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EFEITOS DA EXPOSIÇÃO AO CLORETO DE MERCÚRIO DURANTE A GESTAÇÃO E LACTAÇÃO EM RATAS WISTAR E SUA PROLE: PARÂMETROS BIOQUÍMICOS E DISTRIBUIÇÃO DE MERCÚRIO

TESE DE DOUTORADO

Cláudia Sirlene de Oliveira

Santa Maria, RS, Brasil
2015

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por

Cláudia Sirlene de Oliveira

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brazil

EFEITOS DA EXPOSIÇÃO AO CLORETO DE MERCÚRIO DURANTE A GESTAÇÃO E LACTAÇÃO EM RATAS WISTAR E SUA PROLE: PARÂMETROS BIOQUÍMICOS E DISTRIBUIÇÃO DE MERCÚRIO

Autor: Cláudia Sirlene de Oliveira
Orientadora: Profª. Drª. Maria Ester Pereira
Local e data da defesa: Santa Maria, 8 de Maio de 2015.

O objetivo deste trabalho foi avaliar os efeitos da exposição ao $HgCl_2$ na água de beber em ratas prenhas e/ou lactantes e sua prole. Ainda, avaliar se a exposição intravenosa ao $HgCl_2$ assim como o dano renal induzido pela mesma altera a deposição de mercúrio na prole. O protocolo de exposição ao $HgCl_2$ na água de beber (v.o.) foi: as ratas Wistar foram expostas ao $HgCl_2$ (0, 0,2, 0,5, 10 e 50 µg Hg^{2+}/mL) do dia zero de gestação até o dia 20 ou até o final da lactação. A cada dois dias, as soluções de mercúrio eram trocadas, a ingestão de comida e água e o peso das ratas eram avaliados. A prole foi sacrificada no dia 20 de gestação e nos dias pós-natal 10, 20, 30 e 40. Foram analisados o peso dos órgãos, a homeostase de metais essenciais, os níveis de mercúrio e parâmetros bioquímicos. As tarefas comportamentais foram realizadas nos dias pós-natal 3, 5, 7, 9 e 11 (teste do geotactismo negativo) e 17, 18, 19 e 20 (teste do beaker). O protocolo de exposição ao $HgCl_2$ intravenoso (i.v.) foi: as ratas Wistar foram expostas ao $HgCl_2$ (0,5 e 2,5 µmol $HgCl_2$/kg/2 mL) no dia 20 de gestação e sacrificadas 6 h após a exposição ou no dia 18 de gestação e sacrificadas 48 h após a exposição. Foram avaliados a distribuição do Hg nos organismos materno e fetal e o dano renal através de histologia e marcadores bioquímicos e moleculares. As mães expostas ao $HgCl_2$ v.o. apresentaram diminuição na ingestão de água; a exposição a dose de 50 µg Hg^{2+}/mL causou aumento no peso relativo de rim. As doses de 10 e 50 µg Hg^{2+}/mL causaram aumento dos níveis renais de Cu e hepáticos de Zn e acúmulo de mercúrio em rins nas gestantes; e aumento nos níveis renais de tióis totais e de metalotioneínas nas lactantes. A prole exposta ao $HgCl_2$ apresentou aumento da atividade hepática da porfobilinogênio sintase em fetos e aumento do peso relativo de rim no dia pós-natal 20. As ratas expostas ao $HgCl_2$ i.v. apresentaram maior acúmulo de mercúrio em rins 6 e 48 h após a exposição; embora a 48 h da exposição, os níveis já haviam diminuído em relação a 6 h. A dose de 2,5 µmol $HgCl_2$/kg/2 mL i.v. causou aumento nos níveis séricos de creatinina, aumento da expressão da proteína Kim-1 e alterações na histologia de rim. Os níveis de Hg placentário e fetal não diminuíram com o passar das horas após a exposição; nos órgãos fetais, os níveis de Hg apresentaram aumento dependente da dose e do tempo. Em conclusão, a exposição a baixas doses de $HgCl_2$ na água de beber causou alterações brandas nas mães; e o organismo materno parece ter metabolizado o Hg, evitando danos à prole; provavelmente esta proteção está relacionada ao aumento dos níveis de moléculas detoxificantes (metalotioneínas, por exemplo) durante o período gestacional e lactacional. Ainda, verificamos a incapacidade dos organismos em desenvolvimento (fetos) em excretar/depurar os íons Hg quando as mães foram expostas intravenosamente ao metal.

Palavras-chaves: água de beber; intravenoso; dano renal; metais essenciais; porfobilinogênio sintase; metalotioneínas; ureia; creatinina; tarefas comportamentais

ABSTRACT

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

EFFECTS OF MERCURY CHLORIDE EXPOSURE DURING THE GESTATION AND LACTATION PERIODS IN WISTAR RATS AND THEIR OFFSPRING: BIOCHEMICAL PARAMETERS AND MERCURY DISTRIBUTION

Author: Cláudia Sirlene de Oliveira
Advisor: Maria Ester Pereira
Date and place of the defense: Santa Maria, May 8, 2015.

The objective of this work was to evaluate the effects of $HgCl_2$ exposure in drinking water in pregnant and/or lactating rats and their offspring. Still, it evaluated if the $HgCl_2$ intravenous exposure as well as the renal damage induced by this exposure altered the offspring mercury content. The drinking water (v.o.) $HgCl_2$ exposure protocol was as follows: Female Wistar rats were exposed to $HgCl_2$ (0, 0.2, 0.5, 10 and 50 μg Hg^{2+}/mL) from gestation day 0 until 20 or until the last day of lactation. Every two days, the mercury solutions were changed, food and water intake and rats weight were analyzed. The offspring was killed on gestation day 20 and on the post-natal days 10, 20, 30 and 40. Tissues weight, essential metal homeostasis, mercury content and biochemical parameters were evaluated. Behavioral tasks were carried out on post-natal days 3, 5, 7, 9 and 11 (negative geotaxis test) and 17, 18, 19 and 20 (beaker test). The intravenous (i.v.) $HgCl_2$ exposure protocol was as follows: Female Wistar rats were exposed to $HgCl_2$ (0.5 and 2.5 μmol $HgCl_2$/kg/2 mL) on gestation day 20 and killed 6 h later or on gestation day 18 and killed 48 h later. Hg maternal and fetal distribution and renal damage through histology and biochemical and molecular markers were evaluated. Dams exposed to $HgCl_2$ v.o. presented water intake decreased. The exposure to 50 μg Hg^{2+}/mL caused an increase in relative renal weight. Animals exposed to 10 and 50 μg Hg^{2+}/mL presented an increase in renal and hepatic Cu and Zn levels, respectively, and mercury accumulation (pregnant rats); and, an increase in total thiol and metallothionein renal levels (lactating rats). The offspring only presented an increase in hepatic porfobilinogen-synthase activity (fetuses) and in relative renal weight (post-natal day 20). The pregnant rats exposed i.v. to $HgCl_2$ presented the greater mercury accumulation in kidney in both periods analyzed; although 48 h after the exposure the Hg levels were lower than at 6 h. The exposure to 2.5 μmol $HgCl_2$/kg/2 mL caused an increase in serum creatinine levels and in Kim-1 renal expression as well as renal histology alterations. The placental and fetal Hg did not change in both periods analyzed; the increase in fetal organs Hg levels were dose and time dependent. In conclusion, the exposure to low doses of $HgCl_2$ in drinking water caused mild alterations in dams; also the dam organism was able to handle the Hg avoiding offspring damages; probably, this protection is related with the increase on scavenger molecules (metallothionein, for example) during the pregnancy and lactation. Besides, we verified that when dams were exposed intravenously to $HgCl_2$, the developing organisms (fetuses) were unable to excrete/depurate the Hg.

Keywords: drinking water; intravenous; renal damage; essential metals; porfobilinogen-synthase; metallothionein; urea; creatinine; behavioral tasks

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LISTA DE REDUÇÕES (ABREVIATURAS, SIGLAS E SÍMBOLOS)

°C: graus Celsius;

µCi: microcurie;

µg: micrograma;

µL: microlitro;

µmol: micromol;

a.m.: ante meridiem;

ANOVA: análise de variância;

b.w.: body weight;

BUN: blood urea nitrogen;

Ca: cálcio;

cm: centímetro;

Co: cobalto;

Cu: cobre;

DNA: ácido desoxirribonucléico;

DTNB: ácido 5,5 ditio-bis-2-nitrobenzóico;

ED: experimental day;

EDTA: ácido etilenodiaminotetracético;

Fe: ferro;

g: força gravitacional;

g: grama;

Gapdh: Gliceraldeído 3-fosfato desidrogenase;

GD: gestational day;

GSH: glutationa;

H & E: hematoxilina e eosina;

h: hora(s)

HCl: ácido clorídrico;

HDM: high doses of mercury;

Hg: mercúrio;

Hg^{+}/Hg^{2+}: mercúrio inorgânico, oxidado;

Hg^{0}: mercúrio elementar;

$HgCl_2$: cloreto de mercúrio;

HgO: óxido de mercúrio;

HgS: sulfeto de mercúrio;

HNO_3: ácido nítrico;

i.p.: intraperitoneal;

i.v.: intravenoso;

ISOM: inner stripe of outer medulla;

kg: quilograma;

Kim-1: molécula de lesão renal-1;

L: litro;

LDM: low doses of mercury;

mCi: milicurie;

MeHg/CH_3Hg^+: metilmercúrio;

mg: miligrama;

min: minuto(s);

mL: mililitro;

mm: milímetro;

mM: milimolar;

mmol: milimol;

Mn: manganês;

MT: metalotioneína;

n.d.: não determinado/detectado;

n: número de repetições;

NaCl: cloreto de sódio;

NaH_2PO_4: fosfato monossódico;

NaOH: hidróxido de sódio;

NGAL: lipocalina associada à gelatinase neutrofílica;

nm: nanômetro;

nmol: nanomol;

OSOM: outer stripe of outer medulla;

p.m.: post meridiem;

p: nível de significância;

PBG: porfobilinogênio;

PBG-sintase/PBG-synthase: porfobilinogênio sintase;

PCR: polymerase chain reaction;

pH: potencial hidrogeniônico;

PMSF: fenil metil sulfonil fluoreto;

PND: post-natal day;

RNA: ácido ribonucléico;

rpm: rotações por minuto;

S.E.: erro padrão;

S.E.M.: erro padrão da média;

s: segundo(s);

-SH: grupamento(s) sulfidrílico(s);

TCA: ácido tricloroacético;

v.o.: via oral;

Zn: zinco;

δ-ALA: ácido-δ-aminolevulínico;

δ-ALA-D: δ-aminolevulinato desidratase.

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APRESENTAÇÃO

No item **INTRODUÇÃO** está descrito uma revisão sucinta sobre os temas trabalhados nesta tese. No final deste item estão apresentados os objetivos geral e específicos.

Os **RESULTADOS** estão dispostos na forma de artigo científico e manuscritos submetidos à publicação. As seções Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no artigo e nos manuscritos e representam a integra deste estudo.

No item **DISCUSSÃO** estão apresentados as interpretações e comentários gerais sobre o artigo e os manuscritos científicos apresentados.

No item **CONCLUSÕES** são apresentadas as conclusões gerais do presente trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** apresentadas no final da tese referem-se somente as citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO**.

1.INTRODUÇÃO

Encontrado no grupo ou família 12 (também chamada de 2B) da tabela periódica, o mercúrio é um metal de transição conhecido por apresentar características como: baixa condução térmica, alta condutividade elétrica, alta densidade e baixo ponto de fusão. Além disso, dentre os metais de transição, o mercúrio, no estado de oxidação 0, é o único que é líquido em temperatura ambiente (HEPLER E OLOFSSON, 1975; PARK E ZHENG, 2012; MARTÍN-YERGA et al., 2013). O símbolo atômico do mercúrio é Hg, este é proveniente do nome, em Latim, *Hydragyrum* o qual significa prata líquida. Esta denominação foi escolhida pelo fato do mercúrio possuir coloração prateada e, como mencionado acima, apresentar-se no estado líquido na temperatura ambiente (CLARKSON et al., 2007).

Os primeiros relatos de uso do Hg pela população humana foram creditados aos chineses, os quais utilizavam o cinnabar (HgS) como pigmento para fabricação de tinta vermelha (PARK E ZHENG, 2012). Os alquimistas utilizavam o mercúrio na forma líquida, fazendo amálgamas com o ouro (princípio da mineração), e após a evaporação do mercúrio diziam ter produzido ouro. Com o decorrer do desenvolvimento de novas tecnologias, o Hg passou a ser utilizado na fabricação de produtos, sendo este, parte integrante ou participante no processo de produção (CLARKSON et al., 2007). A partir do aumento do uso deste metal em processos de manufatura e posteriormente na era industrial, foi detectado que a exposição descontrolada ao Hg causa efeitos danosos à saúde. Assim, casos de pesquisadores e trabalhadores que morreram de causas indeterminadas foram relacionados a uma possível exposição ao Hg (GRAEME E POLLACK, 1998). De fato, atualmente, é sabido que o mercúrio é um metal não-essencial altamente tóxico sem qualquer função fisiológica, metabólica ou bioquímica nos organismos em geral (WHO, 2007; ATDSR, 1999).

No ambiente, o mercúrio ocorre naturalmente depositado em rochas. Este chega a superfície por processos naturais como vulcanismo e erosão da crosta terrestre e oceânica. Entretanto, uma das grandes preocupações deve-se ao fato de atividades antropogênicas potencializarem os níveis ambientais de mercúrio (HOROWITZ et al., 2014). Dentre as atividades antropogênicas destacam-se: mineração, queima de combustíveis fósseis, utilização do mercúrio como catalisador na confecção de produtos químicos (ex.: soda cáustica e cloro) ou como componente de produtos químicos (ex.: tintas, fungicidas e bactericidas). Ainda, o descarte descontrolado de materiais que contém mercúrio como, por exemplo, baterias,

termostatos, termômetros e lâmpadas está dentre os processos antropogênicos responsáveis pela liberação de íons Hg no ambiente (DRISCOLL et al., 2013; HOROWITZ et al., 2014).

O mercúrio pode ser encontrado em diferentes formas químicas. Dentre estas destacam-se: a forma inorgânica, a qual inclui o mercúrio elementar (valência 0, Hg^0) e seus dois estados de oxidação (Hg^+, Hg^{2+}); e a forma orgânica (um átomo de mercúrio ligado a um ou mais átomos de carbono, R-C-Hg) (CLARKSON et al., 2007; KIM E ZOH, 2012). Alguns trabalhos classificam o Hg^0 somente como mercúrio elementar ou metálico, ou seja, como uma forma química distinta da forma inorgânica (BERLIN et al., 2007; PARK E ZHENG, 2012). Apesar da divergência quanto a classificação, é consenso entre os pesquisadores que as formas químicas de mercúrio diferem quanto a solubilidade, comportamento no ambiente e toxicocinética (SYVERSEN E KAUR, 2012). O mercúrio de valência 0 é encontrado como um líquido prata e brilhante, extremamente volátil tornando-se rapidamente mercúrio vapor. As formas inorgânica, Hg^+ e Hg^{2+}, geralmente são encontradas ligadas a outros átomos como cloro ($HgCl_2$), enxofre (HgS) e oxigênio (HgO), formando os sais de mercúrio. Os sais de Hg são pouco voláteis, geralmente apresentam-se como um pó branco e inodoro. A forma orgânica, também pode ser chamada de composto organomercurial, possui como principais exemplos o metilmercúrio, etilmercúrio e dimetilmercúrio. Os dois primeiros são sólidos em temperatura ambiente; já o dimetilmercúrio é um líquido incolor e inodoro (ATDSR, 1999).

Assim que as formas de mercúrio atingem a superfície, as mesmas podem ser convertidas entre si através de processos de oxidação/redução e metilação/desmetilação (FIGURA 1). O Hg^0 pode evaporar e retornar a superfície terrestre através da chuva e/ou neve como mercúrio metálico (Hg^0) ou oxidado (Hg^{2+}); o Hg^{2+} pode sofrer metilação por microrganismos (bactérias e fungos) no ambiente aquático (MOREL et al., 1998; KIM E ZOH, 2012). É importante destacar que estas reações químicas são reversíveis. Os íons mercúrio podem entrar na cadeia alimentar; peixes e demais organismos aquáticos contaminam-se com Hg ao alimentarem-se de algas e plantas contaminadas e, no caso dos animais carnívoros, ao ingerirem outros peixes ou invertebrados contaminados (POUILLY et al., 2013). Nos peixes a capacidade de excretar o Hg é baixa e negativamente correlacionada com o tamanho do animal, logo, quanto maior o peixe, menor é a excreção e maior é a concentração de mercúrio (principalmente a forma orgânica) acumulada em órgãos e músculos (TRUDEL E RASMUSSEN, 1997; KENŠOVÁ et al., 2012). Em virtude disto, quanto mais alto o nível trófico da cadeia alimentar analisado, maior será a concentração de

mercúrio depositado, este processo é chamado de bioacumulação/biomagnificação (WARD et al., 2010). Ao alimentar-se de peixes e outros organismos aquáticos, a população humana expõem-se ao mercúrio, principalmente a forma orgânica.

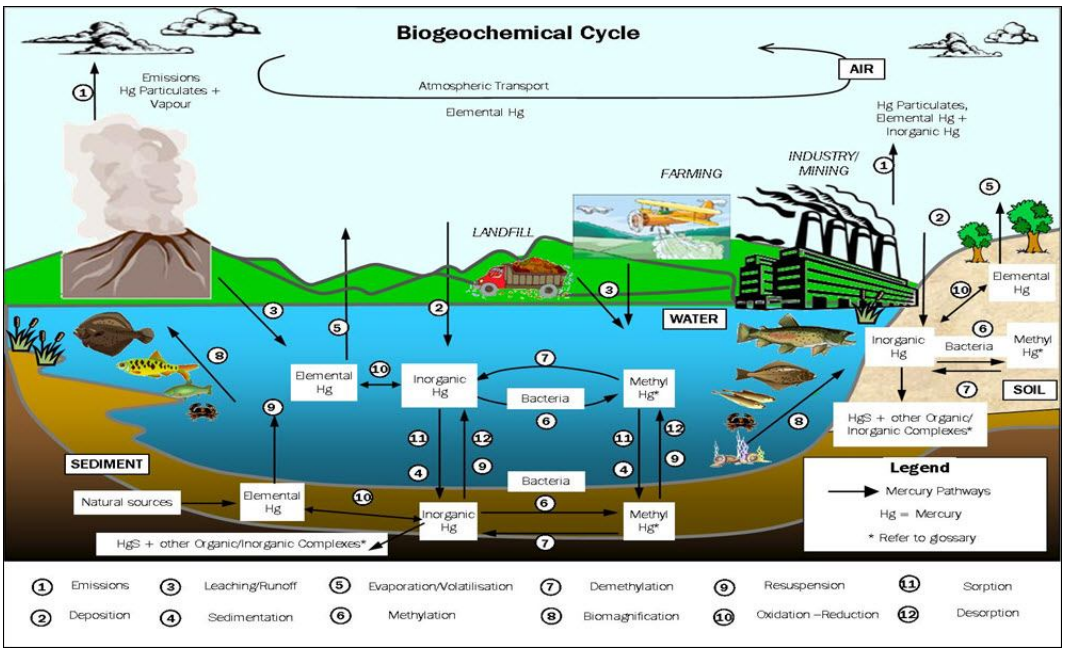

Figura 1: Ciclo biogeoquímico do mercúrio. Retirado de "Mercury inventory for New Zealand: 2008", acessado em 2 de abril de 2015 as 15:41h. (http://www.mfe.govt.nz/publications/waste/mercury-inventory-new-zealand-2008/2-mercury-environment)

Entre as décadas de 50 e 70, no Japão, ocorreu o mais famoso caso de contaminação com mercúrio orgânico. A contaminação ocorreu na Baia de Minamata; em virtude disto, os sintomas apresentados pela população ficaram conhecidos como doença de Minamata. Uma fábrica de acetaldeído da região despejava seus resíduos na baia; estes continham mercúrio inorgânico, o qual acabou sendo incorporado na cadeia alimentar, na forma de metilmercúrio (MeHg) (CLARKSON, 2002; EKINO et al., 2007). Os habitantes da região, os quais se alimentavam basicamente de peixe, foram expostos ao MeHg por um longo período. Após um período de latência, a população da região começou a apresentar elevada irritabilidade, cansaço, visão turva, diminuição da audição, distúrbios olfativos e gustativos e tremores. Entretanto, os danos mais graves foram observados em crianças expostas ao metal no útero e, após o nascimento, através do leite materno. As mesmas apresentaram danos irreversíveis no desenvolvimento motor e mental (EKINO et al., 2007; TSUDA et al., 2009).

Outro caso conhecido de contaminação com mercúrio ocorreu entre os anos de 1971 e 1972, no Iraque. Durante um período de escassez de comida, a população sem alternativa acabou produzindo pão com sementes que seriam utilizadas para o plantio e estavam preservadas com um fungicida que continha MeHg (AL-TIKRITI E AL-MUFTI, 1976; CLARKSON, 2002). Os hospitais da região registraram 6.530 casos de intoxicação, dentre estes, cerca de 459 pessoas morreram. No entanto, acredita-se que o número de contaminados foi muito maior, porém como estes apresentaram sintomas brandos como dor de cabeça e tremores, não houve registros (AL-TIKRITI E AL-MUFTI, 1976). Como este caso de intoxicação foi agudo, ou seja, assim que detectado a fonte do mercúrio, a população parou de ingerir o pão contaminado, as crianças que foram intoxicadas e apresentaram graus de toxicidade do leve ao moderado (os principais sintomas foram fraqueza muscular, danos visuais e ataxia) dois anos após o incidente estavam praticamente recuperadas (AMIN-ZAKI et al., 1978).

Além da exposição ambiental, os seres humanos podem expor-se ao mercúrio ocupacionalmente. A forma de contaminação ocupacional com mercúrio mais estudada está relacionada ao uso de amálgamas de mercúrio em restaurações dentárias; dentistas e seus auxiliares estão constantemente expostos ao vapor de Hg (NGIM et al., 1992; RITCHIE et al., 2002). Rowland et al. (1994) demonstraram que mulheres que trabalharam como auxiliares de dentista apresentaram diminuição na taxa de fertilidade. Ainda, a população que possui restaurações dentárias com amálgamas contendo mercúrio também está exposta ao metal, seja por evaporação ou pelo desgaste ocasionado pela mastigação (LORSCHEIDER et al., 1995). Takahashi et al. (2003) demonstraram correlação positiva entre o conteúdo tecidual de mercúrio (em mães e fetos) e o número de restaurações dentárias.

Atualmente a maioria dos países desenvolvidos aboliu ou reduziu drasticamente o uso de mercúrio, sugerindo, sempre que possível, a troca do Hg por um substituinte menos tóxico. Entretanto, mesmo diminuindo o uso, o mercúrio que foi liberado anteriormente pode estar depositado no solo e ser liberado lentamente no ambiente. Casos de exposição ao mercúrio ainda são registrados no México (TRASANDE et al., 2010; PEREGRINO et al., 2011), China (ZHANG et al., 2010; YASUTAKE et al., 2011), Tailândia (DECHARAT, 2012; DECHARAT et al., 2014) e países da África (GNANDI et al., 2010; PARUCHURI et al., 2010; ADJORLOLO-GASOKPOH et al., 2012; STECKLING et al., 2014). No Brasil, os principais casos de exposição e/ou contaminação com mercúrio encontram-se na região Norte,

onde o mercúrio ainda é utilizado no processo de extração do ouro. Os ribeirinhos são expostos ao metal ao ingerirem peixes e água contaminados (PALHETA E TAYLOR, 1995; MARQUES et al., 2015). Entretanto, é importante ressaltar que estudos realizados na Lagoa dos Patos, Rio Grande do Sul (Região Sul do Brasil) demonstraram que os peixes (NIENCHESKI et al., 2001; KÜTTER et al., 2009) e os sedimentos ao entorno da lagoa apresentaram níveis preocupantes de mercúrio (MIRLEAN et al., 2003, 2009).

As vias de exposição e os órgãos alvos diferem para cada forma química do mercúrio (SYVERSEN E KAUR, 2012). A exposição ao mercúrio metálico ocorre principalmente por inalação, atingindo principalmente as vias respiratórias causando sérios problemas pulmonares. Ainda, o mercúrio vapor é altamente lipofílico e cruza facilmente a barreira hemato-encefálica (PARK E ZHENG, 2012). A forma orgânica de mercúrio também é altamente lipofílica e causa efeitos tóxicos principalmente ao sistema nervoso central; a população é exposta a esta forma de mercúrio principalmente pela via gastrointestinal (alimentos contaminados, ex.: peixes) (HONG et al., 2012; BISEN-HERSH et al., 2014). Diferentemente do mercúrio vapor e dos compostos organomercuriais, as formas inorgânicas oxidadas do mercúrio (Hg^{+} e Hg^{2+}) são altamente nefrotóxicas (NIELSEN et al., 1994; ZALUPS, 2000; BRIDGES et al., 2014). A população é exposta as formas inorgânica através da ingestão de água contaminada (LORSCHEIDER et al., 1995) e do uso de cremes de pele (PEREGRINO et al., 2011). Entretanto, apesar de possuir grande afinidade pelo sistema renal, a forma inorgânica de mercúrio (Hg^{2+}) pode causar danos aos sistemas hepático (FRANCISCATO et al., 2011; UNG et al., 2010), reprodutivo (EL-DESOKY et al., 2013; MARTINEZ et al., 2014), endócrino (ZHU et al., 2000) e nervoso (PEIXOTO et al., 2007a; FRANCISCATO et al., 2009; MORAES-SILVA et al., 2014).

