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TOXICIDADE DO MERCÚRIO EM RATAS VIRGENS, GESTANTES E LACTANTES: EFEITO PROTETOR DO ZINCO E DA N-ACETILCISTEÍNA

Santa Maria, RS, Brasil

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Tese apresentada para o programa de pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para o título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Prof.ª Dr.ª Maria Ester Pereira

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Santa Maria, RS

2016

Dedicatória

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“Tem coisas que tem seu valor
Avaliado em quilates, em cifras e fins
E outras não tem o apreço
Nem pagam o preço que valem pra mim...”
(Gujo Teixeira)

RESUMO

TOXICIDADE DO MERCÚRIO EM RATAS VIRGENS, GESTANTES E LACTANTES: EFEITO PROTETOR DO ZINCO E DA N-ACETILCISTEÍNA

AUTOR: Vitor Antunes de Oliveira

ORIENTADORA: Profª. Dra. Maria Ester Pereira

O mercúrio (Hg) é um metal bivalente encontrado líquido a temperatura ambiente, sem funções biológicas e antropogenicamente liberado em atividades industriais, agricultura e queima de combustíveis fósseis. Os efeitos tóxicos causados pela exposição a esse metal estão relacionados à sua interação com diferentes processos bioquímicos devido a sua afinidade por grupos sulfidrílicos (SH). Estes danos dependem do tempo de exposição e do período de desenvolvimento em que os indivíduos são expostos. Com isso, o objetivo deste trabalho foi avaliar os efeitos do Hg inorgânico, em dose única subcutânea, em ratas virgens, gestantes e lactantes, bem como, a capacidade protetora do zinco (Zn) e da N-acetilcisteína (NAC). Para isso, adotamos três protocolos experimentais: I) Ratas virgens foram tratadas com $ZnCl_2$ (27 mg/kg) e/ou NAC (5 mg/kg) ou salina (0,9%) e 24 horas após com $HgCl_2$ (5 mg/kg) ou salina (artigo 1). II) Ratas gestantes e lactantes tratadas com $ZnCl_2$ (27 mg/kg) e/ou NAC (5 mg/kg) ou salina (0,9%) e 24 horas após com $HgCl_2$ (10 mg/kg) ou salina (manuscrito I). III) Análise renal e hepática de ratas virgens, gestantes e lactantes expostas a uma dose de $HgCl_2$ (5 mg/kg) ou salina (manuscrito II). Em todos os protocolos a eutanásia foi realizada 24 horas após o último tratamento e os tecidos retirados e preparados para as análises. Os protocolos I e II tiveram como foco principal parâmetros bioquímicos em diferentes tecidos e o protocolo III em avaliações morfológicas, expressão proteica em rins e fígado. Ratas virgens expostas ao Hg apresentaram inibição da atividade da δ-aminolevulinato desidratase (δ-ALA-D) em todos os tecidos analisados, alterações em marcadores hepáticos (alanina aminotransferase [ALT] e aspartato aminotransferase [AST]) e renais (creatinina e ureia), além de danos morfológicos e alteração na expressão de proteínas relacionadas ao estresse oxidativo, como a mitofusina 2 (MFN2), óxido nítrico sintetase induzível (iNOS), proteína de choque térmico 27 (HSP27) e proteína reguladora de glicose 75 (GRP75). Ratas gestantes e lactantes expostas ao Hg apresentaram alterações mais brandas que ratas virgens, inclusive sem inibição da δ-ALA-D hepática ou distúrbios em proteínas relacionadas com dano oxidativo, bem como poucos danos morfológicos em rins e fígado. Ratas gestantes e lactantes apresentaram altos níveis hepáticos de metalotioneínas (MT) e aumento no diâmetro glomerular em relação as ratas virgens. Os resultados sugerem maior resistência de ratas gestantes e lactantes ao Hg quando comparadas com ratas virgens. Esta diferença pode ser relacionada ao aumento nos níveis hepáticos de MT induzidos pela gestação e lactação. Essa proteína é sintetizada principalmente no fígado e desempenha importante função quelante, tornando substâncias como o Hg, menos nocivas. Os tratamentos com Zn e NAC, mostraram resultados promissores contra os danos causados pelo Hg, provavelmente pela indução da síntese de MT causada pelo Zn, e pela ação quelante da NAC. Em ambas as situações ocorre a captura do Hg. O metal ligado a MT ou a NAC é neutralizado e consequentemente apresenta menor toxicidade.

Palavras-chave: Metalotioneína, Estresse Oxidativo, Rins, Fígado, δ-aminolevulinato desidratase.

