina Franciscato** como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

COMISSÃO EXAMINADORA:

Profª. Drª. Maria Ester Pereira (Presidente/Orientador)

Prof. Dr. Marcelo Farina

Prof. Dr. Vanderlei Folmer

Profª. Drª. Maribel Antonello Rubin

Prof. Dr. Luiz Fernando Freire Royes

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
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EFEITOS PROTETORES DO ZINCO SOBRE ALTERAÇÕES COMPORTAMENTAIS E BIOQUÍMICAS INDUZIDAS PELO MERCÚRIO EM RATOS JOVENS

Autora: CARINA FRANCISCATO
Orientadora: MARIA ESTER PEREIRA
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O mercúrio é um elemento tóxico capaz de induzir alterações bioquímicas, neurológicas e comportamentais que podem persistir por muito tempo após a exposição ao metal. A contaminação por mercúrio continua sendo um sério problema de saúde pública em países subdesenvolvidos e em desenvolvimento, onde existem garimpos para extração de ouro. Não existe um tratamento totalmente eficaz para os casos de exposições ou intoxicações pelo metal. Assim, pesquisas têm sido desenvolvidas na tentativa de encontrar novas alternativas para casos de intoxicação por mercúrio. Alguns estudos têm demonstrado que o zinco protege contra a toxicidade do mercúrio em ratos jovens. O objetivo deste trabalho foi examinar os efeitos do mercúrio inorgânico sobre parâmetros comportamentais durante e após a exposição e bioquímicos 24 h e 21 dias após a exposição ao metal, e investigar os possíveis efeitos preventivos de zinco sobre as alterações induzidas por mercúrio. Os ratos foram expostos ao $ZnCl_2$ (27 mg/kg/dia, s.c.) do 3º ao 7º dia de vida e ao $HgCl_2$ (5 mg/kg/dia, s.c.) nos 5 dias subseqüentes. Estes animais foram submetidos às seguintes tarefas comportamentais: geotactismo negativo (3º, 5º, 7º, 9º, 11º e 13º dias de idade), imersão da cauda (13º, 20º e 27º dias de idade), locomoção forçada em cilindro giratório (25º e 30º dias de idade), teste do béquer (17º ao 20º dia de idade) e campo aberto (30º e 31º dias de idade). Os animais foram observados diariamente desde o início do tratamento (3 dias de idade) até 33 dias de idade para registrar o número de ratos mortos. Ninhadas sacrificadas aos 13 dias de idade (24 horas após a última dose de mercúrio) foram utilizadas para a dosagem da atividade da acetilcolinesterase e níveis de metais em cérebro e cerebelo. Os animais mortos aos 33 dias de idade (21 dias após o término da exposição ao mercúrio), foram utilizados para analisar a atividade da acetilcolinesterase de cérebro e cerebelo, a atividade da porfobilinogênio-sintase renal e hepática, parâmetros bioquímicos hepáticos e renais; e para a quantificação dos níveis de metais em cérebro, cerebelo, rins, fígado e sangue. Os resultados obtidos após os 13 dias de idade foram divididos em dois grupos de ninhadas que foram definidas ao final do período experimental (33 dias de idade) como ratos menos sensíveis e ratos mais sensíveis ao mercúrio, de acordo com a recuperação do peso corporal até os 33 dias de idade. A exposição ao mercúrio causou acúmulo deste metal em todos os órgãos de todos os ratos tratados com mercúrio. A atividade da acetilcolinesterase de cerebelo de ratos de 13 dias de idade foi diminuída. Além disso, os ratos mais sensíveis ao mercúrio apresentaram prejuízo na função motora e força muscular verificadas no teste do béquer e redução nas atividades locomotora e exploratória no teste do campo aberto; diminuição nos

pesos de fígado e rins; diminuição da atividade de enzima porfobilinogênio-sintase renal; aumento nos níveis séricos de uréia e creatinina e diminuição da atividade da enzima alanina aminotransferase sérica. Este estudo demonstra que os efeitos tóxicos induzidos pelo mercúrio persistem por um longo tempo após o final da intoxicação, e o zinco previne, mesmo que parcialmente, todas as alterações induzidas pelo mercúrio. Ainda, com este trabalho também podemos concluir que existem diferentes tipos de sensibilidade dos animais à toxicidade do mercúrio, que pode ser atribuída à suscetibilidade individual de cada animal, pois alguns animais foram tão sensíveis que morreram antes do final do experimento. Enquanto que outros, apesar de apresentarem aumento do conteúdo de mercúrio nos tecidos, são pouco sensíveis e não apresentaram alterações bioquímicas nem comportamentais.

Palavras-chave: Mercúrio; Zinco; Acetilcolinesterase; Habituação; Atividade locomotora; Porfobilinogênio-sintase; Ratos jovens.

ABSTRACT

Thesis of Doctor's Degree
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PROTECTOR EFFECTS OF ZINC ON BEHAVIORAL AND BIOCHEMICAL CHANGES INDUCED BY MERCURY IN YOUNG RATS

Author: CARINA FRANCISCATO
Advisor: MARIA ESTER PEREIRA
Date and place of the defense: Santa Maria, december, 21, 2009.

Mercury is a toxic element that induces biochemical, neurological and behavioral changes, which can persist for a long time after the metal exposure. The contamination by mercury continues being a serious problem of public health in underdeveloped and in development countries, where mines exist for extraction of gold. There was not a treatment totally effective for the cases of exposure or intoxication by the metal. Thus, researches have been developed in the attempt of finding new alternatives for cases of intoxication by mercury. Studies have demonstrated that zinc protects against mercury toxic effects in young rats. The aim of this work was to evaluate the effects of the inorganic mercury exposure on the behavioral performance of rats during and after the exposure, and on biochemical parameters at 24 h e 21 days after the metal exposure. Still, it was investigated the possible preventive effects of zinc on mercury-induced changes. Pups were exposed from 3rd to 7th postnatal day to $ZnCl_2$ (27 mg/kg/day, s.c.) and subsequently to $HgCl_2$ (5 doses of 5 mg/kg/day, s.c.). The rats were submitted to behavioral tasks: negative geotaxis task (3, 5, 7, 9, 11 and 13 days old), tail immersion (13, 20 and 27 days old) and rotarod tests (25 and 30 days old), beaker test (17 to 20 days old) and open field task (30 and 31 days). The animals were daily observed from start of treatment (3 days) until 33 days old to register the number of rats that died. Litters euthanized at 13 days old (24 hours after the last dose of mercury) were used to acetylcholinesterase activity assays and metal levels determination in cerebrum and cerebellum. The animals killed at 33 days old (21 days after the end of mercury exposure) were used to analyze the cerebrum and cerebellum acetylcholinesterase activity, renal and hepatic porphobilinogen-synthase activity, hepatic and renal biochemical parameters, and to determination of metal levels in cerebrum, cerebellum, kidney, liver and blood. Results obtained after 13 days old were divided in two groups of litters that were defined at the end of experimental period (33 days old) as less sensitive rats to mercury and more sensitive rats to mercury in accordance with the recovery of body weight until 33 days old. The mercury exposure caused accumulation of this metal in all analyzed organs of all mercury treated rats. The cerebellum acetylcholinesterase activity from 13 days old rats was decreased. Besides, the mercury-animals of the more sensitive litters to mercury presented impairment in motor function and muscular strength verified in the beaker test, and reduction of the locomotor and exploratory activities in the open field task; decrease in liver and increase in kidney weights, decrease in renal porphobilinogen-synthase activity, increase in urea and creatinine levels and decrease of alanine amino transferase activity. This study demonstrates that mercury-induced toxic effects persist for a long time after the end of exposure, and zinc prevents, even that

partially, all the alterations induced by mercury. Still, with this work we can also conclude that there are different types of sensibility from the animals to the toxicity of mercury, which can be attributed to the individual susceptibility of each animal, since some animals were so sensitive that died before the end of the experiment; whereas others, in spite of they presented increase of the mercury content in the tissues, they were little sensitive and did not present neither biochemical nor behavioral changes.

Key-words: Mercury; Zinc; Acetylcholinesterase; Habitation; Locomotor activity; Porphobilinogen-synthase; Young rats

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LISTA DE ABREVIATURAS

AChE: acetilcolinesterase (*acetylcholinesterase*);

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*);

AST: aspartato aminotransferase;

ATC: acetiltiocolina iodada (*acetylthiocoline iodide*)

^{o}C: grau Celsius;

CAT: catalase;

Cys: cisteína;

Da: dalton;

DNA: ácido desoxirribonucléico

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*);

EROs: espécies reativas de oxigênio;

g: grama;

g: aceleração da gravidade (força centrífuga);

GABA: ácido gama aminobutírico;

GPx: glutationa peroxidase;

GSH: glutationa;

GST: glutationa-S-transferase;

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;

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;

KOH: hidróxido de potássio;

l: litro;

L: litro;

LDH: lactato desidrogenase (*lactate dehydrogenase*);

LSR: ratos menos sensíveis ao mercúrio (*less sensitive rats*);

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

M: molar;

min: minuto;

MSR: ratos mais sensíveis ao mercúrio (*more sensitive rats*);

MT: metalotioneína(s);

n: número de repetições;

Na: sódio;

$NaBH_4$: tetrahidroborato de sódio;

NaCl: cloreto de sódio;

Na_2HPO_4: fosfato de sódio dibásico;

p: nível de significância;

PBG: porfobilinogênio;

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

pH: potencial hidrogeniônico;

RNA: ácido ribonucléico

Sal: salina;

s. c.: subcutânea; subcutaneamente;

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

SH: grupamento(s) sulfidrílico(s);

SOD: superóxido dismutase;

SNC: sistema nervoso central;

U: unidade;

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

Zn: zinco;

Zn^{2+}: íon zinco;

$ZnCl_2$: cloreto de zinco.

SUMÁRIO

Apresentação

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

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CAPÍTULO 1
1. INTRODUÇÃO

Metais pesados são poluentes ambientais comuns. Entre estes está o mercúrio, um metal tóxico que pode ser encontrado em diferentes formas químicas: mercúrio elementar, inorgânico e orgânico, o que contribui para os seus diversos efeitos tóxicos (Klaassen, 1996).

O uso do mercúrio tanto na indústria quanto na agricultura ficou marcado por desastres ambientais em diferentes partes do mundo. Um exemplo de poluição ambiental causada por este metal ocorreu na década de 50, quando a Baía de Minamata, no Japão, foi contaminada por resíduos provenientes de uma indústria de fertilizantes químicos, resinas sintéticas, plásticos e compostos químicos (Bisinoti & Jardim, 2004), que utilizava mercúrio inorgânico como um catalisador. Ao ser lançada na baía, esta forma do metal foi convertida em metilmercúrio por microorganismos, desencadeando a Doença de Minamata, na qual a população foi contaminada através do consumo de peixes (Klaassen, 1996), e desenvolveu sinais de comprometimento do sistema nervoso central (SNC) (Hamada e Osame, 1996). Outra séria exposição humana ao mercúrio aconteceu na década de 70, no Iraque, quando agricultores e seus familiares foram contaminados com metilmercúrio. Nesta ocasião a forma orgânica do metal foi utilizada como fungicida no tratamento de sementes de trigo, as quais foram usadas como grãos para a produção de pães. Este incidente provocou a intoxicação de mais de 6.000 pessoas, causando mortes e alterações congênitas como retardo mental e desordens motoras (Eisler, 2006).

Apesar de seu uso ter sido reduzido nas últimas décadas em todo o mundo, este metal não essencial continua contaminando rios e solos, pois ainda é utilizado em indústrias e, principalmente, na mineração (Lacerda e Pfeiffer, 1992). Portanto, nos países subdesenvolvidos e em desenvolvimento, onde existem garimpos para extração de ouro, a contaminação por mercúrio continua sendo um sério problema de saúde pública (Wasserman et al., 2003). Neste contexto, parte da população do Brasil continua exposta aos efeitos tóxicos causados pelo mercúrio, tanto de maneira ocupacional (garimpeiros), quanto através do consumo de peixes (população ribeirinha), já que nosso país

tem a maior produção de ouro entre os países da América Latina, e a contaminação mercurial dos rios e lagos decorrentes das atividades garimpeiras de ouro tem sido caracterizada na região da Amazônia brasileira (Pinheiro et al., 2000), pois entre os anos de 1980 e 1990 formam lançados de 1000 a 2000 toneladas de mercúrio no meio ambiente na região Amazônica. A intoxicação ocupacional se dá, principalmente, pela exposição ao vapor de mercúrio; enquanto que a população em geral está exposta ao metilmercúrio, pela ingestão de peixes contaminados (Wasserman et al., 2003). Entretanto, é importante lembrar que as formas elementar e orgânica podem ser convertidas em mercúrio inorgânico (Klaassen, 1996), por isso a importância de se estudar também os efeitos do mercúrio na sua forma inorgânica (cloreto de mercúrio).

Estudos têm demonstrado que a exposição pós-natal de ratos ao mercúrio inorgânico causa alterações comportamentais (Peixoto et al., 2007a) e bioquímicas (Peixoto et al., 2003; Peixoto e Pereira, 2007), pois sabe-se que animais em desenvolvimento são particularmente sensíveis a insultos externos devido ao marcado desenvolvimento e crescimento de órgãos e corpo (Gottlieb et al., 1977). Além disso, os sintomas da toxicidade do mercúrio em humanos persistem por vários anos após o final da exposição (Albers et al., 1988; Kishi et al., 1993).

Este problema torna-se ainda mais grave considerando-se que não existe um tratamento totalmente eficaz para os casos de exposições ou intoxicações pelo metal. Agentes quelantes, como o etilenodiaminotetraacetato de cálcio dissódico, o 2,3-dimercaprol (British Anti-Lewisite, BAL), a D-penicilamina, o ácido meso 2,3-dimercaptosuccínico (DMSA) e o ácido 2,3-dimercapto-1-propanosulfônico (DMPS), são utilizados como tratamento para intoxicações agudas e crônicas por metais pesados. Estes elementos ligam-se aos metais tóxicos diminuindo a toxicidade destes (Domingo, 1995). Entretanto, estas substâncias podem causar sérios efeitos tóxicos como depleção de metais essenciais (Cantilena e Klaassen, 1982) e causar distúrbios hematopoiéticos (Flora e Kumar, 1993). Assim, pesquisas têm sido desenvolvidas na tentativa de encontrar novas alternativas para casos de intoxicação por mercúrio. Alguns estudos já mostraram que a pré-exposição ao zinco é eficaz em prevenir efeitos tóxicos causados pelo mercúrio em ratos jovens (Peixoto et al., 2003; Peixoto e Pereira, 2007).

Considerando a neurotoxicidade exercida pelo mercúrio inorgânico, a deficiência de uma terapia efetiva contra a toxicidade deste metal, os efeitos preventivos do zinco sobre alterações bioquímicas induzidas pelo mercúrio, e que a maioria dos trabalhos têm sido desenvolvidos com ratos jovens, este estudo tem como objetivo avaliar a persistência dos efeitos tóxicos do mercúrio sobre parâmetros bioquímicos e comportamentais, quando a exposição ao metal ocorre em um período de desenvolvimento pós-natal, bem como verificar o efeito preventivo do zinco sobre as alterações imediatas e tardias induzidas pelo cloreto mercúrio.

1.1 Objetivos:

Este trabalho teve como objetivo geral estudar os efeitos imediatos e tardios do cloreto de mercúrio sobre o comportamento e parâmetros bioquímicos de ratos jovens. Além disso, investigar se a pré-exposição ao zinco é capaz de prevenir os efeitos tóxicos do mercúrio.

Dessa forma, os objetivos específicos são:

- observar os efeitos tardios da exposição ao cloreto de mercúrio sobre o desenvolvimento corporal;

- investigar o efeito do cloreto de mercúrio sobre o comportamento de ratos jovens durante e após o término da exposição ao metal em algumas tarefas comportamentais que envolvam comportamento reflexo, sensibilidade a dor, atividade locomotora e memória;

- avaliar os efeitos imediatos e tardios da exposição ao cloreto de mercúrio sobre os níveis de metais e sobre a atividade da enzima acetilcolinesterase de cérebro e cerebelo;

- estudar os efeitos tardios da intoxicação com cloreto de mercúrio sobre o peso de órgãos, sobre o conteúdo de metais nestes órgãos e também no sangue, e sobre a atividade da enzima porfobilinogênio-sintase de fígado e rins;

- investigar os efeitos tardios da intoxicação com cloreto de mercúrio sobre parâmetros marcadores de função renal e hepática;

- e verificar o efeito da pré-exposição ao zinco sobre todos estes parâmetros.

CAPÍTULO 2
2. REVISÃO BIBLIOGRÁFICA

2.1 Mercúrio

2.1.1 Propriedades e fontes de exposição

O mercúrio é um metal pesado de número atômico 80 e massa atômica 200,59, que pertence ao grupo 2B do sistema de classificação periódico, integrando a classe dos metais de transição (Salgado et al., 1996). Na sua forma elementar, este elemento encontra-se na forma líquida em condições normais de pressão e temperatura, mas transforma-se em vapores tóxicos com o aumento da temperatura (Clarkson, 1997). Além da forma elementar, pode também ser encontrado nas formas de sais inorgânicos e compostos orgânicos de mercúrio (Counter e Buchanan, 2004).

As diferentes espécies de mercúrio apresentam solubilidade, reatividade e toxicidade diferentes e, conseqüentemente, comportam-se de maneiras distintas no meio ambiente. O mercúrio elementar (ou vapor de mercúrio ou mercúrio metálico) possui estado de oxidação zero (Hg^0) e é volátil (Salgado et al., 1996). Os sais de mercúrio (ou compostos inorgânicos de mercúrio) existem em dois estados de oxidação, como sais mercurosos monovalentes Hg(I) ou mercúricos bivalentes Hg(II), os quais se combinam com elementos como cloro, enxofre ou oxigênio. Já os compostos orgânicos de mercúrio são formados a partir da ligação covalente do mercúrio a átomos de carbono, originando compostos organomercuriais, dos quais o metilmercúrio é a espécie mais tóxica (Klaassen, 1996).

Na sua forma natural, este metal apresenta-se como mercúrio elementar, podendo ser lançado na atmosfera através de gases vulcânicos, área geotérmica, degradação da crosta terrestre e evaporação de corpos hídricos. Entretanto, existem as fontes antropogênicas de contaminação ambiental por mercúrio, assim como indústrias, mineração e queima de combustíveis fósseis (WHO, 1989). Atualmente, as principais fontes de contato com o mercúrio são termômetros, medidores de pressão arterial, baterias, lâmpadas fluorescentes,

interruptores, amálgamas dentários (Clarkson et al., 2003) e o grande problema da mineração, onde o mercúrio contamina o ambiente e consequentemente o ser humano (Lacerda e Pfeiffer, 1992).

O mercúrio elementar é capaz de dissolver o ouro, a prata, o chumbo e os metais alcalinos, formando ligas relativamente consistentes (amálgamas) (UNEP, 2002). Portanto, no processo da mineração o mercúrio metálico é lançado no meio ambiente e ocorre amalgamação das partículas de ouro. Após esta ligação, a amálgama é queimada para separação do ouro, o que promove a volatilização do mercúrio; o restante do mercúrio é lançado diretamente em rios e solos (Lacerda e Pfeiffer, 1992). Na atmosfera, o mercúrio metálico é oxidado pelo ozônio, formando mercúrio inorgânico divalente, que pode ligar-se a íons como o cloreto e formar o cloreto de mercúrio. Este sal de mercúrio inorgânico deposita-se na água e no solo, onde poderá ser metilado por bactérias anaeróbicas, formando metilmercúrio, ou se volatilizar, retornando ao ambiente (Bisinoti e Jardim, 2004). O mercúrio orgânico acumula-se na cadeia alimentar aquática através do processo denominado bioamplificação, isto é, a concentração do metal aumenta em organismos vivos ao passar de níveis tróficos inferiores (herbívoros) para níveis superiores (carnívoros). Portanto, os peixes consumidos pela população possuem alta concentração do metal (Boening, 2000), um exemplo desta situação é relatado em um trabalho realizado na região amazônica brasileira, onde peixes consumidos pela população local apresentaram níveis de mercúrio total acima do limite recomendável para consumo humano pela Organização Mundial da Saúde, ou seja, superiores a 0,5 μg/g (Akagi et al., 1995).

2.1.2 Toxicidade geral dos compostos de mercúrio

A toxicidade do mercúrio depende da sua forma química, e a intoxicação pode ocorrer através de diferentes rotas de exposição, assim como por absorção cutânea, alimentação ou inalação (Clarkson et al., 2003) (Figura 1).

