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AÇÃO PREVENTIVA DO COBRE SOBRE ALTERAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS INDUZIDAS PELO MERCÚRIO EM RATOS JOVENS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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AÇÃO PREVENTIVA DO COBRE SOBRE ALTERAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS INDUZIDAS PELO MERCÚRIO EM RATOS JOVENS

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

Orientadora: Profª. Drª. Maria Ester Pereira

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elaborada por
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como requisito parcial para a obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**

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À Maria Candida (in memorian), mulher de coração grande, mãe de 10 filhos e mais dois de coração.

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RESUMO

Tese de Doutorado
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AÇÃO PREVENTIVA DO COBRE SOBRE ALTERAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS INDUZIDAS PELO MERCÚRIO EM RATOS JOVENS

Autora: LUCÉLIA MORAES E SILVA
Orientadora: MARIA ESTER PEREIRA
Data e local de Defesa: Santa Maria, 30 de maio de 2014.

Este trabalho investigou o efeito preventivo do cobre contra as alterações bioquímicas e comportamentais induzidas pelo mercúrio em ratos tratados subcutaneamente com salina ou $CuCl_2.2H_2O$ (Cu 2,6 mg/kg/dia) do 3º ao 7º e com salina ou $HgCl_2$ (Hg 3,7 mg/kg/dia) do 8º ao 12º dia de idade. Amostras teciduais de animais mortos 24 h ou 21 dias após o término da exposição ao mercúrio (13 ou 33 dias de idade) foram utilizadas para a análise da atividade das enzimas δ-aminolevulinato desidratase (δ-ALA-D) de sangue, fígado, rim e cérebro; acetilcolinesterase (AChE) de cérebro e cerebelo; parâmetros bioquímicos indicativos de toxicidade hepática e renal; e para a quantificação dos níveis de metalotioneínas (MT) hepática e renal e de metais (Hg, Cu, Zn, Fe e Mg) nos diferentes tecidos. Os animais foram submetidos às tarefas comportamentais: geotactismo negativo (3, 5, 7, 9, 11 e 13 dias de idade), imersão da cauda (13, 20 e 27 dias de idade), teste do béquer (17 aos 20 dias de idade) e locomoção forçada em cilindro giratório (25 e 30 dias de idade). A exposição ao mercúrio reduziu o peso corporal e cerebral e aumentou o peso renal; inibiu a atividade das enzimas δ-ALA-D hepática e renal, AChE de cerebelo e LDH sérica; e aumentou os níveis de uréia e creatinina sérica e MT hepática aos 13 dias. O efeito do mercúrio persistiu sobre o peso corporal e renal, a atividade da δ-ALA-D renal e níveis de uréia verificados aos 33 dias. Ainda, a exposição ao mercúrio causou um acúmulo deste metal em todos os tecidos analisados; aumentou os níveis de Zn e Fe hepático; e diminuiu os níveis

de Fe e aumentou os níveis de Cu renal aos 13 dias. O efeito persistiu sobre os níveis de Hg hepático e sobre os níveis de Hg e Fe renal. Além disso, um decréscimo no peso de fígado e nos níveis de Mg renal e um aumento nos níveis de Zn em cérebro e cerebelo foram observados somente aos 33 dias. Em relação às tarefas comportamentais, ratos expostos ao Hg apresentaram prejuízo na função motora e força muscular verificados nos testes do geotactismo negativo e teste do béquer. A eficiência do cobre como tratamento preventivo foi imediata em parâmetros como atividade da AChE de cerebelo, níveis de creatinina sérica, conteúdo de Hg e homeostase dos níveis de Fe hepático. A prevenção das alterações sobre o peso corporal, renal e hepático, atividade da δ-ALA-D renal, níveis de ureia sérica e homeostase dos níveis renais de Fe e Mg foram observados aos 33 dias. Alterações comportamentais foram totalmente prevenidas. Além disso, a pré-exposição ao cobre causou uma redistribuição do mercúrio, decrescendo os níveis de Hg hepático e sanguíneos e aumentando os níveis renais aos 13 dias. Este efeito ocorreu em paralelo a um aumento dos níveis de MT hepática e renal, sugerindo que a MT hepática pode ligar-se ao Hg e transportar o metal tóxico para o rim a fim de ser excretado. Os resultados do presente estudo sugerem que o cobre pode ser considerado um potencial agente terapêutico em casos de intoxicação por mercúrio, mesmo quando avaliado tardiamente.

Palavras-chave: Cobre. Mercúrio. Metal Essencial. Metalotioneínas. Nefrotoxicidade. Neurotoxicidade. Ratos Recém-Nascidos.

ABSTRACT

Thesis of Doctor's Degree
Graduate Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

PREVENTIVE ACTION OF COPPER ON BIOCHEMICAL AND BEHAVIORAL CHANGES INDUCED BY MERCURY IN YOUNG RATS

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Advisor: MARIA ESTER PEREIRA
Place and data of defense: Santa Maria, may, 30, 2014.

This work examined the effectiveness of Cu pre-exposition on biochemical and behavioral changes induced by Hg exposure in young rats treated subcutaneously with saline or $CuCl_2.2H_2O$ (Cu 2.6 mg/kg/day) from 3 to 7 days old and with saline or $HgCl_2$ (Hg 3.7 mg/kg/day) from 8 to 12 days old. Tissue samples from animals killed 24 h or 21 days after the end of mercury exposure (13 or 33 days old) were used to analyze of blood, liver, kidney and cerebrum δ-aminolevulinic acid dehydratase (δ-ALA-D) activity; cerebrum and cerebellum acetylcholinesterase (AChE) activity; biochemical parameters indicative of hepatic and renal toxicity; and to determination of hepatic and renal metallothionein and metal levels (Hg, Cu, Zn, Fe e Mg) in all tissues studied. The animals also 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), rotarod tests (25 and 30 days old) and beaker test (17 to 20 days old). Mercury exposure reduced body and cerebrum and increased kidney weight; inhibited the hepatic and renal δ-ALA-D, cerebellum AChE and serum LDH activity; and increased serum urea and creatinine and hepatic MT levels at 13 days. The Hg effect persisted on body and renal weight, renal δ-ALA-D activity and urea levels checked after 33 days. Still, Hg exposure caused accumulation of this metal in all tissues analyzed; increased hepatic Zn and Fe levels; and decreased renal Fe and increased Cu levels at 13 days. The effect persisted on hepatic Hg levels; and renal Hg and Fe levels. In addition, a decrease in the liver weight and renal Mg levels; and increase in the

cerebrum and cerebellum Zn levels were observed only at 33 days. In behavioral tasks, rats exposed to Hg presented impartment in motor function and muscular strength verified in the negative geotaxis task and beaker test. The Cu effectiveness as preventive treatment was immediate on parameters such as cerebellum AChE activity, serum creatinine levels, Hg content and homeostasis of hepatic Fe levels. The prevention of altered parameters as body, Kidney and liver weight, renal δ-ALA-D activity, serum urea levels and homeostasis of renal Fe and Mg levels were verified at 33 days. Behavioral changes were completely prevented by Cu pre-exposure. Moreover, Cu pre-exposure caused an important redistribution of Hg decreasing hepatic and sanguine Hg levels and increasing renal levels of 13-day-old rats. This effect occurred in parallel with an increase in MT levels in liver and kidney, suggesting that hepatic MT can bind to Hg and transporting this metal to the kidney in order to be excreted. The results of the present study suggest that Cu can be considered as potential preventive therapeutic agent against Hg toxicity, even when were evaluated later.

Key-words: Copper. Mercury. Essential Metal. Metallothionein. Nephrotoxicity. Neurotoxicity. Newborn Rats.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

ARTIGO 1

MANUSCRITO 1

ARTIGO 2

LISTA DE TABELAS

LISTA DE ABREVIATURAS

ACh: acetilcolina;

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 (*alanine aminotransferase***)**;

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

AST: aspartato aminotransferase (*aspartate aminotransferase*);

ATC: iodeto de acetiltiocolina (*acetylthiocoline iodide*);

BAL: 2,3-dimercaptopropanol, dimercaprol (*2,3-dimercapto-1-propanol*);

^{o}C: grau Celsius;

Da: dalton;

DMPS: ácido 2,3-dimercaptopropano 1-sulfônico (*sodium salt of 2,3-dimercapto-1-propanesulfonic acid*);

DMSA: ácido meso-2,3-dimercaptosuccínico (*meso-2,3-dimercaptosuccinic acid*)

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

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

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

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

EDTA: sal dissódico do ácido etilenodiaminotetraacético (*ethylenediaminetetraacetic acid disodium salt*);

g: grama;

g: aceleração da gravidade (força centrífuga);

GST: glutationa-S-transferase;

h: hora;

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

l: litro;

L: litro;

LDH: lactato desidrogenase (*lactate dehydrogenase*);

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

M: molar;

min: minuto;

MT: metalotioneína(s);

n: número de repetições;

p: nível de significância;

PBG: porfobilinogênio;

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

pH: potencial hidrogeniônico;

Sal: salina;

s.c.: subcutânea; subcutaneamente;

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

SH: grupamento(s) sulfidrílico(s);

SNC: sistema nervoso central;

U: unidade;

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

SUMÁRIO

APRESENTAÇÃO

Esta tese está descrita na seguinte forma: primeiramente são apresentados a introdução e objetivos. A seguir, os resultados são apresentados na forma de artigo e manuscritos, os quais se encontram no item desenvolvimento. Os itens discussão e conclusão, encontradas no final da tese, apresentam interpretações e comentários gerais a respeito dos resultados demonstrados nos artigos e manuscrito contidos neste trabalho. As referências bibliográficas apresentadas no final da tese referem-se somente às citações que aparecem nos itens introdução e discussão.

1. INTRODUÇÃO

O Mercúrio (Hg) e compostos contendo mercúrio são tóxicos para o homem e meio ambiente. Tal como um elemento que ocorre naturalmente, esse metal tóxico sempre esteve presente no ambiente. Contudo, a atividade humana, especialmente com o início da era industrial, mobilizou mercúrio para além das concentrações naturalmente circulantes (UNEP, 2008). A geração de energia ainda com base em combustíveis fósseis, o crescimento industrial e econômico na Ásia e America do Sul, que por sua vez contribuem para a demanda por metais, tem aumentado a mobilização de mercúrio no ambiente, elevando os valores na atmosfera, solos, água doce e oceanos (UNEP, 2013). Atualmente estima-se que 46% das emissões de mercúrio lançado na atmosfera sejam oriundos da queima de combustíveis fósseis, principalmente do carvão utilizado nas indústrias e residências. Estes são seguidos pela mineração artesanal e de ouro em pequena escala (18%), produção de ferro e de metais não ferrosos, incluindo o ouro em grande escala (13%) e produção de cimento (10%). Juntos, a China, a Índia e os EUA são responsáveis por aproximadamente 55% do total das emissões globais de mercúrio originadas principalmente da queima de combustíveis fósseis (Sundseth et al., 2010).

Três espécies químicas desse metal podem ser encontradas no meio ambiente (elementar, inorgânica e orgânica) as quais podem diferir com respeito a seu comportamento no ambiente, bem como com respeito ao seu potencial de interagir em processos biológicos (Syversen & Kaur, 2012). O mercúrio elementar (ou vapor de mercúrio ou mercúrio metálico) possui estado de oxidação zero (Hg^0) e é um líquido de elevada tensão superficial, inodoro e de coloração prateada (Salgado et al., 1996). Compostos inorgânicos de mercúrio, também chamados de sais 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 o mercúrio orgânico é formado quando se liga ao carbono através de uma ligação covalente C-Hg, originando compostos organomercuriais encontrados principalmente no solo e na água (Klaassen, 1996; UNEP, 2002). Na tabela 1

são representadas as várias espécies químicas de mercúrio que tem sido consideradas importantes em processos biológicos e ambientais.

Tabela 1: Principais espécies de mercúrio em amostras ambientais e biológicas (modificado de Morita et al., 1998).

Mercúrio elementar		Hg^0
Espécies de mercúrio inorgânico	Íon mercúrico	Hg^{2+}
	Íon mercuroso	Hg^+
	Sulfeto de mercúrio	HgS
Espécies de mercúrio orgânico	Metilmercúrio	CH_3Hg^+
	Etilmercúrio	$C_2H_5Hg^+$
	Fenilmercúrio	$C_6H_5Hg^+$
	Dimetilmercúrio	$(CH_3)_2Hg$

Na atmosfera, o mercúrio elementar 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 biotransformado por bactérias anaeróbias em mercúrio orgânico (por exemplo, em metilmercúrio – processo conhecido por metilação) ou se volatilizar, retornando ao ambiente (Wasserman et al., 2003; Bisinoti & Jardim, 2004). O processo de biotransformação do mercúrio representa um agravante para o problema da poluição por mercúrio, pois o metilmercúrio acumula-se em cada passo da cadeia alimentar aquática. Uma vez que sua concentração aumenta à medida que avança os diferentes níveis tróficos, esse elemento pode ser encontrado em concentrações elevadas nos peixes, culminando na alimentação humana (Salgado, 1996). Exemplo disso ocorre nas áreas de garimpo brasileiras, onde o mercúrio é utilizado para amalgamar o ouro no processo de extração (Lacerda & Pfeiffer, 1992) e como consequência desse processo o mercúrio é lançado no meio ambiente contaminando peixes, principal fonte de proteína das comunidades ribeirinhas (UNEP, 2002). Além disso, o mercúrio pode ficar retido em sedimentos de rios,

lagos e do mar e lentamente ser convertido em metilmercúrio podendo levar décadas para que sistemas aquáticos alcancem níveis seguros (Randall & Chattopadhyay, 2013).

Todas as formas de mercúrio podem causar efeitos tóxicos em algum grau nos mais diversos tecidos. Esta toxicidade vai depender da forma química do mercúrio, dose, tempo de exposição e da via pela qual a intoxicação ocorre (Zalups, 2000). Casos de intoxicação ocupacional podem ocorrer em decorrência da exposição ao mercúrio elementar principalmente quando respirado como vapor ou partículas de mercúrio, tendo o cérebro e o rim como os locais de maior deposição (Salgado, 1996). Contudo, uma grande preocupação a respeito da poluição pelo mercúrio surge dos efeitos à saúde decorrentes da exposição ao metilmercúrio. Esse metal afeta principalmente o sistema nervoso (Counter & Buchaman, 2004) e pode ser encontrado na água e alimentos aquáticos (Sundseth et al., 2010). Compostos de mercúrio inorgânico também são alvos de inúmeras investigações, destacando-se aqui, o cloreto de mercúrio ($HgCl_2$), o qual será enfatizado no presente estudo. Essa forma de mercúrio pode estar presente no ambiente de trabalho onde são empregados na produção de cloro-álcali, plásticos, fungicidas, germicidas e na formulação de amálgamas dentárias (WHO, 1991; Klassen, 1996). Além disso, a exposição pode ocorrer indiretamente através da interconversão das formas elementar e orgânica de mercúrio para a inorgânica através de processos de biotransformação (Goyer, 1996). O mercúrio metálico pode sofrer oxidação transformando-se em mercúrio divalente envolvendo o sistema enzimático catalase-hidrogênio peroxidase (Salgado, 1996) ou em presença de matéria orgânica, como no ambiente aquático (Goyer, 1996). O metilmercúrio pode ser convertido a mercúrio divalente através do rompimento da ligação C-Hg (WHO, 1991; Vahter et al., 1994).

O mercúrio inorgânico pode causar danos ao organismo afetando vários sistemas, tais como ao sistema renal (Peixoto et al., 2003; Fanciscato et al., 2011), alvo primário da forma inorgânica (Emanueli et al., 1996, Favero, 2014), ao sistema hepático (Kumar et al., 2005; Bashandy et al., 2011) e ao sistema nervoso (Peixoto et al., 2007b; Franciscato et al., 2009b). No meio intracelular pode ligar-se a uma ampla variedade de sistemas enzimáticos, causando alteração na atividade de enzimas e, consequentemente, interferindo no

metabolismo e funcionamento celulares. As enzimas sulfidrílicas são um bom exemplo desse efeito, pois possuem grande afinidade por metais (WHO, 1991).

O rim funciona como o principal órgão excretor de resíduos metabólicos do organismo (Finco, 1997) e devido à alta taxa de fluxo sanguíneo é um dos principais alvos da toxicidade induzida por diversos agentes (Au, 2004). A toxicidade do mercúrio inorgânico sobre o sistema renal está relacionada com sua acumulação nas células epiteliais dos túbulos proximais, e com a sua ligação a grupos sulfídricos, carboxílicos e fosfóricos (Goyer, 1996). O resultado dessas interações pode levar a alterações histopatológicas (Favero et al., 2014), inativação de enzimas e inibição da síntese de proteínas essenciais para a adequada função dos rins. Necrose, túbulos atróficos, deposição de colágeno (Favero et al., 2014) além de estresse oxidativo, peroxidação lipídica, disfunção mitocondrial, mudanças no metabolismo do heme (Zalups, 2000) podem ser observadas nesse órgão. A captação e deposição renal de mercúrio ocorrem de maneira muito rápida podendo ser observada poucas horas após a exposição ao mercúrio (Zalups, 1993). Os efeitos nefrotóxicos da exposição ao mercúrio inorgânico podem ser verificados pelo aumento dos níveis séricos de uréia e creatinina (Peixoto & Pereira, 2007; Franciscato et al., 2011). A uréia representa 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).