Embora os principais casos de contaminação com mercúrio sejam relacionados a forma orgânica (alimentos contaminados) e ao mercúrio vapor (ocupacionalmente/ trabalhadores) a forma química de mercúrio escolhida neste trabalho foi o Hg^{2+} ($HgCl_2$). A escolha da forma inorgânica, Hg^{2+}, deve-se ao fato de que, embora em menores níveis, a população em geral é exposta ao mercúrio inorgânico. Além disso, uma vez em contato com o organismo as formas elementar e orgânica podem ser convertidas a Hg^{2+} (LORSCHEIDER et al., 1995) (FIGURA 2). Por exemplo, no eritrócito o Hg^{0} pode ser oxidado a Hg^{2+} pela ação da enzima catalase (HALBACH E CLARKSON, 1978); já a forma orgânica, principalmente o MeHg, sofre desmetilação (quebra da ligação carbono-mercúrio) no trato gastrointestinal e em

alguns tecidos, como fígado, rim e cérebro (LORSCHEIDER et al., 1995; SHAPIRO E CHAN, 2008). De fato, muitos trabalhos demostraram o acúmulo de Hg^{2+} em diferentes órgãos após a exposição ao mercúrio orgânico (NORSETH E CLARKSON, 1970; OMATA et al., 1980; YAMAMOTO et al., 1986; RODRIGUES et al., 2010) e elementar (ISHITOBI et al., 2010).

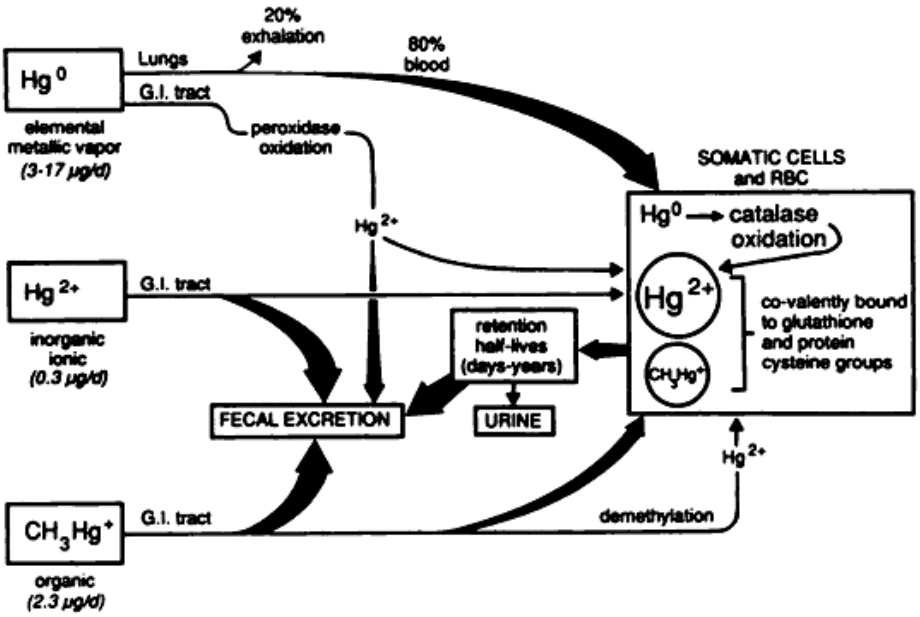

Figura 2: Comportamento das diferentes formas químicas de mercúrio no organismo. Retirado de Lorscheider et al., 1995.

Como observado no caso de Minamata, e em alguns casos menores de contaminação, os efeitos tóxicos da exposição ao mercúrio no período do desenvolvimento pré e pós-natal são pronunciados quando comparados aos efeitos tóxicos apresentados por adultos (SORESEN et al., 1999; AXELRAD et al., 2007; EKINO et al., 2007; TSUDA et al., 2009; MARQUES et al., 2015). Sabe-se que durante o período desenvolvimental, órgãos e membranas ainda não estão completamente formados e são incapazes de processar adequadamente os agentes tóxicos (NIES E SPIELBERG, 1996); isto facilitaria a entrada e acumulação de xenobióticos, como por exemplo, o Hg^{2+}. Logo, é necessário estudar os efeitos da exposição a forma inorgânica de mercúrio e compreender como a mesma interage com os organismos, sejam eles adultos ou jovens.

Estudos do nosso grupo de pesquisa demonstraram que ratos jovens expostos subcutaneamente ao mercúrio inorgânico ($HgCl_2$) por 5 dias (do 8° ao 12° dia de vida pós-

natal) apresentaram alterações comportamentais (PEIXOTO et al., 2007a; MORAES-SILVA et al., 2014), acúmulo de mercúrio em diferentes órgãos e alteração na homeostase de metais essenciais (PEIXOTO et al., 2008; MORAES-SILVA et al., 2014), aumento nos níveis séricos de ureia e creatinina (PEIXOTO E PEREIRA, 2007) e inibição da atividade da enzima porfobilinogênio sintase (PBG-sintase) hepática e renal (PEIXOTO et al., 2003). Ainda, verificou-se que os déficits bioquímicos e comportamentais ocasionados pela exposição ao $HgCl_2$ por 5 dias permaneceram por um longo período após o término da exposição (FRANCISCATO et al., 2009, 2011). Interessantemente, filhotes expostos indiretamente ao $HgCl_2$, via leite materno, do 8° ao 12° dia de vida pós-natal, apresentaram menor peso corporal e acúmulo de Hg em fígado (OLIVEIRA C. et al., 2014) e rim (FAVERO et al., 2014) sem apresentar alterações bioquímicas. Além disso, foi observado que ratas no período lactacional são menos sensíveis ao mercúrio inorgânico quando comparadas com ratas virgens (FAVERO et al., 2014; OLIVEIRA C. et al., 2014; OLIVEIRA V. et al., 2014), resultado este que chama atenção para as mudanças que ocorrem durante o período lactacional e antes deste, o período gestacional, os quais podem estar influenciando os efeitos do Hg.

Durante a gestação e a lactação ocorrem mudanças, tais como, aumento da demanda de nutrientes (DAS et al., 2013; WHO, 1996), alterações na produção e nos níveis de hormônios (MCNEILLY et al., 1983; JEONG, 2010) e aumento do fluxo sanguíneo e da taxa de filtração glomerular (ARTHUR E GREEN, 1982; CHEUNG E LAFAYETTE, 2013). A formação da placenta é a principal mudança anatômica do período gestacional. Este órgão é responsável pela produção de progesterona, hormônio envolvido na manutenção da gestação, e pelas trocas entre mãe e feto (RAMSEY, 1982; AL-SALEH et al., 2010). Durante um longo período acreditou-se que a placenta protegia totalmente o feto contra agentes tóxicos; porém, estudos demonstraram que metais não essenciais como o cádmio (KURIWAKI et al., 2005), o chumbo (CORPAS et al., 2002) e o mercúrio (ASK et al., 2002) ultrapassam a barreira placentária, prejudicando o desenvolvimento fetal. Com relação ao mercúrio, os efeitos da exposição a forma orgânica durante o período gestacional são os mais estudados, sendo bem estabelecido que esta forma de Hg ultrapassa facilmente a barreira placentária (ILBÄCK et al., 1991; ORNAGHI et al., 1993; SAKAMOTO et al., 2002; VINCENTE et al., 2004; NEWLAND et al., 2008; STRINGARI et al., 2008; BRIDGES et al., 2009, 2012). No entanto, quando se trata da forma inorgânica, os estudos são escassos (FENG et al., 2004; CHEHIMI et al., 2012;). Nos primeiros dias/meses de vida pós-natal, o leite materno é o principal responsável pelo fornecimento de nutrientes, vitaminas e anticorpos para a prole. No

entanto, a constituição do leite é diretamente ligada ao estado nutricional e a alimentação da mãe (BALLARD E MORROW, 2013). Assim, se a mãe for exposta a um agente tóxico este pode ser excretado via leite e contaminar a prole. De fato, alguns trabalhos demonstram que substâncias tóxicas como, por exemplo, o mercúrio, são excretadas através do leite materno causando efeitos danosos a prole (SUNDBERG et al., 1999; WINIARSKA-MIECZAN, 2014).

Os roedores, ratos e camundongos, representam os animais de laboratório mais utilizados para estudos de toxicidade reprodutiva; os quais apresentam fases distintas de desenvolvimento em um curto período de tempo. A gestação em ratos varia de 21 a 23 dias (LOHMILLER E SWING, 2006), a qual é dividida em: período de pré-implantação (dia 0 até o dia 5), implantação (dias 5 e 6), organogênese (dia 6 até o dia 15) e desenvolvimento fetal (dia 16 até o dia 21/23) (ROGERS E KAVLOCK, 2012). Após o nascimento os roedores são amamentados durante aproximadamente 21 dias (período neonatal); durante este período ocorrem importantes mudanças no desenvolvimento do neonato, por exemplo, abertura do canal auditivo (dias pós-natal 2-4) e dos olhos (dias pós-natal 14-17) e erupção dos dentes incisivos (dias pós-natal 6-8) (LOHMILLER E SWING, 2006). Além disso, durante o período neonatal ocorre intensa síntese de ácido desoxirribonucléico (DNA), ácido ribonucléico (RNA) e proteína como consequência de intensas divisões celulares, proporcionando crescimento e desenvolvimento rápidos dos órgãos (GOTTLIEB et al., 1987).

O estudo da exposição a agentes tóxicos durante o período gestacional e lactacional é importante para compreensão de como o organismo responde a um insulto tóxico durante períodos de marcadas mudanças fisiológicas e corporais e também para avaliarmos os efeitos da exposição indireta ao tóxico (barreira placentária e leite materno) sobre a prole. Logo, estudos da exposição a forma inorgânica de mercúrio durante a gestação e/ou lactação são necessários uma vez que os primeiros dias de vida pré e pós-natal são de fundamental importância para o desenvolvimento dos jovens e que insultos tóxicos, nos primeiros dias de vida, podem acarretar em danos irreversíveis.

2.OBJETIVOS:

- Avaliar os efeitos da exposição a diferentes doses de $HgCl_2$ na água de beber durante somente a gestação e durante a gestação e lactação sobre parâmetros de desenvolvimento corporal e marcadores de toxicidade nas mães e na prole.

- Avaliar os efeitos da exposição intravenosa a uma dose não nefrotóxica e uma nefrotóxica de $HgCl_2$ sobre a distribuição de mercúrio no organismo materno e fetal.

3.RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo científico e manuscritos submetidos à publicação e em fase de preparação. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos respectivos artigo e manuscritos. Os experimentos foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA-UFSM).

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Zinco como alternativa na prevenção e tratamento da toxicidade induzida pelo mercúrio durante a gestação, lactação e em ratos jovens e adultos".

Numero do Parecer: 096/2011

Pesquisador Responsável: Maria Ester Pereira

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 12 de dezembro de 2011.

Marta Lizandra do Rêgo Leal
Coordenadora da Comissão de Ética no Uso de Animais-UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

3.1 Artigo

Biochemical parameters of pregnant rats and their offspring exposed to different doses of inorganic mercury in drinking water

Cláudia S. Oliveira, Vitor A. Oliveira, Rafael P. Ineu, Lucélia Moraes-Silva e Maria E. Pereira

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Biochemical parameters of pregnant rats and their offspring exposed to different doses of inorganic mercury in drinking water

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Porphobilinogen synthase

ABSTRACT

This work investigated the effects of low and high doses of inorganic mercury in drinking water on biochemical parameters of pregnant rats and their offspring. Female Wistar rats were treated during pregnancy with 0, 0.2, 0.5, 10 or 50 μg Hg^{2+}/mL as $HgCl_2$. Rats were euthanized on day 20 of pregnancy. Pregnant rats presented a decrease in total water intake in all doses of mercury tested. At high doses, a decrease in the total food intake and in body weight gain was observed. Pregnant rats exposed to 50 μg Hg^{2+}/mL presented an increase in kidney relative weight. Mercury exposure did not change serum urea and creatinine levels in any of the doses tested. Moreover, mercury exposure did not change porphobilinogen synthase activity of kidney, liver and placenta from pregnant rats in any of the doses tested, whereas fetuses of pregnant rats exposed to 50 μg Hg^{2+}/mL presented an increase in the hepatic porphobilinogen synthase activity. In general, pregnant rats presented alterations due to $HgCl_2$ exposure in drinking water. However, only the dose 50 μg Hg^{2+}/mL appeared to be enough to cross the blood-placenta barrier, since at this dose the fetuses presented change in the porphobilinogen synthase activity.

1. Introduction

Mercury (Hg) is a non-essential metal without biological function which can be found in three chemicals forms: elemental Hg (Hg°), inorganic mercury (mainly $HgCl_2$) and organic mercury (mainly MeHg). All the forms are toxic to living organisms although they preferentially act on different target organs; $HgCl_2$, for example, is nephrotoxic and MeHg is neurotoxic (Graeme and Pollack, 1998; WHO, 2003; Counter and Buchanan, 2004). The principal exposure sources to mercury are vapor from dental amalgam (Hg°), fish and seafood (organic mercury) and water and air (inorganic mercury) (Lorschieder et al., 1995).

The most famous case of contamination with organic mercury is the Minamata case in the 1950/60s in Japan. People who consumed food, mainly fish, contaminated with MeHg presented several health problems, especially children exposed to the metal in utero (Ekino et al., 2007). Since this case, studies about populations exposed to MeHg contamination have been performed in Canada, the Faroe Islands, the Seychelles Islands, the Amazon Basin, and Iraq (Palheta and Taylor, 1995; Budtz-Jørgensen et al., 2004; Weihe et al., 2005; Debes et al., 2006; Walker et al., 2006; Nevado et al., 2010; Salehi and Esmaili-Sari, 2010). Contamination cases with other forms of Hg (inorganic and elemental) are mainly occupational, e.g., workers in lamp factories, farmers, dentists and their assistants (de Rosis et al., 1985; Shuurs, 1999; Risher et al., 2003; WHO, 2003).

The most known action mechanism of mercury toxicity is its high affinity by sulfhydryl (SH) groups. Hg can bind a variety of biomolecules which have SH groups in their structures, for example, glutathione (GSH) and some enzymes such as sodium–potassium ATPase (Na^+/K^+-ATPase) and porphobilinogen synthase (PBG-synthase) (Graeme and Pollack, 1998; Farina et al., 2003; Omotayo et al., 2011). In fact, PBG-synthase, a cytosolic sulfhydrilic enzyme (Sassa, 1982), is usually utilized as a marker of bivalent metal exposure (Bernard and Lauwerys, 1987).

Studies from our research group have shown that inorganic mercury inhibits renal and hepatic PBG-synthase activity (Peixoto et al., 2007a; Franciscato et al., 2011) from young rats exposed subcutaneously to $HgCl_2$ and euthanized at different times after the exposure. In addition, $HgCl_2$ caused behavior alterations (Peixoto et al., 2007b; Franciscato et al., 2009), a decrease in the body weight gain and an increase in serum urea and creatinine levels (Peixoto and Pereira, 2007; Franciscato et al., 2011).

Regarding adult males, mercury intoxication increases lipid peroxidation in liver, kidney and brain, as well as serum urea and creatinine levels, markers of renal damage. Moreover, it also increase plasma alanine aminotransferase and aspartate aminotransferase activities, markers of hepatic damage (Agarwal et al., 2010; Pal and Ghosh,

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2012). Several papers have shown that male rats are more susceptible to $HgCl_2$-intoxication than female rats (Malagutti et al., 2009; Ekstrand et al., 2010) and this difference is not understood until now. Still, in relation to pregnancy, pregnant rats are less susceptible to MeHg-intoxication than their fetuses (Clarkson, 1992). During the gestation, the placenta plays a central role secreting hormones and enzymes and providing nutrients and oxygen to the fetuses (Al-Saleh et al., 2011). Several studies have shown that this organ acts as a barrier preventing a large amount of toxic substances to pass to the fetuses; however, some of these substances such as cadmium (Kuriwaki et al., 2005), lead (Corpas et al., 2002) and mercury (Ask et al., 2002) are able to cross the blood-placenta barrier and affect the fetal development.

Studies have shown that the organic mercury is able to cross blood-placenta barrier in humans and experimental animals causing consequences for the offspring (Ornaghi et al., 1993; Vicente et al., 2004; Stringari et al., 2008). However, there are few studies about the prolonged inorganic mercury exposure in drinking water to pregnant rats. Taking into account that water is a source of exposure at Hg form and that MeHg can be demethylated in the gastrointestinal tract (Lorschieder et al., 1995), this study evaluated the effects of inorganic mercury exposure via drinking water on the following physiologic parameters of development: body weight gain, total food and water intake. We also investigated serum urea and creatinine levels in pregnant rats as markers of renal damage, and PBG-synthase activity was assessed in liver, kidney and placenta of pregnant and in their fetuses as a marker of Hg effect.

2. Materials and methods

2.1. Chemicals

Mercuric chloride, sodium chloride, potassium phosphate monobasic and dibasic, absolute ethanol, trichloroacetic acid, o-phosphoric acid, perchloric acid and glacial acetic acid were purchased from Merck (Darmstadt, Germany). δ-Aminolevulinic acid (δ-ALA), bovine serum albumin and Coomassie brilliant blue G were obtained from Sigma (St. Louis, MO, USA). ρ-Dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han., Germany). The kits for determination of creatinine and urea were acquired from LABTEST (Lagoa Santa/MG/Brazil).

2.2. Animals

Thirty-four Wistar rats (25 virgin female rats, 7–8 weeks old, 190–220 g; and 9 mature male rats, 14–15 weeks old, 250–300 g) obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12 h light/dark cycle and controlled temperature (23 ± 2 °C). The animals had free access to water and commercial food (Puro Trato, J A Teixeira Veterinária LTDA, Santo Augusto, RS, Brazil; metal contents of diet, in mg/kg dry weight, Ca 12,000, Co 1.5, Cu 10.0, Zn 62.0, Fe 61.0, Mn 62.0). Mercury levels in commercial food and water are below the levels detectable by atomic absorption spectrophotometry. After 2 weeks of adaptation the female and male rats (3:1) were placed in the same cage for the mating. Studies were conducted in accordance with the national and institutional guidelines (University Ethics Committee Guidelines—Process number 096/2011) for experiments with animals.

2.3. Mating

Virgin female Wistar rats (8–10 weeks old, 180–220 g) were placed in animal cages with mature male breeders (15–16 weeks old, 250–300 g). Three female and 1 male were placed in polycarbonate cages during the night (7:00 p.m.–7:00 a.m.); in the morning period (7:00–9:00 a.m.), males were separated from females. Mating was confirmed by the presence of sperm in their vaginal smears, and defined as day 0 of gestation (GD 0). If no sperm were observed in the vaginal smears in any of the three females within 7 days of mating, the male was removed and replaced for another male.

2.4. Treatment

Pregnant rats were placed individually in polycarbonate cages and exposed at different doses of $HgCl_2$ for 20 days, from day 0 until day 20 of gestation (GD 0 to GD 20). $HgCl_2$ was dissolved in distilled water and supplied to females as the unique form of liquid diet during the gestation. The drinking solutions containing mercury were replaced and prepared every 2 days from a stock solution (200 μg Hg^{2+}/mL) prepared weekly and stored (4 °C). Every 2 days, the pregnant rats were weighed and the food and water intake measured. The food and water intake was monitored during the whole experimental period and the food and water intake was calculated per 100 g of body weight per day (Table 1).

The experiments were divided into two protocols:

- *Low doses of mercury (LDM):*
  Pregnant rats received distilled water (control, $n = 3$), 0.2 μg Hg^{2+}/mL (group 0.2, $n = 3$) or 0.5 μg Hg^{2+}/mL (group 0.5, $n = 3$).
- *High doses of mercury (HDM):*
  Pregnant rats received distilled water (control, $n = 7$), 10 μg Hg^{2+}/mL (group 10, $n = 4$) or 50 μg Hg^{2+}/mL (group 50, $n = 5$).

2.5. Sample preparation

On day 20 of gestation rats were euthanized for decapitation. Blood, liver, kidneys, placenta and fetuses were removed from pregnant rats, and liver and kidneys were removed from their fetuses. Serum was obtained by total blood centrifugation at 3000g for 10 min and frozen until analyses (5 days). For PBG-synthase activity assay liver, kidneys and placenta were quickly placed on ice and homogenized in 7, 5 and 10 volumes of NaCl (150 mmol/L, pH 7.4), respectively, with 10 up-and-down strokes at ~1200 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged at 8000g for 30 min at 4 °C and the supernatant fraction was used in the enzyme assay.

2.6. Biochemical determinations

2.6.1. Creatinine

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

2.6.2. Urea

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

2.6.3. PBG-synthase activity

The enzymatic activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen – PBG) formation, except that 76 mmol/L sodium phosphate buffer (pH 6.8) and 2.2 mmol/L δ-ALA were used. The incubation was initiated by adding 200 μL supernatant. Liver, kidney and placenta samples from pregnant rats were incubated for 25, 60 and 120 min, respectively, at 39 °C. For fetuses, liver and kidney samples were incubated for 40 and 120 min, respectively, at 39 °C. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 mol/L and the PBG was measured with Ehrlich's reagent at 555 nm, using the molar absorption coefficient of 6.1×10^4 for Ehrlich-PBG salt. The specific enzymatic activity was expressed as nmol of PBG formed per hour per mg protein.

2.6.4. Protein determination

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

2.7. Statistical analysis

All biochemical assays were carried out in triplicate. The mean of triplicates was used for statistic analyses. The results were analyzed by one-way ANOVA followed by Duncan's multiple range test when appropriated. Each pregnant rat and its litter were considered an experimental *n*.

3. Results

3.1. Low doses of mercury (LDM) exposure

The total body weight gain and food and water intake are shown in Table 1. These results show that LDM did not alter the body weight gain or the food intake. However, the water intake presented as total intake during the 20 days and per 100 g of body

Table 1
Total body weight gain, food, water and Hg^{2+} intake of pregnant rats exposed to low (0.2 and 0.5 μg Hg^{2+}/mL) and high (10 and 50 μg Hg^{2+}/mL) doses of $HgCl_2$ in drinking water during all period of pregnancy.

Groups	Body weight gain[A] (g)	Food intake (g)		Water intake (mL)		Hg^{2+} intake (mg)	
		Total[B]	100 g/day[C]	Total[B]	100 g/da_	Total[B]	100 g/day[C]
LDM							
Control (n = 3)	104.3 ± 7.3	665.0 ± 3.6	5.7 ± 0.3	1342.7 ± 52.8[a]	11.6 ± 0.4[a]	n.d.	n.d
Group 0.2 (n = 3)	86.7 ± 5.2	606.0 ± 53.3	5.5 ± 0.4	956.3 ± 47.3[b]	8.6 ± 0.4[b]	0.19 ± 0.01	0.002 ± 0.001
Group 0.5 (n = 3)	89.3 ± 13.4	595.3 ± 9.0	5.2 ± 0.1	991.3 ± 77.4[b]	8.6 ± 0.5[b]	0.50 ± 0.04	0.004 ± 0.001
HDM							
Control (n = 7)	109.0 ± 6.7[a]	500.0 ± 21.6[a]	4.5 ± 0.1	1225.6 ± 95.9[a]	11.2 ± 0.9[a]	n.d.	n.d
Group 10 (n = 4)	107.5 ± 5.2[a]	496.8 ± 8.7[a]	4.7 ± 0.1	904.3 ± 98.5[b]	8.5 ± 0.8[b]	9.04 ± 0.99	0.085 ± 0.008
Group 50 (n = 5)	81.2 ± 7.8[b]	408.2 ± 28.9[b]	4.0 ± 0.4	591.6 ± 25.7[c]	6.0 ± 0.5[b]	29.58 ± 1.28	0.301 ± 0.024

LDM = low doses of mercury.
HDM = high doses of mercury.
GD = gestation day.
n.d. = not determined.
The results are presented as mean ± SEM. Duncan's multiple range test: different letters confer significant difference between groups ($p < 0.05$).
[A] Total body weight gain = body weight (GD 20) – body weight (GD 0).
[B] Total intake for 20 days of treatment (GD 0 to GD 20).
[C] Food, water and Hg^{2+} intake per 100 g of body weight (B.W.) per day.

Table 2
Total and relative weight from different organs of pregnant rats exposed to high (10 and 50 μg Hg^{2+}/mL) doses of $HgCl_2$ in drinking water during all the period of pregnancy.

	Liver		Kidney		Placenta	
	Total weight (g)	Relative weight (%)	Total weight (g)	Relative weight (%)	Total weight (g)	Relative weight (%)
Control (n = 7)	13.32 ± 0.93	4.87 ± 0.09	1.74 ± 0.11	0.64 ± 0.02	0.54 ± 0.03	0.20 ± 0.01
Group10 (n = 4)	12.15 ± 0.48	4.99 ± 0.24	1.74 ± 0.04	0.72 ± 0.06	0.47 ± 0.02	0.19 ± 0.01
Group 50 (n = 5)	10.94 ± 0.97	4.76 ± 0.17	2.01 ± 0.10	0.89 ± 0.06*	0.43 ± 0.03	0.19 ± 0.02

The results are presented as mean ± SEM. Duncan's multiple range test.
* Differs significantly from the control group ($p < 0.05$).

weight per day was significantly altered by the treatment (one-way ANOVA: $F(2,6) = 12.440$; $p < 0.007$ and $F(2,6) = 18.163$; $p < 0.003$, respectively). The pregnant rats exposed to 0.2 and 0.5 μg Hg^{2+}/mL presented lower total and daily water intake than the control group. Pregnant rats did not present alterations in total body weight gain.