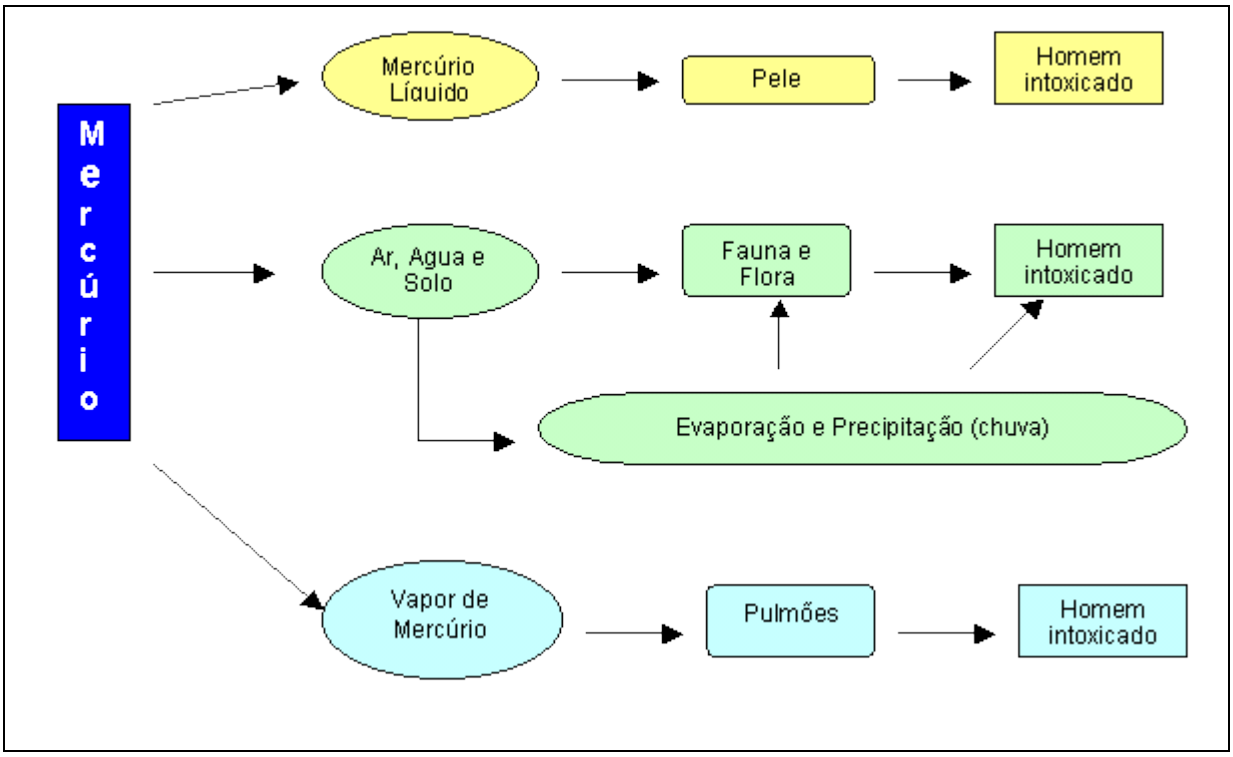

Figura 1 – Esquema do ciclo de intoxicação por mercúrio (http://www.areaseg.com/toxicos/mercúrio.html).

A inalação do vapor de mercúrio causa dispnéia, tosse, podendo progredir para bronquite e pneumonite intersticial (Goyer, 1996). Além disso, o mercúrio vapor, após ser inalado é facilmente absorvido pelos alvéolos e atinge a circulação sanguínea, onde é oxidado ao cátion mercúrico bivalente (Hg^{2+}) nos eritrócitos e depois distribuído para os tecidos (Clifton, 2007). Entretanto, como o mercúrio elementar é capaz de atravessar membranas, uma parte do mercúrio inalado atravessa a barreira hemato-encefálica (Klassen, 1996) ou placentária (Clarkson et al., 1972) antes de ser oxidado. Dessa forma, a exposição ao vapor de mercúrio também causa efeitos tóxicos ao SNC (Goyer, 1996).

Dentre os compostos orgânicos de mercúrio, o metilmercúrio destaca-se como a forma mais tóxica do metal (Ozuah, 2000). Após a ingestão de alimentos contaminados com metilmercúrio, a principal rota de exposição humana, aproximadamente 95% deste é absorvido no trato gastrointestinal.

Após a absorção, esta forma do metal é amplamente distribuída pelo organismo, porém encontra-se mais concentrado no cérebro e no sangue (Guzzi e La Porta, 2008). Na circulação sanguínea, o metilmercúrio atravessa a membrana dos eritrócitos e liga-se à hemoglobina, sendo também oxidado a Hg^{2+}. Mas devido a sua elevada lipossolubilidade, o composto orgânico de mercúrio atravessa facilmente a barreira hemato-encefálica e a placenta (Ozuah, 2000), sendo considerado mais neurotóxico e teratogênico que os sais inorgânicos de mercúrio (Klassen, 1996). Assim, o SNC é o principal alvo do metilmercúrio, e a maior concentração cerebral do composto pode ser encontrada no cerebelo (Sanfeliu et al., 2003), ocorrendo também comprometimento de nervos periféricos. As manifestações clínicas de intoxicação por esta forma do metal incluem retardo mental, cegueira, alteração auditiva, distúrbios da fala, ataxia, parestesia de extremidades e neurite periférica (Ozuah, 2000; Clarkson et al., 2003).

O mercúrio inorgânico exerce seus efeitos em diferentes órgãos, tecidos e células, sendo conhecido por: induzir danos às membranas celulares (Baskin et al., 2003) e ao ácido desoxirribonucléico (DNA), causando danos mutacionais (Bem-Ozer et al., 2000; Schurz et al., 2000); afetar a atividade mitocondrial (Shenker et al., 2000); alterar o transporte de cálcio (Zalups, 2000); causar alterações em estruturas protéicas (Sutton et al., 2002); inibir atividades enzimáticas (Peixoto et al, 2003); interferir com nutrientes essenciais por deslocar e substituir minerais essenciais assim como o zinco ligado a enzimas (Tai e Lim, 2006) e proteínas (Peraza et al., 1998); e ainda, desencadear o processo de apoptose (Shenker et al., 2000; Sutton e Tchounwou, 2007). Essas ações explicam os efeitos deste metal sobre o embrião e também sobre os sistemas reprodutivo, urinário, hematopoiético, imune e neurológico (Sutton et al., 2002; Tchounwou et al., 2003). A exposição aguda ao mercúrio inorgânico pode causar náuseas, vômito e diarréia, mas a cronicidade desta intoxicação resulta em fadiga, perda de peso, parestesia periférica, nefrotoxicidade, hiperpigmentação cutânea e anormalidades psiquiátricas (Clifton, 2007).

2.1.3 Hepatoxicidade

A forma inorgânica do mercúrio pode causar danos hepáticos com alterações histopatológicas assim como vacuolização citoplasmática,

cariorrexia, cariólise, picnose e necrose centrolobular (Kumar et al., 2005). A hepatoxicidade causada pelo mercúrio também aparece refletida pelo aumento sérico de enzimas presente no interior dos hepatócitos, tais como aminotransferases e lactato desidrogenase (LDH) (Kumar et al., 2005; Sener et al., 2007). De fato, a determinação das atividades destas duas enzimas é utilizada como marcador de dano hepatocelular (Meyer et al., 1992).

As transaminases [alanina aminotransferase (ALT) e aspartato aminotransferase (AST)] são enzimas que transferem um grupo amino de um aminoácido a um aceptor α-cetoácido, resultando na formação de um novo aminoácido e um novo cetoácido. Enquanto que a LDH catalisa a reação reversível da conversão de piruvato em lactato (Devlin, 1997). Estas enzimas estão presentes em vários tecidos envolvidos no metabolismo de proteínas e carboidratos, incluindo o tecido hepático. Entretanto, mais recentemente, tem-se verificado que a atividade da LDH não é alterada e da ALT é inibida em soro de ratos jovens expostos ao cloreto de mercúrio (Peixoto e Pereira, 2007).

2.1.4 Nefrotoxicidade

Os rins constituem o órgão alvo primário da toxicidade e acúmulo do mercúrio inorgânico (Clarkson et al., 2003), pois, sendo um órgão altamente perfundido, estão potencialmente expostos a altos níveis de agentes tóxicos (Au, 2004). Além disso, a nefrotoxicidade causada pelos metais tóxicos deve-se, em parte, ao fato de que a eliminação urinária é a principal rota de excreção destes (Fowler, 1992). O mercúrio é reabsorvido e acumulado, predominantemente, nas células dos túbulos proximais. Como os íons mercúricos são altamente reativos, combinam-se intracelularmente com grupamentos sulfidrílicos, causando destruição ou inativação de proteínas e enzimas essenciais para uma adequada função renal. Este metal também promove estresse oxidativo, peroxidação lipídica e disfunção mitocondrial (Zalups, 2000). Ainda, ao se depositar nos rins, causa nefrotoxicidade (Zalups, 2000) induzindo um aumento nos níveis sanguíneos de uréia e creatinina, tanto em ratos jovens (Peixoto e Pereira, 2007) como em animais adultos (Sener et al., 2007).

De fato, um dos mais importantes parâmetros metabólicos utilizados para avaliar a função renal é a análise dos níveis sanguíneos de uréia e creatinina. A

uréia é o principal produto do catabolismo das proteínas, servindo como um mecanismo de excreção da amônia proveniente das reações de desaminação. A creatinina é formada durante o metabolismo normal da musculatura a partir da degradação da fosfocreatina, sendo geralmente produzida em uma taxa constante no organismo (Finco, 1997). Fisiologicamente, estes metabólitos são excretados pelos rins, portanto, o aumento dos seus níveis séricos indica um dano renal que impossibilita a adequada função deste órgão (Ravel, 1997).

2.1.5 Neurotoxicidade

O SNC dos animais em desenvolvimento é particularmente sensível aos efeitos tóxicos causados pelo mercúrio. Sabe-se que o metilmercúrio, que é reconhecido como essencialmente neurotóxico, causa alterações comportamentais em animais expostos ao metal nos primeiros dias de idade (Rocha et al., 1993, 2001; Pereira et al., 1999); e o mercúrio inorgânico, apesar de ser considerado principalmente nefrotóxico, também pode causar alterações comportamentais em animais expostos ao metal durante a gestação (Szász et al., 2002), através da lactação (Franco et al., 2007) ou durante os primeiros dias de vida pós-natal (Rocha et al., 2001; Peixoto et al., 2007a).

O mecanismo da neurotoxicidade do mercúrio não é completamente entendido, mas estudos têm demonstrado que este metal pode causar alterações na homeostasia de alguns neurotransmissores. O metilmercúrio inibe a captação de glutamato pelos astrócitos, causando disfunção neuronal (Aschner et al., 2000; Farina et al., 2003). *In vitro*, tanto o cloreto de mercúrio como o metilmercúrio, interagem com receptores $GABA_A$ através da alquilação de grupos –SH de resíduos cisteinil encontrados nestes receptores (Fonfría et al, 2001). O mercúrio também promove um aumento dos níveis extracelulares de dopamina por liberar este neurotranmissor de suas vesículas de estocagem, inibir a monoamina oxidase, e inibir a recaptação de dopamina, que é o principal mecanismo de eliminação deste neurotranmissor da fenda sináptica (Faro et al., 2007).

O estresse oxidativo também parece ser um importante contribuinte para a neurotoxicidade do mercúrio. Este metal induz a formação de espécies reativas de oxigênio (EROs) (Shanker e Aschner, 2001) e a peroxidação lipídica (Franco et al., 2007). Além disso, altera a atividade das enzimas responsáveis

pela defesa antioxidande celular (Hussain et al., 1997), assim como a catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx) (Benov et al., 1990). O mercúrio, por possuir grande afinidade por grupos –SH (Clarkson, 1997), pode ligar-se a estruturas que contêm estes grupamentos, como a cisteína (Cys) e a glutationa (GSH) (Zalups, 2000), causando diminuição dos níveis celulares de GSH, que é um importante antioxidante celular (Shanker e Aschner, 2001).

O acúmulo de mercúrio no SNC ocorre principalmente no cerebelo, pois as células da camada granular do cerebelo são consideradas um dos principais alvos do mercúrio orgânico. Essa diferença de sensibilidade entre as áreas cerebrais pode ser porque alguns tipos de neurônios específicos têm uma menor capacidade de defesa contra as injúrias causadas pelo mercúrio, o que pode ser o caso das células granulares cerebelares, que possuem baixa eficiência antioxidante (Sanfeliu et al., 2003).

Outra importante característica do mercúrio é que seus efeitos tóxicos persistem por um longo tempo após o término da exposição. Pesquisas têm demonstrado que alterações comportamentais e bioquímicas, bem como lesões no sistema nervoso periférico podem ser identificadas em ratos e camundongos, mesmo que tenha transcorrido um longo período de tempo após o final da intoxicação (Shigematsu, et al., 2000; Rocha et al., 2001; Yoshida et al., 2006; Peixoto et al., 2007a). Além disso, há relatos de que em humanos, os sintomas de intoxicação e os prejuízos neurocomportamentais persistem por vários anos após o final da exposição ao mercúrio (Albers et al, 1988; Kishi et al., 1993).

2.2 Zinco

O zinco também é um metal divalente que pertence ao grupo 2B da tabela periódica, mas ao contrário do mercúrio, este é um metal traço relacionado com funções vitais nos tecidos de mamíferos, estando presente em inúmeras proteínas e enzimas, envolvido em funções estruturais, catalíticas e regulatórias. É um elemento essencial para o crescimento e desenvolvimento, pois é fundamental para proliferação e diferenciação celular. Além disso, é

necessário para o sistema imune, metabolismo intermediário, metabolismo e reparação do DNA, reprodução e apoptose (Maret e Sandstead, 2006).

Também é requerido para a atividade de muitas enzimas críticas para a função cerebral, como aquelas envolvidas na defesa antioxidante, na respiração celular, na síntese de catecolaminas e outras enzimas relacionadas com o crescimento, desenvolvimento e manutenção do sistema nervoso (Mathie et al., 2006), além de funcionar como molécula sinalizadora, que é liberada nos terminais sinápticos e atua sobre proteínas de membrana (Frederickson et al., 2005).

No plasma, o zinco está presente em concentrações aproximadas de 15 μM, sendo transportado ligado à albumina (Mathie et al., 2006), já nos tecidos encontra-se ligado a metalotioneínas, e a concentração do metal é variável. No fígado, por exemplo, sua concentração varia de 168 a 442 μg/g de tecido seco (Rodriguez-Moreno et al., 1997) e é influenciada pelos hormônios adrenocorticotrófico e paratireoideo, e também por endotoxinas (Goyer, 1996). O cérebro é o tecido que possui o mais alto conteúdo de zinco (100-150 μM) quando comparado com os outros tecidos (Mathie et al., 2006). A homeostase deste elemento é regulada por proteínas envolvidas na captação, excreção, armazenamento e transporte intracelular (Chimienti et al., 2003). Estas proteínas funcionam como sensores e proteínas transportadoras de membrana que regulam a entrada e saída do zinco na célula. Existem também metaloproteínas como as metalotioneínas que participam do transporte deste metal entre as organelas, mantendo o zinco disponível em concentrações adequadas (Maret, 2005).

A deficiência de zinco causa um aumento significante na atividade da enzima glutationa-S-transferase (GST) e na peroxidação lipídica no soro, fígado e cérebro de ratos (Yousef et al., 2002). Entretanto, um aumento excessivo intracelular de zinco pode produzir efeitos citotóxicos, causando degeneração neuronal (Choi e Koh, 1998). Portanto, dependendo das concentrações, o zinco intracelular pode ter efeitos neurotóxicos ou neuroprotetores (Cote et al., 2005). O zinco também desempenha importante papel no comportamento e na função cognitiva, tanto que a privação deste elemento durante o período de

desenvolvimento pode causar problemas comportamentais, de aprendizagem e de memória na idade adulta (Mocchegiani et al., 2005).

Este metal possui potencial terapêutico, sendo eficaz no tratamento da Doença de Wilson por induzir a síntese de metalotioneínas, a qual funciona como um ligante intracelular para o cobre (Kitzberger et al., 2005). Também são conhecidos os efeitos preventivos do zinco sobre alterações causadas por diversos agentes, tais como o álcool (Zhou et al., 2005), os organofosforados (Goel et al., 2005; Franco et al., 2009), o lítio (Chadha et al., 2008) e o cádmio (Brzóska et al., 2001).

Estudos recentes têm demonstrado que o zinco pode ser considerado um agente protetor contra a toxicidade do mercúrio. Quando utilizado como pré-tratamento é capaz de prevenir a diminuição no ganho de peso corporal, evitar a inibição da enzima porfobilinogênio-sintase (PBG-sintase) renal e hepática (Peixoto et al., 2003), bem como evitar a insuficiência renal causada pela exposição ao mercúrio (Peixoto e Pereira, 2007). Os efeitos preventivos do zinco podem ser parcialmente atribuídos à síntese de metalotioneínas induzida por este metal (Peixoto et al., 2003; 2007c), já que o zinco é considerado o melhor indutor desta proteína (Eaton et al., 1980; Bracken & Klaassen, 1987). Estas metaloproteínas possuem uma grande afinidade por metais devido ao seu alto conteúdo de aminoácidos sulfidrílicos (Chan et al., 2002), por isso desempenham função de transporte e estocagem de metais essenciais e também de destoxificação de metais não essenciais (Hidalgo et al., 2001).

2.3 Acetilcolinesterase

2.3.1 Distribuição e função

A acetilcolinesterase (AChE; E.C 3.1.1.7) desempenha um importante papel regulatório na transmissão do impulso nervoso (Soreq e Seidman, 2001), pois é responsável por hidrolisar o neurotransmissor acetilcolina (ACh) em colina e acetato (Figura 2), controlando, dessa forma, a ativação sináptica no sistema colinérgico (Taylor, 1996), um sistema envolvido na função cognitiva relacionada à atenção, memória e emoção (Román and Kalaria, 2006).

Esta enzima é amplamente distribuída no sistema nervoso central onde participa na neurotransmissão colinérgica (Taylor, 1996), estando presente nos neurônios colinérgicos e nas adjacências das sinapses colinérgicas. Também é altamente concentrada nas junções neuromusculares (Lefkowitz et al., 1996). Ainda, alguns autores têm relatado a existência de variantes estruturais da AChE distribuídas por diversos tecidos em humanos, sendo que estas variantes possuem atividades não catalíticas, desempenhando funções na neurogênese, sinaptogênese, hematopoiese e osteogênese (Soreq e Seidman, 2001).

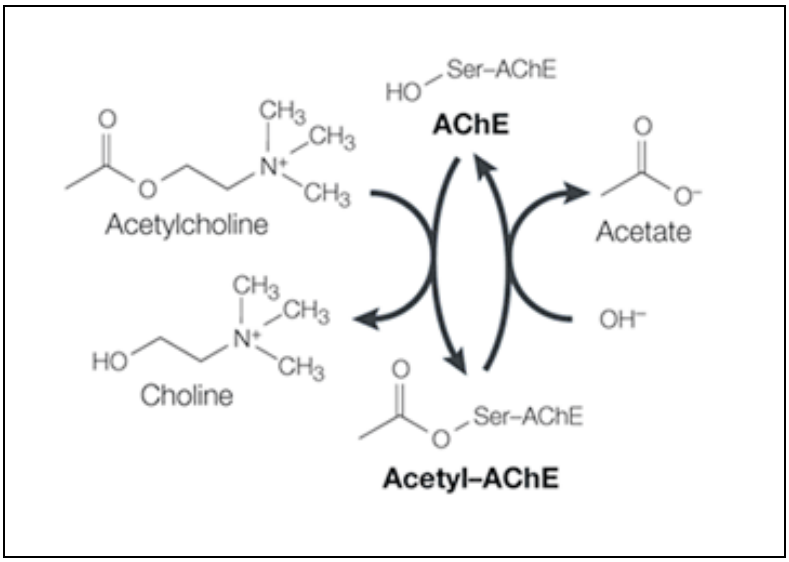

Figura 2 – Hidrólise da ACh pela enzima AChE (Soreq e Seidman, 2001).

2.3.2 Estrutura e mecanismo de ação

A AChE existe em diferentes formas moleculares: homoméricas e heteroméricas (Figura 3). A classe homomérica é constituída de oligômeros de subunidades catalíticas que aparecem como monômeros, dímeros ou tetrâmeros, originando as formas globulares (G): G1, G2 e G4. A classe heteromérica é composta de subunidades catalíticas associadas a subunidades estruturais, onde ocorre a ligação de um, dois ou três tetrâmeros catalíticos a

um filamento de colágeno, resultando nas formas estruturais assimétricas (A): A4, A8 e A12 (Taylor e Brown, 1993).

No tecido nervoso a principal forma encontrada é do tipo globular, predominantemente G4, ligada à membrana. Enquanto que os eritrócitos, linfócitos e plaquetas contêm a forma globular dimérica G2, tetramérica G4 e a assimétrica A12 (Nigg e Knaak, 2000). Na junção neuromuscular predominam as formas heteroméricas de subunidades catalíticas e estruturais, que estão associadas com a lâmina basal externa na sinapse (Taylor & Brown, 1993).