ABSTRACT

MERCURY TOXICITY IN VIRGIN, PREGNANT AND LACTATING RATS: PROTECTIVE EFFECT OF ZINC AND N-ACETILCYSTEINE

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ADVISOR: Maria Ester Pereira

Mercury (Hg) is a divalent metal found liquid at room temperature without biological functions and anthropogenically released in industrial, agricultural activities and burning of fossil fuels. Toxic effects caused by exposure to this metal are related to the interaction of different biochemical processes due to its affinity for sulfhydryl groups (SH). This damage depends on the time of exposure and the development period in which the individuals are exposed. Thus, the aim of this study was to evaluate the effects of a single subcutaneous dose of inorganic Hg in virgin, pregnant and lactating rats, as well as the protective effect of zinc (Zn) and N-acetylcysteine (NAC). For this, three experimental protocols were used: I) Virgin female rats were treated with $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) or saline (0.9%) and 24 hours after with $HgCl_2$ (5 mg/kg) or saline (Article 1). II) Pregnant or lactating rats were treated with $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) or saline (0.9%) and 24 hours after with $HgCl_2$ (10 mg/kg) or saline (manuscript I). III) Renal and hepatic analysis of virgin, pregnant and lactating rats exposed to a dose of $HgCl_2$ (5 mg/kg) or saline (manuscript II). In all protocols euthanasia was performed 24 hours after the last treatment and the tissues removed and prepared for analysis. Protocols I and II focused primarily on biochemical parameters in different tissues and protocol III in morphological evaluations and protein expression in the kidneys and liver. Virgin rats exposed to Hg showed inhibition of the δ-aminolevulinic acid dehydratase (δ-ALA-D) activity in all tissues analyzed, changes in serum markers of hepatic (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and renal (creatinine and urea) damage, morphological damage, and changes in proteins related to oxidative stress expression, for instance, mitofusin 2 (MFN2), inducible nitric oxide synthase (iNOS), heat shock protein 27 (HSP27) and glucose regulated protein 75 (GRP75). Pregnant and lactating rats exposed to mercury showed milder changes than virgin rats, including no inhibition of hepatic δ-ALA-D or alterations of proteins related to oxidative stress and few morphological damage. Pregnant and lactating rats still showed physiologically higher levels of metallothionein (MT) in the liver and larger glomerulus diameter than virgin rats. The results suggest greater resistance of pregnant and lactating rats to Hg compared with virgin rats. This difference may be related to increase of hepatic MT levels induced by pregnancy and lactation. This protein is synthesized in the liver and plays an important chelator role,. making substances, such as Hg, less harmful. The treatment with Zn and NAC showed promising results against damage caused by Hg, probably by induction of MT synthesis caused by Zn and by chelating action of NAC. In both situations occur the capture of Hg. The metal bound to MT or NAC is neutralized and consequently has lower toxicity effects.

Keywords: Metallothionein, Oxidative Stress, Kidneys, Liver, δ-aminolevulinic acid dehydratase.

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Lista de abreviaturas e símbolos

°C: graus Celsius;

δ-ALA-D: δ-aminolevulinic acid dehydratase/ δ-aminolevulinato desidratase;

ALT: alanine alanina aminotransferase/ alanina aminotransferase;

ANOVA: análise de variância;

AST: aspartate alanina aminotransferase/ aspartato aminotransferase;

b.w.: body weight (peso corporal);

DMPS: 2,3-dimercapto-1-propanesulfonic acid/ ácido 2,3-dimercapto-1-propanossulfônico;

DNA: deoxyribonucleic acid (ácido desoxirribonucleico);

EO: estresse oxidativo;

ERO: espécies reativas de oxigênio

g: gravitational force (força gravitacional);

GRP: glucose regulated protein/ proteína reguladora de glicose;

GSH: reduced glutathione/ glutationa reduzida;

HSP: heat shock protein/ proteína de choque térmico;

iNOS: inducible nitric oxide synthase/ óxido nítrico sintetase induzível;

i.p.: intraperitoneal;

i.v.: intravenoso;

MT: metallothionein/ metalotioneína;

MFN2: mitofusin 2/ mitofusina 2;

n: número de repetições;

N.D.: não determinado/detectado;

NAC: N-acetylcystein**/** N-acetilcisteína;

NO: nitric oxide/ óxido nítrico;

NOS: nitric oxide synthases/ óxido nítrico sintase;

Nrf2: nuclear factor erythroid 2/ fator nuclear eritróide 2;

OMS: Organização Mundial da Saúde;

$ONOO^-$: ânion peroxinitrito;

p: nível de significância;

PBG: porfobilinogênio;

PBG-synthase/ PBG-sintase: porphobilinogen synthase/ porfobilinogênio sintase;

PMSF: phenylmethylsulfonyl fluoride (fenil-metil-sulfonil fluoreto);

ROS: reactive oxygen species (espécies reativas de oxigênio);

rpm: rotação/minuto;

S1: fração sobrenadante;

s.c.: subcutânea;

S.E.: standard error (erro padrão);

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

-SH: sulfhydryl group(s)/ grupo(s) sulfidrílico(s);

T: thionein /tioneína;

TCA: trichloroacetic acid (ácido tricloroacético);

v/v: volume/volume;

SUMÁRIO

Apresentação

No item **Introdução** está descrito uma revisão sucinta sobre os temas abordados nesta tese. No final deste item estão apresentados os objetivos.

Os **Resultados** estão dispostos na forma de artigo científico e manuscritos submetidos à publicação. As seções Introdução, Materiais e Métodos, Resultados, Discussão e Referências 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 **Conclusão** são apresentadas as conclusões gerais do presente trabalho.

As **Referências** apresentadas no final da tese referem-se somente as citações que aparecem nos itens **Introdução** e **Discussão**.