No sistema hepático, a exposição ao mercúrio inorgânico pode causar alterações histopatológicas, vacuolização citoplasmática, cariorrexia, cariólise, picnose e necrose centro tubular (Kumar et al., 2005; Bashandy et al., 2011; Favero, 2011). A análise sérica dos níveis das enzimas alanina aminotransferase (ALT), aspartato aminotransferase (AST) e lactato desidrogenase (LDH) tem servido de marcador da hepatoxicidade causada pela exposição ao mercúrio inorgânico (Kumar et al., 2005; El-Shenawy & Hassan, 2008; Bashandy et al., 2011). Os níveis séricos dessas enzimas aumentam quando liberadas a partir de tecidos lesados. Isto permite inferir a

localização e a natureza das variações patológicas em alguns órgãos tais como o fígado. A ALT e AST são enzimas intracelulares presentes em grande quantidade no citoplasma do hepatócito e seus níveis plasmáticos estão elevados em quase todas as doenças hepáticas (Motta, 2003). A ALT é mais específica que a AST para doenças hepáticas, mas essa última é mais sensível, pois o fígado contém maiores quantidades de AST (Champe et al., 2006). A LDH está presente no citoplasma de todas as células e organismos principalmente no miocárdio, fígado, músculo esquelético, rim e eritrócitos; e, embora não específica, em conjunto com a atividade da ALT é considerada marcador de lesão hepática (Meyer et al., 1992; Kopperschlager & Kirchberger, 1996). Alguns estudos, no entanto, tem verificado que a atividade da ALT sérica pode apresentar-se inibida em ratos jovens expostos ao cloreto de mercúrio (Peixoto & Pereira, 2007; Moraes-Silva et al., 2012). Estudos *in vitro* também tem observado que o cloreto de mercúrio (Moraes-Silva et al., 2012) e o p-cloro-mercuriobenzoato (pCMB) (Vedavathi et al., 2004; Zheng et al., 2002; 2003) inibem a atividade dessa enzima e também da LDH em diferentes tecidos. O efeito inibitório sobre essas enzimas parece estar relacionado a uma interação exclusiva do mercúrio a grupos–SH dessas enzimas (Vedavathi et al., 2004; Zheng et al., 2002; 2003).

No SNC, o estresse oxidativo verificado pelo aumento nos níveis de peróxido (Franco et al., 2007b) e alterações de enzimas antioxidantes (Sumathi et al., 2012) podem ser observados como consequência da intoxicação por mercúrio. Mudanças na homeostase do cálcio (Sirois & Atchisson, 2000), glutamato (Aschner et al., 2000; Farina et al., 2003b), alterações no sistema dopaminérgico (Pereira et al., 1999) e colinérgico (Gill et al., 1990; Franciscato et al., 2009b) também foram observados. Embora o mercúrio inorgânico não atravesse facilmente a barreira hemato-encefálica (Klassen, 1996), evidências mostram que essa forma de mercúrio pode ser transportada por um ou mais transportadores de aminoácidos, particularmente da cisteína, o que pode contribuir para o seu acúmulo no cérebro (Bernhoft, 2012). O estudo dos efeitos tóxicos do mercúrio inorgânico sobre o SNC também torna-se importante quando considerado que a intoxicação por mercúrio nas suas formas elementar e orgânica resulta no acumulo de mercúrio inorgânico no cérebro (Vahter et al., 1994). Alterações comportamentais foram observadas na

prole de animais expostos a essa forma de mercúrio durante a gestação (Szász et al., 2002, Chehimi et al., 2012), através da lactação (Franco et al., 2007a) ou durante os primeiros dias de vida pós-natal (Rocha et al., 2001; Peixoto et al., 2007b; Franciscato et al., 2009b). Recentemente um estudo também demonstrou que alterações comportamentais podem ser observadas em animais adultos expostos cronicamente a baixas doses de $HgCl_2$ (Mello-Carpes et al., 2013). Alguns estudos têm observado que a exposição ao mercúrio inorgânico pode inibir a atividade da acetilcolinesterase (AChE) (Franciscato et al., 2009b, Richetti et al., 2011) provavelmente devido à ligação do mercúrio a grupos sulfidrílicos livres na enzima (Frasco et al., 2007). A AChE tem como principal papel biológico a terminação da transmissão do impulso nervoso nas sinapses colinérgicas através da hidrólise rápida do neurotransmissor acetilcolina (ACh) em colina e acetato (Figura 1) (Dvir et al., 2010). Essa enzima também está presente em altas concentrações nas junções neuromusculares (Lefkowitz et al., 1996).

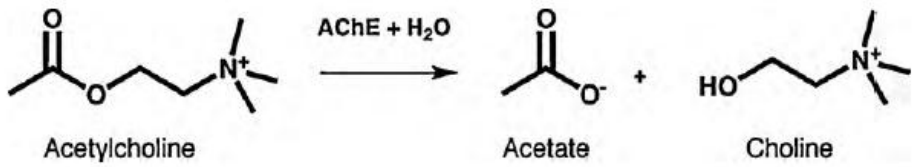

Figura 1: Hidrólise enzimática da ACh pela AChE (modificado de Dvir et al., 2010)

A transmissão do sinal neural é dependente do neurotransmissor excitatório ACh. Receptores da ACh estão localizados na membrana pós-sináptica permitindo a abertura de canais de íons Na^+ e consequente despolarização da membrana proporcionando um potencial de ação e consequente propagação do impulso nervoso. A AChE é vital nesse processo, pois sem a hidrólise da ACh o sinal neural não pode ser desligado (Bhattacgarya, 2001). Quando a atividade da AChE é inibida não ocorre hidrólise da ACh nas sinapses nervosas assim como nas junções musculares, causando um acúmulo anormal do neurotransmissor ACh e consequente

aumento do seu tempo de permanência na sinapse, o que permite novas ligações do neurotransmissor nos múltiplos receptores colinérgicos. Dessa forma o acúmulo deste neurotransmissor provoca hiperexcitação dos neurônios pós-sinápticos (Taylor, 1996).

Além dos sistemas descritos acima, enzimas sulfidrílicas tal como a δ-aminolevulinato desidratase (δ-ALA-D), também conhecida como porfobilinogênio sintase (PBG-sintase), são suscetíveis à toxicidade do mercúrio inorgânico (Rocha et al., 1995; 2001; Peixoto et al., 2003; 2007a; Franciscato et al., 2011). Os resíduos sulfidrílicos presentes na enzima tem alta afinidade por metais divalentes (Gibson et al., 1955), levando a inibição da enzima por substâncias que possuam propriedade química de oxidar grupos –SH, como por exemplo o mercúrio (Emanuelli et al., 1996; Rocha et al., 1995; Peixoto et al., 2003). Essa enzima está presente na via de biossíntese dos compostos tetrapirrólicos tais como o heme, corrinas, bilinas e clorofila (Shemin, 1976). Estes compostos são grupamentos prostéticos de proteínas, as quais desempenham importantes funções no transporte e armazenamento de oxigênio (hemoglobina e mioglobina), transporte de elétrons (citocromo a, b e c), biotransformação de xenobióticos (citrocomo P450) e reações de oxi-redução (catalase e peroxidases) (Timbrell, 1991). A reação de condensação de duas moléculas do ácido δ-aminolevulínico (δ-ALA) para formar porfobilinogênio pela enzima δ-ALA-D (Figura 2) é extremamente sensível à inibição por íons de metais tóxicos. Essa inibição é, em parte, responsável pela elevação do δ-ALA e pela anemia observada no envenenamento por chumbo (Champe et al., 2006). O acúmulo de δ-ALA pode estar relacionado com a superprodução de espécies reativas de oxigênio (Monteiro et al., 1989; Bechara et al., 1993) e a efeitos neurotóxicos (Emanueli et al. 2000; 2001).

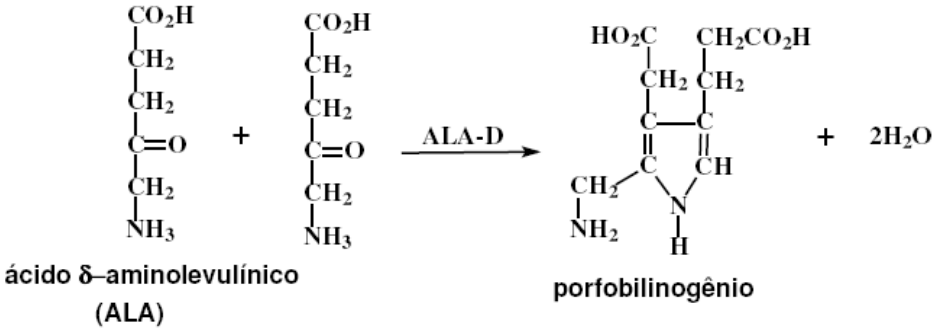

Figura 2: Condensação assimétrica de 2 moléculas do δ-ALA catalisada pela enzima δ-ALA-D (modificado de Shemin, 1976).

A sensibilidade de animais jovens a vários compostos (incluindo metais) pode diferir daquela observada em animais adultos (Jugo, 1976; Kostial et al., 1978; Walsh, 1982; Webb & Holt, 1982; Pereira et al., 1999). Animais em desenvolvimento são mais susceptíveis a agentes químicos devido à imaturidade dos órgãos e membranas e consequente incapacidade de processar adequadamente os mesmos (Nies & Spielberg, 1996). Em roedores, o período pós-natal pode ser dividido em três fases (0-6, 8-12, 17-23 dias de idade) as quais se situam entre o nascimento até o desmame. Durante esses intervalos de desenvolvimento rápido, ocorre um aumento mais pronunciado do peso da maioria dos órgãos quando comparado com as taxas de desenvolvimento que se verificam imediatamente antes e depois desses intervalos. Esse marcado desenvolvimento e crescimento são atribuídos à intensa síntese de proteínas e de DNA presentes nos órgãos (Gottlieb et al. 1977). Alguns estudos tem investigado a consequência de insultos em diferentes fases de desenvolvimento pós-natal em roedores (Sakamoto et al., 1993; Sakamoto & Nakano, 1995; Peixoto et al., 2007ab). A segunda fase parece ser mais sensível ao mercúrio, visto que os ratos apresentaram maiores alterações no peso corporal, de cérebro e de rim, inibição expressiva na atividade hepática e renal da δ-ALA-D (Peixoto et al., 2007a) além de alterações comportamentais durante as duas primeiras fases (Peixoto et al., 2007b).

Alternativas de desintoxicação tem sido estudadas, uma vez que, não existe um tratamento totalmente eficaz em casos de contaminação por esse

metal. Agentes quelantes tais como os compostos sulfidrílicos 2,3-dimercaprol (British Anti-Lewisite, BAL), a D-penicilamina, o ácido meso 2,3-demercaptosuccínico (DMSA) e o ácido 2,3-dimercapto-1-propanosulfônico (DMPS), são bastante utilizados na terapêutica contra as intoxicações por metais (Domingo, 1995). Entretanto, é bem conhecida a falta de especificidade destes agentes ao mercúrio, provocando a eliminação também de metais essenciais e a redistribuição de metais tóxicos (Domingo, 1995; Roza et al., 2005). Assim, faz-se necessário buscar alternativas de tratamento ou mesmo de prevenção dos efeitos deletérios do mercúrio principalmente às populações que se encontram expostas ambientalmente e/ou ocupacionalmente ao metal.

Existem evidências crescentes de que terapias envolvendo micronutrientes podem levar a efeitos significativos sobre a toxicidade causada por vários químicos (Peraza et al., 1998). Dados da literatura relatam o efeito terapêutico de alguns micronutrientes tais como o selênio (Magos & Webb, 1980; El-Demerdash, 2001; Farina et al., 2003a), a vitamina E (Welsh & Soares, 1976; Zaidi & Banu, 2004; Agarwal et al., 2010) e o zinco (Peixoto et al., 2003, 2007c, 2008; Franciscato et al., 2009b, 2011; Moraes-Silva et al., 2012) no tratamento da toxicidade do mercúrio.

Alguns estudos têm verificado o efeito protetor da suplementação com cobre contra estresse oxidativo em ratas prenhas e seus fetos (Enli et al., 2010) e redução da taxa de mortalidade e anemia em ovelhas e cordeiros (Peraza et al., 1998) induzidos pela exposição ao cádmio. O cobre, assim como o zinco, é um elemento traço essencial encontrado em pequenas quantidades em uma variedade de células e tecidos (Mathie et al., 2006). Esse metal essencial é um componente intrínseco do centro catalítico das enzimas pertencentes à família das oxirredutases (Schümann et al., 2002). O seu papel nas atividades enzimáticas de oxidação/redução é consequência da sua capacidade para funcionar como um intermediário na transferência de elétrons (WHO; 1998). Deste modo, ele está presente nas enzimas envolvidas na respiração celular, na defesa contra radicais livres, síntese de melanina, síntese de tecido conjuntivo, no metabolismo celular do ferro e na função de neurotransmissão (WHO, 1998; Schümann et al., 2002). Em alguns casos o cobre é necessário como cofator, como no caso da Cu/Zn-superóxido dismutase (Cu/Zn-SOD), da citrocomo-c oxidase, da ceruplasmina e da tirosina-hidroxilase. Além disso, a

atividade oxidase da ceruplasmina e da Cu/Zn-SOD requer especificamente a presença de cobre. Em outros casos, o cobre parece atuar como componente alostérico de algumas enzimas, conferindo-lhes uma estrutura apropriada para suas atividades catalíticas (Uauy et al., 1998; WHO, 1998).

Em humanos os tecidos que apresentam maiores concentrações de cobre são o fígado, cérebro, baço, osso e músculo esquelético; sendo o fígado e o baço considerado órgãos de reserva (Mason, 1979), cujas concentrações observadas tem sido inversamente proporcionais à idade (Williams, 1983). Até o final da gestação o feto poderá armazenar aproximadamente 15 mg de cobre encontrando-se no fígado cerca de 9 mg do total armazenado (Uauy et al. 1998). Após o nascimento, a concentração de cobre no fígado cai constantemente, pois a oferta de cobre a partir da dieta raramente satisfaz os requisitos para um rápido crescimento típico dessa fase (Mason, 1982; Olivares et al., 2000). Além disso, ocorre um aumento da excreção biliar aumentando as perdas fecais (Olivares et al., 2000). Em roedores as concentrações de cobre hepáticas também são dependentes da idade com altas concentrações entre o segundo e décimo segundo dia após o nascimento e subsequente declínio até a idade adulta (Manson, 1981).

Distúrbios neurológicos tem sido relatados em animais com deficiência de cobre, tais como diminuição de mielinização associada à ataxia, alterações histológicas no cérebro e no corpo estriado (Prohaska, 1990). Pesquisas também tem indicado que a deficiência de cobre na dieta de ratos ocasiona um aumento da fragilidade osmótica nos eritrócitos, sendo evidenciada também uma redução na meia vida dessas células e aumento de viscosidade por alterações na membrana (Williams, 1983).

Tanto em humanos como em roedores o cobre é absorvido principalmente no intestino delgado, e uma pequena parte, no estômago (Tapiero et al., 2003). O cobre é transportado das células intestinais até o fígado, via circulação portal, ligado principalmente à albumina e a transcupreína. A maior parte deste cobre chega rapidamente aos hepatócitos (Linder & Hazegh-Azam, 1996), tornando o fígado o maior órgão distribuidor de cobre. Após a captação hepática, o cobre pode ser armazenado no hepatócito, secretado no plasma ou excretado na bile. Apenas quando a ceruloplasmina é sintetizada e secretada no plasma se inicia uma incorporação apreciável de

cobre em outros tecidos por meio de receptores específicos, onde ocorre a liberação para o interior da célula. O cobre retido no hepatócito está ligado a proteínas ligantes de metal, principalmente metalotioneínas (MT), ou incorporado a cuproenzimas (Luza & Speisky, 1996). As MT tem sua síntese, que ocorre fundamentalmente no fígado e rins, induzida por vários fatores tais como a exposição a vários íons de metais, tal como, cobre, zinco, cádmio, mercúrio entre outros (Dunn et al., 1987; Tapiero et al., 2003). Essas metaloproteínas possuem grande afinidade por metais devido ao seu alto conteúdo de aminoácidos sulfidrílicos (Chan et al., 2002), por isso desempenham função na homeostase de metais essenciais tais como o zinco e o cobre e na destoxificação de metais não essenciais tais como o mercúrio (Hidalgo, 2001; Peixoto et al., 2003).

Estudos investigando os benefícios da exposição preventiva ao cobre contra a toxicidade do mercúrio são incomuns e estudos investigando os benefícios desses efeitos sobre estágios específicos de desenvolvimento pós-natal são raros. As consequências da exposição ao mercúrio durante o período de desenvolvimento podem ser observadas logo após (Peixoto et al., 2003, 2007abc, 2008; Peixoto & Pereira 2007) ou mesmo muito tempo depois do término da intoxicação (Franciscato et al., 2009b; 2011). Nesse contexto, considerando que o tratamento farmacológico prescrito para a intoxicação por mercúrio apresenta diversos efeitos colaterais; e que muitos estudos são realizados apenas 24 h após a intoxicação, o estudo dos efeitos preventivos do cobre, mesmo que tardios, podem ter um efeito relevante no tratamento de casos de intoxicação por mercúrio.

2. OBJETIVO

Investigar a possível ação preventiva do cloreto de cobre contra alterações bioquímicas e comportamentais induzidas pelo mercúrio.