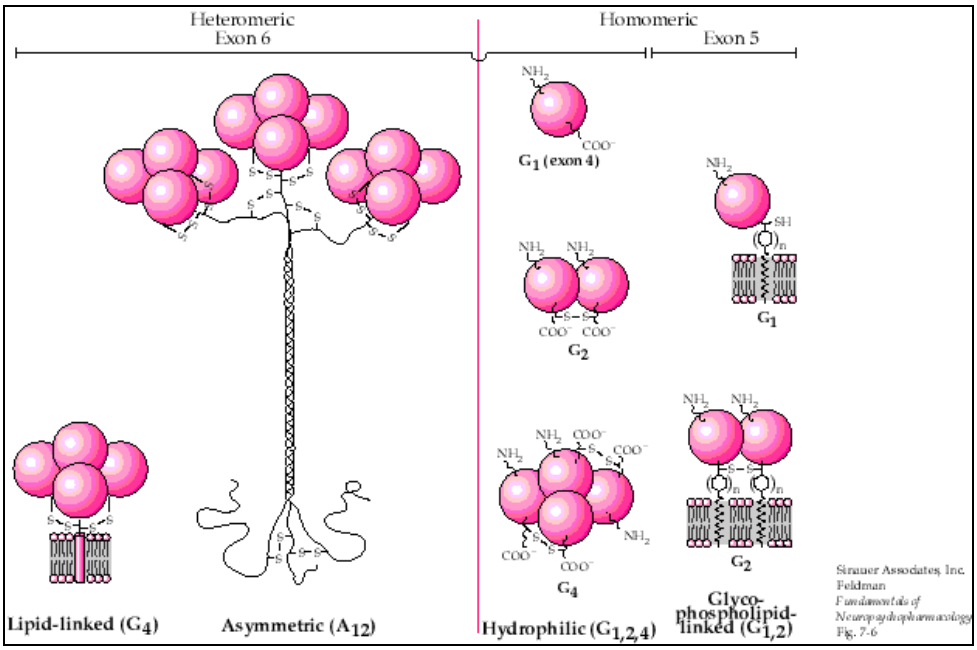

Figura 3 – Isoformas da enzima AChE (Taylor e Brown, 1993)

O sítio ativo da AChE contém dois subsítios (Figura 4): um sítio esterásico que contém os verdadeiros resíduos catalíticos, onde se liga o grupamento éster e carbonila da ACh; e um sítio aniônico (carregado negativamente), no qual se liga a cadeia de nitrogênio quaternário da ACh carregada positivamente (Sussman et al., 1991). Um segundo sítio aniônico, conhecido como sítio aniônico periférico (peripheral anionic site – PAS), parece

regular a catálise de modo alostérico, podendo estar envolvido na ação de alguns inibidores da enzima ou na inibição por excesso de substrato (Marcel et al., 1998). A ocupação deste sítio afeta a conformação do centro ativo e também a afinidade dos compostos ligados a ele (Berman et al., 1981).

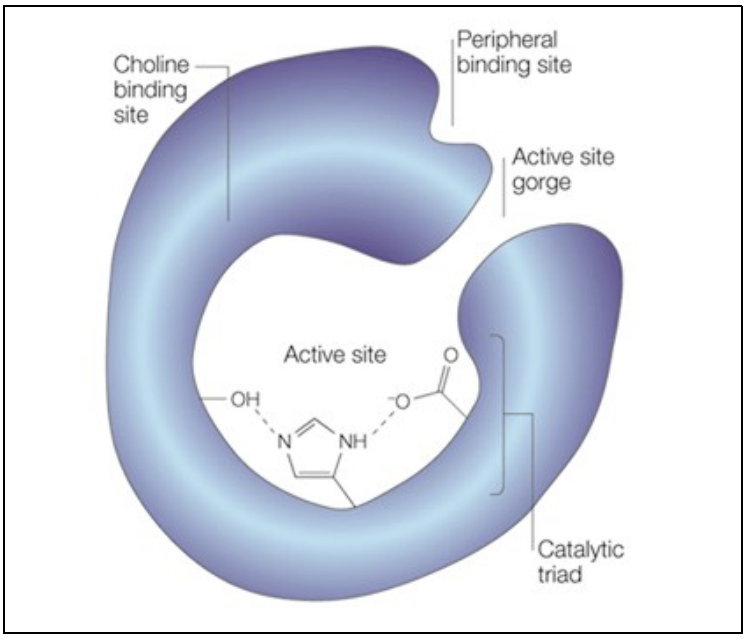

Figura 4 – Sítio ativo da AChE (Soreq e Seidman, 2001).

Esta enzima é definida como uma serina hidrolase. No momento da ligação enzima-substrato ocorre o ataque nucleofílico da hidroxila da serina do local esterásico sobre o grupamento éster-carbonila do substrato, causando a quebra da ligação éster. Durante o ataque enzimático sobre o éster, é formado um intermediário tetraédrico entre a enzima e o éster, que se rompe e forma um conjugado acil-enzima, com a liberação concomitante da colina. O complexo acetil-enzima formado sofre hidrólise, resultando na liberação de acetato e na regeneração da enzima ativa (Taylor, 1996).

2.3.3 Importância toxicológica

A atividade da AChE pode ser usada como um índice da função colinérgica pois mudanças na sua atividade podem indicar alterações na disponibilidade de ACh ao nível de receptores muscarínicos e nicotínicos, podendo, então, atuar como biomarcador para diferentes agentes tóxicos, assim como pesticidas (Taylor, 1996), metais pesados (Gill et al., 1990, Frasco et al., 2005, 2007) e nicotina (Jósê et al., 2009).

Alguns estudos revelaram inibição da atividade da AChE de cérebro de ratos intoxicados com cloreto de mercúrio (El-Demerdash, 2001) e também com metilmercúrio (Sood et al., 1993). Entretanto, Moretto et al. (2004) demonstraram que a exposição ao cloreto de mercúrio produz um aumento na atividade da AChE cerebral.

A ação do zinco sobre a atividade da AChE tem sido estudada, mas o mecanismo de ação destes metais sobre a enzima não está elucidado. Experimentos *in vitro* demonstraram que o zinco não afeta a atividade da AChE (Corsi et al., 2004; Senger et al., 2006), mas há relatos de que a exposição ao zinco causa diminuição na atividade desta enzima em peixes (Suresh et al., 1992) e ratos (Brocardo et al., 2005).

Os inibidores da AChE potencializam a ação da ACh liberada pelo estímulo nervoso, devido ao acúmulo do neurotransmissor em todos os locais onde este é liberado, pois com a inibição da AChE, o tempo de permanência da ACh na sinapse aumenta, permitindo a nova ligação do neurotransmissor nos múltiplos receptores colinérgicos. Assim, a resposta à ACh liberada pelos impulsos colinérgicos ou espontaneamente liberada na terminação nervosa fica exacerbada (Taylor, 1996).

2.4 Porfobilinogênio-Sintase

2.4.1 Distribuição e função

A porfobilinogênio-sintase (PBG-sintase; E.C. 4.2.1.24), também conhecida como δ-aminolevulinato desidratase (δ-ALA-D), é uma enzima citosólica, expressa em muitos tecidos, sendo seus maiores níveis encontrados em tecidos como medula óssea, fígado, rins e eritrócitos (Dessypris, 1998).

Esta enzima está presente na via de biossíntese de compostos tetrapirrólicos (corrinas, bilinas, clorofilas e heme) (Shemin, 1976). Estes compostos são grupamentos prostéticos de proteínas, as quais desempenham importantes funções como transporte e armazenamento de oxigênio (hemoglobina e mioglobina), transporte de elétrons (citocromos a, b e c), biotransformação de xenobióticos (citocromo P450), e reações de oxi-redução (catalases e peroxidases) (Timbrell, 1991).

A biossíntese do heme ocorre parcialmente na mitocôndria e no citosol. O ácido δ-aminolevulínico (ácido 5-aminolevulínico, δ-ALA) é sintetizado mitocondrialmente pela condensação da succinil-CoA com a glicina em uma reação catalisada pela enzima ALA-sintase. Posteriormente, o δ-ALA é transportado para o citosol, onde está presente a enzima PBG-sintase que vai catalisar a segunda reação da síntese dos pirróis (Figura 5) (Dessypris, 1998), promovendo a condensação assimétrica de duas moléculas de δ-ALA, com a concomitante perda de duas moléculas de água, para formar porfobilinogênio (PBG) (Figura 6) (Shemin, 1976).

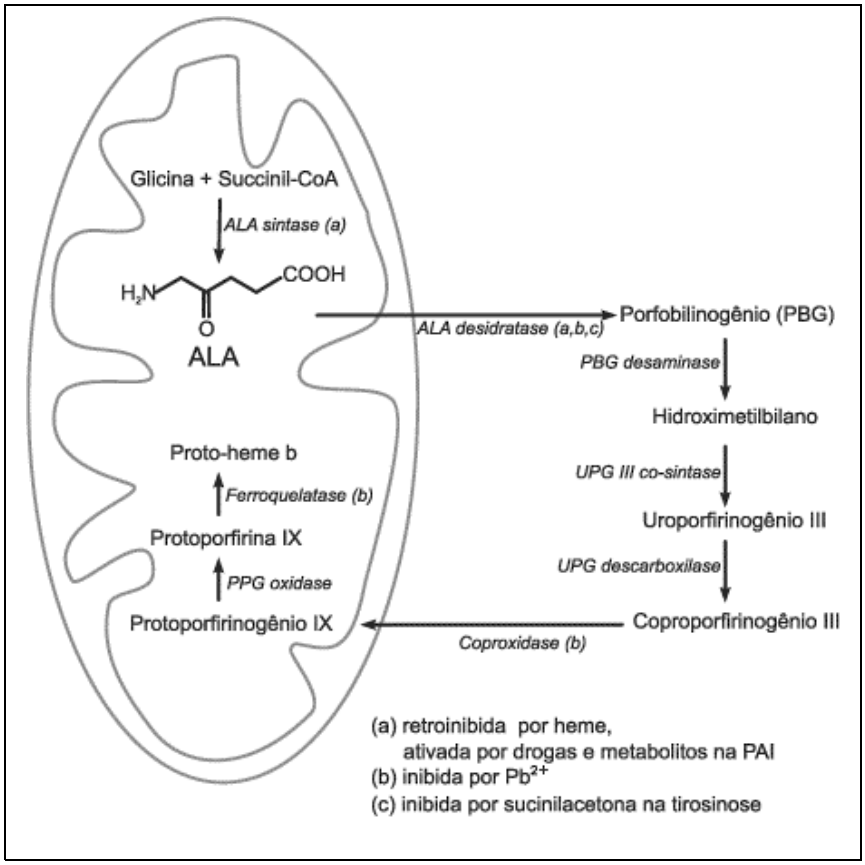

Figura 5 – Via biossintética do grupamento heme (Dutra e Bechara, 2005).

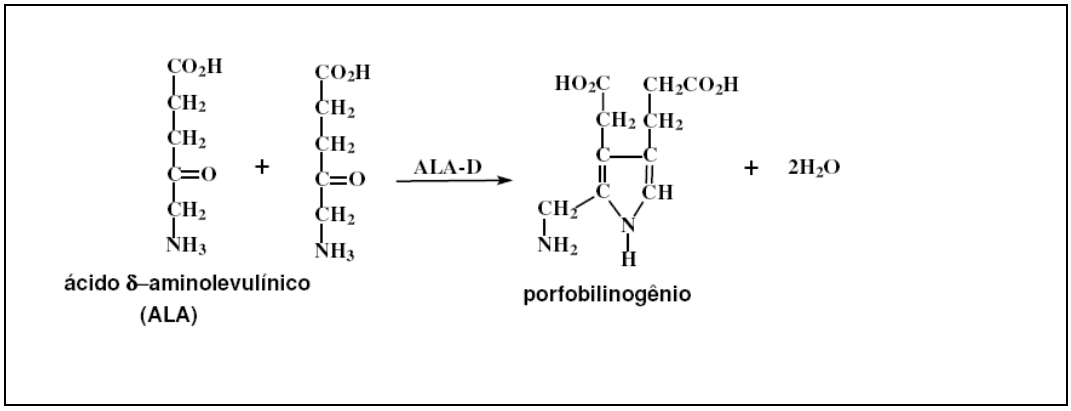

Figura 6 - Condensação assimétrica de 2 moléculas do δ-ALA, catalisada pela enzima PBG-sintase (Shemin, 1976).

2.4.2 Estrutura e atividade catalítica

A PBG-sintase é uma proteína octamérica com peso molecular de 280.000 Da (Dessypris, 1998), que contém oito grupos (subunidades) sulfidrílicos (Tsukamoto, et al., 1980). Portanto, devido a sua natureza sulfidrílica, é uma enzima que posssui grande afinidade por metais, principalmente metais divalentes (Scheuhammer, 1987). Estes metais quando ligados aos grupamentos sulfidrílicos vão alterar a atividade catalítica da enzima, podendo ser ativada quando o metal ligante for o zinco (Thompson et al., 1977; Nelson, 1981), ou inibida quando ligada ao chumbo (Goering, 1993), mercúrio (Rocha et al., 1993; 1995; 2001; Peixoto et al., 2003; 2007b), cádmio (Peixoto et al., 2004) ou cobre (Thompson et al., 1977; Nelson, 1981).

É considerada uma metaloenzima que requer zinco para sua estabilidade e atividade. Entretanto, o papel do zinco na atividade da PBG-sintase não é completamente entendido (Hasnian et al., 1985), mas sabe-se que a enzima possui um sítio de ligação ao zinco importante para a estabilidade de sua estrutura octamérica (Dent et al., 1990), e que após a remoção deste metal por quelantes como o EDTA, os grupos –SH da enzima são facilmente oxidados, com concomitante perda da atividade enzimática (Bevan et al., 1980).

Os aminoácidos lisina, histidina e cisteína têm sido identificados como constituintes da enzima, envolvidos no sítio ativo (Erskine et al., 1999; Tsukamoto et al., 1979). Cada monômero da enzima possui dois sítios catalíticos, denominados P e A, onde se ligam duas moléculas do substrato, sendo que a ligação destes substratos é ordenada. A primeira molécula de δ-ALA liga-se ao sítio P da enzima e forma a metade propionato do produto PBG, com seu nitrogênio amino sendo incorporado ao anel pirrol; enquanto que a segunda molécula de substrato liga-se ao sítio A formando a metade acetato do PBG, retendo seu grupo amino (Jordan e Gibbs, 1985).

Há um alto grau de homologia entre a PBG-sintase de diversas fontes. Uma das principais diferenças que existe é referente ao cofator metálico, pois embora todas requeiram um cátion divalente para sua atividade, a enzima de animais, leveduras e bactérias é dependente de zinco (Chen e Neilands, 1973), enquanto a de plantas utiliza o magnésio (Tamai et al., 1979).

2.4.3 Importância toxicológica

A PBG-sintase é uma enzima sulfidrílica, portanto, é utilizada como biomarcador da exposição a metais pesados. A inibição da atividade da PBG-sintase de eritrócitos humanos, por exemplo, é bastante utilizada como índice de exposição ao chumbo (Goering, 1993). Além disso, sabe-se que a inibição da atividade desta enzima também reflete a intoxicação por mercúrio (Rocha et al., 1993; 1995; 2001; Peixoto et al., 2003; 2007b).

Uma das conseqüências da atividade da PBG-sintase diminuida é inibição da via biossintética do heme, com a diminuição na produção do PBG e, consequentemente, do grupamento heme, o que pode causar anemia, como ocorre em intoxicações por chumbo (Jin et al., 2008) e alumínio (Zaman et al, 1993). Outra alteração na via biossintética dos compostos tetrapirrólicos, causada pela inibição da atividade da PBG-sintase, é a superprodução e acúmulo do substrato δ-ALA, o qual possui atividade pró-oxidante por induzir a produção de espécies reativas de oxigênio (Bechara et al., 1993), e também é neurotóxico por afetar vários sistemas neurotransmissores (Emanuelli et al. 2000; 2001).

2.5 Animais em desenvolvimento

Após o nascimento os roedores apresentam um aumento significante no crescimento dos órgãos e do cérebro, esse período é caracterizado por um de grande aumento no conteúdo cerebral de proteína, DNA, ácido ribonucléico (RNA) e mielina, e pode ser dividido em três fases que incluem aumento no número e no tamanho das células. A 1ª fase (0-6 dias de idade) é caracterizada por hiperplasia cerebral, com síntese de proteína e DNA; na 2ª fase (8-12 dias de idade) o crescimento é mais rápido do que nos dias anteriores e ocorre hipertrofia e hiperplasia; enquanto que na 3ª fase (17-23 dias de idade) ocorre síntese de mielina e hipertrofia cerebral (Gottlieb et al., 1977). Assim, devido a este marcado desenvolvimento e crescimento de órgãos (Winick e Noble, 1965), insultos, mesmo aqueles considerados leves podem causar

pronunciados danos quando aplicados a organismos em desenvolvimento (Smart e Dobbing, 1971). Alguns trabalhos têm relatado uma alta sensibilidade dos animais em relação a insultos externos, tal como a exposição ao mercúrio, impostos durante este período (Rocha et al., 1993; Peixoto et al., 2003; 2007a), sendo que esta sensibilidade depende do estágio de desenvolvimento pós-natal (Peixoto et al., 2004; 2007a).

Estudos com ratos jovens expostos ao cloreto de metilmercúrio têm demonstrado alterações em respostas de orientação, locomoção e catalepsia (Pereira et al., 1999). Ratos da mesma idade tratados com cloreto de mercúrio também apresentam alterações na atividade locomotora (Rocha et al., 2001; Peixoto et al., 2007a). Além disso, a intoxicação por mercúrio em diferentes fases de crescimento durante o período pós-natal produz acúmulo cerebral deste metal e alterações comportamentais (Peixoto et al., 2007a).

CAPÍTULO 3

ARTIGO

$ZnCl_2$ exposure protects against behavioral and acetylcholinesterase changes induced by $HgCl_2$

Carina Franciscato, Fernanda Rodrigues Goulart, Naglezi de Menezes Lovatto, Fabio Andrei Duarte, Érico Marlon de Moraes Flores, Valderi Luis Dressler, Nilce Coelho Peixoto, Maria Ester Pereira

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$ZnCl_2$ exposure protects against behavioral and acetylcholinesterase changes induced by $HgCl_2$

C. Franciscato [a], F.R. Goulart [b], N.M. Lovatto [b], F.A. Duarte [b], E.M.M. Flores [b], V.L. Dressler [b], N.C. Peixoto [a,b], M.E. Pereira [a,b,*]

[a] Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, 97.105-900 Santa Maria, RS, Brazil
[b] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, 97.105-900 Santa Maria, RS, Brazil

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ABSTRACT

This study examined the effects of inorganic mercury exposure on behavioral and biochemical parameters and investigated the possible preventive effects of zinc on the alterations induced by mercury. Pups were exposed from 3rd to 7th postnatal day to $ZnCl_2$ (27 mg/kg/day, s.c.) and subsequently to $HgCl_2$ (5 doses of 5 mg/kg/day, s.c.). Each litter contained two rats for each treatment. The rats were submitted to behavioral task and litters were killed at 13 or 33 days old for acetylcholinesterase activity assays and for the determination of metal levels. Based on the results obtained from 13-day-old rats, they were divided in two groups of litters that were defined at the end of the experimental period (33 days) as less sensitive rats to mercury and more sensitive rats to mercury in accordance with the recovery of body weight until day 33. The mercury exposure caused accumulation of this metal in cerebrum and cerebellum in all mercury treated rats, and inhibited the cerebellum acetylcholinesterase activity from 13-day-old rats. Besides, the mercury-animals of the most sensitive litters to mercury presented impairment in motor function and muscular strength verified in the beaker test, as well as a reduction of the locomotor and exploratory activities in the open field task. Zinc partially prevented all the alterations induced by mercury exposure and reduced the mercury level accumulated in cerebrum and cerebellum. This study confirms the preventive effect of zinc on behavioral alterations induced by mercury in young rats and demonstrates that the mercury behavioral effects are present even for a long time after the end of the exposure.

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1. Introduction

Mercury, a nonessential metal, is an important environmental toxicant since it can cause severe damages on the health of both animals and humans (Clarkson, 1997). It is known that mercury compounds are retained in the tissues for long periods of time (Clarkson, 1983) and induce neurological impairment as behavioral changes (Peixoto et al., 2007a), cognitive deficit and ataxia (Clarkson, 1997). Chronic exposure to it can lead to potentially irreversible neuropsychological deficits and emotional disturbances in children (Carter-Pokras et al., 2007).

This metal, in the inorganic form (e.g. mercury chloride), is mainly nephrotoxic (Goyer, 1995; Emanuelli et al., 1996; Clarkson, 1997; Peixoto et al., 2007b; Peixoto and Pereira, 2007). However, studies have related central nervous system (CNS) alterations by inorganic mercury when animals are exposed during gestation (Szász et al., 2002), during lactation (Franco et al., 2007) or on the first days of postnatal life (Rocha et al., 2001; Peixoto et al., 2007a). Animals exposed on the first days of postnatal life are also very sensitive to behavioral alterations induced by methylmercury (Rocha et al., 1993, 2001; Pereira et al., 1999).

The high sensibility seems to be due to the fact that rodents present an accelerated growth and development of organs during this phase, including the brain. This rapid cerebral postnatal period is divided in three phases. The phase that has received more attention is the second, which is comprised from 8 to 12 days old. This period is characterized by rapid protein, DNA and RNA synthesis (Gottileb et al., 1977).

Recent reports from our research group have demonstrated that most of the effects induced by mercury chloride in rats, such as biochemical and metabolic alterations, were prevented by previous exposure to zinc (Peixoto et al., 2003; Peixoto and Pereira, 2007). Still, we observed that in parallel to these preventive effects, liver and kidney of animals exposed to zinc presented high contents of metallothioneins and a probable transport of mercury from liver to kidney, since they presented a reduction and an

* Corresponding author at: UFSM, CCNE, Departamento de Química, Campus Universitário, Camobi, 97.105-900 Santa Maria, RS, Brazil. Tel.: +55 55 32208799; fax: +55 55 32208799.
E-mail address: pereirame@yahoo.com.br (M.E. Pereira).

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increase of mercury contents in liver and in kidney, respectively (Peixoto et al., 2003, 2007c).