1. INTRODUÇÃO

1.1. MERCÚRIO

O mercúrio (Hg) é um metal bivalente encontrado líquido a temperatura ambiente, sem funções biológicas e antropogenicamente liberado em atividades industriais, agricultura e queima de combustíveis fósseis. Além disso, o Hg pode ser liberado naturalmente na biosfera como resultado de atividades vulcânicas e geotermais (BERLIN et al 2007, LI et al 2009). De acordo com a Organização Mundial da Saúde (OMS), entre 1,5 e 17/1000 crianças, onde peixe é o principal produto da cadeia alimentar, sofrem com danos cognitivos devido à contaminação por Hg. Os efeitos tóxicos causados pela exposição a esse metal são relacionados à sua interação em diferentes processos bioquímicos devido a sua afinidade por grupos sulfidrílicos (SH) (ROONEY 2007). Essa interação pode levar a formação de moléculas chamadas dicisteinilmercúrio, as quais podem passar mais facilmente as membranas celulares e causar danos (ZALUPS 2000).

No Brasil, casos de contaminação por Hg ocorrem principalmente na região amazônica, onde o metal é usado na mineração para amalgamar o ouro. A população ribeirinha está exposta ao Hg ocupacionalmente (mineradores) e através do consumo de água e peixes contaminados (PALHETA e TAYLOR 1995, NEVADO et al. 2010).

1.1.1. MERCÚRIO INORGÂNICO

O Hg inorgânico pode ser encontrado com valência 0 (Hg^0) e em seus dois estados de oxidação (Hg^+ e Hg^{2+}) (BERLIN et al 2007). Além disso, no

organismo a forma orgânica desse metal pode ser desmetilada no trato gastrointestinal resultado na formação de íons Hg^{2+} (LORSCHEIDER 1995).

Estudos do nosso grupo de pesquisa mostram que o Hg inorgânico ($HgCl_2$) inibe a atividade da δ-aminolevulinato desidratase (δ-ALA-D) renal e hepática de ratos jovens expostos subcutaneamente ao metal na segunda fase de desenvolvimento pós-natal ($8^{\underline{0}}$ a $12^{\underline{0}}$ dias de idade) (PEIXOTO et al. 2007a, 2007b, FRANCISCATO et al. 2011). Outros estudos também tem reportado a toxicidade dessa forma do metal no desenvolvimento intrauterino e/ou lactacional e em animais adultos (ILBÄCK et al. 1991, ORNAGHI et al. 1993, VINCENT et al. 2004, SAKAMOTO et al. 2002, NEWLAND et al. 2008, STRINGARI et al. 2008, VASSALO et al. 2011). Entretanto, poucos estudos com Hg inorgânico são realizados, principalmente investigando os efeitos tóxicos em fêmeas. Recentemente, Oliveira et al. (2012) demonstraram que a exposição a baixas doses de $HgCl_2$ na água de beber, durante a gestação, causa uma diminuição na ingestão de comida e ganho de peso corporal (OLIVEIRA et al. 2012). Ainda, Oliveira et al. (2014) sugerem que ratas virgens diferem de ratas lactantes quanto a sensibilidade ao $HgCl_2$ (OLIVEIRA VA et al. 2014).

1.1.2. RINS E FÍGADO: PRINCIPAIS ALVOS DO MERCÚRIO INORGÂNICO

O Hg inorgânico atinge primeiramente os rins, causando danos nos túbulos proximais e lesão glomerular (ZALUPS 2000). Alguns estudos sugerem que poucas horas após exposição a essa forma do metal, cerca de 50% do mesmo encontra-se no tecido renal (ZALUPS e CHERIAN 1992, ZALUPS

2000, BERLIN et al. 2007). Esse processo é facilitado pela formação do dicisteinilmercúrio, que como mencionado anteriormente, consegue circular mais facilmente pelo organismo, transpor as membrana das células renais e dessa forma causar dano (ZALUPS 2000).

Apensar de o sistema renal ser o alvo principal do Hg inorgânico, o tecido hepático também é atingido por esta forma do metal (BERLIN et al. 2007). Estudos utilizando ratos e camundongos tem verificado que o $HgCl_2$ atinge as defesas antioxidantes e aumenta a peroxidação lipídica em fígado tornando o sistema hepático deficitário (FARINA et al. 2003, PEROTTONI et al. 2004, AGARWAL et al. 2010). A hepatotoxicidade dessa forma de mercúrio ainda não está totalmente esclarecida. Entretanto, assim como nos rins, acredita-se ocorrer interação do metal com estruturas orgânicas permitindo ao metal transpor a membrana dos hepatócitos mais facilmente (BERLIN et al. 2007, ROONEY 2007).

1.1.3. MERCÚRIO E ESTRESSE OXIDATIVO

Um dos mecanismos envolvendo a toxicidade renal e hepática do Hg é o estresse oxidativo (EO) (FARINA et al. 2003, BRANDÃO et al. 2009, FREITAS et al. 2009, PAL e GOSH 2012). Os danos oxidativos causados pelos compostos mercuriais tem relação direta com o aumento da peroxidação lipídica (FREITAS et al. 2009), diminuição as defesas antioxidantes não enzimáticas como GSH (SU et al. 2008), tióis totais e não proteicos (FARINA et al. 2003) e inibição de enzimas antioxidantes como a catalase, glutationa peroxidase, glutationa redutase e superóxido dismutase (VICENTE et al. 2004, AUGUSTI et al. 2008, AGARWAL et al. 2010, PAL e GOSH 2012). Além disso, a grande afinidade do Hg por grupamentos favorece a formação de complexos

estáveis os quais podem alterar a função de várias proteínas (BERLIN et al. 2007, ROONEY 2007, FARINA et al. 2011).