The exposure to LDM showed no modifications regarding the number and the weight of fetuses (Table 3), the markers of renal damage (urea and creatinine levels, Table 4) or PBG-synthase activity from different organs (Fig. 1A and C).

3.2. High doses of mercury (HDM) exposure

The total body weight gain as well as food and water intake of pregnant rats exposed to HDM are shown in Table 1. One-way ANOVA revealed a significant effect of the treatment in total body weight gain [$F(2,13) = 4.876$; $p < 0.026$], total food intake [$F(2,13) = 4.932$; $p < 0.025$] and total [$F(2,13) = 14.892$; $p < 0.001$] and daily [$F(2,13) = 11.193$; $p < 0.001$] water intake. The pregnant rats exposed to 10 and 50 μg Hg^{2+}/mL presented lower total and daily water intake and only pregnant rats exposed to 50 μg Hg^{2+}/mL presented lower total body weight gain and total food intake. Furthermore, we assessed the total and relative weight of liver, kidney and placenta and results are shown in Table 2. The pregnant rats exposed to 50 μg Hg^{2+}/mL presented relative weight of kidney significantly higher when compared to the control group [$F(2,13) = 9.427$; $p < 0.003$]. The HDM exposure did not change significantly total or relative weight of liver or placenta.

Two high doses (group 10 and group 50) of inorganic mercury caused an increase in the number of fetuses [$F(2,13) = 9.182$; $p < 0.003$] when compared to the control group, but did not alter the weight of fetuses (Table 3).

Table 3
Number and weight of offspring from pregnant rats exposed to low (0.2 and 0.5 μg Hg^{2+}/mL) and high (10 and 50 μg Hg^{2+}/mL) doses of $HgCl_2$ in drinking water.

Groups	Number of fetus	Weight of fetus (g)
LDM		
Control (n = 3)	11.33 ± 0.33	2.54 ± 0.30
Group 0.2 (n = 3)	12.67 ± 0.88	2.64 ± 0.05
Group 0.5 (n = 3)	12.67 ± 0.33	2.71 ± 0.15
HDM		
Control (n = 7)	9.29 ± 0.47	3.03 ± 0.25
Group 10 (n = 4)	12.50 ± 0.65*	3.14 ± 0.31
Group 50 (n = 5)	12.00 ± 0.71*	2.39 ± 0.10

LDM = low doses of mercury.
HDM = high doses of mercury.
The results are presented as mean ± SEM. Duncan's multiple range test.
* Differs significantly from the control group ($p < 0.05$).

Table 4
Urea and creatinine levels of pregnant rats exposed to low (0.2 and 0.5 μg Hg^{2+}/mL) and high (10 and 50 μg Hg^{2+}/mL) doses of $HgCl_2$ in drinking water during all the period of pregnancy.

Groups	Urea (mg/dL)	Creatinine (mg/dL)
LDM		
Control (n = 3)	41.18 ± 4.48	0.47 ± 0.01
Group 0.2 (n = 3)	55.01 ± 5.71	0.47 ± 0.09
Group 0.5 (n = 3)	51.22 ± 1.30	0.48 ± 0.06
HDM		
Control (n = 7)	43.50 ± 4.45	0.43 ± 0.13
Group 10 (n = 4)	37.65 ± 2.80	0.78 ± 0.15
Group 50 (n = 5)	44.98 ± 6.82	0.46 ± 0.11

LDM = low doses of mercury.
HDM = high doses of mercury.
The results are presented as mean ± SEM.

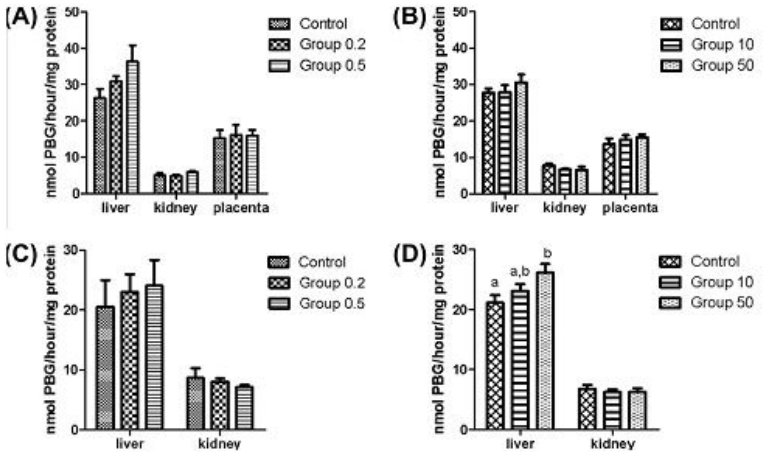

Fig. 1. Porphobilinogen synthase activity from different tissues of pregnant rats exposed to low and high doses of $HgCl_2$ in drinking water during all the period of pregnancy (A and B, respectively) and their offspring exposed to low (0.2 and 0.5 μg Hg^{2+}/mL) and high (10 and 50 μg Hg^{2+}/mL) doses of $HgCl_2$ in utero (C and D, respectively). The results are presented as mean ± SEM (n = 3–7). Duncan's multiple range test: bars labeled with different letters are statistically different ($p < 0.05$).

The biochemical parameters, urea and creatinine levels, and PBG-synthase activity were not significantly changed in pregnant rats as consequence of treatments (Table 4 and Fig. 1B). However, fetuses of pregnant rats exposed to 50 μg Hg^{2+}/mL presented an increase in the hepatic PBG-synthase activity [$F(2,13) = 3.761$; $p < 0.05$] (Fig. 1D).

4. Discussion

Numerous investigators have shown the low and high doses of organic mercury toxicity in humans and experimental animals during the pregnancy and the consequences for the offspring (Ornaghi et al., 1993; Vicente et al., 2004; Stringari et al., 2008). However, studies about the prolonged inorganic mercury exposure in drinking water are scarce in literature. Thus, the aim of this study was to investigate the effect of inorganic mercury exposure in the drinking water during the gestation period. Inorganic mercury exposure caused a dose-dependent decrease in total water intake in both LDM and HDM exposure, probably due to the unpalatability of water caused by the metal, since mercury is known by the metallic taste (WHO, 2003). This result is in agreement with the findings by Orisakwe et al. (2001) who verified a decrease in water intake by male mice exposed to $HgCl_2$ in drinking water. The water-metallic taste also may be the cause of the decrease in food intake and, consequently, of the decrease in the body weight gain of pregnant rats exposed to HDM. In addition, Hg is known not only to cause lack of appetite (Zahir et al., 2005), but also to cause alterations in the absorption of nutrients (Sastry et al., 1982; Farmanfarmaian et al., 1989). Kidney relative weight was significantly increased by HDM exposure in pregnant rats at the dose of 50 μg Hg^{2+}/mL. Some papers have reported alterations in body and kidney weights of animals exposed to this metal (Rocha et al., 1995; Peixoto et al., 2003; Roza et al., 2005; Franciscato et al., 2011). The increase of the kidney size and weight in Hg-exposed rats may be related to structural changes due primarily to an increase in proximal tubule volume (Madsen and Maunsbach, 1981).

In the present study, LDM and HDM exposure did not change the biochemical parameters analyzed in pregnant rats since, PBG-synthase activity as well as serum creatinine and urea levels were not altered. A study with another exposure form (intravenous) has shown alterations in the biochemical parameters in pregnant rats, but these alterations were lesser when compared to non-pregnant rats (Holt and Webb, 1986). Thus, increased levels of endogenous protective molecules during pregnancy may explain the absence of changes in biochemical parameters analyzed in our study. Literature data have shown that rodents during gestational period have increased metallothionein levels (Yoshida et al., 2002), and this could be help in protecting pregnants and fetuses against Hg damage. It has been known that metallothioneins act as detoxifying agents of metal ions (Dabrio et al., 2002), since according to some studies, this protein would sequester the toxic metal turning it unavailable to cause its damage effects (Klaassen et al., 1999; Nath et al., 2000).

In relation to fetuses exposed to inorganic mercury in utero, Feng et al. (2004) observed an increase in Hg levels in kidney, liver and other organs. Holt and Webb (1986) found teratogenic effects caused by Hg, and Atkinson et al. (2001) observed a decrease in total number of pups born in F1 by F0 – male and female $HgCl_2$-exposed. In our study, LDM exposure did not alter the number of fetuses, whereas pregnant rats exposed to HDM presented an increase in the number of fetuses. This finding was not expected and calls for further research. Regarding the PBG-synthase activity we observed only an increase in the hepatic PBG-synthase activity of fetuses which were exposed to 50 μg Hg^{2+}/mL in utero. These results differ from Brandão et al. (2006) and Peixoto et al. (2007a) who observed a decrease in the hepatic PBG-synthase activity in mice and young rats, respectively. This increase in fetal hepatic PBG-synthase activity may be due to a compensatory mechanism, since the fetuses are exposed to inorganic mercury in utero from the beginning of their development, suggesting that fetuses present a distinct metabolism from that observed in young and adults rodents in response to mercury exposure (Rocha et al., 1995; Emanuelli et al., 1996; Peixoto et al., 2003, 2004, 2007a; Roza et al., 2005; Brandão et al., 2006). Similar increase of PBG-synthase activity was related in primary hepatocyte culture from *Hoplias malabaricus*, a wild fish collected in Amazon basin by methyl mercury (Filipak Neto et al., 2008).

In summary, pregnant rats exposed to LDM presented a decrease only in total water intake. On the other hand, pregnant rats exposed to HDM presented a decrease in total water and food intake as well as total body weight gain. Still, fetuses exposed to LDM in utero did not present changes in biochemical parameters, whereas fetuses exposed to HDM in utero presented an increase only in the hepatic PBG-synthase activity. Thus, we conclude that inorganic mercury can cross the blood-placenta barrier at HDM exposure and cause biochemical alterations in fetuses. More studies are necessary to provide information about offspring development (behavior and biochemical parameters) after exposure in utero at these high doses of inorganic mercury.

Conflict of Interest

None declared.

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3.2Manuscrito I

Inorganic mercury exposure in drinking water alters essential metal homeostasis in pregnant rats without altering the newborn behavior

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Abstract: The aim of this work was to investigate the effects of exposure to HgCl2 in the doses of 0, 10 and 50 μg Hg2+/mL in drinking water during the pregnancy period on tissue essential metal homeostasis, as well as the effects of HgCl2 exposure in utero and breast milk on behavioral tasks. Pregnant rats exposed to both inorganic mercury doses presented high renal Hg content and an increase in renal Cu and hepatic Zn levels. Moreover, mercury exposure caused an increase in Hg and essential metal fecal excretion, but not significantly. Pups exposed to inorganic mercury did not present alterations on essential metal homeostasis or on behavioral task markers of motor function. In conclusion, this work showed that the physiologic pregnancy and lactation states protected the offspring from low doses of Hg2+. This protection is likely to be related to the metallothioneins which is an important scavenger molecule.

Inorganic mercury exposure in drinking water alters essential metal homeostasis in pregnant rats without altering the newborn behavior

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Abstract

The aim of this work was to investigate the effects of exposure to $HgCl_2$ in the doses of 0, 10 and 50 µg Hg^{2+}/mL in drinking water during the pregnancy period on tissue essential metal homeostasis, as well as the effects of $HgCl_2$ exposure in utero and breast milk on behavioral tasks. Pregnant rats exposed to both inorganic mercury doses presented high renal Hg content and an increase in renal Cu and hepatic Zn levels. Moreover, mercury exposure caused an increase in Hg and essential metal fecal excretion, but not significantly. Pups exposed to inorganic mercury did not present alterations on essential metal homeostasis or on behavioral task markers of motor function. In conclusion, this work showed that the physiologic pregnancy and lactation states protected the offspring from low doses of Hg^{2+}. This protection is likely to be related to the metallothioneins which is an important scavenger molecule.

Keywords: inorganic mercury; pregnancy; essential metals; metallothionein; behavioral tasks

1. Introductions:

Essential metals such as zinc, iron, cupper, magnesium, and calcium are extremely important to organisms. They participate in a great number of cellular biochemical pathways and some of the essential metals are structural components of macromolecules or enzymatic cofactors. The amount of essential metals requested by the organism depends on the age, gender, genetic factors, and physiologic state (pregnancy and lactation) (Becking et al., 2003; WHO, 1996). During the pregnancy and lactation period, for instance, females have a high nutrient demand, due to the mother's organism being responsible for providing nutrients, such as vitamins, amino acids and essential metals to their offspring via utero or breast milk (Das et al., 2013; WHO, 1996). During the gestation and lactation period, females present biological adaptations to provide nutrients to the offspring, such as increase of food consumption and gastrointestinal absorption efficiency, decrease on urinary and fecal excretion, and mobilization of tissues stores (Prentice, 2003).

Due to the extreme importance of essential metals to the newborn development as well as to a healthy adult life, the World Health Organization (WHO) highlighted the necessity of maintenance of the essential metal homeostasis (WHO, 1996). Studies have shown that essential metal deficiency (e.g. iron) (Garcia et al., 2007), chelating agents (Taubeneck et al., 1992; Mehta et al., 2006; Gabard et al., 1979), and heavy metal intoxication (Cobbina et al., 2015; Peixoto et al., 2008) may alter the essential metal homeostasis. The symptoms from the disturbances in essential metal homeostasis are variable and can differ between the elements, but generally organisms with essential metal deficiency present growth retardation, anaemia, skin lesion, alopecia, osteoporosis (WHO 1996), depression, and anxiety (Młyniec et al., 2014).

Among the toxic metals known for disturbing the essential metal homeostasis, we can highlight cadmium, arsenic, lead, and mercury (Cobbina et al., 2015). Mercury is a non-

essential metal ubiquitously distributed in the environment and it can be found in three chemical forms, namely organic, inorganic and elemental mercury. Indiscriminate and uncontrolled mercury use by humans may increase the release of this metal and consequently increase the risk of contamination. Mercury exposure may occur through pollution inhalation (elemental mercury) or by the ingestion of contaminated food (mainly organic mercury) or water (mainly inorganic mercury) (Rice et al., 2014; Syversen and Kaur, 2012). In the last few decades, developing countries such as Brazil, China, India, and Mexico have been more propitious to Hg and other environmental contaminant effects due to rapid industrialization and increased urbanization without adequate residue and waste control (Horton et al., 2013; Muntean et al., 2014).

Several studies of our research group have demonstrated the effects of subcutaneous inorganic mercury exposure, such as inhibition of sulfhydryl enzymes, nephrotoxicity symptoms (Franciscato et al., 2011; Favero et al., 2014; Peixoto and Pereira, 2007; Oliveira et al., 2014; Fiuza et al., 2014), and behavioral alterations (Peixoto et al., 2007; Fransciscato et al., 2009; Moraes-Silva et al., 2014). Furthermore, pups subcutaneously exposed to $HgCl_2$ presented essential metal level alterations in the liver, kidney (Peixoto et al., 2008) and brain (Moraes-Silva et al., 2014). Consequently, these alterations in essential metal homeostasis may contribute to the toxic effects of mercury. Interestingly, changes in renal and hepatic zinc levels have not been observed when pups were indirectly (via breast milk) exposed to inorganic mercury (Favero et al., 2014; Oliveira et al., 2014).

Taking into consideration the high Hg toxic effects and that drinking water is the main exposure route to inorganic mercury, studies in this area are necessary. Recently, Oliveira et al. (2012) showed that $HgCl_2$ exposure in drinking water during the pregnancy period causes a decrease in food and water intake and body weight gain, without altering the fetus weight. Based on the importance of essential metals to the good development of the offspring during

the gestational and lactation period, the main objective of this work was to verify the effects of $HgCl_2$ exposure in drinking water on essential metal homeostasis in pregnant rats and their fetuses. Moreover, we verified whether pups exposed to $HgCl_2$ in utero and breast milk present behavioral alteration.

2. Material and Methods

2.1 Chemicals

Mercuric chloride, mercury, iron, zinc standard solution to metal dosage, sodium chloride, potassium phosphate monobasic and dibasic, absolute ethanol, sodium hydroxide, nitric acid, ß-mercaptoethanol, sucrose, hydrochloric acid, phenylmethylsulphonylfluoride (PMSF), chloroform, calcium disodium ethylenediaminetetraacetate (EDTA), 5,5 -dithio-*bis*(2-nitrobenzoic acid) (DTNB), and tris(hydroxymethyl) aminomethane (Tris) were purchased from Merck (Darmstadt, Germany).

2.2Animals

Thirty-four Wistar rats (25 virgin female rats, 10–12 weeks old, 190–220 g; and 9 mature male rats, 14–15 weeks old, 250–300 g) obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12 h light/dark cycle and controlled temperature (22 ± 2 ºC). The animals had free access to water and commercial food. After 2 weeks of adaptation the female and male rats (3:1) were placed in the same cage for the mating. Studies were carried out in accordance with the national and institutional guidelines (University Ethics Committee Guidelines—Process number 096/2011) for experiments with animals.

2.3 Mating

Virgin female Wistar rats were placed in animal cages with mature male breeders as described in Oliveira et al. (2012). Briefly, three females and 1 male were placed in the same cage during the night. In the next morning period, males were separated from females. Mating was confirmed by the presence of sperm in the vaginal smears, and defined as day 0 of gestation (GD 0).

2.4 Treatment

Pregnant rats were randomly divided into three groups as described below:

- Control (n=7): animals received only distilled water
- Group 10 (n=9): animals received 10 µg Hg^{2+}/mL in drinking water
- Group 50 (n=7): animals received 50 µg Hg^{2+}/mL in drinking water

Animals were placed individually in polycarbonate cages and exposed to one of the two doses (10 or 50 µg Hg^{2+}/mL) of $HgCl_2$ for 20 days (from day 0 until day 20 of gestation; n= 3 animals per group) or 41 days (from day 0 of gestation until day 21 of lactation; n= 4-6 animals per group) as presented in the Figure 1. $HgCl_2$ was dissolved in distilled water and supplied to females as the unique form of liquid diet during the gestation and/or lactation. Drinking solutions containing mercury were replaced and prepared every 2 days from a stock solution (200µg Hg^{2+}/mL) prepared weekly and stored (4 ºC).

2.5 Sample preparation

On day 19 of gestation, three rats of each group were placed in individual metabolic cages to collect urine and feces for metal determinations. The animals were maintened in the

metabolic cages during 24hs. In the following day (GD 20), pregnant rats (n=3/group) were euthanized for decapitation. Blood, liver, kidneys, placenta and fetuses were removed. In addition, the liver and kidneys were removed from the fetuses. Serum was obtained by total blood centrifugation at 3,000 g for 10 min and frozen until analyses (5 days).

For metallothionein level assays, the tissues from the pregnant rats (liver, kidneys and placenta) 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 a reducing agent. The homogenate was then centrifuged at 16,000 g for 30 min in order to obtain a supernatant containing metallothioneins.

For metal determination pregnant rat tissues (blood, liver, kidneys and placenta), urine and feces, as well as offspring tissues (fetuses: liver and kidney; pups on post-natal day 20: brain) were removed and frozen at -20 ºC until analyses.

2.6 Hg, Cu, Zn and Fe content determination

Metal determination was carried out in inductively coupled plasma atomic emission spectrometry (ICPE-9000; Shimadzu Scientific Instruments). Samples were digested in distilled HNO_3 as described in Ineu et al. (2013) and diluted in distillated water for subsequent Hg, Cu, Zn and Fe level determination. The analytical standards Hg, Cu, Zn and Fe (Merck®) were used to make the curve.

2.7 Metallothionein (MT) level determination

Metallothionein levels were determined by colorimetric method using Ellman`s reagent (Ellman, 1959) as described in Viarengo et al. (1997) and Peixoto et al. (2003). A

standard curve using GSH as reference was constructed in order to express the MT levels in μg of SH per g of tissue.

2.8 Behavioral tasks

Four litters were submitted to negative geotaxis task and beaker test. Each litter contributed with two experimental n.

2.8.1 Negative geotaxis task

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

2.8.2 Beaker test

Animals were submitted to the beaker test from 17 to 20 days old (sessions from 1 to 4) with interval of 24 h between sessions as described by Peixoto et al. (2007). In this task, the ability of rats to balance and move along the rim of 2 L polypropylene beaker was observed. This beaker, with 19.5 cm high X 14 cm diameter, had an outward-curving top edge. The apparatus was placed on a workbench, 1 m from the room floor. A dark refuge box,

inner dimensions 9.5 cm X 5.5 cm X 3.5 cm high, with an entrance platform 5 cm X 5.5 cm, was clamped so that the platform could rest on the spout of the beaker. Each rat was placed on the rim of the beaker, facing the refuge at the furthest distance from it. Time points to reach the refuge, fall or jump off the rim were recorded. A cut off time of 90 s was used for each session (Smart and Dobbing, 1971). Results are presented as mean of latency to access to refuge.

2.9 Statistical analysis

Results were analyzed by one or two-way ANOVA followed by Tukey's multiple range test when appropriate. Effects were considered significant when $p<0.05$.

3. Results

3.1 *Tissue Hg and essential metal levels*

3.1.1 Pregnant rats

The blood, liver, kidneys, and placenta Hg levels as well as essential metal levels from pregnant rats exposed to 0, 10 and 50 μg Hg^{2+}/mL in drinking water are shown in Table 1. One way ANOVA showed significant effect of treatment on renal [$F(2, 8) = 19.17$; $p = 0.0025$] but not on blood, liver and placenta Hg levels. As essential metals, all tissues evaluated presented detectable (Zn, Cu and Fe) levels. However, only renal Cu [$F(2, 8) = 10.12$; $p = 0.0119$] content and hepatic Zn [$F(2, 8) = 5,794$; $p = 0.0397$] content were significantly increased by Hg exposure. Pregnant rats exposed to 10 and 50 μg Hg^{2+}/mL presented a dose dependent increase in kidney Hg and Cu levels. On the other hand, only rats exposed to 50 μg Hg^{2+}/mL presented an increase in hepatic Zn levels when compared to the control group ($p<0.05$, Tukey's multiple comparison test).

3.1.2 Offspring

The renal and hepatic metal levels from fetuses exposed to inorganic mercury in utero were not altered by treatments (Table 3).

The metal content in the brain was analyzed on post-natal day 20 (last day of behavioral tasks). Mercury was not detectable in cerebral tissues. The essential metals (Cu, Zn and Fe) are present in detectable levels and were not altered by mercury exposure in utero and via breast milk (One-way ANOVA; data not showed).

3.2 Hg and essential metal urine and feces excretion

Urine and feces Hg levels as well as essential metal contents are shown in Table 2. Urine Cu and Hg levels were under the detectable limit. In relation to Zn and Fe urine levels, one-way ANOVA showed absence of treatment effect.

Regarding feces metal excretion, Hg was detectable only in the higher Hg dose (50 μg Hg^{2+}/mL) whereas all the essential metals tested were detectable in the feces. One-way ANOVA showed treatment effect only on feces Hg levels [$F(2,8) = 3.992$; $p = 0.05$]. Pregnant rats exposed to 50 μg Hg^{2+}/mL presented an increase in feces Hg excretion when compared to other groups ($p<0.05$, Tukey's multiple comparison test). Additionally, animals Hg-exposed presented a slight increase in Zn, Cu and Fe feces excretion, although not significant.

3.3 Metallothionein (MT) levels

The liver, kidney and placenta MT levels from pregnant rats were not altered by treatments (Table 4).

3.4 Behavioral tasks

3.4.1 Negative geotaxis task

The negative geotaxis of pups exposed to inorganic mercury in uterus and breast milk is shown in Figure 2. The treatment did not interfere in the appearance of the negative geotaxis reflex. All animals presented this reflex on day 5. Two-way ANOVA (3 treatments × 6 sessions) showed a significant effect of session [$F(5,105) = 117.05$; $p = 0.001$] but not of treatment or interaction.

3.4.2 Beaker test

Latency to access to refuge of newborn rats is shown in Table 5. Two-way ANOVA (3 treatments × 4 sessions) revealed a significant effect of session on latency to access to refuge [$F(3, 63) = 6.88$, $p = 0.0004$] but not of treatment or interaction.

4. Discussion:

Studies have shown that the subcutaneous inorganic mercury exposure disrupts essential metal homeostasis (Peixoto et al., 2008; Moraes-Silva et al., 2014). It is uncommon for the population to come into contact with Hg through the subcutaneous route, thus, the importance of understanding how it is handled by the body when administrated in oral route (mainly drinking water) is pivotal. Our study evaluated the effects of gestational exposure to two different inorganic mercury doses in drinking water on the essential metal homeostasis in pregnant rats and their offspring. Moreover, we evaluated whether the inorganic mercury exposure via uterus and breast milk alter the behavioral task markers of motor function.