The essentiality of zinc and other essential metals in the several metabolic functions (Fang et al., 2002) is well known in the literature. In fact, zinc plays an important role in cellular growth and differentiation, specifically in the protein synthesis and DNA replication and transcription (Cherian et al., 2003) as well as in the metallothionein synthesis (Pedersen et al., 1998; Peixoto et al., 2003, 2007c). Zinc is a catalytic and structural element of many proteins (Mathie et al., 2006), and an intercellular signaling messenger that is released from central nerve terminals during synaptic activity (Choi and Koh, 1998). This metal is also involved in the behavior and cognitive functions, and its deficiency may lead to alterations in brain activity, motor development, neuropsychological behavior, attention, learning, and memory (Mocchegiani et al., 2005).

Several neurotransmitter systems, such as glutamatergic, GABAergic, dopaminergic, cholinergic, serotoninergic, and noradrenalinergic, seem to be involved in the cognitive function and other neurological functions (Myhrer, 2003). Among these, the cholinergic system plays a key role in the cognitive function, especially in relation to attention, memory and emotion (Román and Kalaria, 2006). Besides, an appropriate synaptic transmission of this system depends on the activity of the enzyme acetylcholinesterase (AChE) (E.C.3.1.1.7). This enzyme promotes the hydrolysis of the neurotransmitter acetylcholine, resulting in the end of the transmission of the nervous impulse in the synapses. The activity of this enzyme has been used as an index of cholinergic function, since changes in its activity can indicate alterations in the availability of acetylcholine (Taylor, 1996).

The AChE activity can be inhibited by different toxic agents such as pesticides (Taylor, 1996), heavy metals such as mercury (Gill et al., 1990; Frasco et al., 2005, 2007), and nicotine (Jósê et al., 2009). Regarding mercury, some authors have revealed the inhibition of the AChE activity in brain of rats intoxicated with mercury chloride (El-Demerdash, 2001) and also with methylmercury (Sood et al., 1993). Others have related increase of rat brain AChE activity by mercury chloride exposure (Moretto et al., 2004).

In relation to zinc, there are also controversies on their effects on AChE activity. Some studies demonstrate inhibition (Brocardo et al., 2005) or increase (Carageorgiou et al., 2005) of the AChE activity from brain of rats exposed to zinc *in vivo*. *In vitro*, this metal causes inhibition of this enzyme (Frasco et al., 2005). Still, research has shown that zinc affects the enzyme activity neither from *Adamussium colbecki* scallop *in vivo* (Corsi et al., 2004) nor from *Danio rerio* zebrafish brain *in vitro* (Senger et al., 2006).

Considering the information previously described, the inorganic mercury neurotoxicity, the preventive effects of zinc on several biochemical parameters and the high susceptibility of CNS from animals in development to neurotoxicants, this study investigated the effects of mercury chloride on the performance of young rats in several behavioral tasks as well as on cerebrum and cerebellum AChE activities. We have also studied the possible preventive effects of zinc chloride on these behavioral and biochemical alterations induced by mercury.

2. Experimental procedures

2.1. Chemicals

Zinc ($ZnCl_2$), mercury ($HgCl_2$), sodium chloride (NaCl), dibasic (K_2HPO_4) and monobasic (KH_2PO_4) potassium phosphate, dibasic sodium phosphate (Na_2HPO_4), sucrose, *ortho*-phosphoric acid, absolute ethanol, nitric acid (HNO_3), chloridric acid (HCl), and sodium tetrahydridoborate ($NaBH_4$) were obtained from Merck (Rio de Janeiro, RJ, Brazil); acetylthiocholine iodide (ATC), 5-5′-dithiobisnitrobenzoic acid (DTNB), Tris [Tris (hydroxymethyl-d_3) amino-d_2-methane], Coomassie brilliant blue G and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA).

2.2. Animals

The studies were conducted in accordance with the national and institutional guidelines (University Ethics Committee Guidelines—Process number 23081.014805/2007-68) for experiments with animals. Wistar pregnant rats obtained from the General Animal House of the Federal University of Santa Maria were transferred to the colony room and maintained individually in opaque plastic cages at room temperature (23 ± 2 °C). One day after the birth, the number of pups of each litter was reduced to 8. The total number of 34 litters was utilized in the experimental procedure.

2.3. Exposures

The pups were pre-treated from 3rd to 7th postnatal day of life with one daily dose of saline (NaCl 90 mg/kg/day) or $ZnCl_2$ (27 mg/kg/day) and received saline or $HgCl_2$ (5 mg/kg/day) for 5 consecutive days (from 8 to 12 days old). The treatments were administered, randomly, through subcutaneous (*s.c.*) injections in a volume of 10 ml/kg body weight. The animals were weighed daily to adjust the dose. Each litter contained two rats for each treatment. Litters were killed at 13 or 33 days old (24 h or 21 days after the end of mercury exposure) to AChE activity assays and to determination of metals levels. Twenty seven litters were used for behavioral tasks. Litters whose animals of the same treatment died were not used to enzymatic and behavioral experiments. All litters treated were used to calculate the death percentage. It was not possible to conduct blind study since rats treated with mercury presented alopecia in the site of the injection. Results obtained starting at 13 days old performed two groups of litters that were defined at the end of the experimental period (33 days old). These groups were called litter containing more sensitive rats (MSR) to mercury and litter containing less sensitive rats (LSR) to mercury (see Section 3).

2.4. Behavioral tests

Eleven litters were submitted to negative geotaxis task, tail immersion and rotarod tests. Sixteen litters were submitted to beaker test and open field task. Each litter contributed only with one experimental *n* to each treatment. For negative geotaxis, beaker test and tail immersion, each experimental *n* is the mean of the performance of animals with the same treatment per litter. For rotarod and open filed tasks, each litter contributed with only one rat of each treatment (experimental *n*).

2.4.1. Negative geotaxis task

On days 3, 5, 7, 9, 11 and 13 the animals were submitted to behavioral task of negative geotaxis reflex that was carried out on a platform with 30 cm of length, 20 cm of breadth and with an inclination of 30°, where the pups were placed with the head down. The maximum latency for the reflex of negative geotaxis was 60 s for each session. Each trial consisted of the mean latency of 5 consecutive sessions. The trials were made before solution administration. The decrease of latency of negative geotaxis reflex was considered as the improvement of the motor reflex response (Da-Silva et al., 1990). Each experimental *n* is the mean of the performance of animals with the same treatment per litter ($n = 11$).

2.4.2. Tail immersion test

Nociception was assessed in the tail immersion test as described by Tabarelli et al. (2003), with some changes. Rats were wrapped in a towel and 3.5 cm of tail was immersed in water bath (48 ± 1 °C). The time needed for the animal to deflect the tail was used as latency immersion. A cut-off time of 10 s was used to avoid tail tissue damage. The rats were submitted to test at 13, 20 and 27 days old (24 h, 8 and 15 days after the end of mercury exposure). Each experimental *n* is the mean of the performance of animals with the same treatment per litter ($n = 5$ for MSR and $n = 6$ for LSR).

2.4.3. Beaker test

The animals were submitted to beaker test from 17 to 20 days old (5–8 days after the end of mercury exposure) (sessions from 1 to 4) with interval of 24 h between the sessions as described by Peixoto et al. (2007a). In this task, the ability of rats to balance on and move along the rim of 2 L polypropylene beaker was observed. This beaker, with 19.5 cm high × 14 cm diameter, had an outward-curving top edge. The apparatus was placed on a workbench, 1 m from the room floor. A dark refuge box, inner dimensions 9.5 cm × 5.5 cm × 3.5 cm high, with an entrance platform 5 cm × 5.5 cm, was clamped so that the platform could rest on the spout of the beaker. Each rat was placed on the rim of the beaker, facing the refuge at the furthest distance from it. Time points to reach the refuge, fall or jump off the rim were recorded. A cut-off time of 90 s was used for each session (Smart and Dobbing, 1971). The results are presented as mean of latency to access to refuge and as percentage of fall per litter per treatment. Each experimental *n* is the mean of the performance of animals with the same treatment per litter ($n = 10$ for MSR and $n = 6$ for LSR).

2.4.4. Rotarod test

The rotarod apparatus consisted of a cylinder with 3.7 cm of diameter that rotates in a velocity of 8 rpm. Twenty-four hours before the test the rat was

submitted to run in the rotarod for 60 s to avoid the novelty effect. On the day of the test (at 25 and 30 days old, i.e., 13 and 18 days after the end of mercury exposure) the animals were submitted to rotarod and two parameters were evaluated: latency to first fall and the number of falls in 240 s (Tsuda et al., 1996). Each litter contributed with only one rat of each treatment ($n = 5$ for MSR and $n = 6$ for LSR).

2.4.5. Open field task

The open field task was carried out as described by Peixoto et al. (2007a), with some changes. The animals were submitted to open field at 30 and 31 days old (training and test sessions, respectively) with interval of 24 h between sessions. The exploratory behavior was investigated in a circular open field, 58 cm of diameter and 30 cm of high wall, situated on the room floor. Each rat was placed in the central area, in a way not to see the observer. In the succeeding 5 min, the following behaviors were recorded: latency to leave the initial area, crossing (number of areas entered), rearing (incidence of head-lifting on the hind legs either against a vertical surface or unsupported), and number of fecal boluses. The behaviors of exiting the initial area and crossing were considered when the animal placed the four paws in another area. Each litter contributed with only one rat of each treatment ($n = 10$ for MSR and $n = 6$ for LSR).

2.5. Death percentage

The animals were daily observed from the start of treatment (day 3) until 33 days old to register the number of rats that died. Thus, all litters submitted to treatments were considered ($n = 34$ litters). Litters in which all animals of the same treatment died were not used for behavioral test neither for biochemical and metal tissue analyses.

2.6. Tissue preparation

One day after the last administered dose (13 days old) or after the behavioral tests (33 days old) the animals were weighed, anesthetized with ether and euthanized by decapitation. For the determination of the enzymatic activity and analysis of metal contents, brain was removed and cerebrum and cerebellum were separated. Medulla oblongata and pons were discarded.

For the determination of the AChE activity, the cerebrum and cerebellum were homogenized (1:10, w/v) in 10 mM Tris–HCl buffer, pH 7.2 with 160 mM sucrose. The homogenates were frozen at −20 °C until analysis. The enzyme activity was determined in rats from 13-day-old ($n = 5$), 33-day-old MSR ($n = 5$) and LSR ($n = 5$) litters.

For metal content determination, the cerebrum and cerebellum were weighed and frozen at −20 °C until analysis. The metal content was determined in rats from 13-day-old ($n = 3$), 33-day-old MSR ($n = 5$) and LSR ($n = 3$–5) litters. For both AChE and metal analyses each litter contributed with only one rat of each treatment (experimental n).

2.7. Enzyme assay

AChE activity was determined by the method of Ellman et al. (1961), modified as described by Pereira et al. (2004). The mixture assay contained 1.04 mM DTNB, 24 mM potassium phosphate buffer pH 7.2 and 25 μL of enzymatic material. It was pre-incubated for 2 min at 30 °C and the reaction was started with the addition of 0.83 mM ATC. The product from the reaction of thiocholine with DTNB was determined at 412 nm every 30 s during 2 min with an absorption coefficient of 0.0136 M^{-1} cm^{-1}. The specific activity was expressed as μmol ATC hydrolyzed/h/mg protein. All samples were run in triplicate.

2.8. Protein determination

Protein concentrations in the samples were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard. All samples were run in triplicate.

2.9. Metal content determination

The digestion of samples and the determination of zinc and mercury contents were carried out as described in details by Peixoto et al. (2008).

2.9.1. Digestion and quantification of metal procedures

The samples were digested with HNO_3 using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (max 80 bar, 280 °C, Anton Paar, Graz, Austria). After digestion, samples were diluted with water to 25 mL and transferred to graduated polypropylene vials.

Metal analyses were carried out using a Model AAS EA 5 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer with pyrolytic coated tubes for zinc. A batch-operated chemical vapor generation system, HS 5 (Analytik Jena, Jena, Germany), was adapted to this equipment for mercury determinations.

2.10. Statistical analysis

Results were analyzed by one-, two- or three-way ANOVA followed by Duncan's multiple range test or Student t-test when appropriate. The effects were considered significant when $p \leq 0.05$. Each litter contributed with only one n for each experimental group in order to avoid a litter effect (Abbey and Howard, 1973). Pearson correlation coefficients (bivariate correlations, two-tailed significance) were conducted between body weight and metal levels in cerebrum and cerebellum.

3. Results

Results are presented in this way: until 13 days old all litters were considered to compose the total n (each litter contained all experimental treatments). Results obtained starting at 13 days old performed two groups of litters that were defined at the end of the experimental period (33 days old). The first group ($n = 15$) comprised the litters whose mercury exposed rats did not recover the impairment in body weight gain until 33 days old. This group was called litter containing more sensitive rats (MSR) to mercury. The second group ($n = 12$) corresponded to litters whose mercury exposed rats presented a significant recuperation in body weight gain and were called litter containing less sensitive rats (LSR) to mercury. These definitions were confirmed by correlations between body weight and mercury contents in cerebrum and cerebellum (see below).

3.1. Body weight

The effects of the treatments on body weight were analyzed by two-way ANOVA (4 treatments × 14 days) and are illustrated in Fig. 1A (MSR litters) and Fig. 1B (LSR litters). For MSR litters the statistical analysis revealed significant effect of treatment [$F(3,56) = 9.07$, $p < 0.001$], day [$F(13,728) = 1639.57$, $p < 0.001$] and treatments × day interaction [$F(39,728) = 24.52$, $p < 0.001$]. The interaction was significant since the animals treated with Sal-Hg had lower weight gain than other groups. From 12 days (last day of treatment) until the end of experimental period, the Sal-Hg rats presented body weight significantly lower than the other experimental groups (one-way ANOVA, at least $p < 0.03$ for all intervals of age analyzed) (Fig. 1A).

Regarding LSR litters, two-way ANOVA (4 treatments × 14 days) revealed a significant effect of day [$F(13,572) = 2429.01$, $p < 0.001$]. Rats treated with Sal-Hg presented lower body weight only at 12 and 13 days old (day of the last dose and 1 day after) [one-way ANOVA, $F(3,44) = 3.52$, $p < 0.02$ and $F(3,44) = 5.01$, $p < 0.004$, respectively] (Fig. 1B). After these days Sal-Hg rats recovered the body weight.

3.2. Cerebrum and cerebellum weights

One-way ANOVA revealed that the mercury treatment induced a significant reduction of cerebrum and cerebellum weights of 13-day-old rats [$F(3,20) = 6.02$, $p < 0.004$ and $F(3,20) = 8.81$, $p < 0.001$, respectively], but not of 33-day-old rats, for both MSR and LSR litters. The Sal-Hg 13-day-old group presented cerebrum and cerebellum weights significantly lower than the other groups: Duncan's multiple range test ($p < 0.01$) (data not shown).

3.3. Behavioral tests

3.3.1. Negative geotaxis task

The treatments did not interfere in the appearance of the negative geotaxis reflex. All animals presented this reflex on day 5. Two-way ANOVA (4 treatments × 6 sessions) showed a significant effect of session [$F(5,200) = 289.12$, $p < 0.001$] but not of treatment [$F(3,40) = 0.73$, $p = 0.5$] neither of interaction [$F(15,200) = 1,54$,

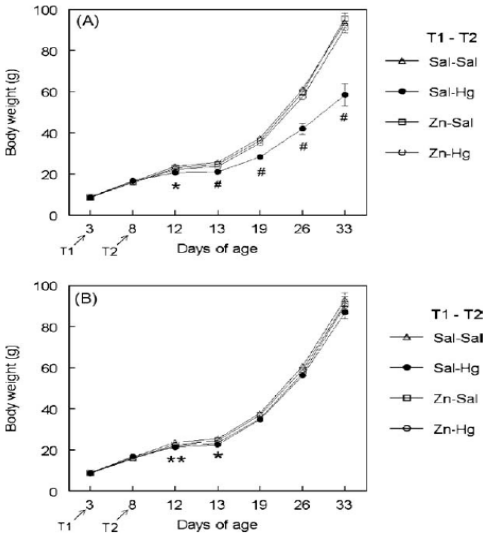

Fig. 1. Body weight of more sensitive (MSR litters, $n = 15$) (A) and less sensitive (LSR litters, $n = 12$) (B) rats treated (s.c.) with saline or $ZnCl_2$ (27 mg/kg/day), from days 3 to 7, and with $HgCl_2$ (5 mg/kg/day), from days 8 to 12. The results are presented as mean ± S.E.M. Duncan's multiple range test: *Sal-Hg differs significantly from Sal-Sal and Zn-Sal groups ($p < 0.05$); #Sal-Hg differs significantly from all other groups ($p < 0.05$); **Sal-Hg differs significantly from Sal-Sal group ($p < 0.05$).

$p = 0.09$]. However, this tendency (treatment × session interaction, $p = 0.09$) was due to the fact that Sal-Hg group presented higher latency than the other groups to perform the complete negative geotaxis reflex in both sessions 5 and 6. In fact, the one-way ANOVA revealed significant effect of treatment in both sessions 5 and 6 [$F(3,40) = 6.58$, $p < 0.001$ and $F(3,40) = 7.18$, $p < 0.001$, respectively], i.e., 24 h after the 3rd and 5th dose of mercury (Fig. 2). The zinc pre-treatment prevented totally the impairment of the performance induced by mercury exposure.

3.3.2. Tail immersion test

The statistical analysis (one-way ANOVA) showed that the treatments did not alter the tail immersion latency of both MSR [on

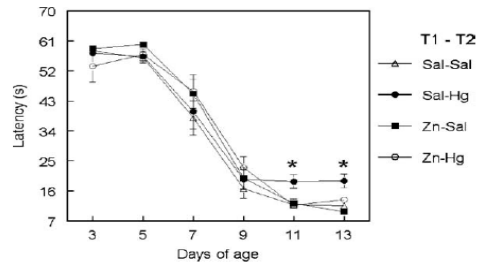

Fig. 2. Latency of negative geotaxis reflex of rats treated as described in the caption of Fig. 1. The results are presented as mean ± S.E.M. ($n = 11$). Duncan's multiple range test: *significant difference from other groups ($p < 0.05$).

Table 1
Tail immersion latency, in water to 48 ± 1 °C, of rats treated as described in the caption of Fig. 1 and submitted to task at 13, 20 and 27 days old.

Animals	Treatment	Tail immersion latency (s)		
		13 days of age	20 days of age	27 days of age
MSR ($n = 5$)	Sal-Sal	4.82 ± 1.01	4.54 ± 0.38	5.16 ± 0.82
	Sal-Hg	4.30 ± 0.54	3.64 ± 0.47	4.16 ± 0.21
	Zn-Sal	4.84 ± 0.67	4.32 ± 0.52	4.80 ± 0.49
	Zn-Hg	5.50 ± 0.86	4.44 ± 0.47	4.52 ± 0.39
LSR ($n = 6$)	Sal-Sal	4.53 ± 0.75	5.56 ± 0.61	4.11 ± 0.70
	Sal-Hg	3.22 ± 0.38	4.40 ± 0.76	4.55 ± 0.85
	Zn-Sal	4.48 ± 0.99	4.68 ± 1.19	4.38 ± 0.66
	Zn-Hg	4.35 ± 1.12	5.17 ± 0.85	3.87 ± 0.55

The results are presented as mean ± S.E.M.

day 13: $F(3,16) = 0.39$, $p = 0.76$; day 20: $F(3,16) = 0.77$, $p = 0.53$; day 27: $F(3,16) = 0.64$, $p = 0.59$] and LSR litters [on day 13: $F(3,20) = 0.53$, $p = 0.67$; day 20: $F(3,20) = 0.34$, $p = 0.79$; day 27: $F(3,20) = 0.18$, $p = 0.91$] (Table 1).

3.3.3. Beaker test

Latency to access to refuge and fall percentage of MSR and LSR are shown in Table 2. Regarding MSR litters, the two-way ANOVA (4 treatments × 4 sessions) revealed a significant effect of treatment and session on latency to access to refuge [$F(3,36) = 3.01$, $p < 0.04$ and $F(3,108) = 26.98$, $p < 0.001$, respectively] as well as on fall percentage [$F(3,36) = 3.29$, $p < 0.03$ and $F(3,108) = 5.59$, $p < 0.001$, respectively]. The treatment was significant because, in general, the animals exposed to mercury alone, presented higher latency to refuge access and higher percentage of fall than other treatment groups. In fact, the one-way ANOVA showed significant effect of treatments in sessions 1 and 4 of latency [$F(3,36) = 3.14$, $p < 0.04$ and $F(3,36) = 3.47$, $p < 0.03$, respectively] and in session 4 of fall percentage [$F(3,36) = 4.34$, $p < 0.01$]. In session 4 (on day 20, 8 days after the end of mercury exposure) the Sal-Hg group presented higher latency than the Sal-Sal and Zn-Sal groups as well as higher fall percentage than the other groups. These both effects were partially and totally prevented by the pre-treatment with zinc, respectively. In relation to LSR litters, statistical analysis showed only a significant effect of the session on latency [$F(3,60) = 25.64$, $p < 0.001$] and on fall percentage [$F(3,60) = 5.46$, $p < 0.002$], but absence of treatment effects.