2.1. Objetivos específicos:

Estudar os efeitos preventivos do cloreto de cobre contra os efeitos imediatos (13 dias) e tardios (33 dias) da exposição ao cloreto de mercúrio sobre:

- o desenvolvimento corporal e peso de órgãos;
- a atividade da enzima δ-ALA-D em rim, fígado, cérebro e sangue como marcadora da exposição a metais tóxicos;
- parâmetros marcadores de função renal e hepática;
- a função colinérgica através da atividade da AChE de cérebro e cerebelo;
- o desempenho comportamental em tarefas que envolvam comportamento reflexo, sensibilidade a dor, força muscular, função cerebelar e coordenação motora;
- os conteúdos de mercúrio e metais essenciais (ferro, magnésio, cobre e zinco) em diferentes tecidos;
- as concentrações de MT hepática e renal como possível mecanismo de ação na prevenção da toxicidade do mercúrio.

3. DESENVOLVIMENTO

3.1. ARTIGO 1

Effectiveness of $CuCl_2$ in protecting against alterations induced by $HgCl_2$ in newborn rats

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Effectiveness of Copper Chloride in Protecting Against Alterations Induced by Mercury Chloride in Newborn Rats

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ABSTRACT: This work investigated the effects of copper as preventive treatment against mercury-induced alterations in young rats. Wistar rats were treated (subcutaneous) with saline or $CuCl_2 \cdot 2H_2O$ (6.9 mg/kg/day) from 3 to 7 days old and with saline or $HgCl_2$ (5.0 mg/kg/day) from 8 to 12 days old. Rats were sacrificed 24 h after the last dose. Mercury-exposed rats presented inhibition of liver (43%) and kidney (52%) porphobilinogen (PBG)-synthase activity and serum lactic dehydrogenase activity (50%). Also, an increase of the serum creatinine and urea levels around threefold and fivefold was observed, respectively. Pre-exposure to copper partially prevented the mercury effect on liver but not on kidney PBG synthase, and prevented the increase of the creatinine levels. Blood and brain PBG synthase and serum alanineaminotransferase activities, as well as glycemia, and liver glycogen content were not altered by treatments. These results show that copper, although being an essential metal, is inefficient as a preventive agent against mercury poisoning in parameters investigated after the end of mercury exposure. © 2012 Wiley Periodicals, Inc. J Biochem Mol Toxicol 26:354–359, 2012; View this article online at wileyonlinelibrary.com. DOI 10:1002/jbt.21429

KEYWORDS: **Copper Chloride; Hepatic Function; Mercury Chloride; Porphobilinogen Synthase; Renal Function; Young Rats**

INTRODUCTION

Mercury, a nonessential and potent toxic metal, occurs in a variety of forms in nature. Its toxicity depends on the chemical form in which the exposure occurs [1,2]. Organic (methylmercury chloride) and metallic forms are principally neurotoxic, while the inorganic form [mercury chloride ($HgCl_2$)] is mainly nephrotoxic [3–5]. However, several studies have related other toxic effects induced by the inorganic form of mercury, such as (1) changes in body and organ weight [5,6], (2) hematological and immunological alterations [7], (3) inhibition of porphobilinogen synthase (PBG-synthase) activity of different tissues in vivo [5,6,8–10] and in vitro [8,9], (4) inhibition of cerebellum acetylcholinesterase [11], and (5) serum alanineaminotransferase (ALT) activities, and (6) increase of serum creatinine and urea levels [4,5]. Most of these alterations were verified in rats exposed to this metal during the second phase of development (from 8 to 12 days old) characterized by rapid protein, DNA and RNA synthesis [12]. This justifies why developing animals are particularly sensitive to extern insults, such as those caused by heavy metals [4–6,8–11,13–18].

Recent studies of our laboratory have demonstrated that zinc chloride protects against several biochemical changes induced in rats by mercury exposure during the second phase of development. Zinc pre-exposure prevented the inhibition of PBG-synthase activity [5,6], a cytosolic enzyme containing sulfhydryl residues that has high affinity for heavy metals and is widely distributed in different tissues [19]. The essential metal also prevented alterations in metabolic parameters related to hepatic and renal function [4,5].

In this context, copper is also an essential metal and plays several biochemical actions in the organism.

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It is a fundamental element to life [20], passes through the placenta by active transport, and is necessary for fetal development [21]. In living organisms, copper can be easily converted between different redox states, such as oxidized Cu(II) and reduced Cu(I) since this metal plays an important role in mitochondrial respiration [22]. In addition, it is used as a cofactor for a variety of metalloenzymes involved in biological processes [23] including antioxidant defense reactions [24]. Studies have indicated that copper deficiency can also modify the cholinergic system, which is involved in learning and memory [25]. This trace metal is present in several tissues, with the highest concentration found in liver [26] and normally binding to proteins. However, if copper is present in excess, it becomes free and may induce the formation of reactive hydroxyl radicals [24] causing damage to cellular components [22]. Moreover, it also shifts other metal cofactors from metalloenzymes, such as replacing zinc in zinc-finger transcription factors inactivating the proteins [23]. Recently, we verified that copper does not alter cerebrum and cerebellum acetylcholinesterase activity or the development of young rats [27]. Despite all the studies involving copper, specific studies on metabolic parameters of young rats are still scarce.

Thus, considering that copper is also an essential metal and the benefit of copper in rat puppies is unknown until now, the aim of the present study was to investigate the effects of a nontoxic dose of copper as preventive treatment against mercury-induced alterations in young rats.

MATERIALS AND METHODS

Chemicals

Copper chloride ($CuCl_2$), $HgCl_2$, and sodium chloride (NaCl); glacial acetic acid, *ortho*-phosphoric acid, nitric acid, perchloric acid; absolute ethanol; ethylic ether; and glycogen were obtained from Merck (Darmstadt, Germany); δ-aminolevulinic acid, Coomassie brilliant blue G250, and bovine serum albumin were obtained from Sigma Chemical Company (St. Louis, MO); trichloroacetic acid was obtained from Reagen; *p*-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Germany); chloride acid, ammonium sulfate, iodine, and potassium iodate (KI) were obtained from Labsynth (Diadema, Brazil); and kits for the determination of creatinine, urea, glucose, ALT, and lactic dehydrogenase (LDH) were obtained from Labtest (Lagoa Santa, Brazil).

Animals

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 were obtained from the General Animal House of the Federal University of Santa Maria, transferred to the colony room, and maintained in opaque plastic cages at room temperature (23 ± 2°C).

Treatment

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. One day after the birth, the number of pups of each litter was reduced to eight. Each litter contributed with only one *n* to each experimental group.

Copper Dose Curve

To investigate the possible preventive effect of copper on damage induced by mercury, we conducted a copper dose curve to select the dose that causes no effect per se. Three-day-old Wistar rats were submitted to copper treatment in the following doses: 6.9, 13.9, and 27.8 mg/kg/day of $CuCl_2 \cdot 2H_2O$ (2.6, 5.2, and 10.4 mg/kg/day of Cu). The percentage of survival was registered for five consecutive days (from days 3 to 7 postnatal) and for more 30 days after the end of treatment. The dose of 6.9 mg/kg/day of $CuCl_2 \cdot 2H_2O$ (2.6 mg/kg/day of Cu) was selected since the animals treated with this dose presented 100% of survival and no visual effect.

Copper and Mercury Exposure

Three-day-old Wistar rats were treated with saline (NaCl 90 mg/kg/day) or $CuCl_2 \cdot 2H_2O$ 6.9 mg/kg/day (2.6 mg/kg/day of Cu) for 5 consecutive days (from 3 to 7 days old). From 8 to 12 days old they received one daily dose of saline or $HgCl_2$ 5 mg/kg/day (3.7 mg/kg/day of Hg). The dose of $HgCl_2$ was selected according to previous studies performed with suckling rats, which demonstrated several biochemical and physiological damages induced by this dose [4–6,11,15,17].

Tissue Preparation

Twenty-four hours after the end of the treatment, the animals were weighed, sedated with ether, and euthanized by decapitation. Blood samples, liver, kidneys, and brain were quickly collected and placed on ice and weighed.

For the PBG-synthase activity determination, liver, kidneys, and brain were homogenized in 7, 5, and 3 volumes of cold (4°C) 150 mM of NaCl, respectively.

Homogenates were centrifuged at 8000 *g* for 30 min at 4°C to obtain the supernatant with the enzymatic material. Heparinized blood samples were hemolyzed in distilled water 1:4 (v/v) with agitation for 10 min in ice bath and used to enzymatic assay.

For glycogen content determination an aliquot of liver was removed, weighed, and stored at −20°C until glycogen extraction and quantitative analysis.

For other biochemical analysis the serum was obtained by centrifugation of blood at 3000 *g* for 10 min and was frozen until analysis (up to 5 days).

Biochemical Determinations

PBG-synthase activity was determined according to the method of Sassa [28] with some modifications [6,18,29]. The incubation was initiated by adding 100 µL of tissue preparation and was carried out for 120, 40, 90, and 180 min for blood, liver, kidney, and brain, respectively, at 39°C. Results were expressed as nmol of PBG formed/h/mg of protein.

Serum ALT(U/mL) and LDH (U/L) activity as well as creatinine (mg/dL), urea (mg/dL), and glucose levels (mg/dL) were determined using the commercial kit Labtest [4].

The glycogen extraction was performed as described by Peixoto and Pereira [4]. The glycogen content was determined by Krisman method [30] and expressed as g of glycogen per 100 g of tissue.

Protein concentrations in the samples were determined by the Coomassie blue method [31] using bovine serum albumin as standard. All samples were run in triplicate.

Statistical Analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Effects were considered significant at least when $p \leq 0.05$.

RESULTS

Survival Rate

Primarily, we determined the dose of copper that does not cause apparent damage to animals using the death percentage and visual inspection as parameters. After, the dose chosen was tested considering its effectiveness in preventing the biochemical mercury alterations. The percentages of survival of rats exposed to different doses of Cu are shown in Figure 1. The doses of Cu 2.6, 5.2, and 10.4 mg/kg/day induced 0%, 8.4%, and 50% of death until the end of the experimental period (30 days) of observation, respectively. The dose 2.6 mg/kg/day was chosen since the animals treated with it showed a percentage of survival similar to the control group and presented no apparent signals of toxicity.

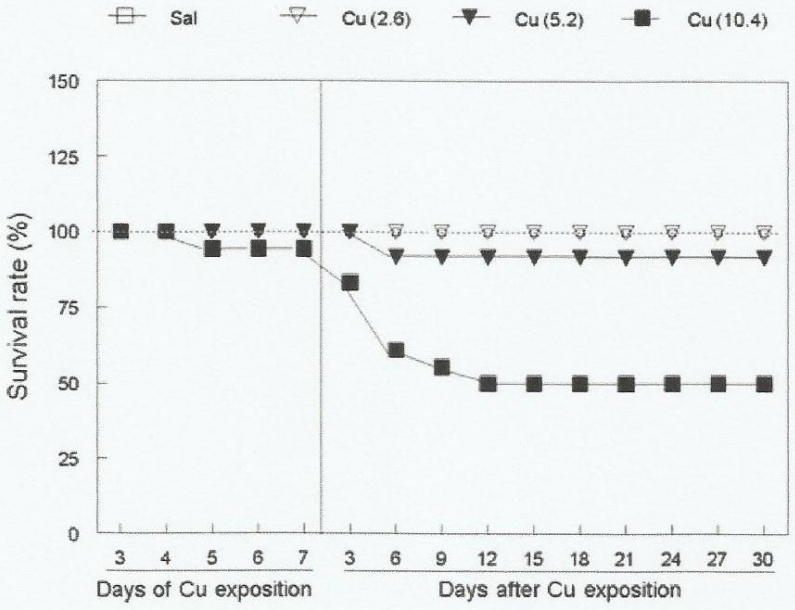

FIGURE 1. Survival rate (%) of young rats exposed to $CuCl_2 \cdot 2H_2O$. Animals were treated with Cu 2.6, 5.2, or 10.4 mg/kg or saline for 5 consecutive days (from 3 to 7 days old) and were euthanized 30 days after the end of treatment ($n = 12–18$).

TABLE 1. Body, Brain, Liver, and Kidney Weights of Young Rats Treated with $CuCl_2.2H_2O$ (Cu 2.6 mg/kg/day; s.c.) for 5 Consecutive Days and Intoxicated with $HgCl_2$ (Hg 3.7 mg/kg/day; s.c.) for 5 Subsequent Days.

	Body Weight (g) (n = 14)			*Organ Weight (g) (n = 5)*		
Treatment Groups	*3 Days Old*	*8 Days Old*	*13 Days Old*	*Brain*	*Liver*	*Kidney*
Sal–Sal	8.55 ± 0.27	16.44 ± 0.57	27.06 ± 0.87^a	1.22 ± 0.02^a	0.84 ± 0.07	0.31 ± 0.02^a
Sal–Hg	8.71 ± 0.26	16.57 ± 0.62	21.39 ± 0.73^b	1.05 ± 0.01^b	0.96 ± 0.05	0.60 ± 0.04^b
Cu–Sal	8.49 ± 0.29	16.15 ± 0.52	26.96 ± 0.77^a	1.20 ± 0.02^a	0.87 ± 0.05	0.31 ± 0.02^a
Cu–Hg	8.66 ± 0.13	16.32 ± 0.58	22.54 ± 0.53^b	1.07 ± 0.03^b	0.93 ± 0.04	0.44 ± 0.06^c

The results are presented as mean ± SEM. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p < 0.05$).

Body and Organ Weights

Body, brain, liver, and kidney weights of young rats treated with Cu (2.6 mg/kg/day; s.c.) for 5 consecutive days and with Hg (3.7 mg/kg/day; s.c.) for 5 subsequent days are shown in Table 1. One-way ANOVA revealed significant treatment effect on body weight at 13 days old (after mercury exposure) [$F(3,52) = 16.14$; $p < 0.001$] as consequence of lower body weight presented by Hg groups (Sal–Hg and Cu–Hg) when compared with other groups (Sal–Sal and Cu–Sal) ($p < 0.05$, Duncan's multiple range test).

In relation to the organ weight, the one-way ANOVA revealed a significant effect of treatment on brain [$F(3,16) = 19.51$, $p < 0.001$] and kidney [$F(3,16) = 13.68$, $p < 0.001$] but not on hepatic weight. Sal–Hg and Cu–Hg groups presented brain weight significantly lower and kidney weight significantly higher than the other groups. Copper pre-exposure prevented partially the mercury effect on renal weight (Duncan's multiple range test: $p < 0.05$).

Glycemia and Glycogen Levels

These parameters were not altered by treatments (data not shown).

PBG-Synthase Activity

The effects of the treatments on PBG-synthase activity from liver, kidney, blood, and brain are shown in Figure 2. One-way ANOVA revealed a significant effect of treatment on liver [$F(3,16) = 3.66$, $p < 0.035$] and kidney [$F(3,16) = 9.30$, $p < 0.001$], but not on brain

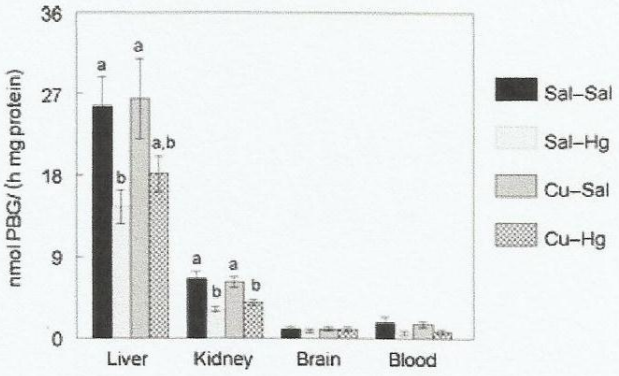

FIGURE 2. Liver, kidney, blood, and brain PBG-synthase activities of young rats treated with saline or $CuCl_2 \cdot 2H_2O$ [Cu 2.6 mg/(kg day); s.c.] from 3 to 7 days old and with saline or $HgCl_2$ [Hg 3.7 mg/(kg day); s.c.] from 8 to 12 days old. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p < 0.05$; $n = 5$).

($p = 0.881$) and blood ($p = 0.116$) enzyme activity. Mercury exposure significantly inhibited both hepatic and renal PBG-synthase activity (Duncan's multiple range test: $p < 0.05$). Cu pre-exposure partially prevented the effect of Hg on enzyme from liver but not from kidney.

Hepatic and Renal Toxicity

ALT and LDH activities and creatinine and urea levels are shown in Table 2. One-way ANOVA revealed a significant effect of treatment on LDH [$F(3,16) = 6.77$, $p < 0.004$] but not on ALT ($p = 0.215$) activity. Sal–Hg and Cu–Hg rats presented LDH activity

TABLE 2. Serum ALT and LDH Activities and Serum Creatinine and Urea Levels of Young Rats Treated as Described in Table 1

Treatment Groups	*ALT (U/mL)*	*LDH (U/mL)*	*Creatinine (mg/dL)*	*Urea (mg/dL)*
Sal–Sal	20.50 ± 3.82	1.55 ± 0.11^a	0.59 ± 0.07^a	47.06 ± 6.83^a
Sal–Hg	12.58 ± 4.48	0.82 ± 0.10^b	1.71 ± 0.38^b	244.4 ± 40.90^b
Cu–Sal	18.42 ± 2.97	1.43 ± 0.18^a	0.77 ± 0.08^a	54.34 ± 9.72^a
Cu–Hg	20.33 ± 2.03	0.90 ± 0.12^b	1.09 ± 0.10^a	189.9 ± 32.21^b

The results are presented as mean ± SEM. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p < 0.05$; $n = 5$).

significantly lower than the other groups (Duncan's multiple range test: $p < 0.05$). In relation to the creatinine and urea levels, the one-way ANOVA revealed a significant effect of treatments [$F(3,16) = 5.97$, $p < 0.006$ and $F(3,16) = 13.66$, $p < 0.001$, respectively]. Hg exposure induced a significant increase of creatinine and urea levels. Also, the copper pre-exposure prevented the Hg effect on creatinine but not on urea levels.