Pregnant rats exposed to inorganic mercury in drinking water presented a significant alteration on Cu and Zn homeostasis and a dose dependent increase in renal Hg levels. It is known that the kidney is the first inorganic mercury target to be reached. For instance, few hours after an intravenous inorganic mercury injection ~50% of administrated dose is burden on total renal mass (Zalups, 1993). In our experimental design (low Hg^{2+} doses in drinking water), mercury was detectable only in the kidney and feces (only 50 µg Hg^{2+}/mL group). Surprisingly, neither group exposed to inorganic mercury presented detectable Hg levels in urine. Our hypothesis is that since pregnant rats are exposed to low inorganic mercury doses during approximately 20 days, the organism, mainly the kidney, might be able to handle the Hg ions without causing the classic inorganic mercury nephrotoxic alterations. Hg is likely to bind to SH groups of scavenger molecules, such as gluthatione (GSH) and metallothionein (MT), forming an inert complex. Therefore, animals exposed to 10 and 50 µg Hg^{2+}/mL presented ~37% and ~22%, respectively, higher renal MT content than the control group. We suggest that this complex Hg-SH group of scavenger molecules may be stocked in renal cells avoiding the necrosis and consequent Hg release in urine. Indeed, we showed that pregnant rats exposed to inorganic mercury in drinking water presented no alterations in serum urea and creatinine levels, which are markers of renal damage (Oliveira et al., 2012).

Regarding essential metal homeostasis, pregnant rats exposed to Hg^{2+} presented an increase in renal Cu content and hepatic Zn content. Studies have shown an increase in renal copper levels after the exposure to toxic elements, such as Hg (Peixoto et al., 2008), arsenic (Birri et al., 2010) and aluminum (Oztürk and Ozdemir, 2013). Recently, García-Sevillano et al. (2013) found that the renal content of Hg and Cu bond to MT (Cu-MT and Hg-MT, respectively) increased in a dose dependent manner after the inorganic mercury exposure. This result suggests that MTs are closely related to renal Cu level increases, as well as to increased kidney Hg levels. Following this point of view, once the liver is one of the organs

responsible for MT synthesis, and Zn is a known inductor of this metalloprotein (Onosaka and Cherian, 1982), the increase in hepatic Zn levels could be an adaptive response, i.e., Zn ions may have been transported to liver to increase the scavenger molecule synthesis.

Despite the increase in Cu and Zn content in the kidneys and liver, respectively, the fecal excretion of these metals was slightly increased (not significantly) by Hg exposure. Thus, Cu and Zn ions accumulated in kidney and liver, respectively, may not have been from the diet. These metals are likely to be redistributed by body stores, for instance, from bones. In agreement with our hypothesis, Bellés et al. (2001) showed that the oral exposure to aluminum during the gestation may cause a decrease in bone Cu and Zn levels and an increase of these essential metals in renal and hepatic tissues. Still, it is important to highlight that oral exposure to inorganic mercury seems to affect the essential metal gastrointestinal absorption once these metals are released in the feces.

Pups subcutaneously exposed to inorganic mercury during the post natal days 8 to 12 present an imbalance in Zn, Cu, Fe and Mg homeostasis, a great Hg accumulation in soft tissues (Peixoto et al., 2008), as well as a severe impairment in motor function (Franciscato et al., 2009). Interestingly, in our work, when the animals were exposed to inorganic mercury in utero and breast milk, the above mentioned alterations were not observed. Still, a previous study from our research group using this same drinking water exposure protocol showed that inorganic mercury did not alter the number and the weight of the fetuses (Oliveira et al., 2012). The absence of behavioral alterations may be related with the absence of cerebral mercury accumulation. Franciscato et al. (2009) found that the damage in the motor function caused by inorganic mercury is probably related with the increase in Hg burden in cerebrum and cerebellum.

Since Hg was administered in relatively low doses, the physiologic states of pregnancy and lactation were likely to be exerting a protection to the offspring against the

toxic agent. The scavenger molecules, mainly MT, seem to be involved in this endogenous protection. Yoshida et al. (2002) showed that pregnant mice have higher tissue MT content than non-pregnant mice. Favero et al. (2014) also showed that lactating rats have a slightly higher renal MT content than non-pregnant rats. In our work, the placenta, which is the organ responsible, among other things, for fetus protection, presented about 5.3 and 2.3 times more MT than the liver and kidney, respectively.

Conclusion:

Inorganic mercury exposure in drinking water during the pregnancy period causes a dose dependent renal Hg accumulation as well as an imbalance in Cu and Zn levels. On the other hand, the offspring exposed to inorganic mercury in utero and breast milk presented no alterations in the essential metal homeostasis and behavioral tasks. In conclusion, the organism during the pregnancy and lactation periods seems to be able to protect the offspring against low doses of inorganic mercury. This protection is likely to be related to an endogenous protection, such as the MT, an important scavenger molecule.

Conflict of interest statement:

The authors declare that there are no conflicts of interest.

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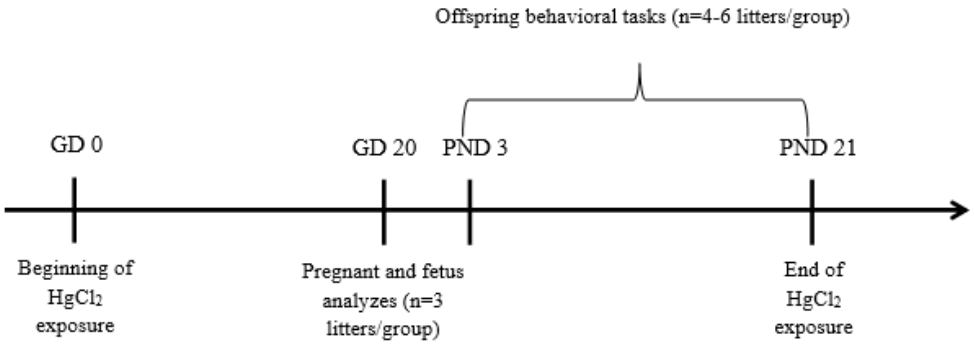

Figure 1: Protocol of $HgCl_2$ exposure and period of metal, biochemistry and behavioral tasks analyzes. Different sets of animals were used for pregnant and fetus analyses and behavioral tasks.

GD= gestation day; PND= post-natal day

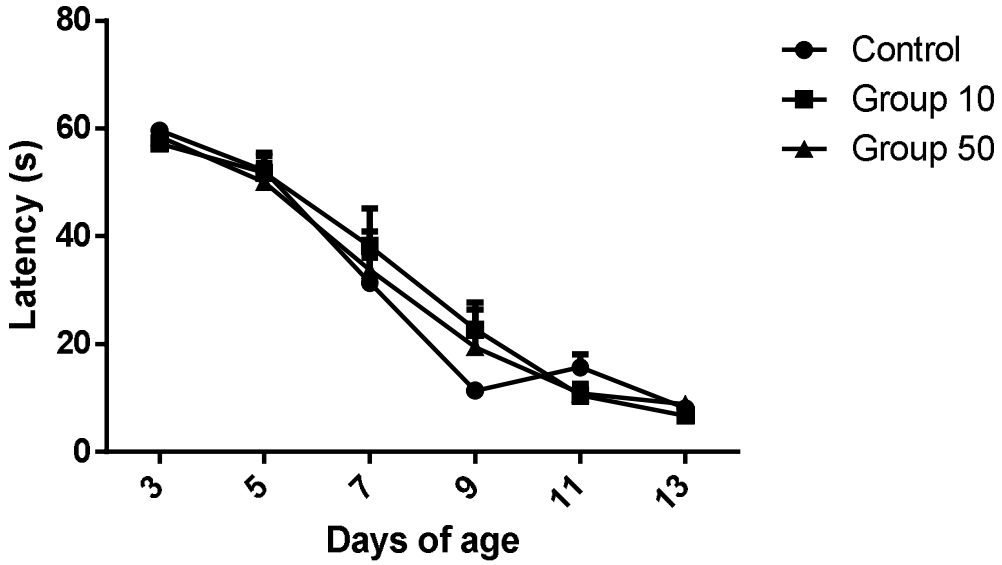

Figure 2: Latency of negative geotaxis reflex of pups exposed to inorganic mercury in utero and via milk from dams exposed to to 10 and 50 µg Hg^{2+}/mL of $HgCl_2$ in drinking water during the pregnancy and lactation periods. The results are presented as mean ± SEM (n=8).

Table 1: Hg, Cu, Zn and Fe levels in different tissues of pregnant rats exposed to 10 and 50 µg Hg^{2+}/mL of $HgCl_2$ in drinking water during all period of pregnancy.

	Hg	Cu	Zn	Fe
Blood (µg/mL)				
Control	n.d.	0.25±0.01	0.37±0.01	36.25±0.15
Group 10	n.d.	0.24±0.01	0.37±0.01	36.85±0.25
Group 50	n.d.	0.24±0.03	0.36±0.03	34.35±3.75
Liver (µg/g)				
Control	n.d.	14.87±9.19	26.25±0.76[a]	146.00±17.70
Group 10	n.d.	7.57±0.50	31.96±2.44[a,b]	190.30±49.90
Group 50	n.d.	6.95±1.61	40.50±4.48[b]	237.90±56.55
Kidney (µg/g)				
Control	n.d.[a]	12.16±1.22[a]	22.00±1.46	56.29±2.47
Group 10	28.59±28.58[b]	21.08±2.92[b]	24.13±1.42	61.17±13.11
Group 50	208.80±34.47[c]	45.67±8.90[c]	27.96±3.08	83.54±33.26
Placenta (µg/g)				
Control	n.d.	4.02±0.84	13.69±0.82	103.01±2.07
Group 10	n.d.	2.44±1.27	14.71±2.03	89.13±9.19
Group 50	n.d.	2.61±0.23	12.89±0.82	88.50±7.39

The results are presented as mean ± SEM (n=3). Tukey's multiple range test: different letters confer significant difference between groups ($p < 0.05$).

n.d. = not detected.

Table 2: Hg, Cu, Zn and Fe excretion in feces and urine of pregnant rats exposed to 10 and 50 µg Hg^{2+}/mL of $HgCl_2$ in drinking water during all period of pregnancy.

	Hg	Cu	Zn	Fe
Urine (µg/mL)				
Control	n.d.	n.d.	0.10±0.02	0.17±0.03
Group 10	n.d.	n.d.	0.09±0.00	0.13±0.01
Group 50	n.d.	n.d.	0.11±0.01	0.19±0.03
Feces (µg/g)				
Control	n.d.[a]	21.13±21.12	190.0±39.69	841.7±124.5
Group 10	n.d.[a]	46.96±1.69	183.8±22.79	552.1±113.6
Group 50	426.2±213.3[b]	61.54±6.89	381.3±76.25	1290.0±116.8

The results are presented as mean ± SEM (n=3) Tukey's multiple range test: different letters confer significant difference between groups ($p < 0.05$).

n.d. = not detected.

Table 3: Hg, Cu, Zn and Fe levels in different tissues of fetuses from pregnant rats exposed to 10 and 50 µg Hg^{2+}/mL of $HgCl_2$ in drinking water during all period of pregnancy.

	Hg	Cu	Zn	Fe
Liver (µg/g)				
Control	n.d.	13.35±1.59	45.29±7.61	299.6±21.32
Group 10	n.d.	18.71±3.68	39.04±5.56	307.5±86.66
Group 50	n.d.	13.07±1.39	47.79±7.62	206.7±11.02
Kidney (µg/g)				
Control	n.d.	n.d.	16.55±3.14	24.11±7.40
Group 10	n.d.	0.70±0.35	15.82±2.18	25.00±4.73
Group 50	n.d.	0.39±0.38	16.44±4.59	23.73±7.95

The results are presented as mean ± SEM (n=3).

n.d. = not detected.

Table 4: Metallothionein levels in different tissues of pregnant rats exposed to 10 and 50 µg Hg^{2+}/mL of $HgCl_2$ in drinking water during all period of pregnancy.

	Liver	**Kidney**	**Placenta**
Control	46.77±3.14	90.13±10.63	329.00±71.58
Group 10	41.15±13.61	124.20±8.45	197.70±28.20
Group 50	50.28±3.87	109.90±17.78	211.20±1.04

The results are presented as mean ± SEM (n=3). The metallothionein content is expressed as µg SH/ g of wet tissue.

Table 5: Latency of access to refuge in the beaker test of pups exposed to inorganic mercury in utero and via milk from dams exposed to 10 and 50 μg Hg^{2+}/mL of $HgCl_2$ in drinking water during the pregnancy and lactation periods.

	Latency of access to refuge (s)			
	Session 1	**Session 2**	**Session 3**	**Session 4**
Control	39.40±9.07	22.80±8.26	27.60±10.50	33.70±12.10
Group 10	54.60±9.08	27.00±4.36	14.20±2.92	21.10±4.71
Group 50	40.20±8.28	39.70±11.30	30.70±9.70	29.40±11.90

The results are presented as mean ± SEM (n=8).

3.3Manuscrito II

Biochemical parameters of female Wistar rats and their offspring exposed to inorganic mercury in drinking water during the gestational and lactational periods

Cláudia S. Oliveira, Vitor A. Oliveira, Taíse F. Pedroso, Mariana M. Fonseca, Lidiane M. Costa, Maria E. Pereira

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Biochemical parameters of female Wistar rats and their offspring exposed to inorganic mercury in drinking water during the gestational and lactational periods

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Abstract

This work investigated the effects of inorganic mercury exposure on biochemical parameters of dams and their offspring exposed to metal in drinking water. Female Wistar rats were exposed to 0, 10 or 50 μg $Hg^{2+/}$mL during 42 days corresponding to gestational (21 days) and lactational (21days) periods. The offspring were sacrificed every 10 days after the birth (postnatal days 10, 20, 30, and 40). Dams exposed to mercury presented a decrease in water intake without alteration in food intake. Still, dams exposed to 50μg Hg^{2+}/mL presented an increase in kidney relative weight without changes on biochemical markers of nephrotoxicity. Moreover, dams presented a slight increase in hepatic and renal metallothionein content after both doses of inorganic mercury exposure. Regarding to offspring, the exposition to inorganic mercury in utero and via breast milk only increased the relative renal weight on postnatal day 20. In conclusion, seems that the dam organism was able to handle the mercury ions avoiding the classic inorganic mercury toxic effects as well as protecting the offspring; we suggested that this protection is related with the hepatic and renal metallothionein content increase.

Keywords: inorganic mercury, breast milk, PBG-synthase, metallothionein

1. Introduction:

Among the non-essential metals, mercury is one of the largest anthropogenic emitted metals. It is released in the environment as a byproduct of fuel combustion, cement production, metals smelting, and silver and gold mining. Besides, the uncontrolled use and discard of Hg-containing products (lamps, batteries, paints and fungicides, medical instruments) contribute to increase on environmental Hg levels (Horowitz et al., 2014). At the environment, mercury specimens can reach the food chain (Liang et al., 2015; Dominik et al., 2014). Humans can be exposed to Hg occupationally (at work) (Ekinci et al., 2014; Decharat et al., 2014) or through the intake of food or water contaminated (WHO, 2007).

Once exposed to mercury, humans and non-human animals present symptoms directly related with the mercury chemical form. Central nervous system is more susceptible to organic mercury (Bisen-Hersh et al., 2014; Sheehan et al., 2014) and renal system is more susceptible to inorganic mercury (Favero et al., 2014; Oliveira et al., 2014; Peixoto and Pereira, 2007). However, the different chemical forms of mercury also can cause toxic effects on hematologic, hepatic, cardiovascular, and reproductive systems (Syversen and Kaur, 2012).

The biggest example of mercury contamination is the Minamata case, in Japan. Population, mainly children, after their parents ate contaminated fish, presented several neurologic symptoms (Ekino et al., 2007; Tsuda et al., 2009). In Brazil, cases of mercury contamination occur mainly in the Amazon region, where mercury is used in mining to amalgamate gold; the riverine are exposed to Hg occupationally (workers) and through the consumption of contaminated fish and water (Marques et al., 2015; Palheta and Taylor, 1995).

Although the largest number of cases of mercury contamination occur with organic forms (diet) and elemental (occupational exposure), studies with inorganic form are important, since the different mercury chemical forms can be interconverted by

methylation/demethylation or oxidation/reduction in the environment (Morel et al., 1998) and within the body (Lorscheider et al., 1995). Hg^0 is convert to Hg^{2+} by intracellular catalase oxidation (Halbach and Clarkson, 1978) and MeHg is demethylated, mainly in the gastrointestinal tract, releasing ions Hg^{2+} (Lorscheider et al., 1995). Thus, not necessarily, the mercury form that the organism is exposed is the same mercury chemical form that is reaching the tissues and causing the damage. In fact, several studies have demonstrated inorganic mercury accumulation in rat tissues after MeHg exposure (Norseth and Clarkson 1970; Omata et al., 1980; Yamamoto et al., 1986; Rodrigues et al., 2010).

As observed in Minamata disease and in others smaller cases of mercury contamination, children are more sensitive to mercury than adults (Soresen et al., 1999; Axelrad et al., 2007; Ekino et al., 2007; Tsuda et al., 2009; Marques et al., 2015). The greater sensitivity of young animals compared to adult animals may be related to immature organs and membranes and their inability to properly process toxic agents (Nies and Spielberg, 1996). Several studies have reported the toxicity caused by organic mercury exposure during intrauterine development and/or during lactation (Bridges et al., 2009, 2012; Newland et al., 2008; Stringari et al., 2008; Vincente et al., 2004; Sakamoto et al., 2002; Ornaghi et al., 1993; Ilbäck et al., 1991). However, few studies are about inorganic mercury exposure (Chehimi et al., 2012; Feng et al., 2004; Oliveira et al., 2012).

Considering the fact that other forms of Hg can be converted to Hg^{2+} and the fact that animals in development are vulnerable to toxic agents, more studies using the inorganic mercury as a toxic agent during the developmental period are necessary. Thus, the aim of this work was to evaluate biochemical markers of toxicity in dams exposed directly to different doses of inorganic mercury in drinking water during the gestational and lactational periods as well as to evaluate the same biochemical markers in offspring indirectly exposed to inorganic mercury, in utero and breast milk.

2. Material and Methods:

2.1 Chemicals

Mercuric chloride, sodium chloride potassium phosphate monobasic and dibasic, absolute ethanol, sodium hydroxide, trichloroacetic acid, o-phosphoric acid, perchloric acid, glacial acetic acid, ß-mercaptoethanol, sucrose, hydrochloric acid, phenylmethylsulphonylfluoride (PMSF), chloroform, calcium disodium ethylenediaminetetraacetate (EDTA), 5,5 -dithio-*bis*(2-nitrobenzoic acid) (DTNB), and tris(hydroxymethyl) aminomethane (Tris) were purchased from Merck (Darmstadt, Germany). δ-ALA, bovine serum albumin and Coomassie brilliant blue G were obtained from Sigma (St Louis, MO, USA). ρ-Dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han, Germany). The commercial kits for biochemical dosages were obtained from Kovalent do Brasil Ltda. (São Gonçalo/ RJ/ Brazil) or Labtest Diagnóstica S.A. (Lagoa Santa/ MG/ Brazil).

2.2 Animals

Thirty-two Wistar rats (24 virgin female rats, 220 ± 20 g; and 8 mature male rats, 300 ± 50 g) obtained from the Animal Facility of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12 h light/dark cycle and controlled temperature (22 ± 2ºC). The animals had free access to water and commercial food. After 2 weeks of adaptation, the female and male rats (3:1) were placed in the same cage for the mating. Studies were conducted in accordance with the national and institutional guidelines (University Ethics Committee Guidelines—Process number 096/2011) for experiments with animals.

2.3 Mating

Three female Wistar rats were placed in animal cages with 1 male breeder during the night as describe in Oliveira et al. (2012). In the next morning period, males were separated from females. Mating was confirmed by the presence of sperm in vaginal smears. The day when the sperm was detected was considered as day 0 of gestation (GD 0).

2.4 Treatment

$HgCl_2$ was dissolved in distilled water and it was supplied to females as the unique form of liquid diet during the gestation and lactation. The drinking solutions containing mercury were replaced and prepared every 2 days from a stock solution (200 µg Hg^{2+}/mL) prepared weekly and stored (4 ºC).

Pregnant rats were randomly divided in three groups as described below:

- Control (n=7): the animals received only distilled water
- Group 10 (n=10): the animals received 10 µg Hg^{2+}/mL in drinking water
- Group 50 (n=7): the animals received 50 µg Hg^{2+}/mL in drinking water

The animals were placed individually in polycarbonate cages and exposed at different doses of $HgCl_2$ from day 0 of gestation until day 21 of lactation (~42 days of exposure). On post-natal day 1, the litters were standardized in 8 pups to avoid undernutrition effect. Dams were killed on day 21 of lactation and every 10 postnatal days (post-natal days: 10, 20, 30 and 40) two newborns were killed. Every 2 days, the dams were weighed and the food and water intake measured.

2.5 Sample preparation

Dams (on lactation day 21) and 2 newborns by litters (on post-natal days 10, 20, 30 and 40) were killed by decapitation. Blood, liver, kidney and brain were collected to biochemical analyzes.

For urea and creatinine analyzes, serum was obtained by total blood centrifugation at 3,000 g for 10 min and it was frozen until analysis (5 days).

For PBG-synthase activity assay liver, kidneys and brain were quickly placed on ice and homogenized in 7, 5 and 3 volumes of NaCl (150 mmol/L, pH 7.4), respectively. The homogenate was centrifuged at 8,000*g* for 30 min at 4 °C and the supernatant fraction was used in the enzyme assay.

For total and non-protein thiol analyzes, tissues were quickly placed on ice and homogenized in 5 volumes of Tris–HCl buffer (10 mM, pH 7.4). The homogenate was centrifuged at 1,050*g* for 20 min at 4 °C and the supernatant fraction (S1) was used for analysis.

For metallothionein level assays, liver and kidneys 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 a reducing agent. The homogenate was then centrifuged at 16,000 g for 30 min to obtain a supernatant containing metallothioneins.

2.6 Biochemical determinations:

2.6.1 Urea and creatinine

Urea and creatinine were determinated using a commercial kit Labtest. Briefly, the estimation of creatinine was carried out by measuring the quantity of product formed, creatinine picrate, and by utilizing creatinine as standard. The absorbance was recorded at 510

nm. The urea was determined by the quantity of indophenol blue formed and it was used as standard. The absorbance was measured at 600 nm.

2.6.2 PBG-synthase activity

The enzymatic activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen – PBG) formation, except that 76 mmol/L sodium phosphate buffer (pH 6.8) and 2.2 mmol/L δ-ALA were used. The incubation was initiated by adding 200 µL supernatant. Liver, kidney and brain samples were incubated for 25, 60 and 180 min, respectively, at 39° C. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 mol/L and the PBG was measured with Ehrlich's reagent at 555 nm, using the molar absorption coefficient of $6.1x10^4$ for Ehrlich-PBG salt. The specific enzymatic activity was expressed as nmol of PBG formed per hour per mg protein. Protein concentrations were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard.

2.6.3 Metallothionein (MT) levels

Metallothionein levels were determined by colorimetric method using Ellman`s reagent (Ellman, 1959) as described in Viarengo et al. (1997) and Peixoto et al. (2003). A standard curve using GSH as reference was constructed in order to express the MT levels in µg of SH per g of wet tissue.

2.6.4. Thiol groups

Thiol groups were determined by the colorimetric method (Ellman, 1959). To determination of the total thiol groups the S1 was used, and to determine non-protein thiol (NPSH) the protein fraction of S1 was precipitated with 200 µL of 4% trichloroacetic acid

followed by centrifugation (1,050*g*, 10 min). The colorimetric assay was carried out in 1 M phosphate buffer, pH 7.4. A standard curve using glutathione was constructed in order to calculate the SH in the tissue samples.

2.7 Statistical analysis

Results were analyzed by one-way ANOVA followed by Tukey's multiple range test or Student's *t*-test when appropriate. The effects were considered significant when $p<0.05$.

3. Results

3.1 Food and water intake

The total food and water intake and the Hg^{2+} intake estimation during the gestation and lactation period are shown on Table 1. Mercury exposure did not alter the total food intake during the gestation or lactation periods. On the other hand, one-way ANOVA showed effect of treatment on total and daily water intake during the gestation [$F(2,19) = 15.84$; $p \leq 0.0001$ and $F(2,21) = 12.71$; $p = 0.0002$, respectively] and lactation [$F(2,19) = 4.619$; $p = 0.024$ and $F(2,21) = 5.309$; $p = 0.0136$, respectively]. Female rats exposed to both inorganic mercury doses presented a decrease in total water intake in both period analyzed (gestation and lactation) when compared to control group ($p<0.05$, Tukey's multiple comparison test). The total Hg^{2+} intake was estimate based in total water intake. Student's *t*-test showed that female rats exposed to 50 μg Hg^{2+}/mL presented an increase in total and daily Hg^{2+} intake during the gestation [$t(15) = 11.84$; $p \leq 0.0001$ and $t(15) = 12.54$; $p \leq 0.0001$] and lactation [$t(15) = 8.081$; $p \leq 0.0001$ and $t(15) = 13.56$; $p \leq 0.0001$] when compared to animals exposed to 10 μg Hg^{2+}/mL.

3.2 Body and organ weights

Dams

The body weight and total and relative liver, kidney, and brain weight of dams exposed to 0, 10 and 50 µg Hg^{2+}/mL during the gestation and lactation are shown on Table 2. The mercury exposure did not alter the total organs weight but altered the relative renal weight (One-way ANOVA; $F(2,11) = 7.289$; $p = 0.0096$). Dams exposed to higher inorganic mercury dose (50 µg Hg^{2+}/mL) presented an increase in renal relative weight when compared to control group.