3.3.4. Rotarod test

The animals of all treatments presented an improvement in the performance in the rotarod test. For the latency to the first fall and for the number of fall in 240 s the two-way ANOVA (4 treatments × 2 sessions) showed a significant session effect for both MSR and LSR litters [latency, $F(1,16) = 11.43$, $p < 0.004$ and $F(1,20) = 19.86$, $p < 0.001$, respectively; number of fall, $F(1,16) = 20.76$, $p < 0.001$ and $F(1,20) = 4.79$, $p < 0.04$, respectively]. The treatments did not alter the two parameters studied in this test (Table 3).

3.3.5. Open field task

The rearing and crossing responses of MSR and LSR litters are shown in Fig. 3. MSR presented significant effects of treatments and session on rearing responses [the two-way ANOVA (4 treatments × 2 sessions): treatment [$F(3,36) = 3.43$, $p < 0.03$]; session [$F(1,36) = 12.68$, $p < 0.001$]]. The treatment effect was consequence of the worst performance of mercury rats in this task. In fact, Sal-Hg rats presented rearing number significantly lower than Sal-Sal rats in the training [one-way ANOVA: $F(3,36) = 2.86$, $p < 0.05$, following by Duncan's *pos hoc* test] (Fig. 3A). Regarding LSR litters, statistical analysis showed only a significant session

Table 2
Latency of access to refuge and fall percentage in the beaker test of rats treated as described in the caption of Fig. 1 and submitted to test from days 17 to 20.

Animals	Treatment	Latency of access to refuge (s)			
		Session 1	Session 2	Session 3	Session 4
MSR ($n = 10$)	Sal-Sal	$78.5 \pm 6.7^{a,b}$	57.1 ± 9.9	51.9 ± 11.6	32.1 ± 10.5^{a}
	Sal-Hg	86.0 ± 4.0^{b}	70.4 ± 8.9	68.4 ± 8.9	63.3 ± 11.3^{b}
	Zn-Sal	60.4 ± 5.7^{a}	45.6 ± 7.2	39.0 ± 7.7	22.8 ± 3.9^{a}
	Zn-Hg	$71.4 \pm 7.5^{a,b}$	53.7 ± 8.9	45.2 ± 10.3	$40.9 \pm 9.7^{a,b}$
LSR ($n = 6$)	Sal-Sal	81.9 ± 3.1	49.3 ± 12.0	36.5 ± 5.9	28.8 ± 6.4
	Sal-Hg	79.2 ± 10.8	61.7 ± 14.1	56.0 ± 11.8	56.7 ± 14.6
	Zn-Sal	70.4 ± 7.7	46.1 ± 14.0	49.1 ± 10.1	30.6 ± 12.0
	Zn-Hg	81.0 ± 5.9	70.1 ± 9.4	55.7 ± 12.9	51.7 ± 14.8
Animals	Treatment	Fall percentage			
		Session 1	Session 2	Session 3	Session 4
MSR ($n = 10$)	Sal-Sal	45.0 ± 15.7	20.0 ± 8.2	35.0 ± 13.0	15.0 ± 10.7^{a}
	Sal-Hg	55.0 ± 15.7	35.0 ± 15.0	50.0 ± 16.7	50.0 ± 16.7^{b}
	Zn-Sal	40.0 ± 10.0	20.0 ± 11.0	10.0 ± 6.7	0.0 ± 0.0^{a}
	Zn-Hg	45.0 ± 13.8	10.0 ± 6.7	10.0 ± 10.0	10.0 ± 6.7^{a}
LSR ($n = 6$)	Sal-Sal	41.7 ± 15.4	25.0 ± 11.2	0.0 ± 0.0	0.0 ± 0.0
	Sal-Hg	50.0 ± 22.4	16.7 ± 16.7	8.3 ± 8.3	16.7 ± 16.7
	Zn-Sal	41.7 ± 15.4	33.3 ± 21.1	25.0 ± 11.2	8.3 ± 8.3
	Zn-Hg	33.3 ± 21.1	50.0 ± 18.2	33.3 ± 16.7	25.0 ± 17.1

The results are presented as mean ± S.E.M. Duncan's multiple range test: MSR litters: different letters confer significant statistical difference among groups ($p < 0.05$). LSR litters: there are not significant statistical differences among groups.

Table 3
Latency to first fall and the number of fall during 240 s in the rotarod task of rats treated as described in the caption of Fig. 1 and submitted to task at 25 and 30 days old.

Animals	Treatment	Latency to first fall (s)		Number of fall at 240 s	
		25 days old	30 days old	25 days old	30 days old
MSR ($n = 5$)	Sal-Sal	61.8 ± 44.7	173.0 ± 44.9	4.60 ± 1.66	1.00 ± 0.77
	Sal-Hg	84.0 ± 43.2	153.4 ± 53.0	1.40 ± 0.51	0.40 ± 0.24
	Zn-Sal	61.6 ± 44.8	121.0 ± 48.8	1.20 ± 0.37	0.60 ± 0.24
	Zn-Hg	45.4 ± 22.6	198.2 ± 41.8	2.20 ± 0.58	0.40 ± 0.40
LSR ($n = 6$)	Sal-Sal	52.2 ± 25.9	171.8 ± 43.1	3.00 ± 1.03	1.67 ± 1.17
	Sal-Hg	52.2 ± 37.7	202.5 ± 37.5	1.00 ± 0.26	0.67 ± 0.67
	Zn-Sal	84.5 ± 49.2	176.0 ± 40.7	1.50 ± 0.76	1.67 ± 1.08
	Zn-Hg	53.0 ± 30.3	137.8 ± 40.5	2.67 ± 0.61	1.00 ± 0.45

The results are presented as mean ± S.E.M.

effect [$F(1,20) = 23.24$, $p < 0.001$]. The session effect indicates that animals improved their performance from the training to the test session.

In relation to crossing responses, the two-way ANOVA of MSR litters revealed a significant treatment [$F(3,36) = 4.93$, $p < 0.006$] and session effects [$F(1,36) = 7.39$, $p < 0.01$]. The treatment effect was consequence of a lower number of crossing responses done by Sal-Hg rats than all other treatment groups in the training session [one-way ANOVA: $F(3,36) = 6.22$, $p < 0.002$, following by Duncan's *pos hoc* test] (Fig. 3B). For LSR litters, statistical analysis showed absence of treatment and session effects (Fig. 3B).

The exit latency of the initial area and number of fecal boluses of the training session of MSR and LSR litters are shown in Table 4. MSR litters presented a significant treatment effect on exit latency [one-way ANOVA: $F(3,36) = 2.77$, $p < 0.05$], but not on the number of fecal boluses. Sal-Hg rats presented greater exit latency than Sal-Sal and Zn-Sal groups. Regarding LSR litters, the treatments did not alter significantly the exit latency or number of fecal boluses.

3.4. Death percentage

The animals treated with Sal-Hg had the highest percentage of death. This occurred mainly in the period subsequent to the end of the treatment (Fig. 4).

3.5. Metal content

Cerebrum and cerebellum mercury levels in 13- and 33-day-old rats are shown in Table 5.

The statistical analyzes were conducted to verify the possible effects of treatment, age, cerebral and cerebellar tissue, as well as

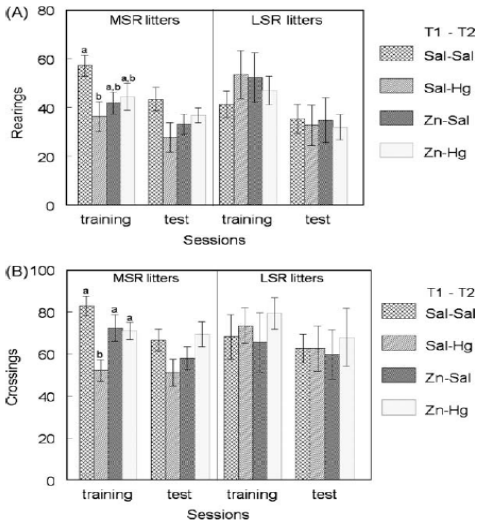

Fig. 3. Rearing (A) and crossing (B) numbers in the open field task of more sensitive (MSR litters, $n = 10$) and less sensitive rats (LSR litters, $n = 6$), treated as described in the caption of Fig. 1, and submitted to training and test at 30 and 31 days old, respectively. The results are presented as mean ± S.E.M. Duncan's multiple range test: MSR litters: different letters confer significant statistical difference among groups ($p < 0.05$); LSR litters: there are not significant statistical difference among groups.

Table 4
Latency of initial area exit and fecal boluses number in the open field of rats treated as described in the caption of Fig. 1 and submitted to training and test at 30 and 31 days old, respectively.

Animals	Treatment	Training	
		Latency of initial area exit (s)	Fecal boluses number
MSR ($n = 10$)	Sal-Sal	2.52 ± 0.44^{a}	3.10 ± 0.79
	Sal-Hg	4.19 ± 0.61^{b}	3.00 ± 0.86
	Zn-Sal	2.73 ± 0.36^{a}	2.90 ± 0.62
	Zn-Hg	$3.04 \pm 0.32^{a,b}$	4.10 ± 0.60
LSR ($n = 6$)	Sal-Sal	4.14 ± 0.94	4.83 ± 0.31
	Sal-Hg	2.46 ± 0.69	3.50 ± 0.92
	Zn-Sal	3.44 ± 0.83	3.67 ± 1.38
	Zn-Hg	4.98 ± 1.22	4.17 ± 0.60

The results are presented as mean ± S.E.M. Duncan's multiple range test: MSR litters: different letters confer significant statistical difference ($p < 0.05$). LSR litters: there are not significant statistical differences among groups.

higher or lower sensitivity to mercury (MSR and LSR litters, 33-day-old rats) on mercury levels.

Three-way ANOVA comparing 13-day-old × 33-day-old MSR litters (4 treatments × 2 ages × 2 tissues) showed a significant effect of treatment [$F(3,24) = 135.48$, $p < 0.001$], age [$F(1,24) = 15.94$, $p < 0.001$], tissue [$F(1,24) = 41.44$, $p < 0.001$], treatment × age interaction [$F(3,24) = 5.87$, $p < 0.004$] and treatment × tissue interaction [$F(3,24) = 27.28$, $p < 0.001$]. Three-way ANOVA comparing 13-day-old × 33-day-old LSR litters (4 treatments × 2 ages × 2 tissues) showed a significant effect of treatment [$F(3,16) = 60.80$, $p < 0.001$], age [$F(3,16) = 21.02$, $p < 0.001$], tissue [$F(3,16) = 20.91$, $p < 0.001$], treatment × age interaction [$F(3,16) = 12.98$, $p < 0.001$] and treatment × tissue interaction [$F(3,16) = 14.42$, $p < 0.001$]. The treatment × age interaction was due to the fact that although all mercury exposed rats presented higher mercury content than control animals, Hg-13-day-old rats presented higher levels of mercury in tissues than the Hg-33-day-old litters. Besides, the treatment × tissue interaction

Table 5
Mercury levels (μg of Hg/g of wet tissue) in cerebrum and cerebellum of 13- and 33-day-old rats treated as described in the caption of Fig. 1.

Animals	Treatment	Cerebrum (μg Hg/g)	Cerebellum (μg Hg/g)
13 days old ($n = 3$)	Sal-Sal	0.06 ± 0.03^{a}	0.03 ± 0.00^{a}
	Sal-Hg	0.80 ± 0.01^{b}	$1.30 \pm 0.09^{b,*}$
	Zn-Sal	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}
	Zn-Hg	0.31 ± 0.04^{c}	0.48 ± 0.18^{c}
33 days old			
MSR ($n = 5$)	Sal-Sal	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}
	Sal-Hg	$0.37 \pm 0.09^{b,\#}$	$1.03 \pm 0.09^{b,*}$
	Zn-Sal	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}
	Zn-Hg	0.19 ± 0.05^{c}	0.25 ± 0.01^{c}
LSR ($n = 3$–5)	Sal-Sal	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}
	Sal-Hg	$0.22 \pm 0.05^{b,\#}$	$0.53 \pm 0.09^{b,*,\#,+}$
	Zn-Sal	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}
	Zn-Hg	0.28 ± 0.08^{b}	0.26 ± 0.01^{c}

The results are presented as mean ± S.E.M. The samples whose Hg concentrations were below the detectable limit of the technique were considered, for statistical analysis, as containing 0.03 μg of Hg/g of wet tissue, which is the minimum measurable quantity.
Duncan's multiple range test: different letters confer significant statistical difference (at least $p < 0.05$) considering the same tissue and age.
* Student t-test (at least $p < 0.05$): differ from cerebrum considering the same age.
# Student t-test (at least $p < 0.05$): differ from 13-day-old rats considering the same tissue.
+ Student t-test (at least $p < 0.05$): differ from 33-day-old MSR litters for the same tissue.

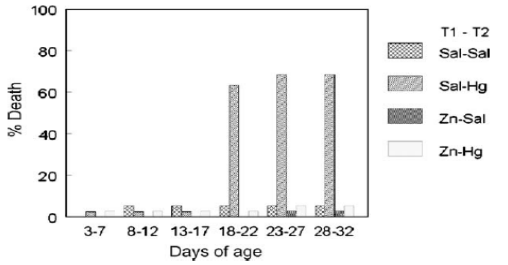

Fig. 4. Death percentage of 34 litters treated as described in the caption of Fig. 1.

was a consequence of the rat exposure to mercury presenting higher mercury content in cerebellum than in cerebrum tissue (see comparisons among groups in Table 5).

The treatment effect on mercury contents in cerebrum and cerebellum was verified when the ages were evaluated separately. The treatment effect was verified on cerebrum and cerebellum mercury content of 13-day-old rats [one-way ANOVA: $F(3,8) = 194.38$, $p < 0.001$ and $F(3,8) = 33.21$, $p < 0.001$, respectively], of 33-day-old MSR [$F(3,16) = 9.64$, $p < 0.001$ and $F(3,16) = 98.07$, $p < 0.001$, respectively] and LSR litters [$F(3,8) = 6.99$, $p < 0.01$ and $F(3,16) = 22.86$, $p < 0.001$, respectively]. Hg-rats (pre-exposed to saline or zinc) presented higher

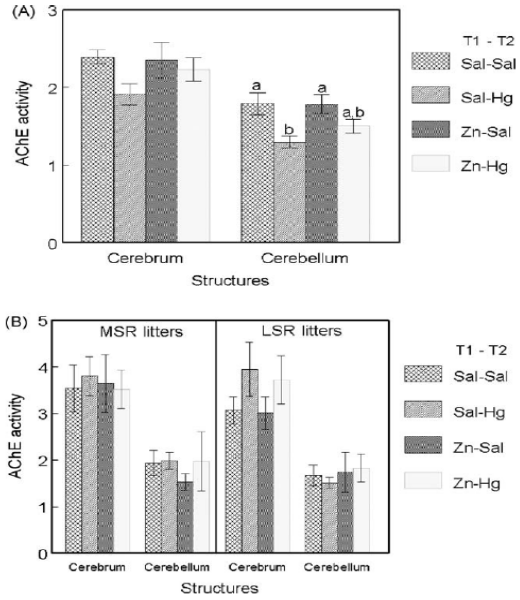

Fig. 5. Cerebrum and cerebellum AChE activities of rats of 13 (A) and 33 (MSR litters and LSR litters) (B) days old treated as described in the caption of Fig. 1. The specific activity was expressed as μmol ATC hydrolyzed/h/mg protein. The results are presented as mean ± S.E.M. ($n = 5$). Duncan's multiple range test: different letters confer significant statistical difference among groups of 13-day-old ($p < 0.05$).

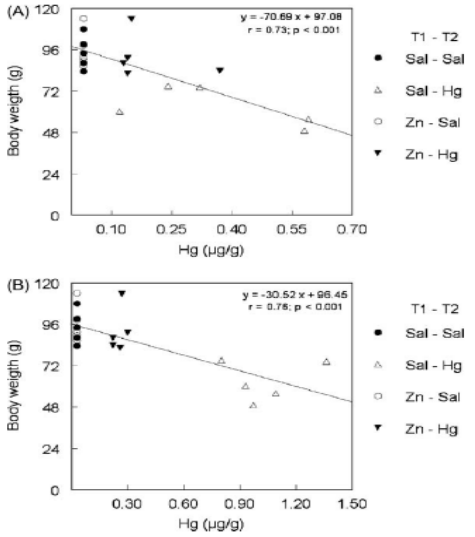

Fig. 6. Relationship between body weight (g) and mercury levels (µg of Hg/g of tissue) in the cerebrum (A) and cerebellum (B) of MSR rats treated as described in the caption of Fig. 1. The results are presented as mean ± S.E.M. ($n = 5$).

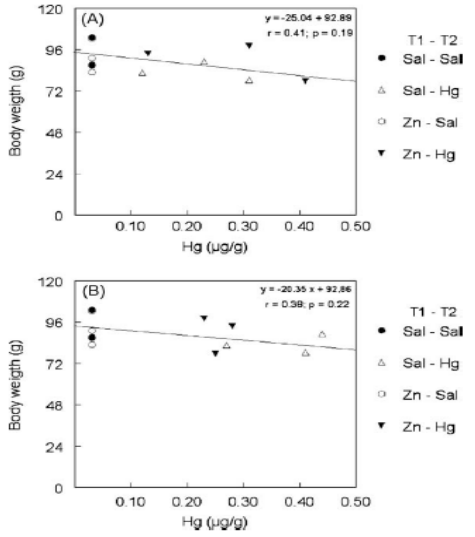

Fig. 7. Relationship between body weight (g) and mercury levels (µg of Hg/g of tissue) in the cerebrum (A) and cerebellum (B) of LSR rats treated as described in the caption of Fig. 1. The results are presented as mean ± S.E.M. ($n = 3$).

levels of this metal than all the other treatment groups. The pre-treatment with zinc partially prevented the increase in the mercury contents induced by mercury exposure in both intervals after treatments (13- and 33-day-old MSR and LSR litters), with exception to cerebrum of Zn-Hg LSR litters.

The zinc content in cerebrum and cerebellum was not altered by treatments in both intervals of age (one-way ANOVA) (data not shown), i.e., the animals treated with zinc presented levels of this metal similar to other groups.

3.6. AChE activity

The one-way ANOVA revealed significant effect of mercury on cerebellum, but not on cerebrum AChE activity from 13-day-old rats [$F(3,16) = 4.92$, $p < 0.01$]. Sal-Hg treated rats presented lower cerebellum AChE activity than Sal-Sal and Zn-Sal rats (Fig. 5A).

The AChE activity of cerebrum from 33-day-old animals of both MSR and LSR litters (Fig. 5B) was not altered by the treatments (one-way ANOVA).

3.7. Relationships between body weight and mercury levels in the cerebrum and cerebellum

For MSR litters, the body weight decreased significantly with the increase in mercury levels in both cerebrum ($r = 0.73$, $p < 0.001$) (Fig. 6A) and cerebellum ($r = 0.76$, $p < 0.001$) (Fig. 6B). However, there was no relationship between these two parameters for LSR litters (Fig. 7A and B).

4. Discussion

This study aimed to investigate the damaging effects of mercury on the behavioral performance of young rats and on cerebrum and cerebellum AChE activities, as well as to verify the protector effects of zinc on the alterations induced by mercury.

Rats exposed to mercury presented reduction of body weight gain at 12 and 13 days old, i.e., after the 4th and 5th dose of the metal (the animals were weighed before administration, from days 8 to 12). The development of rats was observed until 33 days old. The litters were classified as LSR and MSR (less and more sensitive rats to mercury, respectively) considering if Hg-rats presented or not a recuperation of body weight. Reduction of body weight due to mercury exposure has been related to the anorexigenic effect of mercury (Counter and Buchanan, 2004) as well as to the interference of this metal on amino acids and glucose absorption (Farmanfarmaian and Socci, 1985; Farmanfarmaian et al., 1989). We have previously observed that the reduction of body weight gain occurred in parallel to lower glycemia in mercury exposed rats (Peixoto and Pereira, 2007). It is important to observe that animals that received zinc previously to mercury presented no alteration of body weight, neither during the mercury treatment (after the 4th and 5th doses) nor after the end of exposure. These results are in accordance with Peixoto et al. (2003) who verified the preventive effect of zinc on body and organ weight alterations induced by mercury. The different sensitivity of rats to mercury was also verified by Pearson correlation, since the animals that recovered the body weight until 33 days old (LSR litter) presented correlation not significant between body weight and cerebellum or cerebrum metal contents, whereas for MSR litter this correlation was significant.

Behavioral tasks were conducted to evaluate the performance of rats exposed to mercury in the precocious phase of life and submitted to tasks during and after the end of exposure. Besides, we investigated if the possible behavioral alterations induced by mercury could be prevented by the pre-exposure to zinc similarly

to what occurs with biochemical parameters (Peixoto et al., 2003; Peixoto and Pereira, 2007).

On negative geotaxis (3–13 days old) the rats exposed only to mercury presented increase in the latency to complete the response in the sessions 5 and 6 (after the 3rd and 5th doses of mercury). Zinc pre-exposure completely prevented the effects of mercury. A decrease of performance of rats in this task was also verified by Rocha et al. (1993) in young rats exposed to methylmercury during the second stage of postnatal development. This effect seems to involve motor function impairment since this task requires the development of motor force to respond to this reflex (Da-Silva et al., 1990). At 13 days old, the effect occurred in parallel to the decrease in body weight gain suggesting the involvement of a nutritional effect. However, Rocha et al. (1993) demonstrated that rats undernourished during the same phase of growth did not present motor impairment in this task. Besides, Farkas et al. (2009) reported that rats with decrease of body weight by food deprivation in the suckling phase showed no differences in the performance in the negative geotaxis task. Still, in this study we verified that on day 11 the animals did not present decrease in the body weight, however, they already showed impairment in the negative geotaxis task. In session 6 the mercury exposed animals, both saline and zinc pre-exposed, presented a significant increase of mercury contents both in cerebrum and cerebellum. These levels were lower (2.6-fold) when animals were pre-exposed to zinc. These lower levels may be related to the absence of the mercury effect on this task due to zinc pre-exposure.