DISCUSSION

The aims of this work were to investigate the effects of copper on young rats and its possible preventive effects against Hg intoxication. This is important since such effects of copper on young rats are unknown, although it is an essential metal.

Mercury exposure diminished the development of animals and caused a decrease in brain weight, whereas an increase in kidney weight was observed. Cu exposure (Cu–Sal group) did not induce body and organ weight alterations and prevented partially the Hg effect on kidney but not on brain and body weights. Literature data have related this body mercury effect with its ability to induce anorexigenic effect [32]. Similar Hg effects and essential metal preventive effects were observed by Peixoto et al. [6] in rats killed 24 h after the Hg exposure and previously exposed to zinc; these effects persist even after a long time elapsed from mercury intoxication [5,11].

The enzyme PBG-synthase was chosen because it contains sulfhydryl residues, which have high affinity for heavy metals [6,19,29] and it is an important biomarker of metal effects [3,5,6,8–10,33]. Mercury-exposed rats presented liver (43%) and kidney (52%) PBG-synthase activity inhibition. These results are in agreement with other authors who showed a greater sensitivity of liver and kidney enzyme to mercury than other tissues [5,6,8], probably due to the fact that kidney and liver are the organs that more accumulate mercury [5,6,17]. The copper pre-exposure partially prevented the inhibition of PBG-synthase activity from liver, but not from kidney.

ALT and LDH activities as well as creatinine and urea levels were used as markers of hepatic and renal alterations, respectively [34,35]. Serum LDH activities were inhibited by mercury exposure in 51%. The serum creatinine presented increase around threefold and urea levels around fivefold in relation to control values. The previous exposure to copper only prevented the increase of serum creatinine but not the other metabolic parameters. Similar preventive effect on creatinine levels was obtained using zinc as pretreatment [4,5]. Moreover, zinc also prevented the increase of urea level induced by Hg [4,5]. Differently from creatinine, serum urea levels are influenced by changes in protein metabolism. Urea is formed in the liver and represents the principal endpoint product of protein catabolism [36]. In fact, the Hg-intoxicated rats presented lower growth than other groups, which can reflect the increase in the protein catabolism in an attempt to maintain the gluconeogenesis route [37].

In conclusion, exposure to copper 2.6 mg/kg/day caused no toxic effect on young rats. Its preventive effect against mercury poisoning was small since it only prevented the increase on creatinine levels and partially prevented the increase of the kidney weight and the inhibition of liver PBG-synthase activity. Thus, based on these preliminary results further studies are needed to verify the efficiency of copper using other experimental designs, such as other chemical formulation, administration via and even treatment duration.

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3.2. MANUSCRITO 1

Early and late effectiveness of $CuCl_2$ in protecting against biochemical alterations induced by $HgCl_2$ in newborn rats

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Early and late effectiveness of $CuCl_2$ in protecting against biochemical alterations induced by $HgCl_2$ in newborn rats

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Abstract

This work evaluated the effectiveness of Cu against toxic effects caused by Hg exposure and the metallothioneins (MT) participation in this protection in young rats. Wistar rats were treated subcutaneously with saline or $CuCl_2.2H_2O$ (Cu 2.6 mg/kg/day) from 3 to 7 days old and with saline or $HgCl_2$ (Hg 3.7 mg/kg/day) from 8 to 12 days old. Metallothioneins and metal contents were determined at 13- and 33-day-old, and porphobilinogen synthase (PBG-synthase) activity as well as renal and hepatic parameters at 33-day-old. Mercury exposure inhibited the renal PBG-synthase activity (33 days), increased serum urea (33 days), hepatic MT, Hg, Zn and Fe (13 days), renal Cu and Hg (13 days) and blood Hg levels (13 days); and decreased Fe (13 and 33 days) and Mg levels (33 days) in kidney. Copper pre-exposure prevented the alterations induced by Hg, even when were evaluated later (33 days), and caused an important redistribution of Hg decreasing hepatic and sanguine Hg levels and increasing renal levels of 13-day-old rats. This effect occurred in parallel with an increase in MT levels in liver and kidney. These results suggest that hepatic MT can bind to Hg and transporting this metal to the kidney in order to be excreted.

Keywords: Copper; Essential metal; Mercury; Metallothionein; Newborn rats; Renal nephrotoxicity;

1. Introduction

Mercury is a toxic element known mainly due to its effects on humans following acute or prolonged occupational exposures, as well as due to a number of environmental incidents (Holmes et al. 2009). All forms of Hg induce toxic effects, but the extension of toxicity depends on factors such as the chemical form in which the exposure occurs, the route (way) of exposure (Berlin et al. 2007), as well as the specific developing stages to which organisms are exposed (Peixoto et al. 2007b). Inorganic mercury ($HgCl_2$), one of Hg chemical forms found in the environment, is primarily nephrotoxic (Goyer 1995). Moreover, studies have shown that young rats exposed subcutaneously to $HgCl_2$ presented biochemical (Peixoto et al. 2003, 2007ac; Peixoto and Pereira 2007) and behavioral changes (Peixoto et al. 2007b; Franciscato et al. 2009b; Moraes-Silva et al. 2014). The toxicity of inorganic Hg forms is, at least in part, explained by the great affinity for SH groups biomolecules (Goyer 1995). The consequence of these effects is the inhibition of sulfhydryl- containing enzymes such as porphobilinogen synthase (PBG-synthase) (Rocha et al. 1995, 2001; Peixoto et al. 2003, 2007a; Franciscato et al. 2011) which can be used as useful biomarker of metals (Bernard and Lauwerys 1987) and organochalcogen (Ineu et al. 2012) exposition and/or intoxication.

Research have been developed in an attempt to find new alternatives of treatment for Hg poisoning, since there is only limited therapeutic for these cases. A number of chelating agents have been used as recommended procedure in cases of Hg intoxication, increasing the excretion of this toxic metal (Risher and Amler 2005). However, its use has been questioned because they also promote redistribution of Hg and remove essential metals (Domingo 1995). Thus, there is a clear need for the development of more effective treatment protocols not only for victims of Hg poisoning, but also as prevention for the population occupationally exposed.

An alternative in the treatment of metal poisoning has been the use of micronutrients such as Zn. Recent studies from our research team demonstrated that $ZnCl_2$ pre-exposure prevents several biochemical changes induced by $HgCl_2$ in rats exposed during the second phase of postnatal development (8 to 12 days). This essential metal, for example, prevented the inhibition of PBG-synthase activity (Peixoto et al. 2003; Franciscato et al. 2011)

and alterations in metabolite parameters related to the hepatic and renal functions (Peixoto and Pereira 2007; Franciscato et al. 2011). In fact, micronutrients interact with toxic metals at several points in the body and the toxic effects may be potentiated by the trace-element metabolic status of the exposed animal (Peraza et al. 1998) especially in developing animals in which some metals are required in higher quantities due to rapid growth and development (Nies and Spielberg 1996). The most common deficiencies involve Fe, Zn and Cu (Brody 1994; Rivera et al. 2003).

Copper is also an essential trace metal fundamental to life and it is found in all living organisms (Tapiero et al. 2003). This metal is an integral part of many important enzymes involved in a number of vital processes required for growth, development, and maintenance of the nervous system (Mathie et al. 2006). Copper passes through placenta by active transport, is necessary for fetal development (McArdle et al. 2008), and has an important role in enzymes involved in mitochondrial respiration (Tapiero et al. 2003), antioxidant defense and catecholamine synthesis (Mathie et al. 2006). Furthermore, this metal is used as a co-factor for a variety of metalloenzymes involved in biological processes (Bertinato and L'Abbe 2004).

Studies investigating the benefits of preventive Cu exposition on Hg toxicity are uncommon, and studies regarding these effects on specific postnatal stages of development are even rare. Some studies have reported that Cu supplementation may protect against the Cd-induced oxidative stress in pregnant rats and fetuses (Enli et al. 2010) and to reduce the mortality rate and severity of anemia in ewes and lambs (Peraza 1998). We have verified that Cu can prevent increase in creatinine levels and the inhibition of hepatic PBG-synthase activity induced by Hg in 13-day-old rats (Moraes-Silva et al. 2012a), as well as prevent behavioral alterations induced by $HgCl_2$ (Moraes-Silva et al. 2014). However, it is important to verify the efficiency of Cu on other parameters and to clarify the mechanism of protection involved.

Thus, considering that Cu is an essential metal and is involved in several biochemical actions in organism, the objective of this study was to investigate the possible effectiveness of Cu as preventive treatment against biochemical alterations induced by Hg exposure in young rats. Moreover, we studied if metallothioneins (MT) are produced as a consequence of Cu exposure and if

this protein participates in the mechanism of protection induced by Cu treatment.

2. Materials and Methods

2.1. Chemicals

Glacial acetic acid, *ortho*-phosphoric acid, perchloric acid, absolute ethanol, $CuCl_2$, $HgCl_2$, NaCl, K_2HPO_4, KH_2PO_4 and HNO_3, phenylmethylsulphonylfluoride (PMSF), chloroform, calcium disodium ethylenediaminetetraacetate (EDTA), 5,5'-dithio-bis(2-nitrobenzoico acid) (DTNB), and L-cysteine hydrochloride monohydrate were obtained from Merck (Darmstadt/Germany); δ-aminolevulinic acid (δ-ALA), Coomassie brilliant blue G250 and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA); trichloroacetic acid (TCA) was obtained from Reagen (Colombo, PR, Brazil); *ρ*-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han, Germany); and kits for the determination of creatinine, urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were obtained from Labtest (Lagoa Santa, MG, Brazil).

2.2. Animals

Wistar pregnant rats were obtained from the General Animal House of the Federal University of Santa Maria, transferred to the colony room, and maintained in opaque plastic cages at room temperature (23 ± 2°C). One day after birth, the number of pups of each litter was reduced to 8 to avoid undernutrition due to the number of teats. Males and females were used randomly. Each litter contributed with only one *n* to each experimental group. 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.

2.3. Exposures

Pups were pre-treated from postnatal days 3 to 7 with one daily dose of saline (NaCl 90 mg/kg/day) or $CuCl_2.2H_2O$ 6.9 mg/kg/day (2.6 mg/kg/day of Cu). After the pre-treatment, the animals received saline or $HgCl_2$ 5 mg/kg/day (3.7 mg/kg/day of Hg) for 5 consecutive days (from 8 to 12 days old). This dose

of $CuCl_2$ $2H_2O$ was chosen because it does not cause apparent damage to animals and prevents some toxic effects induced by Hg exposure (Franciscato et al. 2009a; Moraes-Silva et al. 2012a). The dose of $HgCl_2$ was selected according to previous studies performed with suckling rats which demonstrated several biochemical, behavioral and physiological damages (Peixoto et al. 2003, 2007c, 2008; Peixoto and Pereira 2007; Franciscato et al. 2009b, 2011; Moraes-Silva et al. 2012ab). 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. Litters were killed at 13 or 33 days old (24 h or 21 days after the end of Hg exposure) to MT and metal content determination. PBG-synthase activity and renal and hepatic parameters were determined in 33 day-old rats.

2.4. Tissue preparation

Twenty four hours or twenty one days after the end of Hg exposure (13- or 33-day-old rats) the animals were weighed and killed by decapitation. Portions of each organ were used for the determination of the enzymatic activity and for the analysis of MT and metal contents. The PBG-synthase and ALT and AST activities as well as urea and creatinine levels were determined in 33-day-old rats. For the PBG-synthase activity determination, the liver and kidneys were homogenized in 7 and 5 volumes of cold (4°C) 150 mM of NaCl, respectively. Homogenates were centrifuged at 8,000 x *g* for 30 min at 4°C to obtain the supernatant with the enzymatic material. Heparinized blood samples were hemolyzed in distilled water 1:4 (v/v) with agitation for 10 min in ice bath and used to enzymatic assay (Peixoto et al. 2003, 2004). For other biochemical analysis (urea, creatinine, ALT and AST) the serum was obtained by centrifugation of blood at 3,000x*g* for 10 min and was frozen until analysis (up to 5 days) (Peixoto and Pereira 2007). Both metals and MT contents were determined in tissues of 13- and 33-day-old rats. For metal content determination, a portion of liver, kidney, and blood (0.2 mL) was removed and frozen at -20 ºC until analysis of metal contents. For MT assay, liver and kidney were homogenized in 4 volumes of 20 mM Tris-HCl buffer, pH 8.6, containing 0.5 mM PMSF as agent antiproteolytic and 0.01% β-mercaptoethanol as

reducing agent. The homogenate was then centrifuged at 17,000 x g for 30 min to obtain a supernatant containing MT (Peixoto et al. 2003).

2.5. Biochemical Determinations

2.5.1. PBG-synthase activity

PBG-synthase activity was determined according to the method of Sassa (1982) with some modifications (Peixoto et al. 2003). The incubation was initiated by adding 200 μL of tissue preparation and was carried out for 120, 40 and 90 min for blood, liver and kidney, respectively, at 39°C. The reaction product was determined using a modified Ehrlich´s reagent at 555 nm with a molar absorption coefficient of 6.1 x 10^4 for the Ehrlich-porphobilinogen salt. Results were expressed as nmol of PBG formed/hour/mg of protein.

2.5.2. Hepatic and renal metabolic parameters

Serum alanine aminotransferase (ALT, U/mL) and aspartate aminotransferase (AST, U/mL) activity as well as creatinine (mg/dL) and urea (mg/dL) levels were determined as described in Peixoto and Pereira (2007) using the commercial kit Labtest.

2.5.3. Protein determination

Protein concentrations were determined by the Coomassie blue method (Bradford 1976) using bovine serum albumin as a standard.

2.6. Metallothionein content

Metallothionein content was assayed as described in Peixoto et al. (2003) utilizing a partially purified metalloprotein fraction obtained by ethanol/chloroform fractionation of the tissue homogenate. Aliquots of 1 ml of supernatant were added with 1.05 ml of cold (-20 ºC) absolute ethanol and 80 μL of chloroform; the samples were then centrifuged at 6000 x g for 10 min. The collected supernatant was combined with three volumes of cold ethanol (-20 ºC), maintained at – 20 ºC for 1 h and centrifuged at 6000 x g for 10 min. The metallothionein-containing pellets were then rinsed with 87% ethanol and 1% of chloroform and centrifuged at 6000 x g for 10 min. The metallothionein content in the pellet was evaluated using the colorimetric method with Ellman’s reagent

(Ellman 1958). Metallothionein concentration was estimated utilizing cysteine as a reference standard and expressed as µg of SH/g of wet weight.

2.7. *Metal content determination*

Hg, Cu, Zn, Fe and Mg contents were determined by inductively coupled plasma atomic emission spectrometry (ICPE-9000; Shimadzu Scientific Instruments). The digestion of samples and the determination of metal content were conducted as described in detail by Prohaska et al (2000) with some modifications (Ineu et al. 2013). Samples were digested with concentrated HNO_3 in an overnight water bath (100 ºC). After digestion, samples were diluted with deionized water to 25 mL and transferred to graduated poly-propylene vials and determined by ICPE-9000. The analytical standard Hg, Cu, Zn, Fe and Mg (Merck®) was used to make the curve and the results were expressed as µg/g of tissue.

2.8. Statistical Analysis

Results were analyzed by one-way ANOVA followed by Duncan's multiple range test. Effects were considered significant at least when $p \leq 0.05$. Each litter contributed with only one *n* for each experimental group in order to avoid the litter effect (Abbey and Howard 1973).

3. Results

3.1. Body, liver and kidney weight

One-way ANOVA revealed a significant effect of the treatment on body [$F(3,52)=16.03$, $p<0.001$], liver [$F(3,52)=16.11$, $p<0.001$] and kidney weight [$F(3,52)=5.37$, $p<0.003$] (Figure 1). Hg-exposed rats presented body and liver weights lower than the control group whereas kidney weight was higher. Copper pre-exposure avoided the Hg effect on body and organ weight (Duncan's multiple range test: $p<0.05$).

3.2. PBG-synthase Activity

PBG-synthase activity from liver, kidney and blood of 33-day-old rats are shown in Figure 2. The enzyme activity from kidney [$F(3,52)=5.27$, $p<0.003$],

but not from other tissues, was significantly inhibited by Hg. Copper pre-exposure totally prevented Hg effect (Duncan's multiple range test: $p<0.05$).

3.3. Renal and hepatic metabolic parameters

Urea and creatinine levels as well as ALT and AST activities from 33 day-old rats are shown in Table 1. Urea [$F(3,48)=7.39$, $p<0.001$], but not creatinine levels, was significantly increased by Hg exposure and Cu pre-exposure prevented this effect (Duncan's multiple range test: $p<0.05$). Although the Sal-Hg group presented serum creatinine levels 55% higher than the control group, this difference was not significant ($p=0.303$). ALT and AST activities were not modified by Hg or Cu exposure.

3.4. Metallothionein content

Table 2 shows the MT levels in liver and kidney. Both hepatic [$F(3,12)=24.54$, $p<0.001$] and renal [$F(3,12)=4.56$, $p<0.024$] MT contents from 13 day-old rats were increased by treatments. Liver from Hg groups presented an increase of MT content in relation to the control group (Sal-Hg, 150%; Cu-Hg, 104%). Kidney from Cu-Hg group presented higher MT content than Sal-Sal group (36%). For 33-day-old rats, MT levels were not altered in the liver and kidney as consequence of any treatment.