Offspring

The body weight and liver, kidney, and brain total and relative weight of pups on postnatal days 10, 20, 30, and 40 are shown on Table 3. Pups exposed to inorganic mercury during the pre and post-natal period did not present body weight alterations in any of the periods analyzed. Regarding to organs weight, one-way ANOVA showed treatment effect only on kidney relative weight at PND 20 [$F(2,11) = 8.301$; $p = 0.0063$]. In fact, animals exposed to 50 µg Hg^{2+}/mL presented an increase of the relative kidney weight when compared to control group.

3.3 Urea and creatinine

Dams

The serum urea and creatinine levels are shown on Table 4. Dams exposed to 10 and 50 µg Hg^{2+}/mL in drinking water did not present alterations on serum urea and creatinine levels.

Offspring

Similar to dams, pups exposed to inorganic mercury did not present alterations on serum urea and creatinine levels (Table 4).

3.4 PBG-synthase activity

Dams

Liver, kidney, and brain PBG-synthase activity are shown on Table 5. Mercury exposure in drinking water during the pregnancy and lactation period did not alter the PBG-synthase activity from different tissues evaluated.

Offspring

As observed in dams, offspring exposed indirectly to inorganic mercury (in utero and breast milk) did not present significant alterations in PBG-synthase activity from different tissues in different periods analyzed (Table 5).

3.5 Metallothionein (MT) levels

Dams

Hepatic and renal MT levels are shown on Table 6. Dams exposed to inorganic mercury (10 and 50 group) presented a slight increase in hepatic (~50%) MT levels. As renal MT levels, mercury exposure altered the MT content [$F(2,16) = 4.052$; $p = 0.0377$]; however, only the animals exposed to 10 μg Hg^{2+}/mL presented a significant renal MT levels increase when compared with the control group.

Offspring

As observed in dams, offspring exposed indirectly to inorganic mercury (in utero and breast milk) did not present significant alterations in hepatic and renal MT levels in the different periods analyzed (Table 6).

3.6 Thiol groups

Dams

The liver, kidney, and brain total and non-protein thiol content are shown on Table 7. One-way ANOVA showed effect of treatment only in renal total thiol levels [$F(2,11) = 4.066$; $p = 0.0476$]. Dams exposed to both inorganic mercury doses (10 and 50 μg Hg^{2+}/mL) showed an increase in renal total thiol levels when compared to control group. Regarding to non-protein thiol, mercury exposure did not alter the non-protein thiol levels in the tissues evaluated.

Offspring

The offspring exposed to inorganic mercury in utero and via breast milk did not present alterations in liver, kidney, and brain total and non-protein thiol content in the different periods analyzed (Table7).

4. Discussion:

Despite the large number of announcements about the toxic effects of mercury exposure (WHO, 2007; ATSDR, 1999), this metal still is used in the manufacture of a great numbers of products or it is released as a byproduct in gold extraction and metal smelters (Horowitz et al., 2014). Thus, studies about the mercury effects in an oral exposure route are necessary, since food and water contaminated are the mainly source of mercury to humans.

In this work, we evaluated the effects of drinking water inorganic mercury exposure during the gestational and lactational periods on tissues weight and biochemical markers of toxicity in dams. Besides, we evaluated the effects of inorganic mercury exposure in uterus and breast milk on body and organs weight as well as on biochemical markers of toxicity in different postnatal periods in offspring.

We observed that inorganic mercury exposure decrease the intake of water in both gestation and lactation periods without alter the food intake. As suggested in a previous work from our lab (Oliveira et al., 2012), this decrease in water intake caused by inorganic mercury

exposure may be is related with the mercury metallic taste (WHO, 2007). Besides, is important to highlight, that the mercury intake in lactation period was about two times higher than the gestation period. This result is interesting, once the inorganic mercury is the main mercury chemical form excreted through the milk (Sundberg et al., 1999).

Several studies have shown that inorganic mercury exposure affects mainly the renal system (Peixoto et al., 2003; Peixoto and Pereira, 2007; Franciscato et al., 2011; Oliveira et al., 2014, Fiuza et al., 2014; Favero et al., 2014; Bridges et al., 2014). The degree of renal damage is related with the exposure model, time of exposure, and how long after the exposure the effects are evaluated. In fact, in our work, only the dams and pups (PND 20) exposed to higher inorganic mercury dose presented relative renal weight increased; however, the well stablished renal markers of toxicity, urea and creatinine serum levels were not alter. Our research group reported the increase in renal weight followed by increased serum urea and creatinine levels in young (Peixoto and Pereira, 2007; Franciscato et al., 2011) and adult (Favero et al., 2014; Oliveira et al., 2014) rats subcutaneously exposed to inorganic mercury. Thus, our hypothesis is that the amount of mercury burden on kidney is causing some proximal tubules damages. However, these damages were not enough to cause a deficit in the clearance of metabolic wastes, as urea and creatinine, mainly because the pregnant and lactating present glomerular filtration rate and renal plasma flow increased (Arthur and Green, 1982; Cheung and Lafayette, 2013). Still, this absent of biochemical effects in dams and offspring exposed to inorganic mercury could be explained by the ligation between ions mercury and thiol groups of scavenger molecules, such as glutathione and metallothionein (MT), forming an inert complex. In agree with it, dams exposed to mercury presented a significant increase in renal total thiol groups (~28%) as well as an increase in renal MT content (72% and 42%, to 10 and 50 μg Hg^{2+}/mL groups, respectively). Moreover, the hepatic MT levels are slight higher when compared to control group in dams exposed to 10 and 50 μg

Hg^{2+}/mL (~50%); this result is interesting since liver is one of the organs responsible by MT synthesis (Onosaka and Cherian, 1982).

Although the Hg can bind to thiol groups of scavengers molecules forming an inert complex, it also can bind to extra and inter cellular biomolecules that containing -SH groups. This can cause alterations on the conformational structure with consequent molecule function alteration, leading to a cellular death following by a possible organ function failure (Graeme and Pollack, 1998; Clarkson, 2003). Enzymes are extremely sensitive to Hg bind with SH groups, for example, mercury is knowing to inhibit the enzymes lactate dehydrogenase (Moraes-Silva et al., 2012), alanine aminotransferase (Peixoto and Pereira, 2007; Franciscato et al., 2011), and PBG-synthase (Fiuza et al., 2014, Peixoto et al., 2003).

PBG-synthase is a sulfhydryl enzyme, participating in the synthesis of the prosthetic group heme (Sassa, 1982). Usually, this enzyme activity is inhibited in different tissues after mercury exposure. In this study, dams and pups did not present PBG-synthase activity alteration. However, pups exposed during the gestation and lactation to inorganic mercury presented a slight increase on hepatic enzyme activity (19% and 31%, 10 and 50µg Hg^{2+}/mL, respectively, on PND 10 and 26 % and 22%, 10 and 50µg Hg^{2+}/mL, respectively, on PND 20). Although not significant, this result call our attention, since Oliveira et al., (2012) observed that fetuses exposed to inorganic mercury (50 µg Hg^{2+}/mL) only during the gestation presented an increase in hepatic PBG-synthase activity, suggesting an organism adaptation to mercury exposure, i. e., the enzyme expression rate is bigger than the enzyme amount that is being inhibited by mercury.

In conclusion, the exposure to inorganic mercury in drinking water during the gestational and lactational period caused alterations mainly to dams exposed to 50 µg Hg^{2+}/mL. These alterations are related to renal system, confirming the inorganic mercury nephrotoxicity. Even the mercury exposure has caused some renal damage, this damage was

slight when compared with other studies (Peixoto and Pereira, 2007; Franciscato et al., 2011; Bridges et al., 2014; Favero et al., 2014; Fiuza et al., 2014; Oliveira et al., 2014). As inorganic mercury was administrated in lower drinking water concentration for a long period, we believe that the dams` body was able to handle the mercury ions, avoiding damage effects and protecting the offspring; probably it is related with the scavenger molecules, glutathione and MT.

Conflict of interest statement:

The authors declare that there are no conflicts of interest.

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Table 1: Food and water intake during the gestation and lactation period of female rats exposed to different doses (10 and 50 µg Hg^{2+}/mL) of $HgCl_2$ in drinking water during the gestation and lactation period.

	Control	Group 10	Group 50
Food intake (g)			
Gestation			
Total*	406.0±48.1	438.4±34.3	407.1±43.3
100g/day#	7.6±0.7	8.1±0.4	7.9±0.8
Lactation			
Total*	945.5±46.6	886.3±41.3	906.3±59.1
100g/day#	16.5±0.6	16.4±0.6	17.6±0.9
Water intake (mL)			
Gestation			
Total*	1001.0±62.3[a]	717.1±38.7[b]	608.3±46.7[b]
100g/day#	19.1±1.4[a]	14.36±0.5[b]	12.38±0.9[b]
Lactation			
Total*	1752.0±100.9[a]	1283.0±95.3[b]	1247.0±145.9[b]
100g/day#	30.8±1.9[a]	24.4±0.9[b]	24.9±1.8[b]
Hg^{2+} intake (mg)			
Gestation			
Total*	n.d.	7.03±0.37	30.41±2.33[$]
100g/day#	n.d.	0.14±0.00	0.62±0.05[$]
Lactation			
Total*	n.d.	12.99±0.87	62.33±7.29[$]
100g/day#	n.d.	0.24±0.01	1.25±0.09[$]

The results are presented as mean ± SEM (n=7-10). Different letters means significant difference among the groups (Tukey's multiple range test; $p < 0.05$). $ differs from the Group 10 (Student's *t*-test; $p < 0.05$).

n.d. = not determinated

*Total intake during the gestation or lactation period.

#Food, water or Hg^{2+} intake per 100 g of body weight per day.

Table 2: Body weight, total and relative weight from different organs of female rats exposed to different doses (10 and 50 μg Hg^{2+}/mL) of $HgCl_2$ in drinking water during the pregnancy and lactation period.

	Control	Group 10	Group 50
BODY WEIGHT (g)			
Initial	221.30±23.39	229.20±12.78	225.50±1457
Last day of gestation	318.30±25.82	329.50±19.12	308.50±19.12
Last day of lactation	259.80±21.80	247.30±10.87	247.00±10.68
LIVER			
Total (g)	11.84±0.79	11.31±0.52	11.15±0.71
Relative (%)	4.42±0.21	4.58±0.24	4.49±0.11
KIDNEY			
Total (g)	1.83±0.14	2.08±0.08	2.34±0.17
Relative (%)	0.71±0.05[a]	0.84±0.03[a,b]	0.94±0.04[b]
BRAIN			
Total (g)	1.65±0.13	1.66±0.06	1.65±0.05
Relative (%)	0.62±0.05	0.67±0.01	0.67±0.02

The results are presented as mean ± SEM (n= 4-6). Different letters means significant difference among the groups (Tukey's multiple range test; $p < 0.05$).

Table 3: Total and relative weight from different organs of pups exposed to inorganic mercury in utero and via milk from dams exposed to different doses (10 and 50 μg Hg^{2+}/mL) of $HgCl_2$ in drinking water during the pregnancy and lactation period.

			LIVER		KIDNEY		BRAIN	
AGE		Body weight (g)	Total (g)	Relative (%)	Total (g)	Relative (%)	Total (g)	Relative (%)
PND10	Control	17.25±0.25	0.50±0.02	2.88±0.09	0.22±0.00	1.28±0.04	0.87±0.02	5.04±0.24
	Group 10	17.10±0.68	0.46±0.03	2.93±0.26	0.21±0.00	1.32±0.07	0.84±0.03	5.30±0.07
	Group 50	16.88±0.82	0.45±0.04	2.65±0.16	0.22±0.01	1.28±0.02	0.84±0.03	5.00±0.26
PND20	Control	37.63±1.53	1.27±0.06	3.41±0.19	0.43±0.02	1.14±0.03[a]	1.29±0.03	3.45±0.05
	Group 10	34.40±2.12	1.20±0.11	3.46±0.18	0.42±0.02	1.22±0.03[a,b]	1.23±0.03	3.62±0.08
	Group 50	35.00±1.87	1.23±0.13	3.46±0.17	0.46±0.03	1.30±0.02[b]	1.29±0.03	3.68±0.20
PND30	Control	70.75±4.79	3.10±0.14	4.49±0.21	0.77±0.02	1.22±0.03	1.47±0.03	2.12±0.05
	Group 10	64.70±6.96	2.68±0.35	4.71±0.22	0.73±0.04	1.35±0.13	1.33±0.05	2.51±0.34
	Group 50	85.38±8.27	2.93±0.28	5.05±0.54	0.80±0.06	1.42±0.24	1.36±0.05	2.43±0.44
PND40	Control	128.50±11.45	5.61±0.52	4.31±0.14	1.21±0.03	0.94±0.05	1.53±0.08	1.19±0.05
	Group 10	112.90±8.78	5.18±0.58	4.45±0.20	1.14±0.09	0.99±0.01	1.49±0.07	1.30±0.05
	Group 50	122.50±6.77	6.15±0.42	4.98±0.20	1.23±0.06	1.00±0.20	1.46±0.04	1.19±0.08

PND: Post-natal day

The results are presented as mean ± SEM (n= 4-6). Different letters means significant difference among the groups (Tukey's multiple range test; $p<0.05$).

Table 4: Serum urea and creatinine levels from dams and pups rats exposed to different doses (10 and 50 μg Hg^{2+}/mL) of $HgCl_2$ as described in Table 1 and 3, respectively.

		UREA (mg/dL)	CREATININE (mg/dL)
DAMS			
	Control	74.85±4.30	0.40±0.15
	Group 10	78.72±3.21	0.50±0.13
	Group 50	63.66±5.30	0.43±0.12
PUPS			
	Control	40.79±2.80	0.33±0.07
PND 10	Group 10	46.27±7.08	0.32±0.09
	Group 50	43.53±6.83	0.24±0.02
	Control	45.46±4.01	0.79±0.47
PND 20	Group 10	56.35±9.74	0.79±0.33
	Group 50	63.14±9.70	0.98±0.49
	Control	52.56±10.07	0.38±0.07
PND 30	Group 10	58.94±7.47	0.32±0.09
	Group 50	48.84±10.07	0.33±0.10
	Control	52.93±6.06	0.34±0.05
PND 40	Group 10	61.49±6.74	0.36±0.04
	Group 50	39.38±4.53	0.38±0.05

PND: Post-natal day

The results are presented as mean ± SEM (n= 4-6).

Table 5: Porphobilinogen synthase activity from different organs of dams and pups rats exposed to different doses (10 and 50 µg Hg^{2+}/mL) of $HgCl_2$ as described in Table 1 and 3, respectively.

		LIVER	KIDNEY	BRAIN
DAMS				
	Control	9.70±1.40	5.26±1.09	1.08±0.22
	Group 10	11.13±1.04	5.11±0.89	1.19±0.24
	Group 50	13.08±1.43	5.71±0.75	1.14±0.11
PUPS				
	Control	19.27±2.59	5.35±1.04	1.58±0.44
PND 10	Group 10	22.92±3.96	6.74±0.70	2.05±0.42
	Group 50	25.24±6.31	5.66±0.55	1.82±0.37
	Control	17.23±1.51	5.33±0.69	1.01±0.14
PND 20	Group 10	21.76±3.22	5.71±0.55	1.35±0.21
	Group 50	21.03±4.78	6.83±0.79	1.11±0.04
	Control	19.24±5.98	9.42±1.24	1.20±0.36
PND 30	Group 10	15.92±1.96	8.58±1.97	1.01±0.27
	Group 50	17.90±3.48	10.65±1.82	1.03±0.21
	Control	17.96±1.70	8.34±0.67	1.23±0.24
PND 40	Group 10	24.24±2.63	8.61±0.34	1.18±0.12
	Group 50	16.16±2.81	8.15±0.66	0.91±0.16

PND: Post-natal day

The results are presented as mean ± SEM (n= 4-6). The enzyme activity is expressed as nmol PBG/h/mg protein

Table 6: Metallothionein from different organs of dams and pups rats exposed to different doses (10 and 50 µg Hg^{2+}/mL) of $HgCl_2$ as described in Table 1 and 3, respectively.

		LIVER	KIDNEY
DAMS			
	Control	88.19±5.88	105.10±5.09[a]
	Group 10	134.60±26.65	181.00±20.66[b]
	Group 50	132.30±16.11	148.90±18.78[a,b]
PUPS			
	Control	136.40±36.65	73.19±14.00
PND 10	Group 10	174.20±33.74	240.60±69.16
	Group 50	127.90±28.67	178.40±38.41
	Control	106.90±18.35	133.00±55.44
PND 20	Group 10	133.00±25.06	146.70±31.68
	Group 50	122.60±16.36	146.20±24.71
	Control	92.91±8.27	93.55±15.51
PND 30	Group 10	106.80±13.03	126.20±8.09
	Group 50	98.85±5.81	110.10±20.45
	Control	111.80±15.17	112.90±9.40
PND 40	Group 10	107.60±6.71	111.80±10.40
	Group 50	101.20±15.75	104.80±5.35

PND: Post-natal day

The results are presented as mean ± SEM (Dams n= 4-8; Pups n= 3-4). Different letters means significant difference among the groups (Tukey's multiple range test; $p<0.05$). The metallothionein content is expressed as µg SH/ g of wet tissue.

Table 7: Total and non-protein thiol from different organs of dams and pups rats exposed to different doses (10 and 50 µg Hg^{2+}/mL) of $HgCl_2$ as described in Table 1 and 3, respectively.

		LIVER		KIDNEY		BRAIN	
		Total SH	NPSH	Total SH	NPSH	Total SH	NPSH
DAMS							
	Control	2.96±0.14	0.70±0.14	1.44±0.06[a]	0.29±0.04	0.69±0.05	0.12±0.01
	Group 10	3.03±0.14	0.70±0.08	1.83±0.13[b]	0.37±0.04	0.87±0.06	0.13±0.01
	Group 50	3.01±0.11	0.74±0.13	1.84±0.06[b]	0.38±0.01	0.83±0.09	0.12±0.03
PUPS							
	Control	2.60±0.38	0.59±0.17	0.74±0.03	0.13±0.02	0.71±0.05	0.13±0.02
PND 10	Group 10	2.82±0.33	0.74±0.16	0.92±0.07	0.25±0.04	0.77±0.06	0.13±0.02
	Group 50	2.60±0.28	0.50±0.10	0.78±0.13	0.17±0.04	0.76±0.03	0.12±0.03
	Control	3.81±0.56	0.62±0.21	1.19±0.14	0.36±0.14	0.89±0.08	0.19±0.04
PND 20	Group 10	2.92±0.38	0.59±0.13	1.07±0.13	0.28±0.07	0.81±0.07	0.15±0.03
	Group 50	2.60±0.10	0.67±0.12	1.27±0.07	0.37±0.12	0.80±0.07	0.16±0.04
	Control	2.94±0.12	0.46±0.09	1.43±0.07	0.20±0.06	0.82±0.03	0.09±0.02
PND 30	Group 10	2.86±0.09	0.55±0.10	1.38±0.09	0.20±0.04	0.84±0.07	0.10±0.02
	Group 50	2.98±0.23	0.60±0.04	1.48±0.02	0.33±0.02	0.88±0.05	0.13±0.01
	Control	3.32±0.32	0.71±0.18	1.27±0.31	0.23±0.07	0.91±0.06	0.12±0.03
PND 40	Group 10	3.36±0.20	0.66±0.11	1.47±0.10	0.28±0.05	0.89±0.08	0.14±0.02
	Group 50	4.02±0.12	0.89±0.19	1.59±0.07	0.33±0.04	0.95±0.06	0.17±0.02

PND: Post-natal day.

The results are presented as mean ± SEM (n= 4-6). Different letters means significant difference among the groups (Tukey's multiple range test; $p<0.05$). The total and non-protein SH content are expressed as mmol SH/g of wet tissue.

3.4 Manuscrito III

Disposition and toxicity of inorganic mercury in pregnant rats and their offspring

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Este manuscrito será submetido ao Periódico *Toxicology*

Disposition and toxicity of inorganic mercury in pregnant rats and their offspring

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Running Title: **Disposition and Toxicity of Hg^{2+} in Pregnant Rats**

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Abstract:

Reproductive toxicants such as methylmercury have been shown to negatively impact fetal health. Methylmercury, however, can be transformed to inorganic mercury (Hg^{2+}) within cells and plasma. Therefore, it is important to understand the handing and disposition of Hg^{2+} in the reproductive system. The purpose of the current study was to assess the disposition and transport of Hg^{2+} in placental and fetal tissues, and to assess the effects of acute renal injury in dams on the disposition of Hg^{2+} in fetal tissues. Pregnant Wistar rats were exposed to nephrotoxic and non-nephrotoxic doses of Hg^{2+} for 6 or 48 h and the disposition of mercuric ions was measured. Accumulation of Hg^{2+} in the placenta was rapid and sustained. Very little Hg^{2+} was removed during the initial 48 h after exposure. Similarly, fetal accumulation of Hg^{2+} increased between 6 h and 48 h. These data suggest that very little Hg^{2+} is removed from placental and fetal tissues. Within fetal organs, the greatest amount of Hg^{2+} was localized in the kidneys, followed by the liver and brain. A dose-dependent increase in the accumulation of Hg^{2+} in fetal organs was observed, suggesting that continued maternal exposure may lead to increased fetal exposure. Taken together, these data indicate that Hg^{2+} is capable of crossing the placenta and gaining access to fetal organs in a dose-dependent manner.

1.Introduction:

Mercury (Hg) exists in three different chemical forms: metallic or elemental mercury (Hg^0), organic (primarily methylmercury, CH_3Hg^+) and inorganic (Hg^{2+}). Humans may be exposed to these forms of Hg through inhalation of Hg vapor or via ingestion of contaminated food and/or water (Bridges and Zalups, 2005; WHO, 2007). Exposure to the various forms of Hg can lead to serious toxicological consequences in the renal, hepatic, cardiovascular, reproductive, and nervous systems (ATSDR, 2008; Bridges and Zalups, 2010).

Of particular concern is the effect of Hg on the reproductive system and the developing fetus. Despite guidelines from the Environmental Protection Agency (EPA), certain populations of pregnant women continue to consume more than the recommended amount of seafood (Nair et al., 2014; Soon et al., 2014; Xu and Newman, 2014). Interestingly, the content of mercury in certain species of fish is increasing (Drevnick et al., 2015) which further increases the risk of Hg-exposure in fish-eating populations. Numerous studies have shown that once ingested, CH_3Hg^+ can readily cross the placenta and accumulate in the fetus (Bridges et al., 2009, 2012; Sakamoto et al., 2013; Yorifuji et al., 2009). The developing neurological system is highly sensitive to the effects of CH_3Hg^+. Indeed, fetal neurotoxicity has been observed at exposure levels that do not result in toxicological effects in the mother (Castoldi et al., 2001; Hong et al., 2012). Prenatal exposure to CH_3Hg^+ has been shown to result in a variety of neurological alterations ranging from cerebral palsy to mild developmental delays (Castoldi et al., 2001). It is important to note that, following exposure to Hg vapor or CH_3Hg^+, these forms of Hg can be converted to Hg^{2+}, either in plasma or in cells of maternal or fetal tissues (Lorscheider et al., 1995; Norseth and Clarkson, 1970a; Norseth and Clarkson, 1971). It has been suggested indirectly that Hg^{2+} is unable to gain access to fetal tissues even though Hg^{2+} has been shown to accumulate in the placenta (Ask et al., 2002; Chehimi et al., 2012; Feng et al., 2004; Oliveira et al., 2012; Yang et al., 1996;

Yoshida, 2002). Given the accumulation of Hg^{2+} in the placenta, it seems possible that Hg^{2+} may also gain access to fetal tissues. Therefore, it is important that we thoroughly understand the way in which Hg^{2+} is handled, not only in maternal organs, but also in placental and fetal tissues.

In adults, the primary site of Hg^{2+} accumulation and toxicity is the kidney, specifically the proximal tubule (Zalups, 2000). In fact, in as little as three hours after intravenous exposure to Hg^{2+} (as $HgCl_2$), approximately 55% of the administrated dose can be detected in the kidneys (Zalups, 1993). In animals exposed to toxic doses of $HgCl_2$, pathological changes such as cellular necrosis, tubular dilatation and atrophy, proteinaceous casts, and interstitial collagen deposition have been identified in and around proximal tubules (Bridges et al., 2014; Favero et al., 2014). Increases in blood urea nitrogen (BUN) and plasma creatinine levels have also been reported and suggest that renal function is reduced following exposure to toxic doses of $HgCl_2$ (Bridges et al., 2014; Zalups et al., 2014). When maternal exposure to $HgCl_2$ is great enough to cause reductions in renal function, it is possible that the maternal burden and disposition of Hg^{2+} will be altered because of an inability to eliminate mercuric ions in urine. Consequently, it is possible that the placental and fetal burden of Hg will also be altered, leading to greater toxicological consequences in the fetus.

Therefore, the purpose of the current study was twofold: 1) To assess the accumulation and transport of Hg^{2+} in placental and fetal tissues; 2) To test the hypothesis that acute renal injury in pregnant dams alters the fetal accumulation of Hg. In the present study, we exposed pregnant Wistar rats to either a non-nephrotoxic or a nephrotoxic dose of $HgCl_2$ and assessed the accumulation and toxicity of mercuric ions in fetal and placental tissues either six or 48 hours after exposure to Hg^{2+}. Understanding how mercuric ions accumulate in placental and fetal tissues will provide insight into the toxicity and handling of mercuric ions in fetal organs.