The animals submitted to behavioral tasks after 13 days were divided in MSR and LSR litters (see above). The mercury rats of LSR litters presented performance in beaker test (17–20 days old) and open field task (30 and 31 days old) similar to their respective controls. Indeed, the treatment with mercury did not interfere in motor and exploratory activities and in spontaneous habituation. However, in relation to MSR litters, rats exposed only to mercury presented a significant worse performance in both tasks. On the beaker task (from 8th to 12th day after the end of exposure) the mercury exposed rats presented higher latency to access to refuge and fall percentage in session 4. These effects were partially and totally prevented by zinc pre-exposure, respectively. On the open field task, mercury exposure decreased the rearing and crossing response numbers in the training session and increased the exit latency of the first area. All effects were partially prevented by zinc pre-exposure. Both tasks are characterized by demanding normal motor function. The effects of mercury on these tasks are in accordance with those by Peixoto et al. (2007a) who observed performance impairment of rats exposed to mercury during the 1st, 2nd or 3rd phases of rapid brain development [1–5, 8–12 or 17–21 days old, Gottileb et al., 1977]. The beaker test assesses the muscular strength and size, as well as the cerebellar function across the rim escape behavior to access to refuge (Smart and Dobbing, 1971). The open field task has, besides locomotor response, the exploratory component (Pereira et al., 1992), mainly in the first session when the ambient is new. This exploratory activity is presented as number of rearing responses and first area exit latency. Sal-Hg group presented lower exploratory activity (lower rearing number and higher first area exit latency), but presented habituation memory, since there was a significant reduction of the rearing response number from the training to the test sessions. However, we need to consider the lower crossing performance done by these animals, since an apparent deficit in the spontaneous exploratory activity may be caused by motor dysfunction (Pereira et al., 1992). Similar effect on open field task was related by Franco et al. (2007), who found a decrease in the crossing number in animals exposed to mercury through the lactation. Other studies have confirmed the induction of neurobehavioral changes when animals are exposed to inorganic mercury during early phases of development, leading to loss of cognitive and motor functions (Rocha et al., 2001; Szász et al., 2002; Franco et al., 2007; Peixoto et al., 2007a). In this work, we also verified that the preventive effect of zinc in the performance impairment induced by mercury occurred in parallel to a reduction of mercury levels in cerebrum and cerebellum in both 13- and 33-day-old rats. Not less important is the fact the Sal-Hg rats presented significant higher mercury content in cerebellum than in cerebrum in all sets of experiment, and although the levels fall from day 13 to day 33 (20 days after the end of mercury exposure), they still remained high when compared with saline or zinc treated rats. Similar results have been published showing that brain mercury levels diminished in the course of days after exposure (Peixoto et al., 2007a). The highest cerebellum mercury level may be related to motor impairment (Baillieux et al., 2008) and to the fact that cerebellar cells are target selective for mercury compounds *in vivo* (Sanfeliu et al., 2003).

Although the Hg rats presented worse performance on beaker test and open field task than the other groups, the impairment in body weight gain seems not to be involved in this behavioral deficit, since Farkas et al. (2009) demonstrated that open field activity was not changed by the decrease of body weight. Furthermore, Alamy et al. (2005) related that undernourished rats exhibited hyperactivity and increased exploratory behavior in the open field.

The rats of both MSR and LSR litters showed no change in nociception assessed by the tail immersion task in three intervals after the end of exposure (13, 20 and 27 days old). Besides, the performance of rats on rotarod task (25 and 30 days old) was not altered by treatments. This absence of effect was not expected when considered that the mercury MSR litters presented impairment of motor function on beaker test, which is featured by forced exploratory activity, and on open field, whose exploratory activity is spontaneous (Smart and Dobbing, 1971; Pereira et al., 1992; Peixoto et al., 2007a). However, although the rotarod task is considered a forced task (Ekambaram and Paul, 2002), the animals probably required less force to keep on cylinder in rotation at 8 rpm (a movement relatively slow). Thus, the motor alteration presented by these mercury rats seems not to be enough to interfere in the performance of this task. In fact, prenatal exposed mice to methylmercury presented a decrease in coordination and/or impaired motor when submitted to accelerating rotarod (3–26 rpm) (Montgomery et al., 2008).

Besides the behavioral parameters, this study investigated the effects of mercury on the AChE activity from cerebrum and cerebellum. This is an important enzyme of cholinergic system responsible by terminus of ACh action due to its hydrolysis (Taylor, 1996). In this work we verified that only the AChE activity from cerebellum of 13-day-old rats was significantly inhibited by the mercury treatment (24 h after the last dose). This inhibition occurred in parallel to higher mercury content in this structure in this interval after treatment. The previous exposure to zinc not only prevented the AChE inhibition induced by mercury but also decreased the mercury content. The absence of mercury effect on cerebrum AChE activity differs from the results obtained by Sood et al. (1993) and El-Demerdash (2001). These authors demonstrated cerebrum AChE inhibition in rats exposed to methylmercury and to inorganic mercury, respectively.

It is important to observe that the mercury treated rats presented a significant higher content of mercury in cerebrum and cerebellum than the other groups. The pre-exposure to zinc partially prevented the increase of mercury content in these structures in all experimental sets with exception of cerebrum from Zn–Hg LSR litter. This finding suggests that the tissue mercury content necessary to inhibit the AChE activity is higher than the content needed to induce behavioral alterations. Besides,

when these levels decreased as a function of zinc pre-exposure, no effects were observed. Mercury levels of the Zn-Hg MSR litter are close to the levels presented by the Sal-Hg LSR litter that did not present biochemical and behavioral alterations. Indeed, mercury levels seem to be insufficient to cause alterations on the behavioral performance as well as on cerebrum and cerebellum AChE activities. LSR litters seem to respond differently to mercury exposure probably due to individual differences. The concrete causes for these differences are not understood and further studies are necessary.

The preventive effects of zinc pre-exposure on damage induced by mercury showed in this work are in accordance with our previous results that showed preventive effects on several biochemical and metabolic alterations induced by mercury (Peixoto et al., 2003; Peixoto and Pereira, 2007). This preventive effect occurred in parallel to high metallothionein content both in liver and kidney (Peixoto et al., 2003, 2007c) as well as high zinc content in these organs (Peixoto et al., 2007c, 2008). Therefore, the preventive effects of zinc can be attributed to the synthesis of metallothioneins induced by this metal (Peixoto et al., 2003), since it is an important inductor of metallothionein synthesis (Eaton et al., 1980; Cosson, 1989). Assuming the high cysteine content in the metallothionein molecule, it is possible that this protein sequesters a high percentage of this toxic metal in an inert complex, making it less available to interact with sensitive organelles or enzymes systems (Peixoto et al., 2003), including those from CNS. This is important when we consider that zinc contents in cerebrum and cerebellum were not altered by zinc exposure.

In summary, these results corroborate with previous results (Peixoto et al., 2007a) showing that the exposure of young rats to mercury from 8th to 12th days old causes behavioral impairment in three tasks, geotactism reflex response, beaker test and open field. This metal also causes cerebellum AChE inhibition on 13-day-old rats (24 h after last dose). Besides, the zinc pre-treatment prevented the decrease in body weight gain and in the AChE activity, as well as behavioral changes induced by mercury. These results show zinc as an important essential metal in the prevention of deleterious effects of mercury on behavioral performance and AChE impairment.

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CAPÍTULO 4

MANUSCRITO

Delayed biochemical changes induced by mercury intoxication prevented by zinc pre-exposure

Carina Franciscato, Lucélia Moraes e Silva, Naglezi de Menezes Lovatto, Fernanda Rodrigues Goulart, Tania Maria Bueno, Fabio Andrei Duarte, Érico Marlon de Moraes Flores, Valderi Luis Dressler, Nilce Coelho Peixoto, Maria Ester Pereira

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Corresponding Author: Dr. Maria Ester Pereira, Ph.D.

Corresponding Author's Institution: Universidade Federal de Santa Maria

First Author: Carina Franciscato, Doctor

Order of Authors: Carina Franciscato, Doctor; Lucélia Moraes-Silva, master; Naglezi M Lovatto, graduate; Fernanda R Goulart, graduate; Tania M Bueno, master; Fábio A Duarte, Doctor; Érico M Flores, Doctor; Valderi L Dressler, Doctor; Nilce C Peixoto, Doctor; Maria Ester Pereira, Ph.D.

Abstract: Abstract

Studies have demonstrated that zinc protects against mercury toxic effects in newborn rats. This work evaluated the effects of mercury in 33-day-old rats exposed to 5 doses of $HgCl_2$ 5 mg/kg/day (s.c.) from postnatal days 8 to 12, and verified the effectiveness of 5 doses of $ZnCl_2$ 27 mg/kg/day (s.c.) from postnatal days 3 to 7 as preventive pre-treatment. Animals were euthanized 21 days after the end of Hg-exposure. Porphobilinogen-synthase activity and the content of metals were determined in liver and kidney. Other biochemical analyses were conduced in plasma and serum. Mercury accumulation in liver and kidney was observed. Hg-animals presented decrease in liver and increase in kidney weights, decrease in renal porphobilinogen-synthase activity, increase in urea and creatinine levels and decrease of alanine amino transferase activity. Zinc prevents all the alterations caused by mercury even those that persist for a long time after the end of exposure.

Delayed biochemical changes induced by mercury intoxication prevented by zinc pre-exposure

Franciscato, C.[a], Moraes-Silva, L.[a], Lovatto, N.M.[b], Goulart, F.R.[b], Bueno, T.M.[a], Duarte, F.A.[b], Flores, E.M.M.[b], Dressler, V.L.[b], Peixoto, N.C.[a,b,c], Pereira, M.E.[a,b,*]

[a]Programa de Pós-Graduação em Bioquímica Toxicológica,

[b]Departamento de Química, Centro de Ciências Naturais e Exatas.

[c]Departamento de Enfermagem, Centro de Educação Superior Norte - RS, Campus de Palmeira das Missões.

Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brasil

Correspondence to:

*Maria Ester Pereira

UFSM, CCNE, Departamento de Química

Campus Universitário, Camobi

97.105-900 Santa Maria, RS, Brazil

Tel/fax: +55 55 32208799

Email: pereirame@yahoo.com.br

Abstract

Studies have demonstrated that zinc protects against mercury toxic effects in newborn rats. This work evaluated the effects of mercury in 33-day-old rats exposed to 5 doses of $HgCl_2$ 5 mg/kg/day (s.c.) from postnatal days 8 to 12, and verified the effectiveness of 5 doses of $ZnCl_2$ 27 mg/kg/day (s.c.) from postnatal days 3 to 7 as preventive pre-treatment. Animals were euthanized 21 days after the end of Hg-exposure. Porphobilinogen-synthase activity and the content of metals were determined in liver and kidney. Other biochemical analyses were conduced in plasma and serum. Mercury accumulation in liver and kidney was observed. Hg-animals presented decrease in liver and increase in kidney weights, decrease in renal porphobilinogen-synthase activity, increase in urea and creatinine levels and decrease of alanine amino transferase activity. Zinc prevents all the alterations caused by mercury even those that persist for a long time after the end of exposure.

Keywords: mercury; zinc; porphobilinogen-synthase; renal insufficiency; young rats; body weight; creatinine; urea; alanine amino transferase.

Ethics

Studies were conducted in accordance with the national and institutional guidelines (University Ethics Committee Guidelines — Process number 23081.014805/2007-68) for experiments with animals.

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1. Introduction

Mercury is a hazardous pollutant and can be found in three different chemical forms: elemental mercury vapor, inorganic mercury and organic mercury. The toxicity of this metal depends on its chemical form (Clarkson, 1997). The exposure to inorganic mercury, which is the object of this study, is mainly occupational-like, occurring in industrial activities and mining (Nevado et al., 2003). In fact, the exposure to this form of mercury causes serious risks to gold miners' health (Barregard, 2008). It is also known that inorganic mercury can undergo methylation in water contaminating the food chain (Wasserman et al., 2003).

The inorganic form such as mercury chloride is known as a nephrotoxic agent (Clarkson, 1997; Emanuelli et al., 1996; Goyer, 1995). Besides causing renal insufficiency (Peixoto and Pereira, 2007), this form produces changes in body and organ weights and decreases the serum alanine amino transferase activity of rats exposed to metal during the second phase of development (Peixoto et al., 2003, 2007a; Peixoto and Pereira, 2007).

Other researchers have demonstrated that inorganic mercury causes hepatotoxicity, since adult mice treated with one dose of $HgCl_2$ (5 mg/kg) presented significant elevation in serum alanine (ALT) and aspartate (AST) amino transferase activities (Kumar et al., 2005). In fact, these enzymes are known as important markers of hepatocellular damage (Meyer et al., 1992).

The large affinity of mercury by sulfhydryl groups is an important contributor to its toxicity (Clarkson, 1997). Therefore, porphobilinogen-synthase [PBG-synthase (E.C.: 4.2.1.24)], an enzyme involved in the heme biosynthesis with high content of sulfhydryl residues (Sassa, 1982), has its activity inhibited

by toxic metals in different tissues from mice (Emanuelli et al., 1996), rats (Peixoto et al., 2003, 2007a; Rocha et al., 1993, 1995, 2001) and humans (Calderón et al., 2003). This enzyme requires zinc for its maximum activity, although the precise function of this metal is not clear (Bevan et al., 1980; Hasnain et al., 1985).

Zinc is an essential metal involved in several metabolic functions (Fang et al., 2002). It is a catalytic and structural element of several proteins (Mathie et al., 2006) and plays an important role in the metallothionein synthesis (Pedersen et al., 1998; Peixoto et al., 2003, 2007b). Our previous results suggest zinc as a protector agent against several mercury toxic effects. In fact, zinc pre-treatment avoids the hepatic and renal PBG-synthase inhibition and the decrease in body weight gain (Peixoto et al., 2003, 2007b). Besides, it prevents the renal insufficiency (Peixoto and Pereira, 2007) in young rats submitted to mercury exposure during an important phase of development (Gottlieb et al., 1977).

In the neonatal phase, young rodents present intervals characterized by remarkable development and growth of organs and body (Gottlieb et al., 1977; Winick and Noble, 1965). Thus, insults, even those considered mild, may cause pronounced damages when applied to developing organisms (Smart and Dobbing, 1971). Therefore, developing animals are particularly sensitive to extern insults such as heavy metal exposure (Franciscato et al., 2009; Peixoto et al., 2003, 2007c; Rocha et al., 1993).

It is known that postnatal exposure to mercury causes behavioral (Peixoto et al., 2007c) and biochemical alterations in rats (Peixoto et al., 2003; Peixoto and Pereira, 2007) even with an elapsed time after exposure (Franciscato et al., 2009; Rocha et al., 2001; Stringari et al., 2006). Recently, we

verified that there are rats more and less sensitive to mercury when exposed to it during developmental period. These subgroups of animals were classified according to body development after the end of mercury exposure. Mercury exposed rats that recovered the body weight gain did not present behavioral alterations (Franciscato et al., 2009).

The aim of this study was to evaluate the toxic effects of mercury in 33-day-old rats exposed to metal during one of the developmental postnatal phases (8 to 12 days old), as well as to verify the effectiveness of zinc as preventive treatment. Still, we investigated if animals present different sensitivities to mercury regarding biochemical parameters. For this, we evaluated body and organ weights, mercury and zinc levels, PBG-synthase, ALT, AST and latic dehydrogenase (LDH) activities, as well as urea and creatinine levels.

2. Materials and Methods

2.1. *Chemicals*

Zinc ($ZnCl_2$), mercury ($HgCl_2$) and sodium chloride (NaCl), glacial acetic acid, *ortho*-phosphoric acid, nitric acid (HNO_3), perchloric acid, absolute ethanol as well as ethylic ether were obtained from Merck (Rio de Janeiro, RJ, Brazil); δ-aminolevulinic acid (δ-ALA), Coomassie brilliant blue G and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA); trichloroacetic acid was obtained from Reagen and *para*-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han, Germany). The kits for the determination of creatinine, urea, glucose, ALT, AST and LDH were obtained from Labtest (Lagoa Santa, MG, Brazil).

2.2. *Animals*

Wistar pregnant rats obtained from the General Animal House of the Federal University of Santa Maria were transferred to the colony room and maintained individually in opaque plastic cages at room temperature (23 ± 2°C). One day after the birth, the number of pups of each litter was reduced to 8.

2.3. *Treatments*

Pups were pre-treated from postnatal days 3 to 7 of life with one daily dose of saline (NaCl 90 mg/kg/day) or $ZnCl_2$ (27 mg/kg/day). After the pre-treatment, the animals received saline or $HgCl_2$ (5 mg/kg/day) for 5 consecutive days (from 8 to 12 days old). Treatments were administered by subcutaneous (s.c.) injections in a constant volume of 10 mL/kg body weight. Animals were weighed daily to adjust the dose. Twenty-seven litters were exposed to treatments. Each litter contributed with only one *n* to each experimental group.

2.4. *Tissue preparation*

Twenty-one days after the end of mercury exposure (33 days old), the animals were weighed, anesthetized and euthanized by decapitation. Liver and kidneys were removed, weighed and minced. Portions of each organ were used for determination of enzymatic activity and for analysis of metal contents.

For the PBG-synthase activity determination, liver and kidneys were homogenized in 7 and 5 volumes of 150 mM of NaCl, respectively. Homogenates were centrifuged at 8,000x*g* for 30 min to obtain the supernatant

with the enzymatic material. For metal contents dosage a portion of each tissue was frozen at -20°C until analysis.

For other biochemical analysis, plasma and serum were obtained from heparinized blood and blood, respectively, by centrifugation at 3,000x*g* for 10 min, and were frozen until analysis (up to 5 days). Another portion of blood was frozen for analysis of metal contents.

2.5. Biochemical determinations

2.5.1. PBG-synthase activity

PBG-synthase activity was determined according to the method of Sassa (1982) by measuring the rate of formation of the product (porphobilinogen), except that 76 mM potassium phosphate buffer (pH 6.8) and 2.2 mM δ-ALA were used (Peixoto et al., 2003). The reaction product was determined using the modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1 x 10^4 for the Ehrlich-porphobilinogen salt. The incubation was initiated by adding 100 μl of tissue preparation and was carried out for 40 and 90 min for liver and kidney, respectively, at 39°C. The specific activity was expressed as nmol of PBG formed per hour per mg of protein. Protein concentrations in the samples were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard. All samples were run in triplicate.

2.5.2. ALT activity

The enzyme activity was determined in a medium containing buffer 55.8 mmol/L, α-ketoglutaric acid 1.67 mmol/L, L-alanine 83.3 mmol/L, sodium azide 12.8 mmol/L and 25 μL of serum incubated at 37°C for 30 min. The reaction was

stopped by adding HCl 0.45 mmol/L. The color reactive (2.4-dinitrophenylhydrazine 0.45 mmol/L) was added and the medium was incubated for 20 min at room temperature. The color was intensified by NaOH 0.33 mmol/L and the absorbance was determined at 505 nm. The activity (in U/mL) was calculated by comparison with a calibration curve utilizing sodium pyruvate as standard.

2.5.3. AST activity

The enzyme activity was determined similarly to ALT enzyme, except that L-aspartic acid 83.3 mmol/L was used as substrate and that the medium was incubated at 37°C for 60 min.

2.5.4. LDH activity

The activity of this enzyme (in U/L) was determined by the formation of NADH. The medium containing buffer 200 mmol/L pH 8.2, lactic acid 260 mmol/L, sodium azide 7.7 mmol/L and 25 μL of plasma was incubated at 37°C for 2 min. After this period, the color reactive (INT 0.64 mmol/L, NAD+ 1.2 mmol/L, phenazine 0.26 mmol/L and sodium azide 1.23 mmol/L) was added and the medium was incubated for another 5 min at 37°C. The reaction was stopped by adding HCl 200 mmol/L and the tubes remained at room temperature for 5 min until the reading of the absorbance at 500 nm.

2.5.5. Creatinine

The determination of creatinine (mg/dL) was carried out by measuring the quantity of creatinine picatre formed at 510 nm. The medium containing picric

acid 7.79 mmol/L, NaOH 145.9 mmol/L and 100 μL of serum was incubated at 37°C for 10 min. After this period of incubation, the first absorbance was determined. The acetic acid 0.4 mmol/L was added and the medium was incubated for another 5 min at room temperature before determining the second absorbance.

2.5.6. Urea

Urea (mg/dL) was determined by the quantity of indophenol blue formed at 600 nm. The medium containing phosphate buffer 19.34 mmol/L, sodium salicylate 58.84 mmol/L, sodium nitroprusside 3.17 mmol/L, urease (≥12.63 UK/L) and 10 μL of serum was incubated at 37°C for 5 min.