1.2. GESTAÇÃO

O período perinatal engloba as fases gestacional e pós-parto e caracteriza-se por importantes mudanças metabólicas no corpo das mães (GUYTON e HALL 2006). Durante a gestação, muitos hormônios, principalmente estrogênios e progesterona, são liberados pela placenta e auxiliam o desenvolvimento dos tecidos envolvidos na amamentação. Além disso, inibem a produção do leite, por hora desnecessária. Com o parto e consequente perda da placenta, os níveis de progesterona caem drasticamente, tornando possível a produção e excreção do leite (PICCIANO 2003, CARVALHO e TAVAREZ 2010, CUMMINGS et al. 2010, CHEUNG e LAFAYETTE 2013, TAN e TAN 2013).

Além das adaptações hormonais envolvidas na gestação, alguns órgãos e sistemas, que não são diretamente envolvidos no desenvolvimento dos fetos sofrem alterações. Durante a gestação, os rins aumentam de tamanho devido à hidronefrose e esse aumento pode permanecer por até seis meses após o parto. Também é possível observar aumento entre 40 a 50% da filtração glomerular, que resulta na diminuição sérica de creatinina, ureia e ácido úrico (CHEUNG e LAFAYETTE 2013, TAN e TAN 2013). Assim, o sistema renal precisa de um rigoroso controle e acompanhamento para suportar estas mudanças metabólicas.

Outro órgão que sofre importantes adaptações durante a gestação é o fígado, porém as alterações não são tão intensas como observada nos rins. Nesse órgão podemos constatar diminuição da produção de alguns metabólitos como bilirrubina, redução na atividade da gama-glutamil transferease e aumento da atividade da fosfatase alcalina. Entretanto, normalmente não há alteração na atividade das principais enzimas hepáticas como a alanina aminotransferase (ALT) e aspartato aminotransferase (AST) e na síntese de ácidos biliares (BACQ, 2000).

Durante a gestação, também é comum que ocorra diminuição do débito cardíaco, aumento do volume sanguíneo, maior vascularização de alguns tecidos, liberação de fatores de coagulação, resistência à insulina, mudança no transito intestinal e farmacocinética de algumas drogas (TAN e TAN 2013).

Como descrito, muitas mudanças ocorrem durante o período gestacional, as quais podem fazer com que as gestantes respondam de forma diferente quando em contato com substâncias químicas, fármacos e agentes tóxicos. Oliveira et al. (2012) notaram que a exposição ao mercúrio inorgânico durante o todo período gestacional de ratas por via oral não causou alterações bioquímicas nas ratas gestantes expostas ao metal. Esse resultado observado por Oliveira et al. (2012) é surpreendente, uma vez que que o mercúrio é sabidamente nocivo (ZALUPS 2000, BERLIN 2007, PEIXOTO et al. 2007a, 2007b, FRANCISCATO et al. 2009, 2011, LI et al. 2009, FIUZA et al. 2014, OLIVEIRA VA et al. 2014). No entanto, não se sabe exatamente o que leva esses animais responderem de forma diferente a esta substância tóxica e compreender isso pode esclarecer tanto os mecanismos de toxicidade do

mercúrio, bem como ajudar na busca de novos compostos contra a sua toxicidade.

1.3. LACTAÇÃO

Após o parto, novas adaptações hormonais são necessárias para a mãe produzir e excretar o leite. Durante esse processo, ocorre à perda da placenta e os níveis de progesterona e estrogênios caem drasticamente. Por outro lado, os níveis de prolactina atingem os níveis mais altos, normalmente 10 vezes maiores que as concentrações encontradas durante a gestação. Após poucos dias do nascimento, os níveis de prolactina retomam os níveis basais, entretanto, cada vez que a prole realiza a sucção do leite, sinais neuronais são enviados ao hipotálamo e os níveis de prolactina aumentam significativamente (KENSINGER 1998, PICCIANO 2003, GUYTON e HALL 2006).

A prolactina é um dos principais hormônios que apresenta níveis alterados durante a lactação e alguns estudos tem investigado o possível efeito benéfico dessa variação hormonal (TORNER e NEUMANN 2002). Além das clássicas funções hormonais, a prolactina apresenta ação como fator de crescimento celular e na resposta imune, por controlar a expressão de mediadores pro-inflamatórios; porém, os mecanismos envolvendo esses efeitos são pouco conhecidos (ZAGA-CLAVELLINA et al. 2014).