2.Materials and methods

Animals

Male and female Wistar rats were obtained from our breeding colony housed in the Mercer University School of Medicine animal facility. Female Wistar rats, weighing 275-300 g, were mated with males Wistar rats in our facility for 36 h in order to obtain pregnant dams. All animals were provided a commercial laboratory diet (Tekland 6% rat diet, Harlan Laboratories) and water *ad libitum* throughout all aspects of experimentation. The animal protocol for the current study was reviewed and approved by the Institutional Animal Care and Use Committee. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Exposure of Animals to $HgCl_2$

Two separate experiments were performed. For the first experiment, pregnant dams were injected intravenously (i.v.) with either a non-nephrotoxic dose of $HgCl_2$ (0.5 μmol • kg^{-1} • 2 mL 0.9% NaCl containing 1 μCi of [$^{203}Hg^{2+}$] per rat) or with a nephrotoxic dose of $HgCl_2$ (2.5 μmol • kg^{-1} • 2 mL saline containing 1 μCi of [$^{203}Hg^{2+}$] per rat) on day 20 of gestation (ED 20). Rats in this group were euthanized 6h after exposure. For the second experiment, pregnant dams were injected i.v. with either 0.5 μmol or 2.5 μmol • kg^{-1} $HgCl_2$ (in 2 mL 0.9% NaCl, containing 1 μCi of [$^{203}Hg^{2+}$] per rat) on ED 18. Rats in this group were euthanized on ED 20, 48h after injection with $HgCl_2$. At the time of injection, each animal was anesthetized with isoflurane and a small incision was made in the skin in the mid-ventral region of the thigh to expose the femoral vein and artery. The dose of $HgCl_2$ was administered into the vein and the wound was closed with two 9-mm wound clips. Animals were housed individually in plastic metabolic cages.

Radioactive Hg [$^{203}Hg^{2+}$]:

Radioactive Hg [$^{203}Hg^{2+}$] was produced by irradiation of mercuric oxide at the Missouri University Research Reactor (MURR) facility as described previously (Belanger et al., 2001; Bridges et al., 2004). Briefly, a 3-mg sample of mercuric oxide was irradiated by neutron activation for 4 weeks at MURR. Following irradiation, sample was dissolved in 1 N HCl and the activity was measured using a Fluka ion chamber. Activities ranged from 30-45 mCi/mL.

Collection of Organs

At the time of euthanasia, rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylazine (70/30 mg • kg^{-1} in 2 mL saline). A 1-mL sample of blood was obtained from the inferior vena cava and placed in a polystyrene tube for estimation of [$^{203}Hg^{2+}$] content. Another sample of blood was placed in blood separation tubes in order to separate plasma from the cellular contents of blood. Total blood volume was estimated to be 6% of body weight (Lee and Blaufox, 1985).

Right and left kidneys were removed and each kidney was trimmed of fat and fascia, weighed, and cut in half along the mid-traverse plane. One-half of the right kidney was placed in fixative (40% formaldehyde, 50% glutaraldehyde in 96.7 mM NaH2PO4 and 67.5 mM NaOH) in preparation for histological analyses. The remaining half was frozen in liquid nitrogen for future RNA analyses. A 3-mm transverse slice of the left kidney was utilized to obtain samples of cortex, outer stripe of outer medulla (OSOM), inner stripe of outer medulla (ISOM) and inner medulla. Each zone of the kidney was weighed and placed in a separate polystyrene tube for estimation of [$^{203}Hg^{2+}$] content. The liver was then excised, weighed, and a 1-g section was removed for determination of [$^{203}Hg^{2+}$] content.

Urine and feces were collected in 24-h increments with the first collection taking place 24 h after the injection with $HgCl_2$. The second 24-h collection occurred 48 h after the injection with $HgCl_2$. Urine from each animal was mixed and a 1-mL sample was weighed and placed in a polystyrene tube for estimation of [$^{203}Hg^{2+}$] content. All of the feces excreted by each animal during each 24-h period were counted to determine accurately the total fecal content of [$^{203}Hg^{2+}$]. The content of [$^{203}Hg^{2+}$] in each sample was determined by counting in a Wallac Wizard 3 automatic gamma counter (Perkin Elmer).

Collection of Fetuses and Placentas:

The uterus of each pregnant rat was removed and each fetus and placenta was extracted. Each placenta was weighed and placed in a polystyrene tube for estimation of [$^{203}Hg^{2+}$] content. Amniotic fluid was collected on a piece of Whatman paper which was placed in a polystyrene tube. Each fetus was weighed, decapitated, and placed in 5 mL of 80% ethanol in a glass scintillation vial. After the entire fetus was counted, the brain, kidneys and liver of each fetus were removed. Each organ was weighed and placed in separate polystyrene tubes. The content of [$^{203}Hg^{2+}$] in each sample was determined by counting in a Wallac Wizard 3 automatic gamma counter.

Measurement of Creatinine and Blood Urea Nitrogen:

For determination of plasma creatinine, 30 µL of plasma was utilized and the concentration of creatinine was assessed using the QuantiChrome creatinine assay (BioAssay). Similarly, using a 5µL sample of plasma, the concentration of BUN was determined using the QuantiChrome Urea Assay (BioAssay).

Real-time PCR

Analysis of kidney injury molecule-1 (Kim-1) was performed with an ABI Prism 7000 Detection System as described previously (Bridges et al., 2014). A Gene Expression Assay was utilized to detect Kim-1 (Kim-1: Rn00597701_m1) in samples. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh; Rn01775763_g1) was used as a reference gene.

Histological Analyses

Following fixation, kidneys were washed twice with normal saline and placed in 70% ethanol. Tissues were processed in a Tissue-Tek VIP processor using the following sequence: 95% ethanol for 30 min (twice); 100% ethanol for 30 min (twice); 100% xylene (twice). Tissue was subsequently embedded in POLY/Fin paraffin (Fisher). Five-µm sections were cut using a Leitz 1512 microtome and were subsequently mounted on glass slides. Sections were stained with hematoxylin and eosin (H & E) and were viewed using an Olympus IX70 microscope. Images were captured with a Jenoptix Progress C12 digital camera.

Data Analyses

Data were analyzed first with the Kolmogorov–Smirnov test for normality and then with Levene's test for homogeneity of variances. Data were then analyzed using a two-way analysis of variance (ANOVA) followed by Tukey's post hoc testing. A p-value of <0.05 was chosen *a priori* to represent statistical significance.

3.Results

Disposition of Hg^{2+} in Maternal Tissues

Content of Hg^{2+} in Total Renal Mass

The amount of Hg^{2+} in the total renal mass of rats exposed to $HgCl_2$ is shown in Figure 1. The burden of Hg (nmol • g^{-1}) in the total renal mass of dams exposed to the

nephrotoxic dose of $HgCl_2$ (2.5 µmol $HgCl_2$ • kg^{-1}) was significantly greater than that of dams exposed to the non-nephrotoxic dose (0.5 µmol $HgCl_2$ • kg^{-1}) after 6 h and 48 h. Interestingly, the renal burden of Hg in dams 48 h after exposure to the 2.5-µmol • kg^{-1} dose was significantly lower than that of dams exposed to the same dose for 6 h.

Content of Hg^{2+} in Renal Zones

The amount of Hg^{2+} (nmol • g^{-1}) detected in the renal cortex (Figure 2A) was significantly greater in dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ than that of dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$ after either 6 h or 48 h. The amount of Hg in the renal cortex of dams 48 h after exposure to the 2.5-µmol • kg^{-1} dose was significantly less than that in the cortex of corresponding dams 6 h after exposure.

The amount of Hg^{2+} (nmol • g^{-1}) detected in the OSOM (Figure 2B) was significantly greater in dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ than that of dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$ after either 6 h or 48 h. The amount of Hg in the OSOM of dams 48 h after exposure to the 2.5-µmol • kg^{-1} dose was significantly less than that in the OSOM of corresponding dams 6 h after exposure. The amount of Hg detected in the ISOM and the inner medulla was minimal (data not shown).

Urinary Excretion of Hg^{2+}

The amount of Hg^{2+} excreted in urine of dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ was significantly greater than that of rats exposed to the 0.5-µmol • kg^{-1} dose at each time point measured (Figure 3). The 24-h time point represents the initial 24 h after injection with $HgCl_2$ while the 48-h time point represents the second 24-h collection period. The urinary excretion of Hg (at either dose) was significantly greater at the 24 h and 48 h time points than at the 6 h time point.

Content of Hg^{2+} in Liver and Blood

The hepatic burden of Hg^{2+} (nmol • g^{-1}) in dams exposed to 2.5 µmol $HgCl_2$ • kg^{-1} $HgCl_2$ for either 6 h or 48 h was significantly greater than that of corresponding dams exposed to 0.5 µmol $HgCl_2$ • kg^{-1} (Figure 4A). The amount of Hg in liver of dams exposed to either dose of $HgCl_2$ was significantly greater after 6 h than of corresponding dams after 48 h.

Similarly, the hematologic burden of Hg^{2+} (nmol • g^{-1}) in dams exposed to 2.5 µmol $HgCl_2$ • kg^{-1} $HgCl_2$ for either 6 h or 48 h was significantly greater than that of corresponding dams exposed to 0.5 µmol $HgCl_2$ • kg^{-1} (Figure 4B). Like that of liver, the amount of Hg in blood of dams exposed to either dose of $HgCl_2$ was significantly greater after 6 h than after 48 h.

Fecal Excretion of Hg^{2+}

There was no difference in the fecal excretion of Hg^{2+} between groups of rats exposed to 0.5 µmol • kg^{-1} $HgCl_2$ or 2.5-µmol • kg^{-1} $HgCl_2$ and euthanized 6 h after exposure (Figure 5). However, in animals exposed to Hg^{2+} for 48 h, the fecal excretion of Hg^{2+} during the first 24-h period was not significantly different in dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$ than in dams exposed to the 2.5-µmol • kg^{-1} dose. In contrast, during the second 24-h period (48 h after exposure), the fecal excretion of Hg^{2+} was significantly greater in dams exposed to the 2.5-µmol • kg^{-1} dose than in those exposed to the 0.5-µmol • kg^{-1} dose. In dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$, the excretion of Hg^{2+} during the first 24-h period was similar to that during the second 24-h period of dams, while in dams exposed to the 2.5-µmol • kg^{-1} dose, the fecal excretion of Hg^{2+} was significantly greater in the second 24-h period than that in the first 24-h period.

Burden of Hg^{2+} in Brain

The amount of Hg^{2+} detected in the brain was low but detectable (Figure 6). The burden of Hg^{2+} in the brain was significantly greater in dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ than that of dams exposed to 0.5 µmol • kg^{-1} in both periods analyzed (6 h or 48 h after exposure).

Content of Hg^{2+} in Uterus and Amniotic Fluid

The uterine content of Hg^{2+} was significantly greater in dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ than in corresponding dams exposed to the 0.5 µmol • kg^{-1} dose (Figure 7A) at 6 h and 48 h after exposure to $HgCl_2$. In dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$, the uterine burden of Hg was significantly lower after 48 h than after 6 h. Interestingly, the content of Hg in uteruses from dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ was not significantly different at the 6-h and 48-h time points.

The content of Hg^{2+} in amniotic fluid was greater in dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ than in corresponding dams exposed to 0.5 µmol • kg^{-1} after 6 h and 48 h (Figure 7B). In dams exposed to either dose of $HgCl_2$, the amount of Hg^{2+} in the amniotic fluid was significantly lower after 48 h than after 6 h.

Content of Hg^{2+} in Placentas and Fetuses

The placental burden of Hg^{2+} per rat is shown in Figure 8A. The burden of Hg^{2+} was significantly greater in placentas from dams exposed to 2.5 µmol • kg^{-1} than in placentas from corresponding dams exposed to the 0.5-µmol • kg^{-1} dose. There was no significant difference in the placental burden of Hg^{2+} at 6 h and 48 h in dams exposed to either dose of $HgCl_2$.

Figure 8B shows the total fetal burden of Hg^{2+} per rat following exposure to 0.5 µmol or 2.5 µmol • kg^{-1} $HgCl_2$. The total fetal burden of Hg^{2+} in dams exposed to the 2.5-µmol • kg^{-}

[1] dose was significantly greater than that of dams exposed to the 0.5-µmol • kg^{-1} dose. In dams exposed to the 0.5 µmol • kg^{-1} dose, the fetal burden of Hg^{2+} was significantly greater in dams exposed for 48 h compared with dams exposed for 6 h. There was no difference in the fetal burden of Hg^{2+} at the 6-h and 48-h time points following the 2.5-µmol • kg^{-1} dose.

Assessment of Hg^{2+}-induced Nephropathy

Real-time PCR analyses (Figure 9) were performed in order to evaluate the expression of Kim-1 in kidneys of dams exposed to 0.5 or 2.5 µmol • kg^{-1} $HgCl_2$. The expression of Kim-1 was significantly greater in kidneys isolated from dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ than in kidneys from corresponding dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$. In addition, the expression of Kim-1 was significantly greater in kidneys of dams exposed for 48 h than in kidneys of corresponding dams exposed for 6 h.

Analyses of plasma creatinine levels (Table 1) support the results of the PCR analyses. Samples of plasma, collected from dams 6 or 48 h after injection with $HgCl_2$, were utilized for analysis of plasma creatinine and BUN. In rats exposed to 0.5 µmol • kg^{-1} $HgCl_2$, plasma creatinine levels were similar to that of control rats (Amini et al., 2012; Bridges et al., 2014; Moeini et al., 2013; Palm and Lundblad, 2005). In dams exposed to 2.5 µmol • kg^{-1} $HgCl_2$ for 6 h, plasma creatinine increased by approximately 50%. When dams were exposed the 2.5-µmol • kg^{-1} dose for 48 h, plasma creatinine increased approximately two-fold. BUN was not altered.

Figure 10 shows the histological features of kidneys from dams exposed to 0.5 µmol or 2.5 µmol • kg^{-1} $HgCl_2$ for 48 h. No histological changes were observed in kidneys from dams exposed to either dose of Hg^{2+} for 6 h. In kidneys of dams exposed to 0.5 µmol • kg^{-1} $HgCl_2$, the renal structures in the cortex (Figure 10A) and OSOM (Figure 10B) appeared normal. Similarly, in kidneys of dams exposed to the 2.5-µmol dose of $HgCl_2$, no pathological

alterations were observed in the cortex (Figure 10C); however, the OSOM (Figure 10D) exhibited significant cellular injury and necrosis throughout, particularly at the cortico-medullary junction. This pattern of injury is similar to that reported previously for $HgCl_2$-induced nephrotoxicity (Zalups, 1988; Zalups and Diamond, 1987a; Zalups and Diamond, 1987b). Injury was characterized by eosinophilic cytoplasm and pyknotic nuclei within the proximal tubules. In addition, tubule lumens were filled with proteinaceous material and numerous leukocytes were present in the interstitial space.

Disposition of Hg^{2+} in Fetal Tissues

The content of Hg^{2+} in fetal kidney, liver and brain is shown in Figures 11A, 11B and 11C, respectively. The amount of Hg^{2+} (Figure 11A) in the total renal mass of fetuses from dams exposed to 2.5 μmol • kg^{-1} $HgCl_2$ was significantly greater than that of fetuses from dams exposed to the 0.5-μmol • kg^{-1} dose in both periods analyzed (6 and 48 h after $HgCl_2$ exposure). Interestingly, the amount of Hg^{2+} in the total renal mass of fetuses from dams exposed to the 2.5-μmol dose of $HgCl_2$ was significantly greater after 48 h than after 6 h.

The amount of Hg^{2+} in liver (Figure 11B) of fetuses from dams exposed to the 2.5-μmol dose of $HgCl_2$ was significantly greater than that of fetuses harvested from corresponding dams exposed to the 0.5-μmol • kg^{-1} dose at either time period. There was no significant difference between the 6 h and 48 h time points at either dose.

The amount of Hg^{2+} in fetal brain (Figure 11C) was greater in fetuses harvested from dams exposed to the 2.5-μmol • kg^{-1} dose of $HgCl_2$ than in fetuses from corresponding dams exposed to the 0.5-μmol • kg^{-1} dose. The amount of Hg in fetal brain was significantly greater after exposure for 48 h than for 6 h for either dose examined. When the burden of Hg was compared in fetal kidney, liver and brain, the greatest amount was detected in the total renal mass (at either time point).

4.Discussion

Toxic substances such as heavy metals can cross the placental barrier and compromise fetal health (ATSDR, 2008; Bridges et al., 2009; Bridges et al., 2012; Chehimi et al., 2012; Myers et al., 2000). Indeed, methylmercury has been shown to readily cross the placenta and accumulate in fetal organs (Bridges et al., 2009, 2012; Castoldi et al., 2001; Clarkson et al., 2003). However, little is known about the ability of Hg^{2+} to cross the placenta. Since methylmercury can be biotransformed to Hg^{2+} within biological systems (Daniel, 1972; Gage, 1964; Norseth and Clarkson, 1970a; Norseth and Clarkson, 1970b), it is important to understand how Hg^{2+} is handled by placental and fetal tissues. Therefore, one aim of the current study was to characterize the handling of Hg^{2+} in maternal and fetal tissues following maternal exposure to Hg^{2+}. In addition, this study was also designed to examine the effects of non-nephrotoxic and nephrotoxic doses of $HgCl_2$ on the maternal and fetal disposition of mercuric ions. To our knowledge, this study represents the first report of the distribution of Hg^{2+} (following a non-nephrotoxic or a nephrotoxic dose) in maternal and fetal tissues following exposure to Hg^{2+}.

One of the most interesting aspects of the current study is the disposition of Hg^{2+} in placental and fetal tissues. The placental burden of Hg^{2+} did not change between 6 and 48 h after exposure to either dose of Hg^{2+}, suggesting that 1) placental accumulation of Hg^{2+} is rapid and/or 2) the placenta does not have efficient mechanisms for the elimination of mercuric ions without the use of a metal complexing agent. Previous studies have shown that the concentration of Hg^{2+} in cord blood was greater than that in maternal blood (Chen et al., 2014; Sakamoto et al., 2013), which suggests that the fetus may be exposed to greater levels of Hg^{2+} than the mother. Similarly, mercuric ions may be retained in the placenta (Ask et al., 2002). It is feasible to suggest that continued maternal exposure could lead to increasing

concentrations of mercuric ions in reproductive and fetal tissues. The lack of elimination from these tissues may lead to significant deleterious effects in the fetuses without clinical symptoms in the mother. It is also interesting to note that maternal exposure to the higher dose of Hg^{2+} led to a dose-dependent increase in the placental burden of Hg^{2+}. This finding, along with that of others (Sakamoto et al., 2013), suggests that the amount of Hg^{2+} in the placenta may be a useful measure of maternal (and consequently, fetal) exposure to Hg^{2+}.

The total fetal burden of Hg was similar to that detected in the placenta. When dams were exposed to the 0.5-μmol • kg^{-1} dose of $HgCl_2$, the fetal accumulation of Hg was significantly greater after 48 h than after 6 h. This finding suggests that fetal accumulation of Hg continues to increase during the initial 48 h after exposure to $HgCl_2$. In addition, the fetal burden of Hg increased in a dose-dependent manner following maternal exposure to the 2.5-μmol • kg^{-1} dose of $HgCl_2$. Based on these findings, we suggest that continued exposure of pregnant women to mercuric compounds, either through the environment or diet, may lead to significant exposure of the fetus to mercury.

When the amount of Hg^{2+} was measured in fetal organs, we found that the total renal mass had the greatest amount of Hg^{2+}, followed by liver and brain. This pattern of accumulation is similar to that observed previously when dams were exposed intravenously to methylmercury (Bridges et al., 2009, 2012). Since the kidney is the primary site of accumulation of Hg^{2+} in dams (Bridges and Zalups, 2010; Favero et al., 2014; Zalups, 2000), it is not surprising that it is also the primary site of accumulation in the fetus. It is important to note that the accumulation of Hg in the fetal kidneys increased in a dose-dependent manner after dams were exposed to 2.5 μmol • kg^{-1} $HgCl_2$. This pattern of increase was also observed in the total placental and fetal burden, suggesting that Hg^{2+} is transferred from the mother to the fetus and fetal organs. A similar pattern of accumulation was observed in fetal liver and

fetal brain. Together, these data are the first to suggest that maternal exposure to inorganic forms of Hg can lead to dose-dependent accumulation in fetal organs.

The disposition of Hg^{2+} in dams was similar to that reported previously in male Wistar rats (Bridges et al., 2008a, 2008b, 2011). It should be noted that when dams were exposed to 0.5 μmol • kg^{-1} $HgCl_2$, the renal accumulation of Hg^{2+} was similar at the 6- and 48-h time points. In other words, very little Hg^{2+} was removed from the kidney during this time-period following exposure to this dose of Hg^{2+}. This accumulation may be due to mercuric ions that are bound to protein and non-protein thiols in the intracellular compartments of renal epithelial cells (Barbier et al., 2005; Zalups, 2000). The use of a metal complexing agent may be necessary to bind and remove these mercuric ions from cells. This finding is significant in that long-term subclinical exposure to mercuric compounds could lead to a significant and constant burden of Hg^{2+} in the kidney. Indeed, recent reviews suggest that chronic exposure to small amounts of mercury may lead to significant long-term health effects (Homme et al., 2014; Hong et al., 2012).

The results of the current study also show clear differences in the handling and disposition of Hg^{2+} following the exposure of dams to non-nephrotoxic or nephrotoxic doses of $HgCl_2$. When dams were exposed to a higher dose of $HgCl_2$ (for either 6 h or 48 h), we observed an increase in the renal accumulation of Hg^{2+}. This increase in accumulation was dose-dependent during the first 6 h after exposure. i.e., renal accumulation following the 2.5-μmol • kg^{-1} dose was 5-fold greater than that following the 0.5-μmol • kg^{-1} dose. In contrast, 48 h after exposure to the 2.5-μmol • kg^{-1} dose, the renal burden of Hg^{2+} was only twofold greater than that of the 0.5-μmol • kg^{-1} dose. The lack of dose-dependence at the 48-h time point may be due to injury and death of proximal tubular cells, which leads to sloughing off of these cells and their contents into the tubular lumen for excretion in urine. Indeed, the amount

of Hg detected in urine at the 48-h time point was significantly greater than that excreted during the first 6 h.

Histological analyses were performed in order to characterize the Hg^{2+}-induced nephropathy in kidneys of dams. Exposure of dams to the 0.5-µmol dose of $HgCl_2$ for 6 or 48 h did not result in renal injury. Similarly, when rats were exposed to the 2.5-µmol dose for 6 h, no histological alterations were observed in maternal kidneys. In contrast, when rats were exposed to the 2.5-µmol dose for 48 h, areas of significant cellular injury and necrosis were evident in the inner cortex and OSOM of the kidneys. These data correspond to our previous findings (Bridges et al., 2014; Zalups and Diamond, 1987b) and suggest that an exposure period of longer than 6 h (at the 2.5-µmol • kg^{-1} dose) is necessary to induce renal injury. The current real-time PCR analyses of Kim-1, a biomarker of renal injury, support this conclusion. The expression of Kim-1 was significantly greater in kidneys of rats exposed to 2.5 µmol • kg^{-1} $HgCl_2$ for 48 h than in other groups of rats.

With regard to the hepatic burden of Hg^{2+}, we found that following exposure to 0.5 µmol • kg^{-1} $HgCl_2$, the amount of Hg^{2+} in the liver was similar in the 6-h and 48-h periods. Like in kidney, this finding suggests that little elimination occurs during this time period and at this dose. The pattern of Hg^{2+} accumulation and excretion following the 2.5-µmol • kg^{-1} dose was similar to that of the kidney. The hepatic burden of Hg^{2+} was less after 48 h than after 6 h while the fecal elimination of Hg^{2+} increased during the 48 h following exposure to $HgCl_2$.

The hematologic burden in blood also increased in a dose-dependent manner when rats were exposed to the 2.5-µmol • kg^{-1} dose of $HgCl_2$. In rats that were euthanized 48 h after exposure to $HgCl_2$, the hematologic burden of Hg was significantly lower than that of rats in the corresponding 6-h group. This reduction is likely due, in part, to filtration of mercuric ions at the site of the glomerulus and eventual excretion in the urine. Similarly, mercuric ions in

hepatic blood may be extracted by hepatocytes and secreted into bile for eventual fecal elimination.

In brain, Hg^{2+} also accumulated in a dose-dependent manner, with animals exposed to the 2.5-μmol • kg^{-1} dose of $HgCl_2$ exhibiting approximately 5-fold greater levels than corresponding rats exposed to the 0.5-μmol dose. Interestingly, the relative amount of Hg^{2+} removed from brain during the period between the 6-h and 48-h time periods was not as much as that removed from other organs. This finding suggests that once Hg^{2+} crosses the blood-brain barrier, its rate of clearance from the brain is slow.

In conclusion, the current study provides novel data suggesting that Hg^{2+} is taken up by the placenta and that this form of mercury is capable of gaining access to fetal organs. In addition, and more importantly, we suggest that the accumulation of Hg^{2+} in fetal tissues follows the pattern of maternal exposure in a dose-dependent manner. Furthermore, the removal of Hg^{2+} from fetal organs does not appear to correlate with the ability of the mother to excrete mercuric ions in urine.