2.5.7. Glucose

The reaction for the determination of the glucose concentration was conducted in a medium containing phosphate buffer 9.9 mmol/L, phenol 9.9 mmol/L, glucose oxidase (≥12,000 U/L), peroxidase (≥1,000 U/L), 4-amino-antipyrine 0.4 mmol/L and 10 μL of serum that was incubated at 37°C for 10 min. The product formed from the complete oxidation of the glucose is a red-violet quinoneimine, whose absorbance was measured at 500 nm.

2.6. *Metal content determination*

The digestion of samples and the determination of Zn and Hg contents were conducted as described in detail by Peixoto et al. (2008).

2.6.1. Digestion and quantification of metals

Samples were digested with HNO_3 using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (max 80 bar, 280°C, Anton Paar, Graz, Austria). After digestion, samples were diluted with water to 25 mL and transferred to graduated polypropylene vials.

Metal analyses were carried out using a Model AAS EA 5 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer with pyrolytic coated tubes for zinc. A batch-operated chemical vapor generation system, HS 5 (Analytik Jena, Jena, Germany), was adapted to this equipment for mercury determinations.

2.7. *Statistical analysis*

Results were analyzed by one- or two-way ANOVA followed by Duncan's multiple range test or Student's *t*-test when appropriate. Effects were considered significant when $p \leq 0.05$.

3. Results

The body weight gain was used as parameter to classify the animals in more or less sensitive to Hg (Franciscato et al., 2009). Thus, animals were divided into two groups. The first group comprised the litters whose mercury exposed rats did not recover the impairment in body weight until 33 days old. This group was called litter containing more sensitive rats to mercury (MSR litters). The second group corresponded to litters whose mercury exposed rats presented a significant recuperation in body weight gain. This group was called litter containing less sensitive rats to mercury (LSR litters).

3.1. Body, liver and kidney weights

Body, liver and kidney weights of the 33-day-old animals are showed in Table 1. Regarding body weight, one-way ANOVA revealed a significant effect of treatment [$F(3,56)=25.05$, $p<0.001$] of MSR litters. Sal-Hg group presented body weight significantly lower than the other groups (Duncan's multiple range test: $p<0.05$). LSR litters did not present changes in body weight at 33 days old (one-way ANOVA).

Regarding liver and kidney weights, one-way ANOVA revealed a significant effect of treatment on liver [$F(3,16)=9.92$, $p<0.001$] and kidney [$F(3,16)=6.85$, $p<0.004$] weights of MSR litters. Sal-Hg group presented liver weight significantly lower and kidney weight significantly higher than the other groups (Duncan's multiple range test: $p<0.05$). Regarding LSR litters, the mercury treatment did not alter these parameters (one-way ANOVA).

3.2. Renal and hepatic PBG-synthase activity

Renal and hepatic PBG-synthase activities are shown in Figure 1. For MSR litters, one-way ANOVA revealed a significant effect of treatment on renal [$F(3,16)=8.92$, $p<0.001$] but not on hepatic PBG-synthase activity. Duncan's multiple range test showed that the Sal-Hg rats presented a significant inhibition in renal PBG-synthase activity (about 50%) when compared to the other groups ($p<0.05$). For LSR litters, renal and hepatic enzyme activities were similar between groups (one-way ANOVA).

3.3. Hepatic toxicity

Parameters of hepatic toxicity (ALT, AST and LDH activities) are shown in Table 2. For MSR litters, one-way ANOVA revealed a significant effect of the

treatment on ALT activity [$F(3,16)=14.52$, $p<0.001$]. Sal-Hg rats presented ALT activity significantly lower than the other groups (Duncan's multiple range test: $p<0.05$). AST and LDH activities were not altered by treatments (one-way ANOVA). For LSR litters, enzyme activities were similar between groups (one-way ANOVA).

3.4. Renal toxicity

Parameters of renal function (creatinine and urea levels) are shown in Table 3. For MSR litters, one-way ANOVA revealed a significant effect of treatment on creatinine and urea levels [$F(3,20)=13.24$, $p<0.001$ and [$F(3,20)=32.14$, $p<0.001$, respectively]. Sal-Hg rats presented creatinine and urea levels significantly higher than the other groups (Duncan's multiple range test: $p<0.05$). For LSR litters, statistical analysis showed absence of mercury effect on these metabolite levels (one-way ANOVA).

3.5. Glucose

Treatments altered serum glucose levels neither for MSR nor LSR litters (one-way ANOVA) (Table 3).

3.6. Metal content

3.6.1 Mercury levels

Hepatic and renal mercury contents are shown in Figure 2. For MSR litters (Fig. 2A), one-way ANOVA showed significant effect of treatment on hepatic [$F(3,20)=7.43$, $p<0.002$] and renal [$F(3,20)=30.95$, $p<0.001$] Hg contents. Sal-Hg rats presented hepatic mercury content greater than all the

other treatment groups. Zn-Hg rats presented a short increase of these metal levels; however, this group was not significantly different from Sal-Sal and Zn-Sal, showing that zinc pre-treatment prevented the increase in the mercury contents induced by mercury exposure (Duncan's multiple range test: $p<0.05$). Regarding renal mercury levels, Hg-rats (pre-exposed to saline or zinc) presented greater renal mercury content than the other treatment groups. However, the pre-treatment with zinc induced an additional increase in renal mercury levels (Duncan's multiple range test: $p<0.05$).

For LSR litters (Fig. 2B), one-way ANOVA showed a significant effect of treatment on hepatic [$F(3,8)=19.03$, $p<0.001$] and renal [$F(3,8)=13.95$, $p<0.002$] Hg contents. Both Sal-Hg and Hg-Zn groups presented hepatic and renal mercury levels greater than the other treatment groups (Duncan's multiple range test: $p<0.05$).

Treatments altered the blood mercury levels neither for MSR nor for LSR litter (one-way ANOVA) (data not shown).

3.6.2. Zinc content

Treatments altered blood, hepatic, and renal zinc contents neither for MSR nor for LSR litters (one-way ANOVA) (Table 4).

4. Discussion

This research investigated the delayed effect of mercury in rats exposed to this metal in the developmental phase postnatal, evaluating body weight gain, organ weights, PBG-synthase activity, mercury and zinc contents, as well as

hepatic and renal damage. Moreover, we verified the effectiveness of zinc in protecting against toxic effects caused by mercury.

In this study, we verified that there are rats more and less sensitive to mercury for both biochemical and physiological parameters. Previously, using the same protocol of intoxication, we observed that young rats exposed to mercury presented different sensitivity regarding behavioral alterations (Franciscato et al., 2009). At 12 and 13 days old, i.e., after the 4th and 5th dose of the metal, all Hg-exposed rats presented body weight significantly lower than other treatment groups (data not shown). However, at 33 days old, it was observed that there are litters whose Hg-animals recovered the body weight gain [called litter containing less sensitive rats (LSR) to mercury] and litters whose Hg-animals did not recover the body weight gain [called litter containing more sensitive rats (MSR) to mercury] (Franciscato et al., 2009).

Body weight alterations in MSR litters occurred in parallel to a decrease in liver weight and an increase in kidney weight. These effects had already been verified 24 h after Hg-exposure (Peixoto et al., 2003). We can observe that these effects persist even after a long time elapsed from mercury intoxication. Interestingly, these mercury effects are totally prevented by previous exposure to zinc, when the parameters are evaluated at 33 days old as well as at 13 days old (Peixoto et al., 2003) (21 days or 24 h after mercury exposure, respectively).

In this study, we utilized the PBG-synthase activity to assess the extension of mercury intoxication in order to determine if this enzyme could serve as biomarker of heavy metal exposure (Bernard and Lauwerys, 1987; Oskarsson and Fowler, 1987) even a long time after the end of exposure. This is an important aspect, since in most of the studies the animals are euthanized 24

h after exposure (Peixoto et al., 2003, 2007a). Results showed that only renal PBG-synthase activity from MSR litters, but not from LSR litters, presented inhibition by mercury and that this inhibitory effect was prevented by zinc pre-exposure. For liver, the absence of PBG-synthase inhibition can be due to the fact that this organ presented short mercury content (see below). However, mercury content does not explain the absence of Hg effect on renal enzyme activity from LSR litters (Hg exposed rats presented high Hg content) as well as of Zn-Hg rats from MSR litters (Hg content higher than Sal-Hg rats).

Amino transferases and LDH enzymes are sensitive indicators of hepatocellular damage; therefore an increase in these serum activities can represent liver lesion (Devlin, 1997; Meyer et al., 1992). Regarding MSR litters, results revealed that AST as well as LDH activities were not altered by mercury exposure. However, we verified a reduction of 63.3% in ALT activity 21 days after the mercury intoxication, being that this effect was totally prevented when the animals were pre-exposed to zinc. Previously, Peixoto and Pereira (2007) showed a reduction of 40% in this enzyme activity 24 h after Hg-exposure, which was prevented by zinc exposure. Thus, we can conclude that mercury effect continues for a long time after metal intoxication and that this alteration does not indicate hepatic damage. Our results differ from those reported by Kumar et al. (2005) that found elevation in serum ALT and AST activities of adult mice 1, 3, 7, 15 and 30 days after the exposure to one dose of $HgCl_2$ (5 mg/kg) intraperitonially, revealing hepatotoxicity. Still, we verified that these parameters were not modified in LSR litters that also presented a short Hg-hepatic content (see below).

Differently from hepatic parameters, the renal function of MSR litters, but not of LSR litters, was significantly altered by mercury exposure, reinforcing the previous finding indicating mercury as a potent nephrotoxic agent (Clarkson, 1997; Emanuelli et al., 1996; Goyer, 1995; Peixoto and Pereira, 2007). Twenty-one days after the end of mercury exposure, rats presented an increase in serum creatinine and urea levels of 185.5% and 203.6%, respectively. When animals were pre-exposed to zinc, no nephrotoxic effect was verified, indicating zinc pre-exposure as an important preventive treatment against mercury-induced nephrotoxicity. Similar results about renal toxicity and zinc preventive action were also obtained when animals were euthanized 24 h after the end of mercury exposure (Peixoto and Pereira, 2007).

Even 21 days after the end of exposure, MSR as well as LSR litters, presented increase of mercury content in both liver and kidney tissues. However, it is interesting to observe that the level of mercury found in kidney was around 3.6-fold higher than that found in liver. This result differs from those verified when animals were euthanized 24 h after exposure, which presented liver mercury levels higher than renal mercury levels (Peixoto et al., 2003, 2007b). Still, we can observe that the content of mercury in both tissues was reduced in function of long interval after exposure (at 24 h, ~40 and ~70 μg/g, kidney and liver, respectively; at 21 days, ~5 and ~1.5 μg/g, kidney and liver, respectively). This fact can be related to an attempt of the organism to eliminate the metal. In fact, several studies have demonstrated the formation of complexes such as metallothionein-Hg (Goering and Klassen, 1983; Peixoto et al., 2007b), glutathione-Hg and cysteine-Hg-cysteine. Besides, mercury bound

to these small-molecular-weight thiols is easily uptaken by kidney, causing accumulation of the metal in this organ (Zalups, 1998).

Blood mercury contents were similar among all groups, showing that the high blood Hg level presented 24 h after exposure (Peixoto et al., 2007b) is reduced after 21 days. This reduction probably is due to the elimination of this metal from the bloodstream by renal filtration and tissue reuptake, which occurs during this long interval (21 days) between intoxication and euthanasia. In fact, mercury is eliminated from the body in the urine and feces, and its half-life is around 20 days (Timbrell, 2008).

Exposure to zinc chloride induces an important increase of zinc content in the liver and kidney, but not in the blood, when rats are euthanized 6 days after exposure to zinc (Peixoto et al., 2008). However, no alteration in these levels was found after a long time (26 days) elapsed from zinc exposure.

Zinc is able to reduce the hepatic mercury content; however, it causes increase of renal mercury levels (Peixoto et al., 2003, 2007a, 2008). Similarly, despite the lower zinc levels found in 33-day-old rats, zinc-exposure caused a reduction of 4-fold in hepatic mercury levels and an increase of 2-fold in renal mercury levels. Literature data suggest that this zinc effect can be attributed to metallothionein synthesis induced by zinc pre-exposure (Peixoto et al., 2003, 2007a). In fact, an increase in liver and kidney metallothionein contents was verified in rats exposed to zinc (Peixoto et al., 2007a), being that liver is the main organ involved in the synthesis of this metalloprotein (Peixoto et al., 2003; Tandon et al., 2001). Thus, the mercury bound to metallothionein synthesized in the liver can be transported to kidney where it continues deposited. Although rats pre-exposed to zinc presented larger kidney mercury levels, this toxic metal

seems to be unavailable to cause effects, probably due its bind to metallothionein, explaining the protective effect of zinc.

Regarding LSR litters, Hg-exposed rats pre-exposed or not to zinc presented high hepatic and renal Hg content, but absence of effect on all parameters studied. The real mechanisms involved in the results are unknown. However, we have already verified that rats exposed to mercury could be less sensitive to it when evaluated in behavioral tasks (Franciscato et al., 2009).

This study shows that mercury accumulates in the tissues and its toxic effects persist for a long time after the end of exposure, causing renal insufficiency and inhibition of the PBG-synthase activity. Besides, zinc is an effective protector against mercury toxicity even when these analyses are conducted a long time after exposure.

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Legends

Fig.1. Hepatic and renal PBG-synthase activity of more sensitive (MSR litters, n=5) (**A**) and less sensitive (LSR litters, n=4) (**B**) rats treated as described in Table 1. The results are presented as mean ± S.E.M. Duncan's multiple range test: *significant difference from other groups ($p<0.05$).

Fig.2. Hepatic and renal mercury content of more sensitive (MSR litters, n=6) (**A**) and less sensitive (LSR litters, n=3) (**B**) rats treated as described in Table 1. The results are presented as mean ± S.E.M. Duncan's multiple range test: different letters confer significant statistical difference in the same session ($p<0.05$).

Table 1. Body, liver and kidney weights of rats treated (s.c.) with saline or $ZnCl_2$ (27 mg/kg/day), from 3rd to 7th, and with saline or $HgCl_2$ (5 mg/kg/day), from 8th to 12th day old.

Animals	Treatment	Weight (g) Body	Liver	Kidney
		(n=15)	(n=5)	(n=5)
	Sal-Sal	93.6 ± 2.73	4.89 ± 0.25	0.91 ± 0.04
	Sal-Hg	58.4 ± 5.41*	3.22 ± 0.27*	1.67 ± 0.24*
MSR	Zn-Sal	95.5 ± 2.68	4.91 ± 0.19	0.99 ± 0.06
	Zn-Hg	90.9 ± 2.31	4.74 ± 0.31	1.08 ± 0.07
		(n=12)	(n=4)	(n=4)
	Sal-Sal	93.2 ± 3.14	4.78 ± 0.33	0.95 ± 0.09
	Sal-Hg	86.9 ± 3.06	4.35 ± 0.15	0.99 ± 0.07
LSR	Zn-Sal	91.0 ± 3.32	4.91 ± 0.25	1.03 ± 0.07
	Zn-Hg	90.1 ± 3.32	4.76 ± 0.18	1.03 ± 0.09

The results are presented as mean ± S.E.M. Duncan's multiple range test: *significant difference from other treatment groups ($p<0.05$).

Table 2. Serum ALT and AST and plasma LDH activity of rats treated as described in Table 1.

Animals	*Treatment*	*ALT (U/mL)*	*AST (U/mL)*	*LDH (U/L)*
MSR (n=5-6)	Sal-Sal	68.6 ± 3.61	125.8 ± 2.85	243.6 ± 34.5
	Sal-Hg	25.2 ± 3.34*	120.2 ± 2.69	197.4 ± 49.5
	Zn-Sal	67.2 ± 7.73	124.2 ± 6.08	236.3 ± 34.8
	Zn-Hg	66.0 ± 6.18	131.2 ± 3.69	275.4 ± 28.8
LSR (n=3-4)	Sal-Sal	52.7 ± 6.37	111.0 ± 4.92	252.2 ± 83.6
	Sal-Hg	52.8 ± 6.16	116.0 ± 5.11	218.3 ± 53.8
	Zn-Sal	51.5 ± 9.04	109.7 ± 5.31	250.6 ± 45.1
	Zn-Hg	53.2 ± 4.42	118.5 ± 4.17	287.9 ± 87.9

The results are presented as mean ± S.E.M. Duncan's multiple range test: *significant difference from other treatment groups ($p<0.05$).

Table 3. Serum creatinine, urea and glucose of rats treated as described in Table 1.

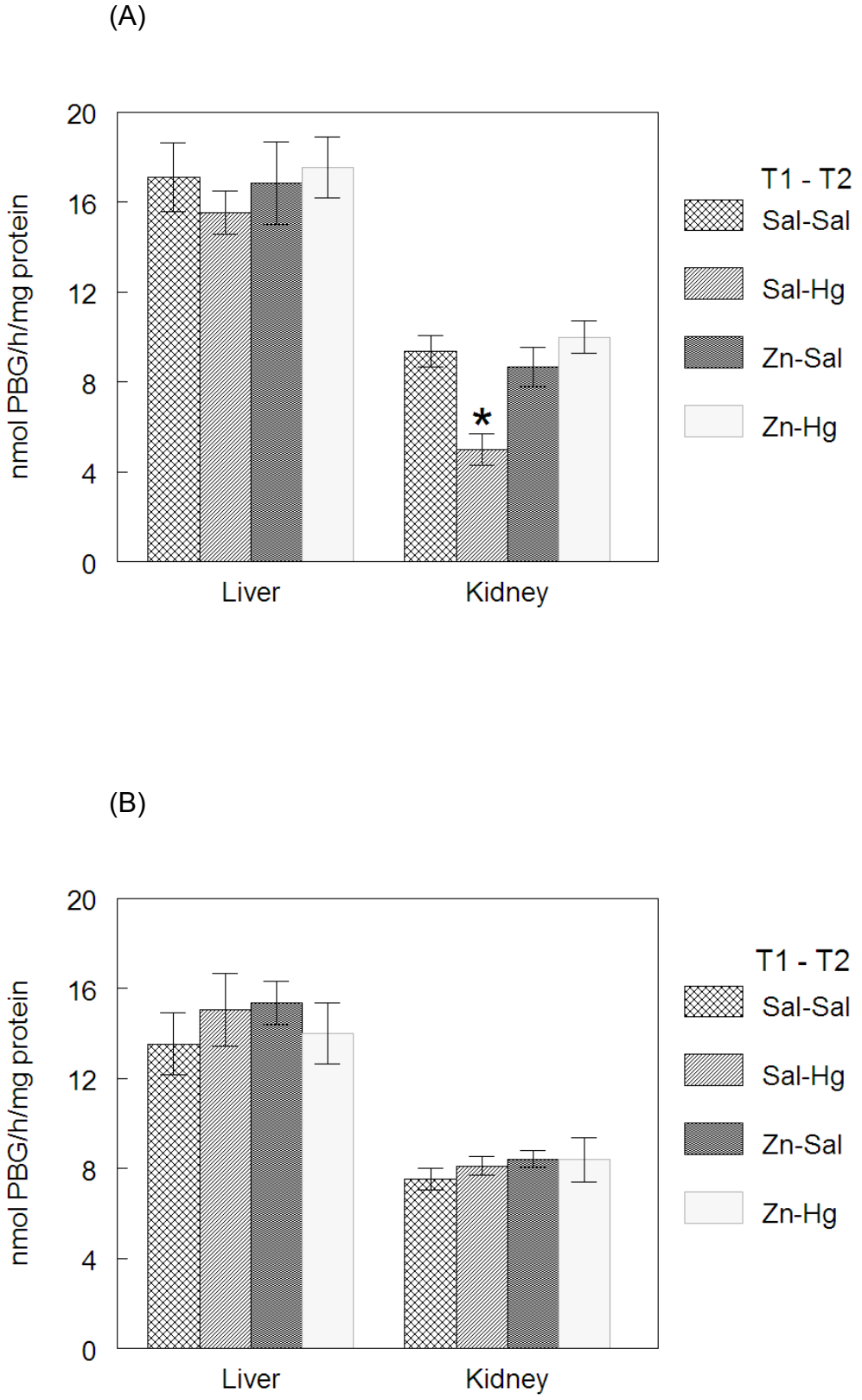
Animals	*Treatment*	*Creatinine (mg/dL)*	*Urea (mg/dL)*	*Glucose (mg/dL)*
MSR (n=6)	Sal-Sal	0.62 ± 0.13	47.3 ± 1.98	155.2 ± 11.65
	Sal-Hg	1.77 ± 0.24*	142.7 ± 14.33*	127.6 ± 14.20
	Zn-Sal	0.69 ± 0.06	45.5 ± 3.54	139.7 ± 15.25
	Zn-Hg	0.76 ± 0.11	65.9 ± 6.28	139.4 ± 7.80
LSR (n=4)	Sal-Sal	0.44 ± 0.04	49.0 ± 4.76	142.3 ± 13.97
	Sal-Hg	0.51 ± 0.08	57.4 ± 6.73	165.8 ± 26.87
	Zn-Sal	0.75 ± 0.17	50.0 ± 1.64	159.7 ± 10.01
	Zn-Hg	0.63 ± 0.09	64.0 ± 4.96	142.3 ± 9.10

The results are presented as mean ± S.E.M. Duncan's multiple range test: *significant difference from other treatment groups ($p<0.05$).

Table 4. Blood, hepatic and renal zinc content (µg of Zn/g of wet tissue) of rats treated as described in Table 1.