Assim como na gestação, as alterações hormonais, adaptações metabólicas e morfológicas que ocorrem durante a lactação fazem com que esses animais respondam diferentemente a substâncias químicas e agentes tóxicos. Entre essas mudanças, podemos destacar: aumento do volume sanguíneo e plasmático (SUZUKI et al. 1993) e aumento da osmolaridade

plasmática (SUZUKI et al. 2000). Também é possível observar aumento do fluxo sanguíneo, principalmente nas glândulas mamárias e tecido hepático (HANWELL e LINZELL 1973). Solaiman et al. (2001) observaram que a lactação aumenta os níveis de metalotioneínas (MT) em diferentes órgãos de camundongos. Essa proteína atenua o efeito tóxico de várias substâncias e assim aumenta a resistência (SALAIMAN et al. 2001). Greenwood et al. (1973) e Prester et al. (1994) mostraram que durante a lactação a meia-vida biológica do mercúrio pode diminuir de 40 a 50% (GREENWOOD et al. 1973, PRESTER et al. 1994). Nesse contexto, Franco et al. (2007) mostraram uma possível resistência de ratas lactantes ao mercúrio (FRANCO et al. 2007).

Estudos recentes do nosso grupo de pesquisa mostraram que ratas lactantes expostas ao $HgCl_2$ durante 5 dias consecutivos não apresentam inibição da δ-ALA-D renal, diferentemente de ratas virgens expostas a mesma dose (FAVERO et al. 2014). Em outro estudo observou-se que ratas virgens expostas ao mercúrio, de forma aguda, são mais sensíveis que ratas lactantes (OLIVEIRA VA et al. 2014). Analisando esses resultados, nós podemos imaginar que as mudanças metabólicas envolvendo o período lactacional possibilitam a esses animais uma maior proteção contra substâncias tóxicas como o mercúrio. Entretanto, os mecanismos que envolvem essa possível proteção ainda não estão claros e por isso esse trabalho pode ajudar a elucidar importantes dúvidas que ainda persistem sobre esse tema.

1.4. ZINCO

O zinco (Zn) é um metal bivalente e é o segundo elemento traço mais abundante no organismo com diferentes funções vitais. Esse metal essencial

está envolvido na atividade e estrutura de muitas enzimas, realiza um importante papel na divisão e crescimento celular e expressão gênica (STEHBENS 2003, SANDSTEAD e AU 2007, SHAH 2011). Além das funções vitais, o Zn está diretamente envolvido na proteção contra a intoxicação e estresse oxidativo induzido por clorpirifós (MANSOUR e MOSSA 2011), cádmio (MESSAOUDI et al. 2010) e mercúrio (TANDON et al. 2001). Nas últimas décadas, as pesquisas relacionando Zn e a proteômica tiveram destaque no cenário científico. Uma das proteínas dependentes de Zn mais estudada é a MT (Figura 1), que tem sua síntese modulada pela quantidade de desse metal, como mostra a figura 2. Em resumo, o aumento da quantidade disponível de Zn induz a síntese de tioneina (T) através da ação de fatores de transcrição Zn-dependentes e como consequência leva a formação de MT. Se a quantidade disponível de Zn é baixa e o metal é necessário para alguma função vital, ele é liberado da MT e ocorre a formação de T (MARET, 2000, GUNNAR et al. 2007).

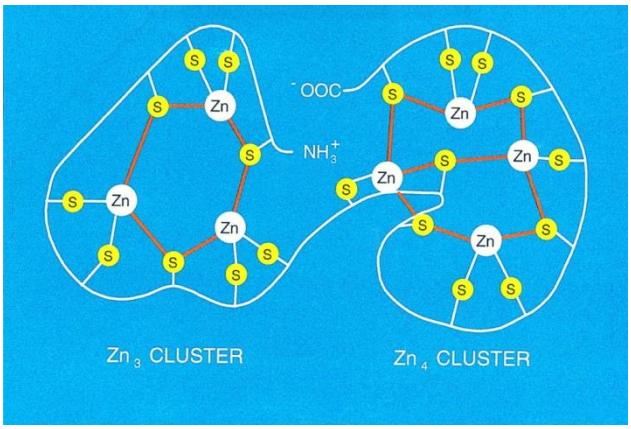

Figura 1: Esquema estrutural da metalotioneína (OTTAVIANI 2014).

Outro mecanismo de proteção mediado pelo Zn é através da ativação do fator nuclear eritróide 2- relacionado ao fator 2 (Nrf2) que induz o aumento da

expressão de moléculas antioxidantes, como a glutationa reduzida (GSH) e a própria MT, as quais tem um importante papel contra a toxicidade do Hg (CHEN e SHAIKH 2009, OTEIZA 2012).

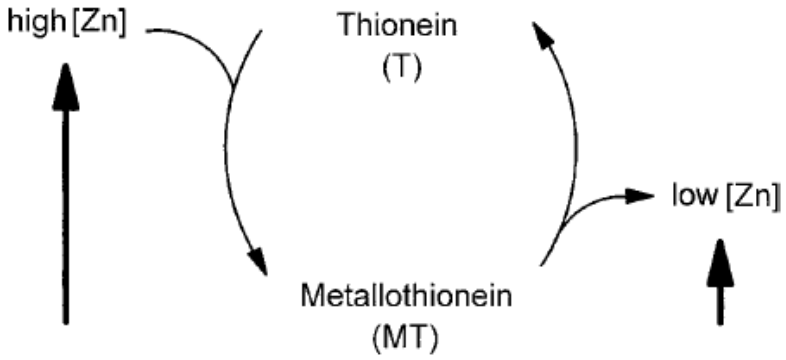

Figura 2: Relação entre os níveis de Zn e a síntese de MT. Adaptado de MARET 2000.