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Figure Legends

Figure 1: Renal burden of Hg 6 h or 48 h after intravenous injection of pregnant Wistar dams with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. Data represent mean ± SE of three or six dams. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 2: Content of Hg in cortex (A) and outer stripe of outer medulla (OSOM) (B) of kidneys from pregnant Wistar dams injected intravenously with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. Cortex and OSOM were dissected from kidneys harvested 6 h or 48 h after injection with $HgCl_2$. Data represent mean ± SE of three or six dams. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 3: Content of Hg in urine of pregnant Wistar dams injected intravenously with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. Urine was collected 6, 24, and/or 48 h after injection with $HgCl_2$. Data represent mean ± SE of three or six dams. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 4: Hepatic (A) and hematologic (B) burden of Hg 6 h or 48 h after intravenous injection of pregnant Wistar dams with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. Data represent mean ± SE of three or six dams. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. +

Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 5: Content of Hg in feces of pregnant Wistar dams injected intravenously with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. Feces were collected 6, 24, and/or 48 h after injection with $HgCl_2$. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h. # Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 24 h.

Figure 6: Content of Hg in brain of pregnant Wistar dams injected intravenously with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 7: Content of Hg in uterus (A) and amniotic fluid (B) of pregnant Wistar dams injected intravenously with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 8: Total placental (A) and fetal (B) burden of Hg 6 h or 48 h after intravenous injection of pregnant Wistar dams with 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL. Data represent mean ± SE of three or six dams. * Significantly different ($p < 0.05$) from the mean for the corresponding group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1}. + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 9: Real-time PCR analyses of Kim-1 in RNA isolated from kidneys of pregnant Wistar dams injected intravenously with 0.5 μmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 μmol $HgCl_2$ • kg^{-1} • 2 mL. *Significantly different from the mean for the group of rats exposed to 0.5 μmol $HgCl_2$ • kg^{-1} ($p < 0.05$). + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Figure 10: Histological analyses of kidneys RNA isolated from kidneys of pregnant Wistar dams injected intravenously with 0.5 μmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 μmol $HgCl_2$ • kg^{-1} • 2 mL. In kidneys of rats injected with 0.5 μmol $HgCl_2$ • kg^{-1} • 2 mL, no histological alterations or pathological changes were observed in the cortex (A) or outer stripe of the outer medulla (B). In kidneys of rats injected with 2.5 μmol $HgCl_2$ • kg^{-1} • 2 mL, the cortex (C) appeared normal; however, significant areas of necrosis were evident in the outer stripe of the outer medulla (D).

Figure 11: Content of Hg in total renal mass (A), liver (B), and brain (C) of fetuses from Wistar dams injected intravenously with 0.5 μmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 μmol $HgCl_2$ • kg^{-1} • 2 mL. Kidneys, liver and brain were obtained from fetuses that were harvested 6 or 48 h after dams were injected with $HgCl_2$. Data represent mean ± SE of fetuses harvested from three or six dams. *Significantly different from the mean for the group of rats exposed to 0.5 μmol $HgCl_2$ • kg^{-1} ($p < 0.05$). + Significantly different ($p < 0.05$) from the mean for the group of rats exposed to the same dose for 6 h.

Table 1: Plasma creatinine and BUN levels. Pregnant Wistar rats were exposed intravenously to 0.5 µmol $HgCl_2$ • kg^{-1} • 2 mL or 2.5 µmol $HgCl_2$ • kg^{-1} • 2 mL for 6 or 48 h.

	6 h		48 h	
Dose	**Creatinine**	**BUN**	**Creatinine**	**BUN**
0.5 µmol $HgCl_2$	0.35±0.08	22.23±1.28	0.38±0.04	23.33±4.18
2.5 µmol $HgCl_2$	0.54±0.05	19.12±2.00	0.74±0.06*	19.09±2.94

*Significantly different from the mean for the group of rats exposed to 0.5 µmol $HgCl_2$ • kg^{-1} ($p<0.05$).

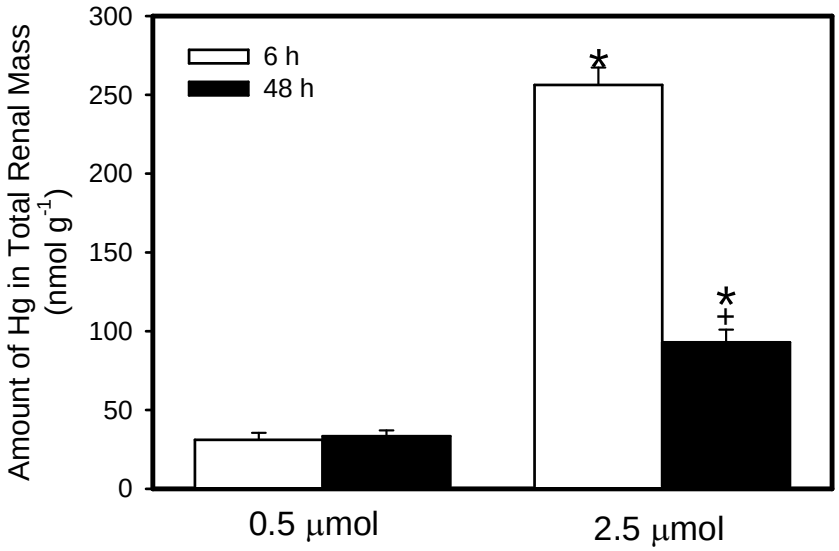
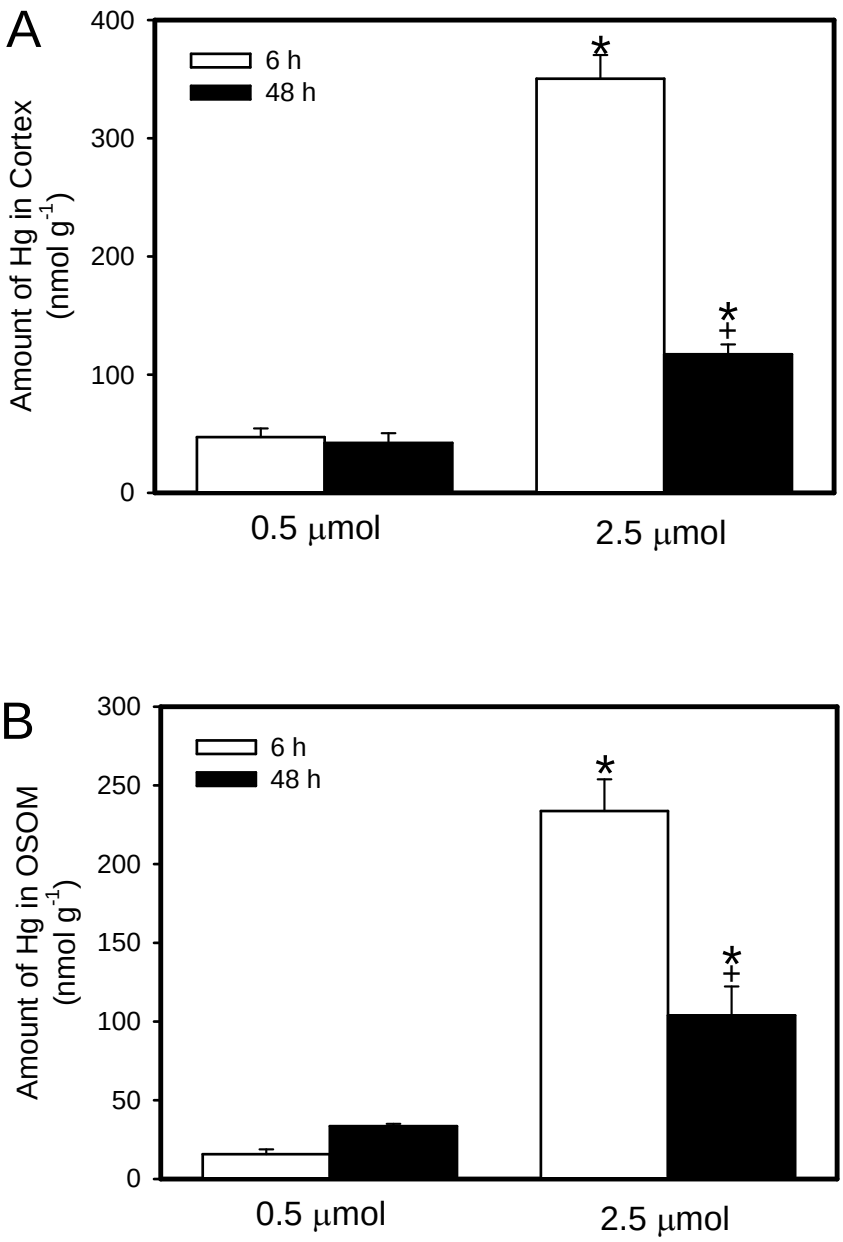


Figure 1



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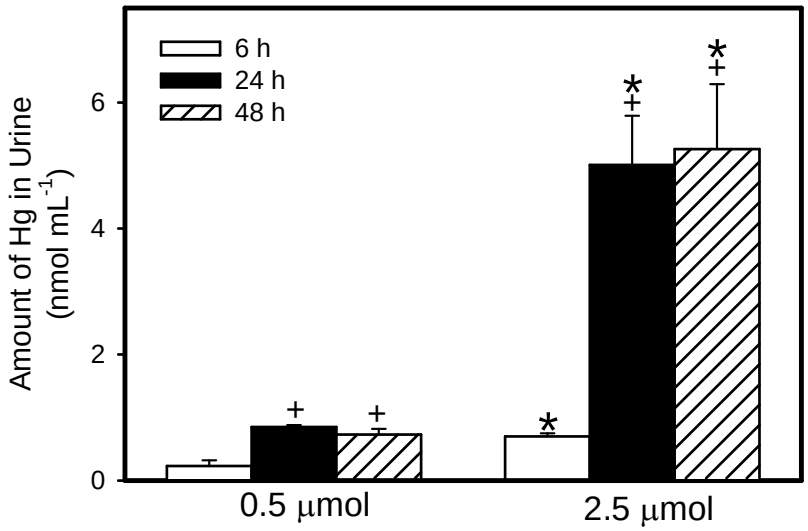