Animals	Treatment	Blood (µg/g)	Hepatic (µg/g)	Renal (µg/g)
MSR (n=5-6)	Sal-Sal	4.18 ± 0.20	24.87 ± 0.47	20.03 ± 1.89
	Sal-Hg	3.69 ± 0.21	29.82 ± 2.49	16.77 ± 2.82
	Zn-Sal	4.09 ± 0.22	25.48 ± 0.74	20.83 ± 2.07
	Zn-Hg	4.19 ± 0.07	25.17 ± 0.75	24.52 ± 2.78
LSR (n=3)	Sal-Sal	4.92 ± 0.38	25.33 ± 1.63	24.77 ± 0.68
	Sal-Hg	4.89 ± 0.10	25.13 ± 0.59	22.57 ± 4.46
	Zn-Sal	4.82 ± 0.25	27.17 ± 1.92	25.37 ± 0.39
	Zn-Hg	4.93 ± 0.07	24.30 ± 1.40	26.20 ± 1.08

The results are presented as mean ± S.E.M.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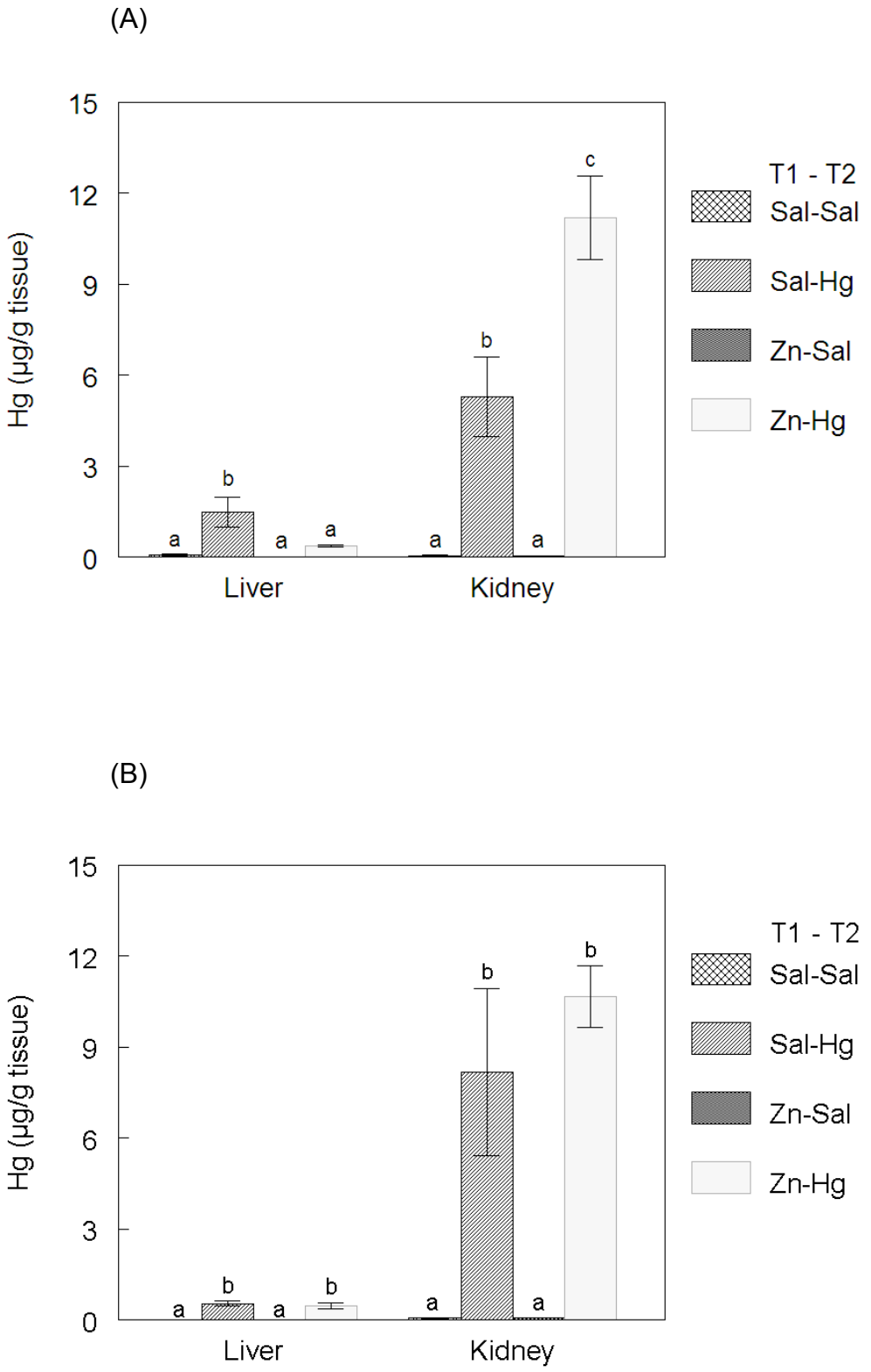
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Zn-Sal
Zn-Hg

Fig. 2.

CAPÍTULO 5
DISCUSSÃO

O mercúrio inorgânico é essencialmente nefrotóxico, mas existem relatos de que esta forma do metal também pode causar neurotoxicidade (Peixoto et al., 2007a; Franco et al., 2007). Outra característica deste metal é que os sinais clínicos de sua intoxicação são persistentes (Albers et al, 1988; Kishi et al., 1993). Por outro lado, estudos têm demonstrado que alterações bioquímicas severas induzidas por este metal são prevenidas pela pré-exposição ao zinco em ratos jovens (Peixoto et al., 2003; Peixoto e Pereira 2007). Considerando as informações descritas acima, este trabalho teve como objetivo estudar os efeitos imediatos e tardios do mercúrio sobre o comportamento e parâmetros bioquímicos de ratos; e ainda investigar a possível ação preventiva do zinco sobre alterações comportamentais imediatas e tardias e sobre as mudanças bioquímicas tardias causadas pelo mercúrio.

Ratos expostos ao mercúrio apresentaram redução no ganho de peso corporal nos dias 12 e 13 (após a 4ª e 5ª dose do metal). Os resultados obtidos a partir dos 13 dias justificaram a divisão das ninhadas em dois grupos. O primeiro grupo correspondeu às ninhadas cujos animais tratados com mercúrio não recuperaram o prejuízo no ganho de peso corporal até os 33 dias de idade, estas ninhadas foram classificadas como ninhadas contendo os ratos mais sensíveis ao mercúrio (“more sensitive rats” = MSR litters). O segundo grupo correspondeu às ninhadas cujos animais tratados com mercúrio recuperaram o prejuízo no ganho de peso corporal, e foram denominadas como ninhadas contendo os ratos menos sensíveis ao mercúrio (“less sensitive rats” = LSR litters). Estas classificações foram confirmadas por análises de correlações entre o peso corporal e os conteúdos de mercúrio no cérebro e cerebelo.

Nas ninhadas MSR, a intoxicação com cloreto de mercúrio provocou uma diminuição no ganho de peso corporal a partir do 12º dia de idade (após a 4ª dose do metal) até 33º dia de idade, ou seja, o prejuízo sobre o ganho de peso persistiu durante 21 dias após o término da exposição ao metal. No final deste período também foi observada uma diminuição no peso de fígado e um aumento no peso dos rins. Entretanto, houve uma diminuição nos peso de cérebro e cerebelo somente aos 13 dias de idade (24 horas após o final da

exposição), não ocorrendo alterações no peso destes órgãos aos 33 dias de idade.

Os efeitos do mercúrio sobre o peso corporal e sobre os pesos de fígado e rins, que ocorreram nas ninhadas MSR, já foram verificados quando ratos tratados com o mesmo protocolo eram sacrificados 24 horas após o final da exposição (Peixoto et al., 2003), portanto não ocorre recuperação no ganho de peso após o final da intoxicação. O prejuízo sobre o ganho de peso corporal pode ser atribuído ao efeito anorexigênico deste metal (Counter and Buchanan, 2004). Houve uma relação significativa entre o peso corporal e peso de fígado dos animais (dados não mostrados), confirmando que a diminuição no peso de fígado corresponde à diminuição do peso corporal. Enquanto que o aumento no peso e tamanho de rim pode ser devido às alterações estruturais neste órgão causadas pelo mercúrio, como aumento de volume dos túbulos proximais, dilatação do lúmem tubular e aumento de volume das células dos túbulos proximais (Madsen e Maunsbach, 1981). Portanto, o aumento do tamanho dos rins ocorre de maneira compensatória devido à redução do número de néfrons funcionais causada pelo mercúrio (Zalups, 2000).

Todos os animais que receberam mercúrio apresentaram aumento nos níveis deste metal em todos os órgãos estudados, quando comparados com o controle. Apesar de ser essencialmente nefrotóxico (Clarkson et al., 2003), o mercúrio inorgânico também acumulou-se no SNC, sendo que sua concentração no cerebelo foi 2,9 vezes maior que no cérebro para as ninhadas MSR, mostrando que o cerebelo é um tecido alvo deste metal no SNC (Sanfeliu et al., 2003). Assim, podemos relacionar a diminuição da atividade da AChE de cerebelo dos animais de 13 dias de idade ao aumento do conteúdo de mercúrio neste tecido. Os altos níveis de mercúrio encontrados no cerebelo também podem estar relacionados ao prejuízo motor observado nas tarefas descritas a seguir. Mas é importante lembrar que nem todos os grupos que tiveram alterações comportamentais apresentaram inibição na atividade desta enzima. Isto sugere que o conteúdo de mercúrio necessário para inibir a atividade da enzima é maior do que aquele necessário para causar déficits comportamentais.

As tarefas comportamentais foram realizadas durante e após o final da exposição aos metais. Desta forma, foram avaliados os efeitos imediatos e

tardios do mercúrio sobre parâmetros comportamentais. Os animais submetidos às tarefas comportamentais após os 13 dias de idade foram divididos em ninhadas MSR e LSR, sendo que os tratamentos não causaram alterações sobre o desempenho comportamental das ninhadas LSR.

A tarefa do reflexo de geotactismo negativo foi conduzida dos 3 aos 13 dias de idade, portanto, as ninhadas ainda não estavam divididas em MSR e LSR. Os ratos expostos somente ao mercúrio apresentaram um aumento na latência para completar a resposta de geotactismo negativo nas sessões 5 e 6 (após 3ª e 5ª doses de mercúrio).

Em relação ao teste do béquer, os animais das ninhadas MSR expostos ao mercúrio apresentaram maior latência para acessar o refúgio e maior porcentagem de queda na 4ª sessão; enquanto que no teste do campo aberto, estes animais mostraram diminuição no número de respostas de orientação e no número de cruzamentos na sessão de treino, e ainda, apresentaram um aumento na latência de saída da primeira área. Portanto o mercúrio alterou o desempenho dos animais nessas três tarefas comportamentais: a função motora necessária para que o animal possa responder ao reflexo do geotactismo negativo (Da-Silva et al., 1990) ficou prejudicada; a força muscular e função cerebelar necessárias para equilibrar-se e alcançar o refúgio no teste do béquer (Smart e Dobbing, 1971), bem como a atividade locomotora e exploratória no teste do campo aberto (Pereira et al., 1992) foram diminuídas. Apesar disso, os animais apresentaram memória de habituação, pois houve uma redução no número de respostas de orientação da sessão de teste em relação ao treino.

Alguns trabalhos tem descrito alterações comportamentais em animais expostos ao mercúrio inorgânico (Rocha et al., 2001; Szász et al., 2002; Franco et al., 2007; Peixoto et al., 2007a). No presente trabalho, os animais começam a apresentar déficits comportamentais logo no início da intoxicação com mercúrio, e estes problemas continuam por um longo período após o término da exposição. Esta situação concorda com Albers et al. (1988) e Kishi et al. (1993) que analisaram pessoas que tiveram exposição ocupacional ao mercúrio e observaram prejuízos neurocomportamentais, mesmo após vários anos do final da exposição ao metal.

Apesar dos ratos que demonstraram pior desempenho nos testes do

béquer e do campo aberto terem apresentado prejuízo no ganho de peso corporal, esta diminuição no peso parece não estar envolvida no prejuízo comportamental, pois estudos têm demonstrado que a atividade no campo aberto não é alterada pela diminuição no peso corporal (Farkas et al., 2009), e outros têm relatado que a desnutrição causa hiperatividade e aumento do comportamento exploratório no campo aberto (Alamy et al., 2005). Assim, não podemos atribuir o prejuízo no desempenho comportamental ao menor ganho de peso destes animais.

A sensibilidade à dor foi avaliada através do teste de imersão da cauda, no qual a resposta de hiperalgesia é geralmente atribuída a mecanismos centrais (Ramabadran et al., 1989), mas os resultados mostraram que os tratamentos não alteram a resposta nociceptiva. Os diferentes tratamentos também não afetaram o desempenho dos animais no teste da locomoção forçada em cilindro giratório (rotarod), usado para avaliar a coordenação motora (Ekambaram and Paul, 2002). A ausência de efeito nesse teste não era esperada, já que os animais das ninhadas MSR tratados com mercúrio apresentaram diminuição da força motora no teste do béquer. Entretanto, os animais provavelmente necessitam de uma menor força para se manterem sobre o cilindro em rotação baixa (8 rpm). Assim, o prejuízo motor causado pela intoxicação por mercúrio não interferiu no comportamento dos animais neste teste.

Níveis elevados de mercúrio no tecido hepático foram encontrados em animais intoxicados por mercúrio 24 horas após o final da intoxicação, sendo que estes animais também apresentaram inibição na atividade da enzima PBG-sintase hepática (Peixoto et al., 2003). No presente trabalho, podemos observar que 21 dias após o final da exposição ao metal a atividade da PBG-sintase hepática de ambos os ratos MSR e LSR não encontra-se inibida, provavelmente devido a uma diminuição (em torno de 40 vezes, MSR) dos níveis de mercúrio encontrados aos 21 dias (≈1,5 μg/g) em comparação a 24 h após o término da exposição (≈60 μg/g) (Peixoto et al., 2003); isto é, os níveis hepáticos de mercúrio diminuíram e o efeito inibitório sobre a enzima desapareceu. Entretanto, após 21 dias os níveis séricos da ALT dos ratos MSR continuam diminuídos similarmente ao efeito encontrado 24 h após a exposição (Peixoto e Pereira, 2007). Mas esta alteração não indica lesão hepática, pois em uma

lesão dos hepatócitos ocorre o extravasamento desta enzima com o consequente aumento dos seus níveis séricos (Meyer et al., 1992).

Em relação à PBG-sintase renal, verificou-se que mesmo após 21 dias da exposição, os animais MSR apresentam inibição da atividade da enzima, mostrando que o efeito inibitório do mercúrio sobre a PBG-sintase verificado aos 13 dias de idade (24 horas após o final da intoxicação) (Peixoto et al., 2003) persiste após um longo período, embora o conteúdo renal de mercúrio tenha diminuído (≈5 μg/g e ≈30 μg/g para 21 dias e 24 h, respectivamente). Ainda, observou-se que a concentração renal de mercúrio foi maior do que nos outros tecidos, mostrando a alta afinidade da forma inorgânica do metal pelo tecido renal (Clarkson et al., 2003). Esses fatos indicam que após o final da exposição, o mercúrio foi transportado até os rins, por onde certa quantidade do metal foi eliminada, mas uma porção ficou armazenada neste tecido refletindo a ligação do mercúrio a grupamentos tióis que são facilmente captados e depositados nos rins (Zalups, 2000).

Por depositar-se em grandes quantidades nos rins e causar importantes alterações celulares neste tecido (Zalups, 2000), sabe-se que o mercúrio causa insuficiência renal (Franco et al., 1997). Os níveis séricos de uréia e creatinina das ninhadas MSR apresentaram-se aumentados 21 dias após o final da exposição ao mercúrio, confirmando esta situação. Estes dados demonstram que a insuficiência renal verificada em animais 24 horas após o final da exposição ao metal (Peixoto e Pereira, 2007) é irreversível. Entretanto, os ratos LSR também apresentaram alto conteúdo de Hg renal e ausência de qualquer efeito deletério do mercúrio sobre esse órgão.

O pré-tratamento com zinco preveniu o prejuízo no ganho de peso corporal; reduziu o conteúdo de mercúrio no cérebro e cerebelo, prevenindo assim as alterações comportamentais causados pelo metal tóxico. Ainda, preveniu, mesmo que parcialmente, a inibição da AChE de cerebelo. No fígado, este pré-tratamento também reduziu o acúmulo de mercúrio, e evitou a diminuição dos níveis séricos de ALT. Entretanto, causou aumento dos níveis renais de mercúrio, mas mesmo assim preveniu a inibição da atividade da enzima PBG-sintase e a insuficiência renal. A elevação do conteúdo renal de mercúrio pode ser explicada pelo fato de que a metalotioneína, cuja síntese é induzida pelo zinco, liga-se ao mercúrio transportando-o até os rins, onde este é

absorvido e armazenado (Zalups, 2000). Mas apesar dos ratos pré-expostos ao zinco apresentarem elevado conteúdo renal de mercúrio, este metal tóxico parece não estar disponível para causar seus efeitos nocivos, provavelmente, devido a sua ligação às metalotioneínas.

O efeito preventivo do zinco sobre alguns efeitos tóxicos causados pelo mercúrio (acúmulo tecidual do metal pesado, inibição da atividade da enzima PBG-sintase e insuficiência renal) já foi demonstrado quando os animais são mortos 24 horas após o final da exposição ao mercúrio (Peixoto et al., 2003; Peixoto e Pereira, 2007). Assim, podemos confirmar que o efeito protetor do zinco persiste mesmo quando o parâmetro é analisado muito tempo depois do pré-tratamento. O efeito preventivo do zinco pode ser atribuído à síntese de metalotioneínas induzida por este metal (Peixoto et al., 2003), pois um alto conteúdo destas proteínas já foi identificado em fígado e rins de ratos tratados com zinco (Peixoto et al., 2007c), sendo que o fígado é o principal produtor destas (Peixoto et al., 2003; Tandon et al., 2001).

Além dos animais terem sido classificados em MSR e LSR devido à diferença quanto à sensibilidade ao mercúrio, outros animais tratados com mercúrio foram ainda mais sensíveis, pois morreram ao longo dos 21 dias após o final do tratamento. A diferença de sensibilidade ao mercúrio apresentada pelos animais pode ser atribuída à suscetibilidade individual de cada animal, pois alguns trabalhos têm relatado que a vulnerabilidade às intoxicações pode variar de acordo com o efeito de polimorfismos genéticos que afetam negativamente a capacidade de destoxificação do indivíduo (Rose et al., 2008).

Pesquisas já demonstraram que existe polimorfismo em um gene humano que codifica a enzima coproporfirinogênio oxidase (uma enzima da via biossintética do heme), o que modifica o efeito do mercúrio sobre a excreção urinária de porfirinas (Woods et al., 2005) e também aumenta a vulnerabilidade à neurotoxicidade causada pelo mercúrio em humanos (Echeverria et al., 2006). Ainda, há relatos de que o polimorfismo genético em crianças com autismo pode aumentar a vulnerabilidade à toxicidade ao chumbo durante períodos críticos de neurodesenvolvimento pré e pós-natal (Rose et al., 2008). Portanto, uma predisposição genética pode alterar a resposta biológica aos metais tóxicos em humanos. Assim, outros estudos são necessários para elucidar a causa da diferença de sensibilidade ao mercúrio apresentada pelos animais

deste trabalho.

CAPÍTULO 6
CONCLUSÕES

Observando-se os resultados descritos, podemos concluir que:

1. os animais podem apresentar diferentes sensibilidades à toxicidade do mercúrio que podem ser atribuídas à suscetibilidade individual:

a) alguns animais foram tão sensíveis que morreram antes do final do experimento;

b) outros animais, apesar de apresentarem aumento do conteúdo de mercúrio nos tecidos, não apresentaram alterações bioquímicas nem comportamentais;

c) em um nível intermediário, houve animais cuja exposição ao mercúrio causou alterações como:

-prejuízo no ganho de peso corporal, afetando o desenvolvimento e crescimento corporal;

-alterações no desempenho comportamental durante e após o tratamento por um período de 21 dias, prejudicando a função motora, força muscular, função cerebelar, atividade locomotora e exploratória;

-aumento no conteúdo de mercúrio em cérebro, cerebelo, fígado e rins;

-diminuição do peso de fígado, que está relacionada ao prejuízo no ganho de peso corporal;

-aumento do peso renal, que pode ser de forma compensatória devido à perda de néfrons funcionais;

-inibição da atividade da enzima PBG-sintase renal;

-insuficiência renal mesmo quando avaliada 21 dias após o final da exposição ao metal;

-diminuição da atividade sérica da enzima ALT, que não pode ser considerada como reflexo de uma lesão hepática;

- a inibição da atividade da enzima acetilcolinesterase de cerebelo verificada 24 horas após o final da exposição não foi verificada aos 21 dias após o término da intoxicação;

2. o zinco é efetivo em prevenir os efeitos deletérios imediatos e tardios causados pelo mercúrio, pois:

a) reduziu os níveis de mercúrio no cérebro, cerebelo e fígado, e aumentou os níveis renais, de todos os animais expostos ao cloreto de mercúrio. Sendo que o mercúrio permaneceu acumulado no tecido renal sem causar toxicidade;

b) impediu os prejuízos comportamentais induzidos pelo mercúrio tanto durante quanto após o final da exposição ao metal tóxico;

c) evitou as alterações bioquímicas e a insuficiência renal.

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