Baseado nesses aspectos, nosso grupo de pesquisa vem, nos últimos anos, testando o Zn como tratamento contra a toxicidade do Hg em diferentes modelos. O Zn atenuou alterações bioquímicas e comportamentais em ratos jovens expostos ao Hg (FRANCISCATO et al. 2009, 2011). Além disso, Peixoto et al. (2003, 2007b) observaram que o pré-tratamento com $ZnCl_2$ protegeu da inibição da δ-aminolevulinato desidratase (δ-ALA-D) em rins e fígado de ratos jovens induzida pelo Hg e ainda aumentou a síntese de MT (Peixoto et al. 2003, 2007b). Mais recentemente Oliveira et al. (2014, 2015) e Favero et al. (2014) observaram que o Zn também protege das alterações bioquímicas causadas pelo Hg em fêmeas adultas e sugerem que essa proteção possa estar relacionada a síntese de MT e GSH induzida pelo Zn (OLIVEIRA VA et al. 2014, 2015, FAVERO et. 2014)

O Zn está envolvido em diferentes aspectos do desenvolvimento e manutenção da homeostase dos animais, por isso é fundamental manter a

demanda diária desse mineral. Tomat et al. (2011) relatam que a deficiência de zinco no período intra-uterino e pós-parto, aumenta a peroxidação lipídica e diminui as defesas antioxidantes em rins e causa aumento da pressão arterial (TOMAT et al. 2011). Apesar disso, ainda há muitas dúvidas sobre os mecanismos envolvendo a participação do Zn no desenvolvimento dos organismos, especialmente durante a gestação e lactação. Por essa razão, estudos como os apresentados nessa tese pode clarear as lacunas existentes relacionadas às funções desempenhadas pelo Zn.

1.5. N-ACETILCISTEÍNA

A N-acetilcisteína (NAC) é um agente mucolítico usado há muito tempo no tratamento de várias patologias, inclusive doenças renais (DRAGER et al. 2004, FISHBANE 2008, KINBARA et al. 2010). Entretanto, a NAC é usada principalmente no tratamento de intoxicação hepática causada por paracetamol (PRESCOTT 2005, KONDALA et al. 2007). A NAC deriva do aminoácido L-cisteína e é de fácil absorção. Além disso, é um dos responsáveis pela regulação da biossíntese da GSH, um importante antioxidante que protege as células das espécies reativas de oxigênio (EROs) as quais encontra-se elevadas nos casos de intoxicação por Hg (Falluel-Morel et al. 2012, ATKURI et al. 2007, BERK et al. 2013). De fato, resultados com a NAC são promissores no tratamento contra a intoxicação por Hg. Estudos i*n vitro* mostram que quando adicionado Hg e NAC em uma solução há a formação de um complexo entre esses dois compostos (Figura 3). Além disso, a NAC administrada oralmente aumenta a excreção urinária de Hg de maneira mais eficiente que quelantes conhecidos como o ácido 2,3-dimercapto-1- propanossulfônico

(DMPS) (JALILEHVAND et al. 2013). Esses resultados podem explicar, pelo menos em parte, o efeito protetor da NAC frente ao Hg, mas muitas incertezas permanecem. Brandão et al. (2006) observaram que a combinação de NAC e Hg aumenta os níveis de ureia e creatinina em soro, indicando dano renal e sugere que isso ocorre pela formação de um complexo Hg-NAC que facilita o transporte do metal para os rins (BRANDÃO et al. 2006). Por outro lado, nesse mesmo estudo os autores observam que a NAC aumenta os níveis hepáticos de MT, o que poderia diminuir a toxicidade do Hg com o passar do tempo.

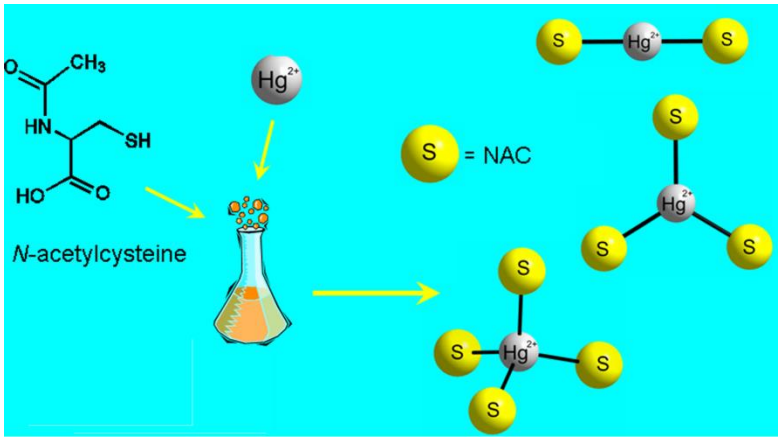

Figura 3: Formação do complexo NAC-Hg em solução. Adaptado de JALILEHVAND et al. 2013

Alguns estudos recentes mostram que a NAC reduz drasticamente os riscos de nefropatia (KAY et al. 2003, DREGER et al. 2004, FISHBANE 2008), previne alterações causadas pela insuficiência renal (KINBARA et al. 2010) e aumenta a excreção de creatinina em pacientes com transplante renal (RUIZ FUENTES et al. 2008). Acredita-se que o mecanismo primário envolvido nesses efeitos benéficos estão relacionados aos efeitos antioxidantes da NAC e redução de EROs produzidos durante a desordem renal. Apesar disso, há poucos estudos de triagem clínica relacionando esse composto e danos renais para que se possa utilizar a NAC de forma eficaz e segura nesses casos.