3.5. Mercury and essential element content

3.5.1. Mercury content

Blood, liver and kidney Hg contents are shown in Table 3. Tissues which presented absence of Hg were not included in the statistical analysis (liver from Sal-Sal and Cu-Sal and kidney from Cu-Sal groups of 13-day-old rats; liver from Sal-Sal, Cu-Sal and Cu-Hg, kidney from Sal-Sal and Cu-Sal groups of 33-day-old rats). The presence of Hg in the blood from Sal-Sal and Cu-Sal groups at 13 and 33 days, and in the kidney from Sal-Sal group at 13 days may be consequence of a small contamination since animals of all treatments were in the same home box (each litter contained two rats for each treatment). For 13-day-old rats, the Hg exposed rats for both Hg groups presented significant higher Hg content than the other groups [blood, $F(3,8)=47.38$, $p<0.001$; kidney, $F(3,9)=17.95$, $p<0.001$; liver (not included in the statistical analysis) (see

above)]. Copper pre-exposure partially decreased the blood Hg levels and increased the renal Hg levels. For 33-day-old rats, an increase of Hg content was detected in liver from Sal-Hg group and in kidney from Sal-Hg and Cu-Hg groups.

3.5.2. Zinc content

Blood, liver and kidney Zn content are shown in Table 3. Only liver Zn content of 13-day-old rats presented significant effect of treatments [$F(3,12)=8.40$, $p<0.003$]. Both groups exposed to Hg (Sal-Hg and Cu-Hg) presented higher Zn levels than the other groups.

3.5.3. Copper content

For 13-day-old rats, Cu levels in liver and kidney were significantly altered by treatments [$F(3,12)=30.46$, $p<0.001$ and $F(3,12)=9.77$, $p<0.002$, respectively]. Higher Cu levels in the liver of Cu-Sal and Cu-Hg rats and in the kidney of Cu-Hg rats than the other groups were verified. Copper levels of 33-day-old rats were not altered as consequence of any treatment (Table 3).

3.5.4. Iron content

Liver and kidney Fe contents from 13-day-old rats (Table 4) were significantly altered by treatments [$F(3,12)=5.26$, $p<0.015$; $F(3,12)=4.87$, $p<0.019$, respectively]. Both Hg exposed groups presented an increase of Fe contents in liver; and Cu pre-treatment partially prevented this effect. For kidney, all metal groups presented lower Fe levels than the Sal-Sal group (Duncan's multiple range test: $p<0.05$). For 33-day-old rats, the Hg exposure decreased kidney Fe contents [$F(3,8)=0.12$, $p<0.008$] and Cu pre-treatment partially prevented the Hg effect (Duncan's multiple range test: $p<0.05$).

3.5.5. Magnesium content

Liver and kidney Mg contents are shown in Table 4. Only kidney Mg content of 33-day-old rats presented significant effect of treatments [$F(3,8)=20.13$, $p<0.001$]. Both Hg groups presented lower Mg levels than the other groups, although Cu pre-treatment partially prevented the effect of Hg on renal tissue (Duncan's multiple range test: $p<0.05$).

4. Discussion

This research investigated the effectiveness of Cu as a preventive treatment against biochemical alterations induced by Hg exposure as well as Hg and Cu effects on essential metal levels.

The results of this work demonstrate that Hg exposure reduced body and liver weight, increased kidney weight, inhibited renal PBG-synthase activity (30%), and increased urea (138%) and creatinine (55%, not significant) levels in 33-day-old rats. These alterations agree with other works from our research group that show hepatic and renal $HgCl_2$ toxicity in both adults and young rats (Peixoto et al. 2003; Peixoto and Pereira 2007; Franciscato et al. 2011; Moraes-Silva et al. 2012a; Favero et al. 2014; Oliveira et al. 2014b). The new findings in this paper is that the previous exposure to Cu (3 to 7 postnatal days) prevented essential metal homeostasis and biochemical alterations caused by $HgCl_2$ exposure in newborn rats, even when these parameters were evaluated several days after the end of exposure.

Several studies have shown that the Hg exposure promotes Hg accumulation in different organs (Peixoto et al. 2008; Franciscato et al. 2011; Favero et al. 2014; Oliveira et al. 2014a; Oliveira et al. 2014b). In our study, 24h after Hg exposure the Sal-Hg group presented high hepatic Hg levels followed by kidneys and blood. However, 21 days after Hg exposition, kidney presented higher Hg levels than liver and both organs presented a decrease in Hg levels when compared to levels presented 24h after the end of exposure. This result shows that after the end of exposure, Hg is naturally excreted from the body; however is important to note, that even with the Hg levels diminished the biochemical alterations remain. On the other hand, Cu pre-exposure (Cu-Hg group) caused an important Hg redistribution, decreasing liver and blood Hg levels and increasing renal Hg levels 24h after Hg exposure. Interesting, Moraes-Silva et al. (2012a) showed that Cu pretreatment prevented the increase in serum creatinine levels 24h after $HgCl_2$ exposure.

Glutathione and MT, two important intracellular thiols, appear to be involved in regulation the renal accumulation of mercury and, ultimately, the susceptibility to mercury-induced renal cellular injury (Zalups 2000). Zalups and Cherian (1992ab) demonstrated that the induction of renal MT is associated

with increased renal accumulation of mercury and decreased severity of the nephropathy induced by both organic and inorganic mercury.

Metallothioneins (MT) are sulfhydrylic protein which can sequester toxic metals like Cd and Hg and reduce their cellular toxicity (Cherian and Nordberg, 1983). The MT synthesis is induced by several factors, including essential and toxic metals (Pedersen et al. 1998; Peixoto et al. 2003, 2007c; Irato and Albergone 2005). Indeed, our results suggest that the increase in hepatic MT in 13 days old rats is a consequence of hepatic Hg and Zn retention (see below) caused by Hg exposure. Moreover, the redistribution of Hg as consequence of Cu exposure infers that the Cu together with MT is involved in the mechanism of Hg elimination. That way, we suggested that Hg redistribution is related to the formation of non-toxic metallothionein-Hg (MT-Hg) complex in liver which is transported to the kidney, and after it is excreted (Chan et al. 1993). The liver and kidney MT levels presented no alteration at 33 days probably due to the low levels of Hg found in these tissues and homeostasis of Zn hepatic levels.

During the first days of postnatal life the nutrients, for example, essential metals, are required in high quantities due to increase in cell division to promote the rapid growth and development (Nies and Spielberg 1996). Indeed, we observed that, independently of treatment, both hepatic Zn and Cu levels at 33 days were lower than those observed at 13 days regardless of treatment. These results are in agreement with Mason (1982) who found that the content of these metals in liver from Wistar rats were age-dependent with maximum increase of Zn and Cu concentration around day 2 and 12 after birth, respectively, and subsequently declining until adulthood. Moreover, animals pre-exposed to Cu (Cu-Sal and Cu-Hg groups) presented a pronounced increase of Cu levels in liver (~265%, at 13 days). This effect is probably related to the fact that liver is the first site of Cu deposition after it enters the blood, and may be stored within hepatocytes, secreted into plasma, or excreted in bile (Gaetke and Chow 2003). Twenty-one days after the end of Hg exposure the Cu-Hg group still presented Cu levels about 3.6 fold higher than the control group. Renal Cu levels also presented increased in Cu-Hg group at 13-day-old. This Cu accumulation in renal and hepatic tissues appears not to be toxic since Cu did not alter the renal and hepatic markers both at 33-day-old (Table 1) and 13-day-old (Moraes-Silva et al. 2012a).

The exposure to toxic agents can promote alterations in essential metals homeostasis (Peixoto et al. 2008; Feng et al. 2004), mainly during the first days of life, because the organs and membranes are in development (Nies and Spielberg 1996). In this way, hepatic Zn levels increased in 13 day-old-rats as a result of $HgCl_2$ exposure, showing that the Hg present in the liver caused changes in the Zn homeostasis. Induction of hepatic MT synthesis, in some cases, has been attributed to enhanced hepatic uptake of Zn (Brady and Bunger 1979; Goering et al. 1985).

Hg exposure also caused change in Fe and Mg homeostasis. A hepatic increase at 13 days and a renal decrease of Fe levels in the rats exposed to Hg at 13 and 33 days was observed. Copper administration partially avoided the increase of hepatic and decrease of renal Fe levels at 13 and 33 days, respectively. Though Fe is indispensable for life, when present in excess can cause tissues damage and organ failure (Papanikolaou and Pantopoulos 2005). Regarding to Mg, Cu pretreatment prevented the decrease in hepatic and renal Mg levels induced by Hg exposure 21 days after the end of exposure. This preventive effect is important since Mg is a cofactor of several enzymes (Brody 1994). The deficiency of this essential metal has been suggested as a contributor to the prevalence cardiovascular disease, skeletal disorders and hypertension (Vormann 2003; Sontia and Touyz 2007). We inferred that this alteration in Fe and Mg homeostasis contributed to Hg mechanism of toxicity, however this result call for more research.

In summary, the effectiveness of Cu as a preventive treatment against the toxic effects caused by Hg exposure was observed in parameters such as content of Hg and homeostasis of Fe at 13 days. The preventive effect of Cu on various parameters such as body and organ weight, PBG-synthase activity, urea levels, and homeostasis of Mg levels was observed later, at 33 days. This is an important aspect, since in most of the studies these parameters are analyzed 24 h after Hg exposure. Copper pre-exposure caused no alterations in levels of essential metals (Cu, Zn, Fe, and Mg) and therefore appears to be an effective method for victims of Hg poisoning when compared with the chelating agents, which have a low metal selectivity. Furthermore, the present study suggests that the protective effect of Cu may be related to an increase in the

renal MT levels accompanied by Hg sequestration in hepatic tissue for the kidney as a consequence of Cu previous exposure.

Conflicts of interests

The authors declare that there are no conflicts of interest.

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Legends for figures

Figure 1. Body, liver and kidney weights of 33-day-old rats treated (s.c.) with saline or $CuCl_2.2H_2O$ (Cu 2.6 mg/kg/day; s.c.) from 3 to 7 days old and with saline or $HgCl_2$ (Hg 3.7 mg/kg/day; s.c.) from 8 to 12 days old. Results are presented as mean ± S.E.M. (*n*=14). Duncan's multiple range test: different letters confer significant statistical difference between groups ($p<0.05$).

Figure 2. Liver, kidney and blood PBG-synthase activities of 33-day-old rats treated as described in the caption of Figure 1. Results are presented as mean ± S.E.M. (*n*=14). Duncan's multiple range test: different letters confer significant statistical difference between groups ($p<0.05$).

Table 1. Serum urea and creatinine levels and ALT and AST activity of 33-day-old rats treated as described in the caption of Figure 1.

Treatment	Urea (mg/dL)	Creatinine (mg/dL)	ALT (U/mL)	AST (U/mL)
Sal-Sal	48.30 ± 2.53[a]	0.69 ± 0.19	71.0 ± 10.0	145.7 ± 25.8
Sal-Hg	115.0 ± 20.4[b]	1.07 ± 0.26	75.0 ± 13.6	123.3 ± 18.2
Cu-Sal	53.01 ± 5.62[a]	0.62 ± 0.13	63.4 ± 5.20	127.0 ± 15.3
Cu-Hg	69.19 ± 6.87[a]	0.65 ± 0.15	68.3 ± 6.35	125.5 ± 21.8

The results are presented as mean ± S.E.M. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p<0.05$; n=8-13).

Table 2. Metallothionein content (MT) in liver and kidney of 13- and 33-day-old rats treated as described in the caption of Figure 1.

Treatments	MT levels (mg of SH/g of tissue)			
	13 days old		33 days old	
	Hepatic	Renal	Hepatic	Renal
Sal-Sal	0.138 ± 0.02 [a]	0.105 ± 0.008 [a]	0.177 ± 0.04	0.167 ± 0.03
Sal-Hg	0.346 ± 0.01 [b]	0.125 ± 0.004 [ab]	0.172 ± 0.05	0.136 ± 0.02
Cu-Sal	0.183 ± 0.01 [a]	0.127 ± 0.010 [ab]	0.161 ± 0.03	0.167 ± 0.02
Cu-Hg	0.282 ± 0.02 [c]	0.143 ± 0.005 [b]	0.127 ± 0.02	0.173 ± 0.03

The results are presented as mean ± S.E.M. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p<0.05$; $n=3$-4).

Table 3. Mercury, copper and zinc levels in blood, liver and kidney of 13- and 33-day-old rats treated as described in the caption of Figure 1.

Treatments	Hg levels (µg/g of tissue)			Cu levels (µg/g of tissue)			Zn levels (µg/g of tissue)		
	Blood	Liver	Kidney	Blood	Liver	Kidney	Blood	Liver	Kidney
13-old-day rats									
Sal-Sal	1.7 ± 0.3 [a]	N.D	2.6 ± 2.4 [a]	3.8 ± 1.7	41.4 ± 11.2[a]	0.1 ± 0.1 [a]	6.3 ± 0.70	24.7 ± 3.9[a]	17.2 ± 2.8
Sal-Hg	11.3 ± 1.2 [b]	73.7 ± 8.1	19.9 ± 3.9 [b]	3.0 ± 0.4	29.6 ± 10.7 [a]	1.9 ± 0.9 [a]	5.7 ± 1.05	46.7 ± 2.5 [b]	16.9 ± 1.3
Cu-Sal	1.6 ± 0.1 [a]	N.D.	N.D.	2.8 ± 0.3	152.0 ± 8.1 [b]	0.02 ± 0.02 [a]	6.0 ± 0.57	28.3 ± 3.9 [a]	15.5 ± 2.6
Cu-Hg	4.6 ± 0.4 [c]	60.8 ± 6.5	32.2 ± 3.9 [c]	2.2 ± 0.5	147.8 ± 16.4 [b]	4.9 ± 1.2 [b]	5.2 ± 1.51	47.8 ± 5.8 [b]	17.7 ± 1.1
33-old-day rats									
Sal-Sal	1.8 ± 0.2	N.D.	N.D.	3.0 ± 0.5	3.7 ± 1.3	0.02 ± 0.01	6.5 ± 1.4	19.8 ± 0.9	12.8 ± 1.4
Sal-Hg	2.2 ± 0.1	0.5 ± 0.4	2.0 ± 0.9	2.7 ± 0.2	7.6 ± 3.2	0.3 ± 0.3	5.1 ± 0.6	18.7 ± 0.8	12.9 ± 3.1
Cu-Sal	1.6 ± 0.1	N.D.	N.D.	2.6 ± 0.4	9.8 ± 5.6	0.6 ± 0.3	5.8 ± 1.3	20.0 ± 2.0	13.4 ± 1.4
Cu-Hg	1.4 ± 0.3	N.D.	5.9 ± 1.0	2.1 ± 0.7	13.6 ± 5.2	2.5 ± 1.3	4.5 ± 1.5	18.8 ± 0.5	12.6 ± 2.5

The results are presented as mean ± S.E.M. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p<0.05$; n=3-4).

N.D.: not detected.

Table 4. Iron and magnesium levels in liver and kidney of 13- and 33-day-old rats treated as described in the caption of Figure 1.

Treatments	Fe levels (µg/g of tissue)		Mg levels (µg/g of tissue)	
	Liver	Kidney	Liver	Kidney
13-old-day rats				
Sal-Sal	55.6 ± 15.2 [a]	22.9 ± 1.6 [a]	204.4 ± 13.1	174.4 ± 30.9
Sal-Hg	92.5 ± 4.1 [b]	14.2 ± 0.8 [b]	182.8 ± 4.7	175.4 ± 40.0
Cu-Sal	46.9 ± 8.0 [a]	16.5 ± 2.4 [b]	189.9 ± 3.1	152.3 ± 25.1
Cu-Hg	77.2 ± 3.5 [ab]	17.1 ± 1.6 [b]	192.5 ± 8.2	162.6 ± 10.5
33-old-day rats				
Sal-Sal	48.1 ± 8.5	24.2 ± 1.9 [a]	200.1 ± 4.1	173.8 ± 4.4 [a]
Sal-Hg	48.8 ± 2.8	8.6 ± 2.4 [b]	187.7± 10.3	111.0 ± 10.1 [b]
Cu-Sal	54.9 ± 14.7	22.4 ± 3.5 [a]	217.3 ± 3.7	166.4 ± 5.0 [a]
Cu-Hg	49.5 ± 6.1	14.3 ± 1.9 [c]	184.2 ± 7.2	143.6 ± 3.3 [c]

The results are presented as mean ± S.E.M. Duncan's multiple range test: different letters confer significant statistical difference among groups ($p<0.05$; n=3-4).