Figure 3

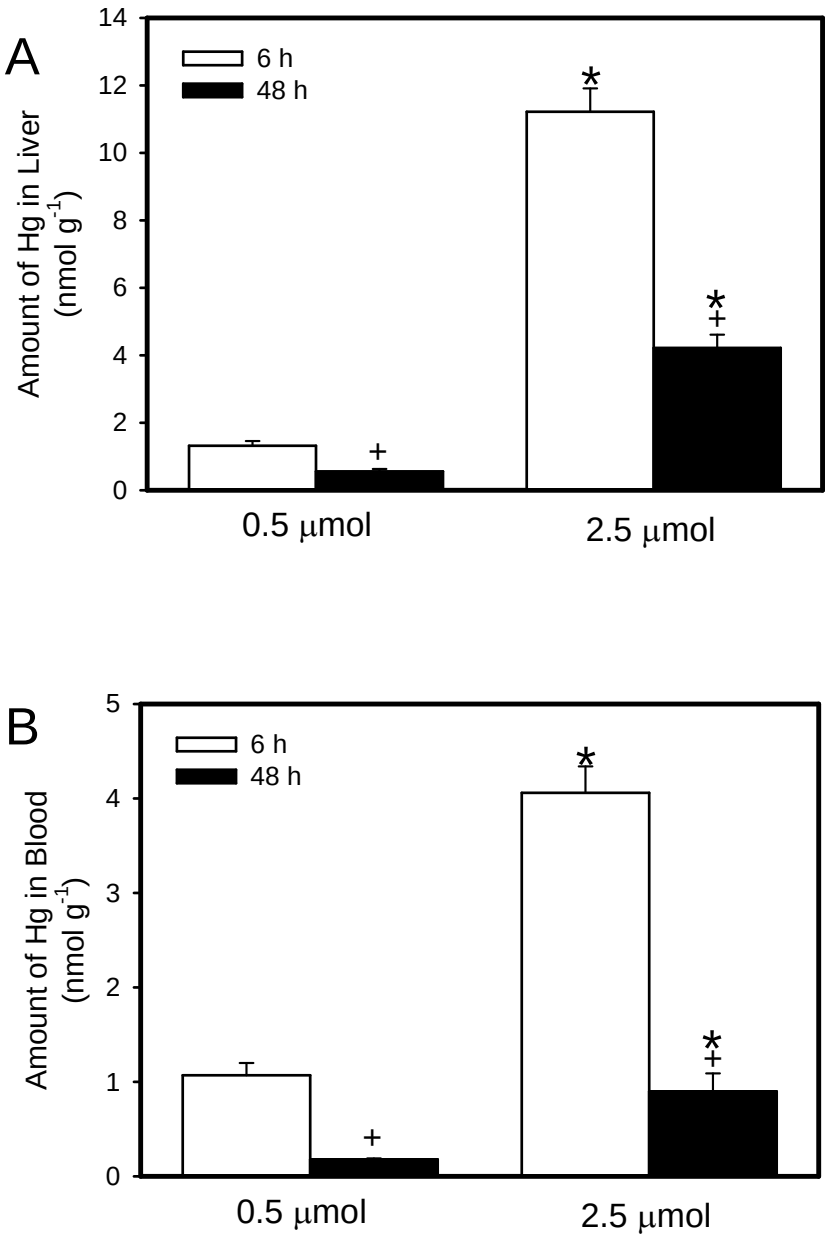


Figure 4

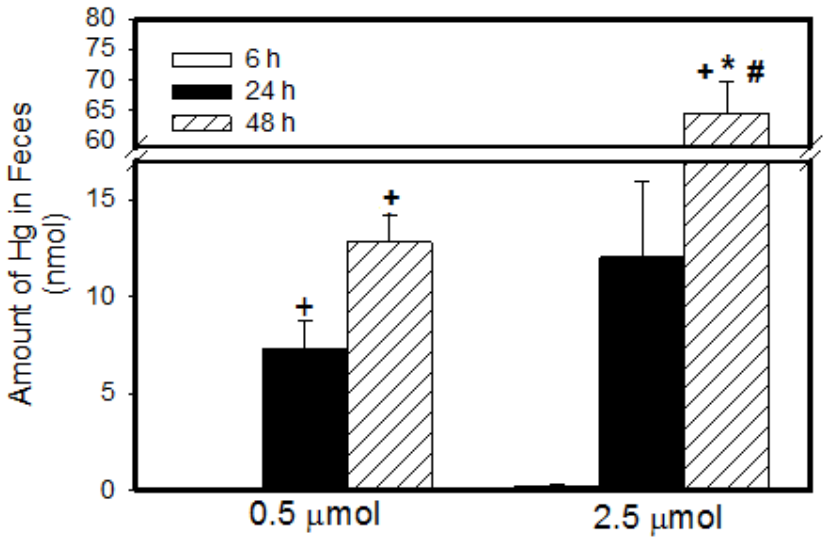


Figure 5

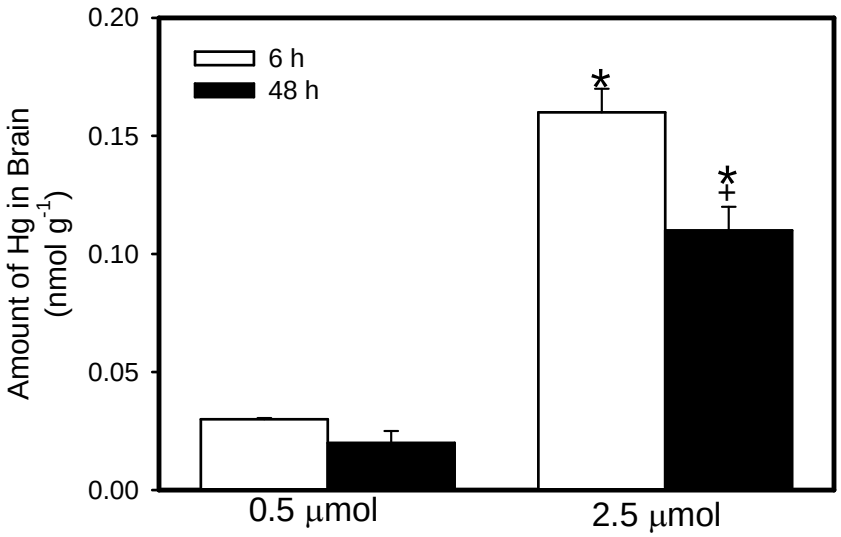


Figure 6

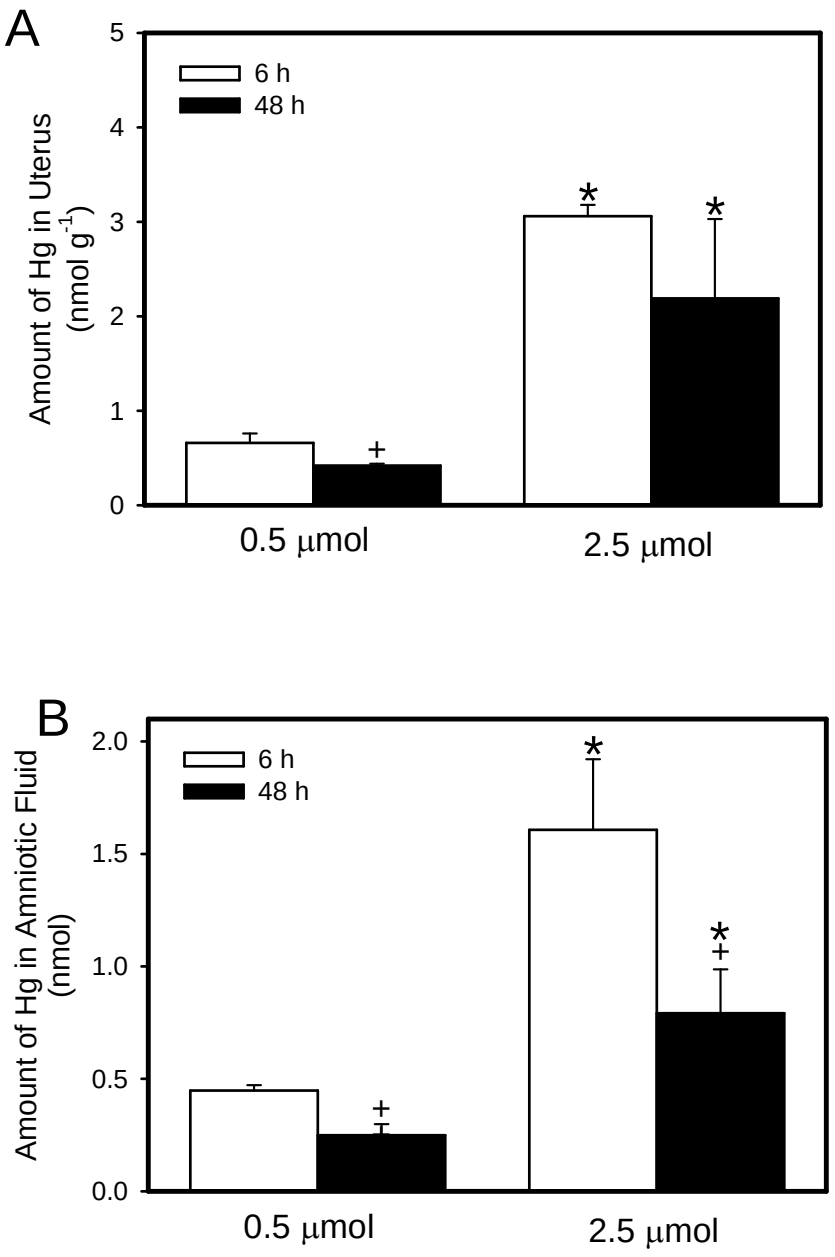


Figure 7

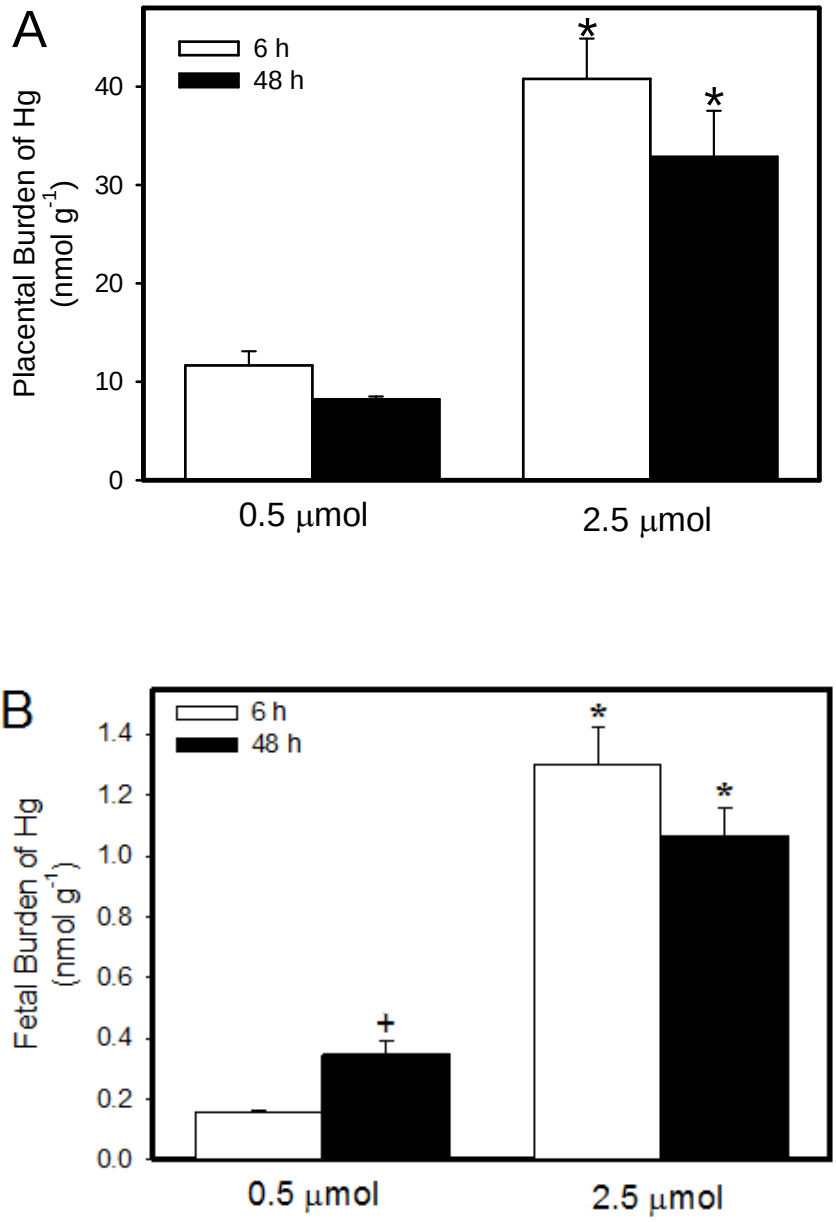


Figure 8

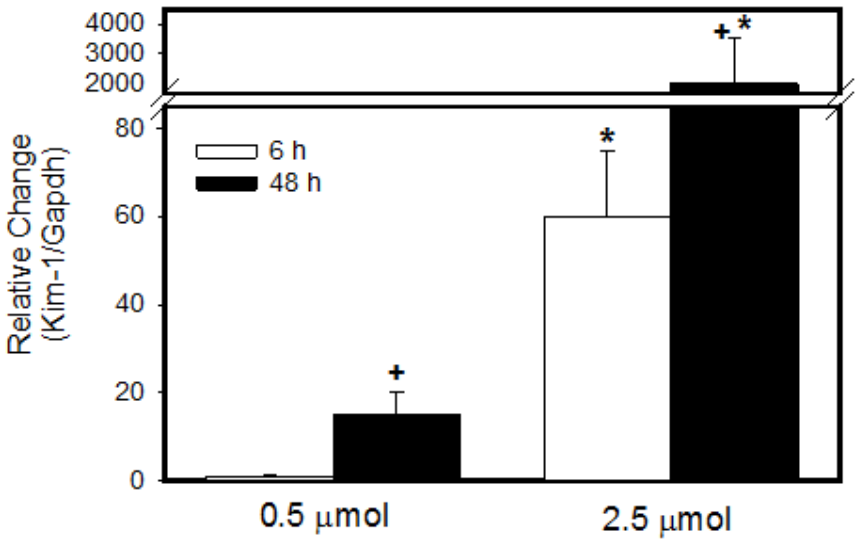


Figure 9

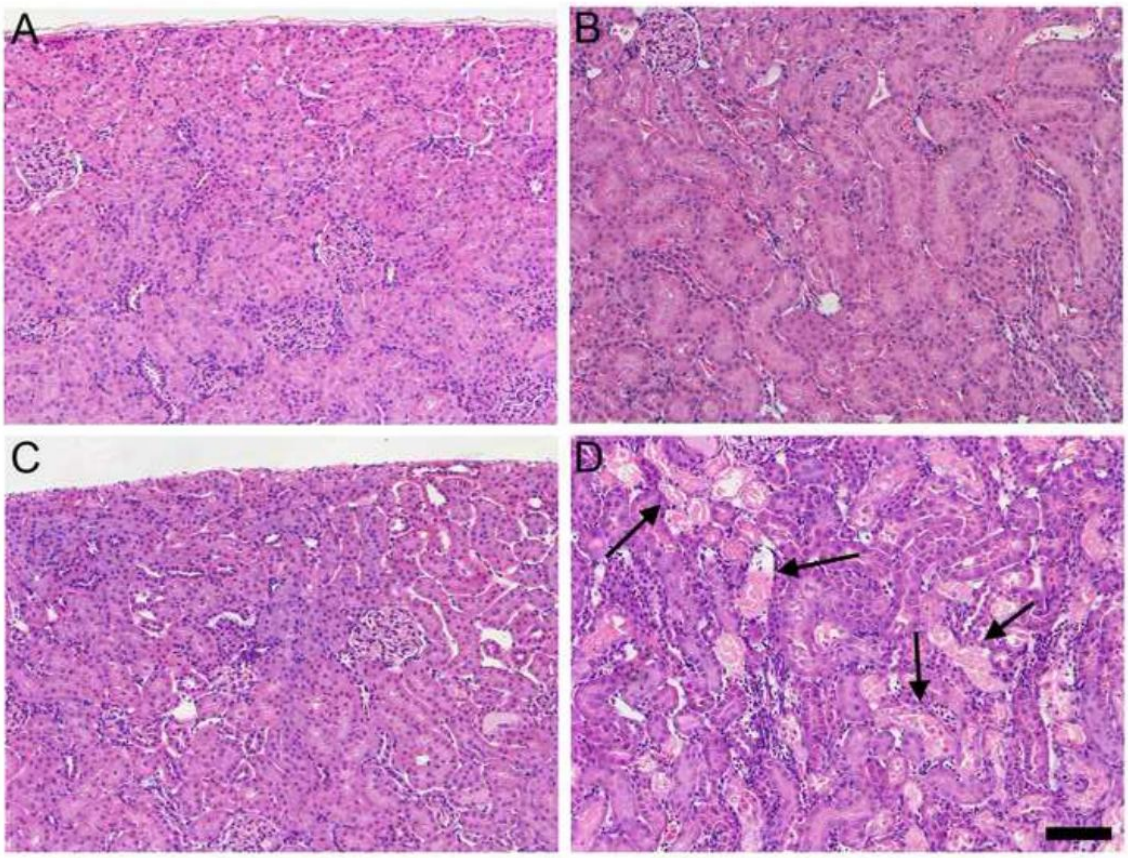
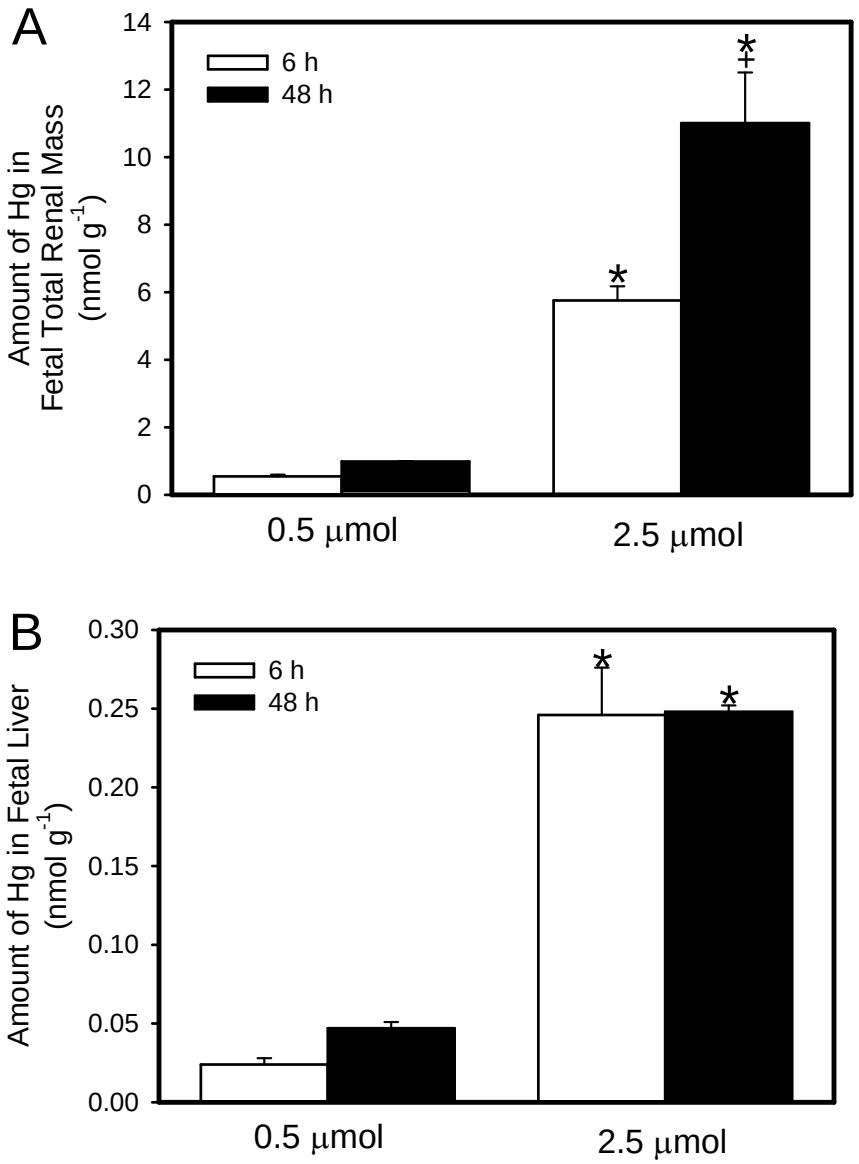


Figure 10



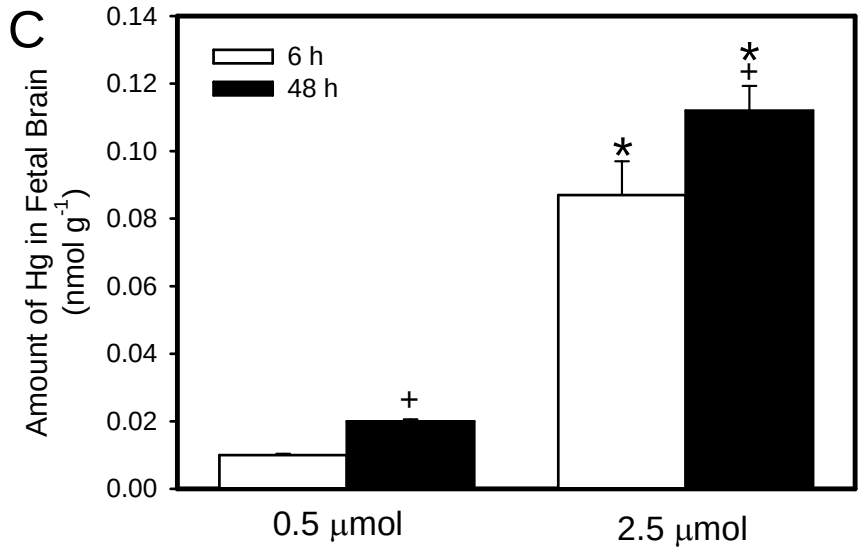


Figure 11

4.DISCUSSÃO

Estudos da exposição a agentes tóxicos durante os períodos gestacional e lactacional são de grande importância na busca pela compreensão de como xenobióticos são processados pelo organismo durante períodos de marcadas mudanças anatômicas e fisiológicas. Ainda, é importante observar se o agente tóxico está sendo transferido para a prole e se os efeitos são mais ou menos pronunciados nos organismos em desenvolvimento. Dentre os inúmeros agentes tóxicos conhecidos, esta tese buscou contribuir com dados sobre a exposição a forma inorgânica de mercúrio. Assim como, avaliar os efeitos do metal sobre marcadores de exposição e moléculas detoxificantes com um enfoque em marcadores de dano renal, visto que, a forma inorgânica de mercúrio afeta principalmente o sistema renal.

Este estudo mostra que a exposição ao mercúrio inorgânico na água de beber (uma das principais vias pela qual a população em geral é exposta), nas doses testadas, causou alterações bioquímicas brandas nas mães com ausência de alterações tóxicas na prole. As fêmeas expostas ao mercúrio inorgânico apresentaram uma diminuição na ingestão de água, tanto no período gestacional (**artigo**) quanto no lactacional (**manuscrito II**); acredita-se que a inclusão do metal na água tornou-a impalatável, devido ao gosto metálico, característico dos sais de mercúrio (WHO, 2007). A ingestão de íons Hg^{2+} por 100 g de rata por dia (média dos resultados obtidos no **artigo** e no **manuscrito II**) foi de ~0,11 mg Hg^{2+} para os animais expostos a dose de 10 µg Hg^{2+}/mL e de ~0,46 mg Hg^{2+} para os animais expostos a dose de 50 µg Hg^{2+}/mL durante o período gestacional; durante o período lactacional estes valores aumentaram aproximadamente 2,5 vezes (~0,24 mg Hg^{2+} e ~1,25 mg Hg^{2+} para as ratas lactantes expostas as doses de 10 e 50 µg Hg^{2+}/mL, respectivamente). O aumento da ingestão de água e de comida durante o período de lactação, deve-se ao aumento da demanda energética do animal para a produção do leite (PICCIANO, 2003). Consequentemente, por ingerirem mais água durante a lactação os animais ingeriram mais mercúrio; este dado é importante e chama atenção para o cuidado que gestantes e principalmente lactantes devem ter com a água e os alimentos que ingerem, pois em áreas de contaminação com mercúrio mulheres no período lactacional podem potencializar a ingestão deste metal.

O mercúrio é transportado na corrente sanguínea e para o interior das células principalmente ligado a grupamentos sulfídricos (-SH) de biomoléculas (ROONEY, 2007). A afinidade do mercúrio pelos grupamentos -SH também está diretamente relacionada a toxicidade deste metal. Uma vez realizada a ligação Hg-SH, a mesma é difícil de ser rompida.

Com a inserção de um átomo de Hg na molécula, a mesma perde sua conformação normal e consequentemente a sua função. Se a intoxicação com Hg continuar ou não for tratada, pode ocorrer a morte celular seguida de falência dos órgãos atingidos (GRAEME E POLLACK, 1998; CLARKSON, 2002). As principais biomoléculas atingidas pelo mercúrio são as enzimas, já foi demonstrado que o mercúrio interage com o grupamento -SH das enzimas lactato desidrogenase (MORAES-SILVA et al., 2012), alanina aminotransferase (PEIXOTO E PEREIRA, 2007; FRANCISCATO et al., 2011) e porfobilinogênio sintase (PEIXOTO et al., 2003, 2007b; FAVERO et al., 2014; OLIVEIRA V. et al., 2014).

A porfobilinogênio sintase (PBG-sintase), também chamada de δ-aminolevulinato desidratase (δ-ALA-D), é uma enzima sulfidrílica encontrada no citosol das células de praticamente todos os tecidos (SASSA, 1982). Esta enzima participa da rota de síntese do grupamento prostético, heme; ela catalisa a condensação assimétrica de duas moléculas do ácido aminolevulínico obtendo como produtos o composto monopirrólico, porfobilinogênio, e duas moléculas de água (SHEMIN, 1976). A PBG-sintase é usada como marcador de exposição a produtos tóxicos tanto em animais de laboratório (PEIXOTO et al., 2003, 2007b; FRANCISCATO et al., 2011; FAVERO et al., 2014; OLIVEIRA V. et al., 2014) como animais selvagens (contaminação ambiental) (COMPANY et al., 2011; ESPÍN et al., 2015). Os metais divalentes possuem grande afinidade pelos grupamentos sulfidrílicos da enzima. Em um estudo anterior desse laboratório (PEIXOTO et al., 2004) observou-se que a atividade da enzima de sangue e cérebro é mais sensível a exposição aos metais divalentes do que a enzima de fígado e rim, *in vitro*. Outros trabalhos do nosso grupo de pesquisa demonstram que animais expostos subcutaneamente ao $HgCl_2$ apresentaram inibição da atividade da enzima de diferentes tecidos (PEIXOTO et al., 2003, 2007b; FRANCISCATO et al., 2011; FAVERO et al., 2014; OLIVEIRA V. et al., 2014). Ao contrário dos dados acima, neste trabalho foi observado um aumento significativo na atividade hepática da PBG-sintase de fetos expostos intrauterinamente a maior dose de mercúrio inorgânico testada (50 µg Hg^{2+}/mL) (**artigo**). E após o nascimento a atividade da enzima de fígado continua levemente aumentada em relação ao grupo controle (19% e 31%, nos grupos 10 e 50 µg Hg^{2+}/mL, respectivamente, no dia pós natal 10; 26 % e 22%, nos grupos 10 e 50 µg Hg^{2+}/mL, respectivamente, no dia pós natal 20) (**manuscrito II**). Quando avaliada após o término da exposição (nos dias pós natal 30 e 40) a atividade da enzima dos animais expostos ao Hg^{2+} (durante a gestação e lactação) retorna aos níveis do grupo controle. Nós sugerimos que o aumento na atividade da enzima é uma resposta adaptativa do organismo a exposição ao

mercúrio, visto que os filhotes foram expostos ao metal durante todo o período gestacional e lactacional, ou seja, o organismo pode estar compensando uma leve inibição enzimática causada pelo metal, expressando mais enzima. Recentemente, Oliveira C. et al. (2014) demonstraram aumento na atividade hepática da PBG-sintase em ratas lactantes expostas subcutaneamente ao mercúrio inorgânico. Entretanto, pouco se sabe sobre este mecanismo até o momento.

É amplamente aceito entre os pesquisadores que o principal órgão atingido pelo mercúrio inorgânico é o rim. O mercúrio presente na corrente sanguínea é filtrado pelos glomérulos depositando-se principalmente nas células dos túbulos proximais (ZALUPS, 2000). O acúmulo de Hg nos rins pode causar o aumento do volume das células e dos túbulos proximais assim como a dilatação do lúmen tubular, causando o aumento do volume renal (MADSEN E MAUNSBACH, 1981). De fato, neste trabalho, as ratas expostas ao mercúrio na água de beber (50 μg Hg^{2+}/mL) apresentaram aumento no peso relativo de rim quando expostas durante 20 dias (gestação) (**artigo**) e durante ~42 dias (gestação e lactação) (**manuscrito II**). Entretanto, mesmo o aumento do peso de rim sendo um indicativo de dano renal, os níveis séricos de ureia e creatinina, marcadores de nefrotoxicidade, não foram alterados. Interessantemente, quando as ratas prenhas foram expostas intravenosamente a uma dose nefrotóxica de $HgCl_2$ (2,5 μmol $HgCl_2$/kg/2mL equivalente a ~0,5 mg Hg^{2+}/kg/2mL) somente os níveis de creatinina foram alterados 48 h após a exposição (**manuscrito III**). O fato de durante a gestação e a lactação a taxa de filtração glomerular ser aumentada (ARTHUR E GREEN, 1982; CHEUNG E LAFAYETTE, 2013) quando comparada a animais não gestantes ou lactantes pode ajudar a explicar a ausência de efeito nos níveis de ureia e creatinina (animais expostos na água de beber) e ureia (animais expostos intravenosamente a dose nefrotóxica).

A ureia é produto do catabolismo de proteínas, sendo a principal forma pela qual os mamíferos excretam o excesso de nitrogênio; já a creatinina é o produto da degradação da fosfocreatina (BAUM et al., 1975; CHAMPE et al., 2006). Em organismos saudáveis estes dois metabólitos são transportados para o rim e excretados através da urina. O aumento do nível sérico destes metabólitos é um indicativo de dano renal severo (BAUM et al., 1975). Logo, marcadores de dano renal mais sensíveis tem se destacado como, por exemplo, a lipocalina associada à gelatinase neutrofílica (NGAL, sigla do inglês neutrophil gelatinase-associated lipocalin) e a molécula de lesão renal-1 (Kim-1, sigla do inglês kidney injury

molecule-1). A Kim-1 é uma glicoproteína transmembrana expressa nas células dos túbulos proximais; acredita-se que a mesma está envolvida no processo de reparação e renovação celular renal após insultos tóxicos (ICHIMURA et al., 1998), porém a função desta proteína ainda não está bem descrita. Sabe-se que em animais saudáveis a expressão da Kim-1 é baixa, praticamente nula; porém, logo após a exposição a um agente nefrotóxico a expressão desta proteína aumenta consideravelmente (OBERMÜLLER et al., 2014). De fato, no nosso estudo, as ratas gestantes que foram expostas a dose considerada não nefrotóxica de $HgCl_2$ (0,5 μmol $HgCl_2$/kg/2mL equivalente a ~0,1 mg Hg^{2+}/kg/2mL) não apresentaram alterações nos níveis de ureia e creatinina, porém, apresentaram aumento na expressão renal de Kim-1 (**manuscrito III**); ressaltando a maior sensibilidade desta proteína a insultos tóxicos.

As ratas prenhas que foram expostas ao mercúrio na água de beber apresentaram níveis detectáveis de Hg somente nos rins e nas fezes (**manuscrito I**). Quando os animais foram expostos intravenosamente ao metal, e uma técnica de detecção de Hg mais sensível foi utilizada, foi possível avaliar a distribuição do mercúrio no organismo materno; e o rim continuou sendo o órgão que apresentou os maiores níveis de mercúrio (**manuscrito III**). Curiosamente, não foi detectado Hg na urina das ratas expostas ao metal na água de beber (**manuscrito I**). Nossa hipótese é de que como o organismo está sendo exposto ao mercúrio a doses relativamente baixas por um longo período, ele está sendo hábil a processar os íons Hg^{2+} provavelmente formando um complexo inerte com grupamentos -SH de moléculas presentes nas células renais (ZALUPS, 2000; BARBIER et al., 2005), assim como moléculas detoxificantes (discutido abaixo). Corroborando com esta hipótese as ratas gestantes expostas intravenosamente a dose não nefrotóxica de $HgCl_2$ apresentaram os mesmos níveis renais de mercúrio 6 e 48 h após a exposição, sem alterações histológicas e com baixa taxa de excreção urinária de Hg. Já as gestantes expostas a dose nefrotóxica de $HgCl_2$ apresentaram uma diminuição nos níveis renais de Hg 48h após a exposição quando comparado com os níveis renais de Hg 6 h após a exposição, isto provavelmente está relacionado as lesões histopatológicas (necrose e acúmulo de proteína) nas células dos túbulos proximais acelerando a eliminação do metal através da urina (**manuscrito III**).

A alteração renal influencia na homeostase corporal e em como o organismo processa metabólitos, sejam eles tóxicos ou não. Entretanto, neste trabalho o dano renal causado pelo mercúrio nas ratas expostas intravenosamente ao metal não alterou a passagem de Hg para a prole (**manuscrito III**). Sabe-se que animais em desenvolvimento são mais sensíveis a

insultos tóxicos devido a imaturidade de órgãos e membranas (NIES E SPIELBERG, 1996). Esta imaturidade permite que os agentes tóxicos atinjam facilmente os órgãos causando danos muitas vezes irreversíveis. Quando a concentração de Hg em diferentes órgãos de fetos cujas mães foram expostas intravenosamente ao $HgCl_2$ no dia 18 de gestação foi avaliada, observou-se um acúmulo dose e tempo dependente do metal (rim>fígado>cérebro) (**manuscrito III**); este resultado corrobora com o fato de órgãos ainda em formação serem incapazes de processar agentes tóxicos. Enquanto nas mães, 48 h após a exposição os níveis teciduais de Hg diminuíram em relação a 6 h após a exposição (a única exceção foram os níveis de Hg renal das fêmeas expostas a dose não nefrotóxica, discutido anteriormente); nos tecidos fetais a concentração de Hg 48h após a exposição estava aumentada em relação ao observado 6 h após a exposição; comprovando a incapacidade de órgãos em desenvolvimento de excretar/depurar agentes tóxicos.

Por sua vez, a prole cujas mães foram expostas ao Hg^{2+} na água de beber apenas apresentou aumento da atividade da PBG-sintase hepática (discutido anteriormente) e um leve aumento no peso relativo de rim no dia pós-natal 20 (**manuscrito II**). Porém, não apresentou alteração: (1) no peso corporal (**manuscrito II**), (2) nos marcadores de dano motor (teste do geotactismo negativo e teste do beacker) (**manuscrito I**), (3) nos parâmetros bioquímicos avaliados (ureia e creatinina, tios totais e não protéico e metalotioneínas) (**manuscrito II**), (4) alteração na homeostase de metais essenciais (**manuscrito I**) e (5) níveis detectáveis de Hg em fígado, rim e cérebro (**manuscrito I**). Embora se saiba que organismos em desenvolvimento são mais sensíveis a insultos tóxicos, quando as mães foram expostas a doses consideravelmente baixas de Hg^{2+}, por um longo período na água de beber, o organismo materno processou o xenobiótico evitando que o mesmo chegasse até a prole. Provavelmente, esta proteção endógena está relacionada a moléculas detoxificantes como glutationa e metalotioneína.

As metalotioneínas (MT) são proteínas citoplasmáticas de baixo peso molecular ricas no aminoácido cisteína (CHERIAN E GOYER, 1978). Esta proteína é responsável pela detoxificação de metais não essenciais e pela homeostase de metais essenciais; os metais formam uma ligação estável com os grupamentos –SH presentes na proteína (ROESIJADI, 1996). A síntese das metalotioneínas é induzida por metais como Zn, Cu, Ag, Cd e Hg (CHERIAN E GOYER, 1978; ONOSAKA E CHERIAN, 1981) e espécies reativas de oxigênio (BABULA et al., 2012; RUTTKAY-NEDECKY et al., 2013). Yoshida et al. (2002)

observaram aumento no conteúdo de metalotioneínas em diferentes tecidos de camundongas gestantes quando comparadas a animais não gestantes; nesta mesma linha, Favero et al. (2014) observaram um leve aumento nos níveis renais de metalotioneínas em ratas lactantes em comparação com ratas não lactantes. Estes dois estudos corroboram com a nossa hipótese de que o organismo das ratas gestantes e lactantes processam os íons mercúrio provavelmente formando um complexo inerte com as metalotioneínas (Hg-MT), o qual é transportado para o rim, evitando que o metal atinja a prole e também que cause danos severos as mães. De fato, as ratas prenhas e lactantes expostas, ao mercúrio na água de beber apresentaram os níveis renais de MT aumentados quando comparadas ao grupo controle (**manuscritos I e II**). Entretanto mais estudos são necessários para elucidar o papel das MT em uma possível proteção contra a exposição a baixas doses de mercúrio inorgânico durante a gestação e/ou lactação.

5.CONCLUSÕES:

Observando os resultados descritos, podemos concluir que:

1) A exposição ao $HgCl_2$ na água de beber causou:
   - → Diminuição na ingestão de água devido a alterações na palatabilidade causada pela adição do $HgCl_2$;
   - → Os efeitos da exposição ao $HgCl_2$ foram leves, porém pronunciados nas mães, visto que estas apresentaram aumento do peso relativo de rim, acúmulo renal de Hg e alteração na homeostase de Cu e Zn; também, observou-se aumento nos níveis renais de metalotioneínas e tióis totais;
   - → A prole somente apresentou aumento da atividade hepática da PBG-sintase (fetos) e aumento do peso relativo de rim (dia 20 pós-natal).

Como o mercúrio foi administrado em baixas doses, na água de beber, durante um longo período, o organismo materno foi capaz de processar os íons Hg evitando danos a prole. Provavelmente, esta proteção esta relacionada com as moléculas detoxificantes, principalmente as metalotioneínas, as quais tem a síntese aumentada durante a gestação e lactação. Nós sugerimos que as metalotioneínas ligam-se ao Hg formando um complexo inerte, transportando o metal para o rim a fim de ser excretado, evitando que este cause danos severos a prole e ao próprio organismo materno.

2) A exposição intravenosa ao $HgCl_2$ causou:
   - → Acúmulo de Hg em todos os órgãos avaliados, principalmente nos rins das ratas prenhas;
   - → 48 h após a exposição os níveis de Hg nos órgãos maternos diminuíram em relação aos níveis observados 6 h após a exposição;
   - → 48 h após a exposição as ratas prenhas expostas a dose de 2,5 µmol $HgCl_2$/kg/2 mL apresentaram dano renal, o qual foi determinado através do aumento dos níveis séricos de creatinina e da expressão da proteína renal Kim-1 e de alterações histopatológicas;
   - → Os níveis de Hg após 6 e 48 h são similares tanto na placenta quanto nos fetos;
   - → Os níveis de Hg nos órgãos fetais aumentaram de maneira dependente da dose e do tempo.

A exposição intravenosa a dose de 2,5 μmol $HgCl_2$/kg/2 mL causou dano renal nas mães 48 h após a exposição, entretanto, este não interferiu na deposição de mercúrio na prole. Independentemente da dose de $HgCl_2$ a que as mães foram expostas, os fetos não eliminaram o mercúrio acumulado nos órgãos com o passar das horas após a exposição.

PERSPECTIVAS:

- Nas ratas Wistar gestantes e lactantes, assim como, na prole expostas ao mercúrio inorgânico na água de beber as perspectivas são:
  - → Avaliar a expressão gênica em rim e fígado de moléculas detoxificantes, principalmente metalotioneínas;
  - → Avaliar marcadores mais sensíveis de toxicidade renal, por exemplo, a expressão da gênica da proteína Kim-1;
  - → Avaliar possíveis danos histopatológicos.

- Nos animais expostos intravenosamente ao mercúrio inorgânico durante a gestação as perspectivas são:
  - → Avaliar os níveis de mercúrio durante o período neonatal;
  - → Avaliar parâmetros bioquímicos marcadores de dano renal e hepático na prole durante o período neonatal;
  - → Avaliar o desempenho da prole em tarefas comportamentais que envolvam desenvolvimento motor e memória;

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