Recentemente, motivados por essas propriedades antioxidantes, vários estudos associam a NAC à expressão de óxido nítrico sintase (NOS) (BERGAMINI et al. 2001, MAJANO et al. 2004, SOUZA et al. 2015). A NOS produz óxido nítrico (NO), que reage com o oxigênio formando o ânion peroxinitrito ($ONOO^-$), que danifica as células através da peroxidação lipídica e promove a apoptose (CERQUEIRA E YOSHIDA 2002). Entretanto, Çaglıkülekci et al. (2004) mostraram que a NAC protege as células desse processo, pois reduz a expressão de NOS quando a peroxidação lipídica é causada por lipopolissacarídeos. Por outro lado, nesse mesmo estudo a NAC falhou na tentativa de prevenir a expressão de NOS e a peroxidação lipídica quando já está estabelecida a icterícia obstrutiva (ÇAGLIKÜLEKCI et al. 2004).

Como visto, resultados utilizando a NAC são promissores em diferentes aspectos clínicos; entretanto, ainda há muitas incertezas sobre o uso terapêutico desse composto contra o Hg. Assim, pesquisas como essa, que testam a NAC podem ajudar a esclarecer as dúvidas relacionadas aos seus efeitos benéficos.

2. OBJETIVO GERAL

Avaliar o efeito da exposição ao $HgCl_2$ em ratas virgens, gestantes e lactantes, bem como, a capacidade protetora do $ZnCl_2$ e da NAC contra os danos causados pelo Hg.

3. OBJETIVOS ESPECÍFICOS

1. Avaliar os efeitos da exposição ao $HgCl_2$ através de marcadores de toxicidade, análise morfológica, características ultra-estruturais dos tecidos e expressão de proteínas relacionadas ao estresse oxidativo.
2. Avaliar a capacidade preventiva do $ZnCl_2$, NAC ou a combinação de ambos sobre as alterações causadas pelo $HgCl_2$.

4. DESENVOLVIMENTO

Os resultados estão apresentados em forma de artigo científico e manuscritos submetidos. Os itens Introdução, Material e Métodos, Resultados, Discussão e Referências estão no artigo e manuscritos. Os experimentos foram aprovados pelo Comitê de Ética para Uso de Animais da Universidade Federal de Santa Maria (CEUA-UFSM) 2011/096 (Anexo 1).

4.1. ARTIGO

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Zinc and *N*-acetylcysteine modify mercury distribution and promote increase in hepatic metallothionein levels

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ABSTRACT

This study investigated the ability of zinc (Zn) and *N*-acetylcysteine (NAC) in preventing the biochemical alterations caused by mercury (Hg) and the retention of this metal in different organs. Adult female rats received $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) or saline (0.9%) subcutaneously and after 24 h they received $HgCl_2$ (5 mg/kg) or saline (0.9%). Twenty-four hours after, they were sacrificed and analyses were performed. Hg inhibited hepatic, renal, and blood δ-aminolevulinic acid dehydratase (δ-ALA-D) activity, decreased renal total thiol levels, as well as increased serum creatinine and urea levels and aspartate aminotransferase activity. $HgCl_2$-exposed groups presented an important retention of Hg in all the tissues analyzed. All pre-treatments demonstrated tendency in preventing hepatic δ-ALA-D inhibition, whereas only $ZnCl_2$ showed this effect on blood enzyme. Moreover, the combination of these compounds completely prevented liver and blood Hg retention. The exposure to Zn and Hg increased hepatic metallothionein levels. These results show that Zn and NAC presented promising effects against the toxicity caused by $HgCl_2$.

1. Introduction

Mercury (Hg) is a toxic metal, which is liquid at room temperature and presents peculiar characteristics such as low thermal conduction and good electrical conductivity. Due to these characteristics, Hg (mainly inorganic Hg) has been widely used in industrial activities for the production of different products, which may cause people occupational exposure and consequently suffer the effects of its toxicity [1,2]. Another major source of Hg contamination is through dental amalgams, which are still widely used by dentists all over the world [1–3]. Exposure to Hg occurs in the organic and inorganic forms, whose primary target are the central nervous and renal systems, respectively [3]. Inorganic mercury also affects liver, blood, intestine, thyroid and testicles [3,4]. In fact, recent studies have demonstrated that animals exposed to mercury chloride ($HgCl_2$) (5 mg/kg) present renal [5–8], hepatic [9–12], testicular [13], behavioral [14,15] and cardiovascular [16] alterations.