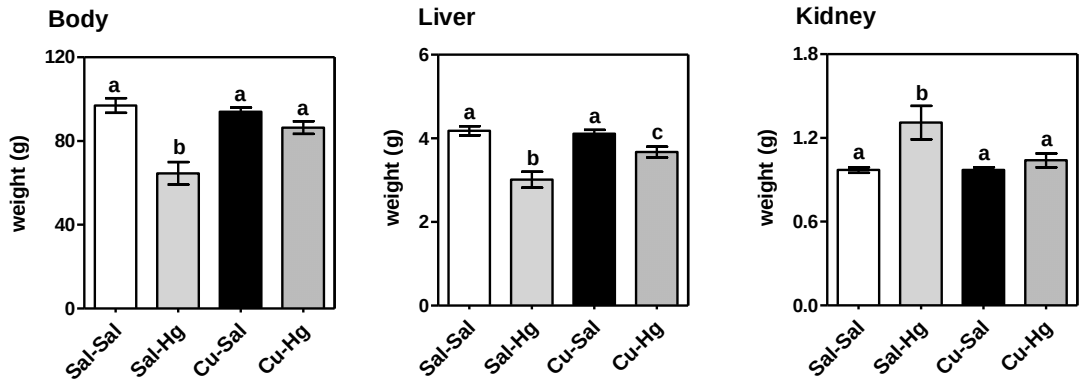
Body
Liver
Kidney
weight (g)
120
80
40
0
6
4
2
0
1.8
1.2
0.6
0.0
a
b
a
a
a
b
a
c
a
b
a
a
Sal-Sal
Sal-Hg
Cu-Sal
Cu-Hg

Figure 1

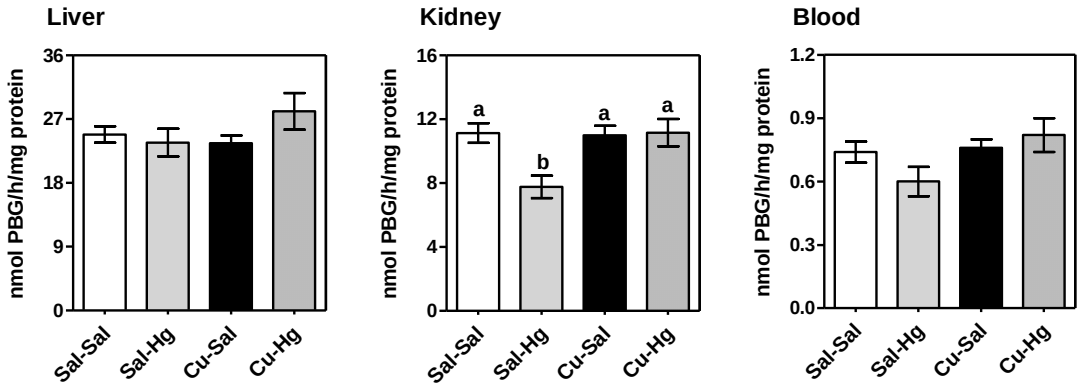
Liver
Kidney
Blood
nmol PBG/h/mg protein
36
27
18
9
0
16
12
8
4
1.2
0.9
0.6
0.3
0.0
a
b
a
a
Sal-Sal
Sal-Hg
Cu-Sal
Cu-Hg

Figure 2

3.3. ARTIGO 2

Preventive effect of $CuCl_2$ on behavioral alterations and mercury accumulation in central nervous system induced by $HgCl_2$ in newborn rats

Lucélia Moraes-Silva, Lucieli Flores de Siqueira, Vitor Antunes de Oliveira, Cláudia Sirlene de Oliveira, Rafael Porto Ineu, Taíse Fonseca Pedroso, Mariana Mesquita Fonseca, Maria Ester Pereira

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Preventive Effect of $CuCl_2$ on Behavioral Alterations and Mercury Accumulation in Central Nervous System Induced by $HgCl_2$ in Newborn Rats

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ABSTRACT: This study investigated the benefits of Cu preexposition on Hg effects on behavioral tests, acetylcholinesterase (AChE) activity and Hg, and essential metal contents in the cerebrum and cerebellum of neonate rats. Wistar rats received (subcutaneous) saline or $CuCl_2 \cdot 2H_2O$ (6.9 mg/kg/day) when they were 3 to 7 days old and saline or $HgCl_2$ (5.0 mg/kg/day) when they were 8 to 12 days old. Mercury exposure reduced the performance of rats in the negative geotaxis (3–13 days) and beaker test (17–20 days), inhibited cerebellum AChE activity (13 days), increased cerebrum and cerebellum Hg (13 days), cerebrum Cu (13 days), and cerebrum and cerebellum Zn levels (33 days). The performance of rats in the tail immersion and rotarod tests as well as Fe and Mg levels were not altered by treatments. Copper prevented all alterations induced by mercury. These results are important to open a new perspective of prevention and/or therapy for mercury exposure. © 2014 Wiley Periodicals, Inc. J. Biochem. Mol. Toxicol. 00:1–8, 2014; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.21569

KEYWORDS: **Copper; Mercury; Essential Metal; Newborn Rats; Motor Activity; Neurotoxicity**

INTRODUCTION

It is well known that Hg compounds can lead to irreversible neuropsychological deficits and emotional disturbances in children [1]. The central nervous system (CNS) is extremely sensitive to injury from toxic agents applied during ontogeny of the developmental process because it is a critical period for the formation of the basic circuit of the nervous system, while in the adults the CNS already has a developed blood–brain barrier to give much more protection [2].

The different varieties of Hg (elemental mercury vapor, inorganic mercury salts, and organic mercury) differ with respect to their behavior in the environment as well as with respect to their potential to interact with biological processes [3]. Inorganic mercury ($HgCl_2$) is primarily nephrotoxic [4–6]; however, its effect on CNS during early periods of development is well documented. In fact, we recently demonstrated that $HgCl_2$ induces behavioral impairment and accumulation of this metal in the cerebrum and cerebellum [7–9].

Toxic damage to the nervous system can alter the synthesis and release of neurotransmitters, which may be associated with behavioral changes [10]. The cholinergic neurotransmission in the CNS plays an essential role in modulating the cognitive process such as learning, memory, arousal and sleep, as well as in modulating locomotor activity [11]. The appropriate synaptic transmission of the cholinergic system is partially controlled by the acetylcholinesterase (AChE) enzyme (E.C. 3.1.1.7). This enzyme catalyzes the hydrolysis of neurotransmitter acetylcholine, thereby interrupting cholinergic activity in the synapse [12]. Inhibition of AChE activity is widely used as a specific biomarker for organophosphorus and carbamates pesticides [13], and studies have linked AChE inhibition with behavioral alterations using mammalian [14] and aquatic species [15]. More recently, some studies on mercury [9] and cadmium [16] toxicity in the brain of rats have

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demonstrated a decrease in AChE activity and behavioral impairment have also been observed.

Studies investigating the benefits of preventive Cu exposition on Hg toxicity are uncommon, and studies investigating these effects on specific postnatal stages of development are rare. We have already verified that Cu can prevent some of the metabolic damage caused by Hg in young rats such as an increase in serum creatinine [17]. Copper is an essential metal and plays several biochemical actions in the organism. This metal is an integral part of many important enzymes involved in a number of vital processes required for growth, development, and maintenance of the nervous system [18]. Studies have indicated that Cu deficiency can also modify the cholinergic system, which is involved in learning and memory [19]. This trace metal is present in low levels in several tissues, with the highest concentration found in the liver [20].

In the present investigation, we examined the effectiveness of Cu as a preventive treatment against behavioral alterations induced by $HgCl_2$ exposure in young rats. The AChE activity and levels of Hg and essential metals in the cerebrum and cerebellum were determined at the end of Hg exposure (in 13-day-old rats) and after behavioral studies (in 33-day-old rats).

MATERIALS AND METHODS

Chemicals

Reagents used were obtained from Sigma (St. Louis, MO) and standard commercial suppliers.

Animals

Wistar pregnant rats were obtained from the General Animal House of the Federal University of Santa Maria, Brazil, transferred to the colony room, and maintained in opaque plastic cages at room temperature (23 ± 2°C). One day after birth, the number of pups of each litter was reduced to eight to avoid undernutrition due to the number of teats. Males and females were used randomly. Each litter contributed to only one *n* to each experimental group. 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.

Exposures

Pups were pretreated from the postnatal day 3–7 with a daily subcutaneous (s.c.) injection of saline (NaCl 90 mg/kg/day) or $CuCl_2 \cdot 2H_2O$ 6.9 mg/kg/day (2.6 mg/kg/day of Cu). After the pretreatment, animals received (s.c.) saline (NaCl 90 mg/kg/day) or $HgCl_2$ 5 mg/kg/day (3.7 mg/kg/day of Hg) for 5 more consecutive days (when they were 8 to 12 days old). Figure 1 shows the experimental layout and timeline. The dose of $CuCl_2$ was chosen because it did not cause apparent damage to animals and prevent some toxic effects induced by Hg exposure [17, 21]. The dose of $HgCl_2$ was selected according to previous studies performed with suckling rats [5, 9, 17, 22–26]. Animals were weighed daily to adjust the dose and were

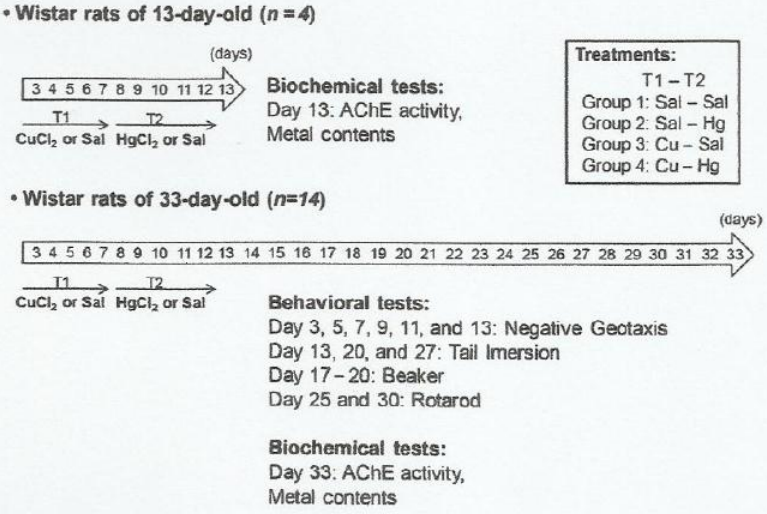

FIGURE 1. Experimental design: young rats were treated with saline or $CuCl_2 \cdot 2H_2O$ (Cu 2.6 mg/kg/day; s.c.) from 3 to 7 days old (T1) and with saline or $HgCl_2$ (Hg 3.7 mg/kg/day; s.c.) from 8 to 12 days old (T2). Litters were killed when 13 or 33 days old (24 h or 21 days after the end of Hg exposure). Four litters were used for the determination of AChE activity and metal levels when 13 days old. Fourteen litters were used for behavioral tasks and, at 33 days old, were used for the determination of AChE activity and metal levels.

administered with a constant volume of 10 mL/kg body weight. Behavioral tests were performed between the days 3 and 27. Each litter contained two rats for each treatment. For all behavior tests (negative geotaxis, tail immersion, beaker, and rotarod tests), the experimental n is the mean of the performance of two animals with the same treatment per litter. For both AChE and metal analyses, each litter contributed to only one rat of each treatment (experimental n). It was not possible to conduct a blind study because rats treated with Hg presented alopecia at the site of the injection. Litters were killed when they were 13 or 33 days old (24 h or 21 days after the end of Hg exposure). Litters whose animals of the same treatment died were not used for enzymatic and behavioral experiments.

Behavioral Tests

The negative geotaxis task was conducted on 3, 5, 7, 9, 11, and 13 days old rats and was carried out on a platform with 30 cm of length, 20 cm of breaker, and with an inclination of 30°. On each trial, the rat was placed head downward on the inclined plane and its latency to turn 180° to an upright position was recorded in seconds. This negative geotaxis reaction (reflex) was stimulated by the abnormal position of the head and body, initiated by vestibular and postural systems, and required organized motor movement for successful completion [27]. The maximum latency for the reflex of negative geotaxis was 60 s for each session. Each trial consisted of the mean latency of five consecutive sessions. The trials were made before solution administration [9]. The decrease in latency of negative geotaxis reflex was considered as the improvement of the motor reflex response [28].

Nociception was assessed in the tail immersion test as described by Franciscato et al. [9]. Rats were wrapped in a towel and 3.5 cm of tail was immersed in a water bath (48°C). The time needed for the animal to deflect the tail was used as latency immersion. A cutoff time of 10 s was used to avoid tail tissue damage. The rats were submitted to test when they were 13, 20, and 27 days old (24 h, 8 and 15 days after the end of mercury exposure), and three sessions per day with an interval of 10 s between the sessions were performed.

In the beaker test, the ability of rats to balance on and move along the rim of the 2-L polypropylene beaker was observed from 17 to 20 days (5–8 days after the end of mercury exposure) (sessions from 1 to 4) with an interval of 24 h between the sessions. 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 farthest distance from it [8]. Time points to reach the refuge, fall, or jump off the rim were recorded. On each occasion when the animal fell or jumped off the rim, the timer was stopped. The animal was placed in the original position, and the timer was started again. Whenever the animal entered the refuge box, the timer was stopped and recorded finalizing the session. A cutoff time of 90 s was used for each session. The results are presented as a mean of latency to access to refuge and as a percentage of fall per litter per treatment [29].

The rotarod test was carried out as described by Franciscato et al. [9]. In this test, two parameters were evaluated: latency to first fall and the number of falls in a total of 240 s [30]. Twenty-four hours before the test, the rats were submitted to run in the rotarod for 60 s to avoid the novelty effect. The rats were submitted to test at 25 and 30 days (13 and 18 days after the end of mercury exposure).

Tissue Preparation

One (13-day-old) or 21 (33-day-old) days after the last administered dose, the animals were weighed and killed by decapitation. For the determination of the enzymatic activity and analysis of metal contents, the brain was removed and the cerebrum and cerebellum were separated. The 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 activity was determined in tissues of 13- to 33-day-old rats.

For the metal content determination, the cerebrum and cerebellum from 13- to 33-day-old rats were removed and frozen at −20 °C until analysis.

Enzyme Assay

AChE activity was determined by the method of Ellman et al. [31], modified as described by Pereira et al. [32]. The tissue preparation was preincubated for 2 min at 30°C. The product from the reaction of thiocholine with 5-5′-dithiobisnitrobenzoic acid (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. Protein concentrations were determined by the Coomassie blue method [33] using bovine serum albumin as a standard.

Metal Content Determination

The content of Hg, Cu, Zn, Fe, and Mg was determined by inductively coupled plasma atomic emission spectrometry (ICPE-9000; Shimadzu, Tokyo, Japan). The digestion of samples and the determination of metal content were conducted as described in detail by Prohaska et al. [34] with some modifications [35]. Samples were digested with concentrated HNO_3 in a water bath (100°C) overnight. After digestion, samples were diluted with deionized water to 25 mL and transferred to graduated polypropylene vials and determined by an ICPE-9000 spectrometer. The analytical standard Hg, Cu, Zn, Fe, and Mg (Merck, Darmstadt, Germany) was used to make the curve, and the results were expressed as $\mu g/g$ of tissue.

Statistical Analysis

Results were analyzed by one- or two-way analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's *t*-test when appropriate. The effects were considered significant at least when $p \leq 0.05$. Each litter contributed to only one *n* for each experimental group to avoid the litter effect [36].

RESULTS

Behavioral Tests

In the negative geotaxis task, the treatment did not interfere with the appearance of the reflex since all the treatment groups presented a similar negative geotaxis reaction (reflex) until session 4 (9 days). However, the Sal–Hg group presented higher latency (11 and 13 days) than the other groups to perform the complete negative geotaxis task (Figure 2). The two-way ANOVA (four treatments × six sessions) showed a significant effect on session [$F(5,260) = 505.34$, $p < 0.001$] and treatment × sessions interaction [$F(15,260) = 2.50$, $p < 0.002$]. The interaction was due to the fact that although all groups presented an increase in the performance during the sessions, the Sal–Hg group presented higher latency than the other groups to perform the complete negative geotaxis in both sessions 5 and 6 (24 h after the third and fifth dose of mercury) {one-way ANOVA from sessions 5 [$F(3,52) = 6.95$, $p < 0.001$] and 6 [$F(3,52) = 19.30$, $p < 0.001$]}. The Cu pretreatment totally prevented the impairment of the performance induced by mercury exposure.

Analysis of tail immersion latency (one-way ANOVA) revealed no differences between groups (data not shown).

For the beaker test, we found that Hg-exposed rats presented worse performance than other treatment groups (Table 1). The two-way ANOVA (four treatments × four sessions) revealed a significant effect of treatment and session on latency to access to refuge [$F(3,52) = 12.68$, $p < 0.001$ and $F(3,156) = 122.82$, $p < 0.001$, respectively], and a significant effect of session on the fall percentage [$F(3,156) = 8.06$, $p < 0.001$]. The treatment was significant because the animals exposed to Hg alone presented higher latency to refuge

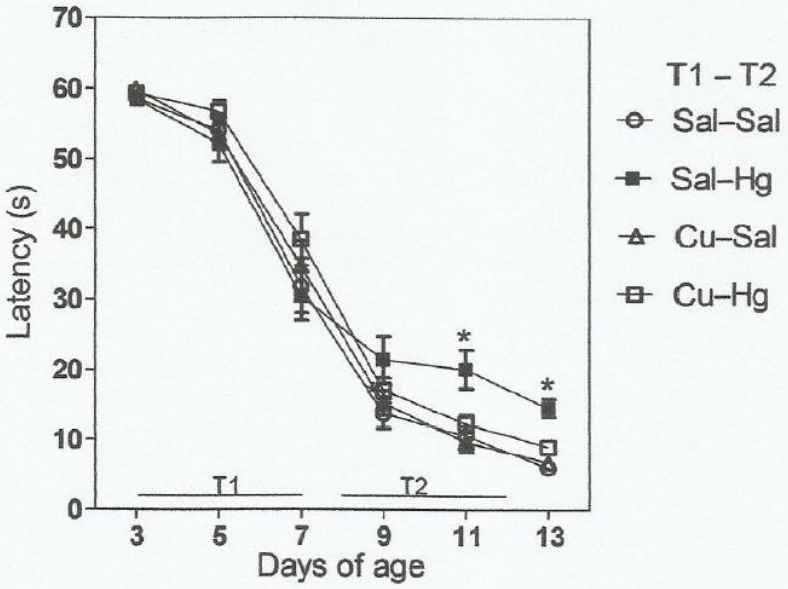

FIGURE 2. Latency of negative geotaxis reflex of rats treated as described in the caption of Figure 1 and submitted to test at days 3, 5, 7, 9, 11, and 13. The results are presented as mean ± SEM ($n = 14$ per treatment group per session). Duncan's multiple range test: *significant difference from other groups ($p \leq 0.05$).

TABLE 1. Latency of Access to Refuge and Fall Percentage in the Beaker Test of Young Rats Treated as Described in the Caption of Figure 1 and Submitted to Test from Days 17 to 20

Animal	*Treatment*	*Session 1*	*Session 2*	*Session 3*	*Session 4*
			Latency of access to refuge (s)		
($n = 14$)	Sal–Sal	52.5 ± 4.0[a]	34.6 ± 5.1[a]	18.3 ± 2.6[a]	11.8 ± 1.2[a]
	Sal–Hg	81.0 ± 3.8[b]	74.1 ± 5.1[b]	57.1 ± 6.7[b]	47.6 ± 7.3[b]
	Cu–Sal	52.5 ± 4.2[a]	37.8 ± 5.0[a]	22.2 ± 3.8[a]	16.2 ± 2.9[a]
	Cu–Hg	59.7 ± 6.7[a]	46.5 ± 7.0[a]	30.9 ± 6.8[a]	22.6 ± 6.4[a]
			Fall percentage		
	Sal–Sal	21.4 ± 10.1	17.9 ± 6.6	7.1 ± 4.8[a]	0.0 ± 0.0
	Sal–Hg	22.9 ± 7.4	38.4 ± 7.7	30.0 ± 7.3[b]	16.8 ± 6.3
	Cu–Sal	32.1 ± 8.5	21.4 ± 8.6	7.1 ± 4.8[a]	7.1 ± 4.8
	Cu–Hg	32.1 ± 8.5	32.1 ± 11.2	25.0 ± 10.1[ab]	6.8 ± 10.1

The results are presented as mean ± SEM. Duncan's multiple range test: different letters confer a significant statistical differences among groups ($p < 0.05$; $n = 14$ per treatment group per session).

access than other treatment groups. In fact, the one-way ANOVA showed a significant effect of treatment in all (1–4) sessions of latency [$F(3,52) = 7.71, p < 0.001$; $F(3,52) = 10.13, p < 0.001$; $F(3,52) = 10.88, p < 0.001$; and $F(3,52) = 9.85, p < 0.001$, respectively]. This effect was totally prevented by the pretreatment with Cu. In relation to the fall percentage, one-way ANOVA revealed that this parameter was significantly altered by metals in session 3 [$F(3,52) = 2.79, p < 0.05$]. The Hg-intoxicated rats presented an increase of 4.2-fold in the fall percentage when compared to the control group, and Cu exposure partially prevented the Hg effect.

The rotarod test revealed an improvement in the motor performance at all treatments. Two-way ANOVA (four treatments × two sessions) revealed a significant effect on the session of latency to the first fall [$F(1,52) = 42.87, p < 0.001$] and on the number of fall [$F(1,52) = 13.04, p < 0.001$]. The treatments did not alter the two parameters studied in this test (data not shown).

AChE Activity

AChE activity from the cerebrum and cerebellum of 13-day-old rats presented slightly inhibited by Hg exposure (37% and 43% lower than the control group, respectively); however, Student's t-test revealed that the inhibition only was significant for the enzyme from the cerebellum [$t(6) = 2.81, p < 0.031$] of rats exposed to Hg (the Sal–Hg group) when compared to the Sal–Sal group (Figure 3). The Cu–Hg group did not differ from the Sal–Sal group in this tissue [$t(6) = 0.67, p = 0.53$]. Enzyme activity from tissues of 33-day-old rats was not altered by the treatments (one-way ANOVA).

Hg and Essential Elements Content

The content of Hg, Cu, Zn, Fe, and Mg in the cerebrum and cerebellum is presented in Table 2. Tissues that presented the absence of the Hg or Cu content were not included in the statistical analysis. Rats exposed only to mercury (Sal–Hg) presented an elevated Hg level in the both cerebrum and cerebellum in 13-day-old rats (24 h after Hg exposition), whereas it was not elevated in the Cu–Hg group. Mercury levels in 33-day-old rats were not altered as a consequence of any treatment.

The Cu content in the cerebellum of 13- and 33-day-old rats was slightly elevated in the Cu–Hg group and in the cerebrum of 13-day-old rats of the Sal–Hg group.

The Zn content was altered by treatment only at 33 days [one-way ANOVA: cerebrum, $F(3,8) = 3.96$, $p < 0.05$; cerebellum $F(3,8) = 3.33$, $p < 0.04$, respectively]. The Sal–Hg group presented Zn levels slightly more elevated than the saline group (Duncan's multiple range test: $p < 0.05$), and Cu exposure prevented partially the Hg effect.

Fe and Mg content was not altered in the cerebrum and cerebellum as a consequence of any treatment.

DISCUSSION

Behavioral tasks, metals determination, and AChE activity were conducted to investigate whether the alterations in these parameters induced by Hg could be prevented by preexposure to Cu.

Studies have reported that the exposure to $HgCl_2$ is able to induce behavioral alterations during brain developmental periods [7, 9, 37, 38] and, more recently, in adult rats [39]. In the present study, utilizing the two behavioral tests, we verified that the motor function, muscular strength and size, and cerebellar function were damaged in young rats exposed to Hg. In addition, we observed ameliorative effects of Cu treatment against motor impartment induced by Hg exposure.

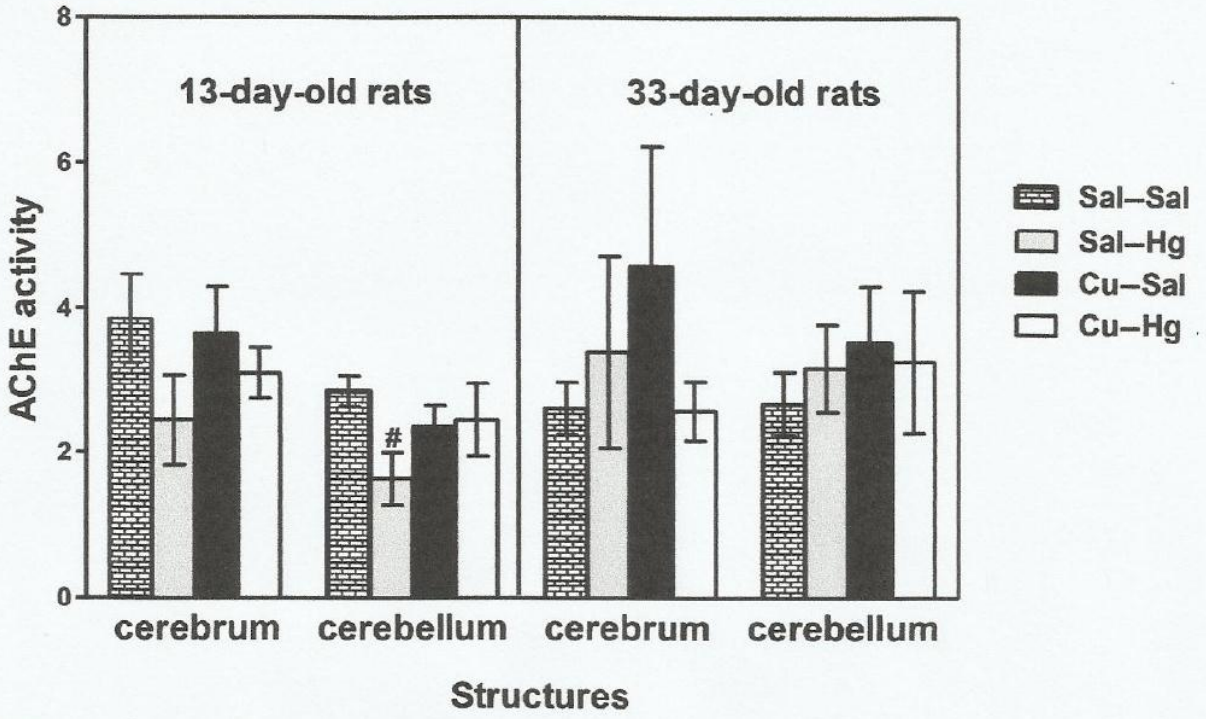

FIGURE 3. Cerebrum and cerebellum AChE activities from 13 and 33 days old rats treated as described in the caption of Figure 1. The specific activity was expressed as μmol ATC hydrolyzed/h/mg protein (mean ± SEM, $n = 4$–6 per treatment group); Student's t-test: $^{\#}p < 0.05$ differs from the Sal–Sal group.

TABLE 2. Mercury, Copper, Zinc, Iron and Magnesium Levels in Cerebrum and Cerebellum of 13- and 33-Day-Old Rats Treated as Described in the Caption of Figure 1

	Hg Level (μg/g of Tissue)		*Cu Level (μg/g of Tissue)*		*Zn Level (μg/g of Tissue)*		*Fe Level (μg/g of Tissue)*		*Mg Level (μg/g of Tissue)*	
Treatment	*Cerebrum*	*Cerebellum*	*Cerebrum*	*Cerebellum*	*Cerebrum*	*Cerebellum*	*Cerebrum*	*Cerebellum*	*Cerebrum*	*Cerebellum*
13-Day-old rats ($n = 3$)										
Sal–Sal	ND	ND	ND	ND	9.0 ± 0.2	9.3 ± 1.2	13.0 ± 1.5	12.6 ± 1.7	137.2 ± 2.8	131.3 ± 5.4
Sal–Hg	0.4 ± 0.3	1.3 ± 0.7	0.1 ± 0.1	ND	9.4 ± 0.2	9.9 ± 1.3	15.8 ± 1.4	14.9 ± 1.4	132.9 ± 5.9	134.2 ± 25.6
Cu–Sal	ND	ND	ND	ND	8.3 ± 0.1	7.9 ± 0.3	16.1 ± 4.7	16.5 ± 3.0	137.1 ± 2.4	128.4 ± 5.1
Cu–Hg	ND	ND	ND	2.2 ± 2.0	9.2 ± 0.8	9.3 ± 1.3	12.1 ± 1.9	17.2 ± 2.3	125.2 ± 3.0	112.4 ± 11.9
33-Day-old rats ($n = 3$)										
Sal–Sal	ND	ND	ND	ND	9.8 ± 0.7^{a}	7.7 ± 0.2^{a}	14.7 ± 3.6	18.5 ± 4.1	132.1 ± 11.9	127.6 ± 9.4
Sal–Hg	ND	ND	ND	ND	11.7 ± 0.3^{b}	11.1 ± 1.0^{b}	19.3 ± 2.1	20.2 ± 4.8	135.0 ± 4.6	132.9 ± 4.7
Cu–Sal	ND	ND	ND	ND	10.4 ± 0.3^{ab}	9.3 ± 0.3^{ab}	14.1 ± 0.6	13.0 ± 0.8	133.2 ± 5.5	133.5 ± 1.1
Cu–Hg	ND	ND	0.2 ± 0.2	0.1 ± 0.03	11.1 ± 0.3^{ab}	9.7 ± 0.8^{ab}	15.7 ± 1.7	16.9 ± 0.82	130.1 ± 4.3	128.5 ± 14.7

Abbreviation: ND, not detected.
The results are presented as mean ± SEM. Duncan's multiple range test: different letters confer a significant statistical difference among groups ($p \leq 0.05$; $n = 3$ per treatment group).

This study also investigated the effects of Hg on the AChE activity, an important enzyme used as an index of the cholinergic function [12]. Literature data have shown that cholinesterase activity is sensitive to Hg poisoning because AChE activity in the CNS is related to inhibition of both $HgCl_2$ [9, 40] and metylmercury [41] exposure. In the case of the inorganic form, behavioral impairment was accompanied by cerebellar AChE inhibition [9]. Newly here the inhibition on AChE activity from 13-day-old rats was totally prevented by Cu preexposition. This effect can be related, at least in part, to behavioral impairment (balance and motor force) observed in rats exposed to mercury, because the cerebellum is the brain structure most critically involved in reflex adjustments and acquisition of fine and sequential motor control [42].

Mercury exposure promoted deposition of this metal mainly in the cerebellum followed by the cerebrum in 13-day-old rats. The higher deposition of Hg in the cerebellum (3.2-fold) when compared to the cerebrum of Hg-treated rats accompanied the motor impairment [43] and cerebellum AChE activity inhibition [9], suggesting that the level of Hg accumulation may directly be associated with the neurotoxic effect of Hg. This hypothesis is reinforced by the fact that Cu treatment completely prevented both motor impartment and AChE inhibition activity in parallel to the reduction of the cerebellar Hg level.

The essential metals imbalance can be detrimental to the CNS contributing to pathoetiology of several neurodegenerative disorders [44]. However, the increase in Cu levels in cerebrum (13-day-old rats) and cerebellum (13- and 33-day-old rats) of Cu–Hg rats appears not to be neurotoxic because these exposed groups did not present any alterations in the parameters evaluated. In fact, Cu preexposure totally prevented the behavioral alterations induced by mercury. The Zn content in the cerebrum and cerebellum (33-day-old) was altered by Sal–Hg treatment. Alterations in zinc homeostasis are linked to impairment of neuronal metabolism through inhibition of key enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase [44], and AChE [45]. However, in this study, 33-day-old Hg-exposed rats presented no alterations on AChE activity in the cerebrum and cerebellum.

It has been previously reported that Cu supplementation may protect against the Cd-induced oxidative stress in pregnant rats and fetuses [46] and reduce the mortality rate and severity of anemia in ewes and lambs [47]. However, studies investigating the benefits of preventive Cu exposition on Hg toxicity are uncommon. We have verified that Cu can prevent some damage induced by Hg [17], such as an increase in the creatinine levels and inhibition of PBG-synthase activity in the liver. The present study adds more data on benefits of Cu preventive exposition on Hg toxicity, and these are important to open up a new perspective of prevention and/or therapy for mercury exposure.

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4. DISCUSSÃO

Animais expostos a insultos externos durante o período de desenvolvimento podem ser mais vulneráveis em relação àqueles que já passaram por essa fase (Rice & Barone, 2000). Estudos tem demonstrado os vários efeitos tóxicos relacionados à exposição ao mercúrio inorgânico ($HgCl_2$) em ratos neonatos (Peixoto et al., 2003, 2007abc, 2008; Peixoto & Pereira 2007; Franciscato et al., 2009b, 2011; Moraes-Silva et al., 2012). A forma mais efetiva de tratamento contra intoxicação por metais ainda consiste na utilização de agentes quelantes que ao se ligarem aos metais tóxicos facilitariam sua eliminação (Vamnes et al., 2003; Syversen & Kaur, 2012). Entretanto, é bem conhecida a falta de especificidade destes agentes ao mercúrio, provocando a eliminação também de metais essenciais e a redistribuição de metais tóxicos a outros órgãos (Domingo, 1995; Rooney, 2007). Dessa forma faz-se necessário buscar alternativas de tratamento ou mesmo de prevenção dos efeitos deletérios do mercúrio considerando que existem populações ocupacional e ambientalmente expostas. Considerando as informações acima descritas este trabalho investigou a ação preventiva do $CuCl_2$ sobre alterações bioquímicas e comportamentais induzidas pela exposição ao $HgCl_2$ em ratos jovens.

O cobre é um micronutriente requerido por todos os organismos vivos e é utilizado como cofator para uma variedade de metaloenzimas envolvidas nos mais diversos processos biológicos. Contudo, quando em excesso pode atuar como um pró-oxidante, favorecendo a geração de radicais livres com subsequente dano celular (Vulpe & Packamn, 1995 *apud* Morgan et al., 2004). No presente estudo, primeiramente nós determinamos a dose de cobre que não causa danos aparentes aos animais. As doses de cobre de 2,6, 5,2 e 10,4 mg/kg/dia foram administradas durante cinco dias (do 3º ao 7º dia de idade) e induziram 0%, 8,4% e 50% de mortes, respectivamente, até o fim do período experimental (30 dias). A dose de 2.6 mg/kg/dia foi escolhida uma vez que a mesma apresentou 0% de mortes e nenhuma alteração por inspeção visual. Esta dose também não afetou o ganho de peso corporal, os pesos de cérebro e

cerebelo, nem a atividade da AChE nesses tecidos 24h após exposição (Franciscato et al., 2009a).

Os rins são considerados alvos primários da intoxicação por mercúrio inorgânico (Clarkson et al., 2003). Alterações histológicas (Stacchiotti et al., 2003) bem como aumento dos níveis de uréia e creatinina são descritos na literatura (Peixoto & Pereira 2007; Franciscato et al., 2011; Favero et al., 2014). Embora o fígado não seja definido como o órgão mais sensível as alterações por mercúrio, estudos tem revelado que a exposição ao metal tóxico pode causar aumento na atividade de enzimas marcadoras de hepatotoxicidade (Kumar et al., 2005; Peixoto & Pereira, 2007; Bashandy et al., 2011; Oliveira et al., 2014). Alterações relacionadas ao metabolismo primário como a diminuição dos níveis de glicose (Peixoto & Pereira, 2007), além do aumento na atividade gliconeogênica no fígado também foram observados (Moraes-Silva et al., 2012). Mercúrio causa alterações na regulação do apetite (Counter & Buchanan, 2004) e alterações no peso corporal bem como no peso de órgãos (Rocha et al., 2001; Emanuelli et al., 1996; Peixoto et al., 2003; Franciscato et al., 2011; Moraes-Silva et al., 2012). A inibição da atividade da δ-ALA-D, uma importante enzima sulfidrílica marcadora da exposição a metais, também ocorre após a exposição de metais divalentes tal como o $HgCl_2$ (Rocha et al., 1995; Emanuelli, 1996; Peixoto et al., 2003; Franciscato et al., 2011). Entre os compostos de mercúrio, o metilmercúrio é considerado o principal responsável por alterações neurológica em humanos e animais experimentais (Azevedo et al., 2012). Entretanto, vários estudos tem apontado o $HgCl_2$ como indutor de danos ao SNC de ratos neonatos (Rocha te al., 2001; Peixoto et al., 2007; Franciscato et al., 2009b).

No presente estudo nós verificamos que a exposição a uma dose (s.c.) diária de 5,0 mg/kg de $HgCl_2$ (equivalente a 3,7 mg/kg de Hg^{2+}) durante cinco dias consecutivos (do 8º ao 12º dia de idade) causou várias alterações verificadas 24 h ou 21 dias após a administração da última dose de mercúrio (aos 13 ou 33 dias de idade).

A exposição ao mercúrio alterou o desenvolvimento uma vez que os animais apresentaram menor peso corporal e peso de cérebro e um aumento no peso de rim aos 13 dias de idade. A persistência dos efeitos foi verificada sobre o peso corporal e peso de rim além de um decréscimo no peso de fígado

aos 33 dias, isto é, 21 dias após o término da exposição. Um decréscimo na atividade da δ-ALA-D renal e hepática também foi observado enquanto a atividade da enzima de cérebro e sangue não foi alterada aos 13 dias de idade. O resultado sobre a atividade da δ-ALA-D renal persistiu após 21 dias da exposição ao mercúrio. A função renal, avaliada pelo aumento da creatinina e uréia sérica foi alterada aos 13 dias de idade, bem como aos 33 dias, sobre os níveis de uréia. Quanto à função hepática, verificada pela atividade das enzimas séricas ALT, LDH e AST, observou-se uma diminuição na atividade da LDH aos 13 dias de idade. Contudo a inibição na atividade dessas enzimas não tem expressão clínica, visto que a hepatotoxicidade é caracterizada pela elevação de suas atividades e não pela diminuição (Champe et al., 2006).

O efeito anorexígeno do mercúrio (Counter & Buchanan, 2004) pode, pelo menos em parte, explicar o menor peso corporal, e este justificar o menor peso de cérebro e fígado dos animais expostos. Já o aumento no peso e tamanho de rim pode estar relacionado a alterações estruturais neste órgão causadas pelo mercúrio, como o aumento de volume dos túbulos proximais (Madsen & Maunsbach, 1981). A inibição da atividade da δ-ALA-D tem sido relacionada aos efeitos pró-oxidantes do mercúrio nos grupos sulfidrílicos essenciais localizados no sítio ativo da enzima (Emanuelli et al., 1996). O dano renal observado neste estudo pelo aumento dos níveis de ureia e creatinina é um efeito comumente associado à exposição ao mercúrio inorgânico devido a sua ação nefrotóxica (Zalups & Lash, 1994).

A prévia exposição a uma dose (s.c.) diária de 6,9 mg/kg de $CuCl_2.2H_2O$ (equivalente a 2,6 mg/kg de Cu) por cinco dias consecutivos (do 3º ao 7º dia de idade) preveniu várias alterações verificadas 24 h ou 21 dias após a administração da última dose de mercúrio (aos 13 ou 33 dias de idade).

Os parâmetros avaliados 24 h após a exposição ao mercúrio mostram que a prévia exposição ao cobre preveniu parcialmente o aumento do peso renal, a inibição da atividade da δ-ALA-D hepática e totalmente o aumento nos níveis de creatinina. Já aos 33 dias o cobre preveniu a perda de peso corporal, o menor peso de fígado e o aumento de peso renal. Também foi possível observar o efeito preventivo do cobre sobre a inibição da atividade da δ-ALA-D

renal e sobre o aumento dos níveis de uréia sérica, os quais não foram prevenidos aos 13 dias de idade.

A exposição ao $HgCl_2$ causou um aumento nos níveis deste metal no fígado, rim e sangue aos 13 e 33 dias de idade e no cérebro e cerebelo aos 13 dias. O tecido hepático apresentou níveis de mercúrio mais elevados em relação aos demais tecidos aos 13 dias, enquanto que aos 33 dias, os níveis renais desse metal tóxico foram maiores que no fígado, porém reduzidos em comparação aos níveis encontrados aos 13 dias. Ainda, diferentemente do que ocorrem aos 13 dias, os níveis de mercúrio no sangue foram similares entre todos os grupos e não detectados em cérebro e cerebelo aos 33 dias. Os resultados referentes aos níveis de mercúrio em diferentes tecidos confirmam os relatos sobre o mercúrio inorgânico o qual é caracterizado pela sua distribuição muito heterogênea, contudo presente principalmente no fígado e rins (WHO, 1991; Peixoto et al., 2007c).

Os animais previamente expostos ao cobre (grupo Cu-Hg) apresentaram diferente distribuição de mercúrio nos tecidos. Os animais tratados com cobre apresentaram menores níveis hepáticos e maiores níveis renais aos 13 dias de idade. Ainda observamos que, embora a prévia exposição ao cobre tenha contribuído para um aumento nos níveis renais de mercúrio, esse efeito não parece ser nefrotóxico, desde que os animais apresentaram atenuação de efeitos deletérios do mercúrio. Dois importantes tiois intracelulares, glutationa e MT parecem estar envolvidos na regulação do acúmulo de mercúrio renal e, em última instância, a suscetibilidade a lesão celular induzida por esse metal (Zalups, 2000). Estudos tem documentado que o aumento da MT renal está relacionado com o aumento de mercúrio nesse tecido e diminuição da gravidade da nefropatia induzida por mercúrio (Zalups & Cherian, 1992ab; Peixoto et al., 2003). De fato, MT são proteínas sulfidrílicas as quais podem sequestar metais tóxicos como Cd e Hg e reduzir a toxicidade desses (Cherian & Nordberg, 1983). A síntese destas proteínas, que ocorre primariamente no fígado, pode ser induzida por vários fatores tais como a exposição a vários íons de metais, tal como, cobre, zinco, cádmio, mercúrio entre outros (Dunn et al., 1987; Tapiero et al., 2003). Zinco é um conhecido indutor da síntese de metalotioneínas (Pedersen et al., 1998; Peixoto et al., 2003; 2007c) e alguns estudos tem correlacionado o aumento da captação de Zn hepático a indução

da síntese de metalotioneínas após a exposição a uma variedade de agentes químicos e insultos ambientais (Brady & Bunger, 1979; Goering et al., 1985). No presente estudo, nós sugerimos que as MT foram produzidas como conseqüência da retenção de Hg e Zn no fígado dos animais expostos ao mercúrio (Sal-Hg e Cu-Hg) aos 13 dias. A prévia exposição ao cobre nos animais tratados com Hg influenciou na distribuição desta proteína, levando a uma diminuição da concentração da mesma no fígado e aumento nos rins em relação ao grupo Sal-Hg. O aumento da MT renal acompanhado pelo aumento da acumulação de Hg nesse mesmo tecido, como descrito anteriormente, pode estar relacionado a um mecanismo no qual a metaloproteína forma um complexo não tóxico com o mercúrio (MT-Hg) hepático para ser transportado para o rim e após ser excretado (Goering & Klaassen, 1983). Os níveis de MT hepática e renal não apresentaram alterações aos 33 dias de idade, provavelmente devido aos baixos níveis de Hg hepático e homeostase dos níveis de Zn nesse tecido.

A exposição ao mercúrio também causou alterações na homeostase dos níveis hepáticos de Fe, os quais apresentaram níveis aumentados aos 13 dias. Já os níveis renais de Fe aos 13 e 33 dias, assim como, os níveis de Mg aos 33 dias foram menores que aqueles encontrados no grupo controle. Os níveis de Cu em cérebro aos 13 dias, assim como, os níveis de Zn em cérebro e cerebelo aos 33 dias foram aumentados.

O metabolismo normal do Fe é altamente regulado e é extremamente importante na manutenção da função celular. O aumento nos níveis de Fe hepático pode ser tóxico e mediado por estresse oxidativo, uma vez que, esse metal pode causar a formação de espécies reativas através de reações do tipo Fenton (Fontecave & Pierre, 1993) e contribuir para o aumento dos efeitos tóxicos do mercúrio. A redução nos níveis de Mg pode contribuir para o prejuízo da função celular normal considerando que esse metal é um importante cofator de várias enzimas (Brody, 1994). Ainda, alterações de metais essenciais no SNC podem contribuir para ocorrência de desordens neurodegenerativas. O aumento nos níveis de Zn no cérebro tem implicado no prejuízo do metabolismo neuronal através da inibição de enzimas como a gliceraldeído-3-fosfato desidrogenase (GAPDH), a fosfofrutoquinase (Kozlowski et al., 2009) e a AChE (Brocardo et al., 2005). Contudo, no presente estudo só

foi observado alterações na homeostase desse metal aos 33 dias, intervalo após a exposição ao mercúrio em que a atividade da AChE de cérebro e cerebelo não foi alterada.

A pré-exposição ao cobre contribuiu para a homeostase de metais essenciais. A presença de níveis detectáveis de Cu em cérebro (13 dias) e cerebelo (13 e 33 dias) foi observada em animais Cu-Hg, contudo, parece não ser indicativo de toxicidade uma vez que alterações comportamentais foram totalmente prevenidas por esse metal.

Alterações comportamentais foram verificadas durante e após a exposição ao mercúrio. Os animais tratados com Hg apresentaram déficits comportamentais na tarefa de reflexo de geotactismo negativo e teste do béquer. A tarefa do reflexo de geotactismo foi conduzida dos 3 aos 13 dias de idade. Nessa tarefa o desenvolvimento motor para responder ao reflexo de geotactismo é requerido pelo animal (Da-Silva et al., 1990). Contudo, animais expostos ao mercúrio apresentaram um aumento na latência para completar a resposta de geotactismo negativo nas sessões 5 e 6 (24 horas após a 3ª e 5ª dose de mercúrio). O teste do béquer foi realizado dos 17 aos 20 dias de idade (5 a 8 dias após o fim da exposição ao mercúrio). Nessa tarefa, a força muscular bem como a função cerebelar são necessárias (Smart & Dobbing, 1971). Contudo, quando expostos ao mercúrio os animais apresentaram maior latência para acessar o refúgio e maior porcentagem de queda na 3ª sessão refletindo prejuízo no desempenho do animal. Ainda, a dose de mercúrio usada não mostrou mudanças em relação à sensibilidade à dor, observado no teste de imersão da cauda realizado aos 13, 20 e 27 dias de idade, nem em relação à coordenação motora, observado no teste da locomoção forçada em cilindro giratório (rotarod) realizado aos 25 e 30 dias de idade. Esses resultados concordam com os de Franciscato et al. (2009b) que analisaram os efeitos da exposição ao mercúrio nessa mesma fase de desenvolvimento.

Existem evidências de que células cerebelares são alvos seletivos à exposição ao MeHg (Sanfeliu et al., 2003); e a relação entre os déficits motores e o dano cerebelar são fenômenos bastante relatados (Sakamoto et al., 1993; Franco et al., 2007a; Carvalho et al., 2007), uma vez que, o cerebelo é uma estrutura fundamentalmente envolvida no controle motor (Shutoh et al., 2006). Estudos comportamentais mostram déficits motores em animais expostos ao

$HgCl_2$ tanto quando expostos durante os primeiros dias de vida pós natal (Rocha et al., 2001; Franco et al., 2007a; Franciscato et al., 2009) bem como quando expostos na idade adulta (Mello-Carpes et al., 2013). Embora o mecanismo neurotóxico do $HgCl_2$ não seja bem entendido, a inibição da atividade da AChE (Franciscato et al., 2009b) e aumento da peroxidação lipídica (Franco et al., 2007a) em cerebelo podem contribuir para os distúrbios neurológicos causados pela exposição a esse metal.

No presente estudo, um aumento nos níveis de Hg no cerebelo de ratos expostos ao Hg foi verificado aos 13 dias de idade e esses foram 3,2 vezes maiores que aqueles encontrados no cérebro desses animais. A associação do aumento dos níveis de mercúrio em cerebelo e a inibição da atividade da AChE nesse mesmo tecido, também observada aos 13 dias, parece estar diretamente relacionada com o aparecimento dos danos comportamentais, o que pode explicar, pelo menos em parte, o mecanismo neurotóxico do mercúrio. Essa hipótese é reforçada quando considerado o fato de que a exposição ao cobre preveniu completamente o prejuízo motor, bem como a inibição da AChE cerebelar e em paralelo reduziu os níveis de mercúrio, apontando uma ligação entre os fenômenos.

De forma geral, este estudo demonstrou que a exposição ao cobre preveniu diversos efeitos causados pela exposição ao metal tóxico. A prevenção foi imediata (24 horas após o fim da intoxicação por mercúrio) e também persistente, ou seja, o efeito preventivo ocorre mesmo quando o parâmetro é analisado muito tempo depois da exposição. Em outros casos o efeito preventivo foi observado somente tardiamente (21 dias após o fim da intoxicação por mercúrio). Dessa forma, a eficiência do cobre como tratamento preventivo foi observado em parâmetros como a atividade da δ-ALA-D hepática, níveis de creatinina sérica, conteúdo de Hg, homeostase dos níveis de Fe hepático e atividade da AChE de cerebelo aos 13 dias. Já parâmetros como peso corporal, renal e hepático, atividade da δ-ALA-D renal, níveis de ureia sérica e homeostase dos níveis renais de Fe e Mg foram observados aos 33 dias. Alterações comportamentais também foram totalmente prevenidas.

Dessa forma, o cobre pode ser considerado um potencial agente terapêutico em casos de intoxicação por mercúrio e quem sabe, no futuro,

utilizado como estratégia terapêutica para casos de intoxicação ocupacional ou ambiental por mercúrio em humanos.

5. CONCLUSÕES

Considerando os resultados descritos, podemos concluir que:

1. A exposição a uma dose (s.c.) diária de 5,0 mg/kg de $HgCl_2$ por cinco dias consecutivos (do 8º ao 12º dia de idade):

a) afeta o crescimento e desenvolvimento dos animais, como verificado pela perda de peso corporal (13 e 33 dias), de cérebro (13 dias), fígado (33 dias) e aumento do peso renal (13 e 33 dias);

b) reduz a atividade da enzima δ-ALA-D hepática (13 dias) e renal (13 e 33 dias); cuja atividade serve para o biomonitoramento da exposição a metais divalentes;

c) prejudica a função renal uma vez que os níveis de ureia (13 e 33 dias) e creatinina (13 dias) encontram-se elevados;

d) causa uma elevação nos teores de mercúrio em fígado, rim e sangue (13 e 33 dias); e em cérebro e cerebelo (13 dias);

e) promove uma alteração endógena de metais verificada pelo aumento dos níveis de Zn e Fe hepático (13 dias), um decréscimo nos níveis de Fe (13 e 33 dias) e Mg (33 dias) e aumento nos níveis de Cu (13 dias) no rim, bem como, um aumento nos níveis de Zn (33 dias) em cérebro e cerebelo;

f) prejudica o desempenho dos animais nas tarefas comportamentais como verificado nos testes do geotactismo negativo e teste do béquer; assim como reduz a atividade da AChE de cerebelo;

2. O tratamento (do 3º ao 7º dia de idade) com $CuCl_2.2H_2O$ (s.c.) na dose de 6,9 mg/kg/dia aplicado aos animais durante cinco dias que antecedem a exposição ao mercúrio:

a) preveniu parcialmente o aumento do peso renal aos 13 dias e totalmente a alteração do peso corporal e peso de órgãos como fígado e rim aos 33 dias;

b) evitou, mesmo que parcialmente, a inibição na atividade da enzima δ-ALA-D hepática (13 dias); e totalmente a inibição da enzima renal (33 dias);

c) preveniu o aumento dos níveis de creatinina utilizada como índice de insuficiência renal aos 13 dias; bem como, o aumento nos níveis de uréia sérica aos 33 dias;

d) diminui a retenção de mercúrio no cérebro e cerebelo; e causou uma redistribuição do mercúrio decrescendo os níveis de mercúrio hepáticos e sanguíneos e aumentando os níveis renais em paralelo a um aumento dos níveis de MT renal aos 13 dias, sugerindo que o cobre, juntamente com as MT, podem estar envolvidos no mecanismo de eliminação de Hg;

e) contribuiu para a homeostase de metais essenciais como o Fe no fígado (13 e 33 dias) e rim (33 dias), Mg no rim (aos 33 dias), bem como, na homeostase dos níveis de Zn em cérebro e cerebelo alterados pela exposição ao mercúrio aos 33 dias;

f) preveniu as deficiências comportamentais tanto durante quanto após o final da exposição ao mercúrio em paralelo a prevenção da inibição da AChE de cerebelo, a qual, também teve sua atividade similar ao controle quando os animais foram pré-expostos ao cobre anteriormente a intoxicação por mercúrio.

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