It is known that 99% of Hg circulating in the plasma is bound to sulfhydryl groups (SH) of proteins and the toxicity of this metal is mainly due to its affinity for these SH-groups that facilitate their entry into the cells [4]. Although the distribution of inorganic Hg in the body varies greatly among organs, the main target is the renal system followed by the liver [3].

Hg affinity by SH groups may enhance its toxicity due to Hg binding to SH containing endogenous molecules; such a feature offers the scientific community great prospects to be studied, since the exogenous substances containing SH may prevent damage caused by Hg. One of these substances that is already being studied as a possible protective agent against Hg effects is the *N*-acetylcysteine (NAC) [17,18]. Studies have indicated NAC as an important antioxidant. Moreover, it is known that it also works as a precursor of cysteine and glutathione, which act as antioxidants [19,20]. However, little is known about its interaction with Hg [4,21].

Recently, *in vitro* experiments have indicated that NAC and Hg could form complexes [22]. Moreover, *in vivo* studies have shown that NAC increases reduced glutathione (GSH) and non-protein thiols [23,24]. At a physiological pH, NAC may also chelate Hg and thus act as an antidote against $HgCl_2$ poisoning [21].

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0946-672X/

Another compound that also plays an important activity against the toxicity of $HgCl_2$ is zinc (Zn), which binds to SH groups preventing their oxidation by Hg. The Zn is also involved in the synthesis of molecules rich in SH groups, such as reduced glutathione (GSH) and metallothionein (MT), which play a role against the toxicity of Hg [4,25]. Studies have shown that Zn can activate nuclear factor erythroid 2-related factor 2 (Nrf2) that acts on DNA and promotes the expression of antioxidant molecules such as GSH and MT [25,26]. Nevertheless, further studies are still needed for clarifying the actual role of Zn as an antioxidant and in the detoxification processes.

Considering the few studies in the literature indicating the ability of Zn and NAC to minimize the inorganic Hg toxicity, the present study aims to investigate the effect of these compounds on the toxic action of $HgCl_2$ in rats through the redistribution of Hg and biochemical parameters.

2. Material and methods

2.1. Chemicals

Mercuric chloride, zinc chloride, mercury and zinc solutions, sodium chloride, potassium phosphate monobasic and dibasic, absolute ethanol, sodium hydroxide, trichloroacetic acid, nitric acid, sulfuric acid, *o*-phosphoric acid, perchloric acid and glacial acetic acid were purchased from Merck (Darmstadt, Germany). δ-ALA, *N*-acetylcysteine, 5,5'-ditiobis(2-nitrobenzoico) acid (Ellman's reagent), 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

Adult female Wistar rats were obtained from the animal facility of the Federal University of Santa Maria, and maintained on a 12 h light/dark cycle and at a controlled temperature (22 ± 2 °C). The animals had free access to water and commercial food (GUABI, RS, Brazil) and were handled according to the guidelines of the Ethical code and care of Experimental Animal, Federal University of Santa Maria, Brazil.

2.3. Exposure to metals

Rats were randomly distributed in eight groups with six animals each ($N = 6$ per group). The doses of $ZnCl_2$ and $HgCl_2$ were selected according to previous works from our research group [8,9], and the non-toxic NAC dose was chosen by a pilot study carried out in our laboratory. The animals were weighed and subcutaneously (s.c.) injected with 0.9% NaCl (saline solution), or $ZnCl_2$ (27 mg/kg), or *N*-acetylcysteine (NAC) (5 mg/kg) or combined treatment with $ZnCl_2$ (27 mg/kg) and NAC (5 mg/kg). After 24 h, the animals received saline or $HgCl_2$ (5 mg/kg) (s.c.). $ZnCl_2$, NAC and $HgCl_2$ were dissolved in a saline solution and injected at a volume of 1 mL/kg body weight (b.w.).

According to Sandstead and Au [27], the excretion of zinc is slow, and the metal may remain in the body for months. Moreover, Rodenstein et al. [28] 24 h after NAC administration, found high concentration of this compost and concluded that it is rapidly absorbed and slowly excreted. Thus being, we elaborated the following experimental design:

Group 1 (Sal–Sal; $N = 6$): saline and 24 h after saline.
Group 2 (Zn–Sal; $N = 6$): $ZnCl_2$ and 24 h after saline.
Group 3 (NAC–Sal; $N = 6$): NAC and 24 h after saline.
Group 4 (Zn + NAC–Sal; $N = 6$): $ZnCl_2$ + NAC and 24 h after saline.
Group 5 (Sal–Hg; $N = 6$): saline and 24 h after $HgCl_2$.
Group 6 (Zn–Hg; $N = 6$): $ZnCl_2$ and 24 h after $HgCl_2$.
Group 7 (NAC–Hg; $N = 6$): NAC and 24 h after $HgCl_2$.
Group 8 (Zn + NAC–Hg; $N = 6$): $ZnCl_2$ + NAC and 24 h after $HgCl_2$.
*$ZnCl_2$ 27 mg/kg; NAC 5 mg/kg; $HgCl_2$ 5 mg/kg.

2.4. Tissue preparation

Twenty-four hours after the administration of saline or $HgCl_2$, rats were weighed and sacrificed by decapitation. Blood samples were collected in tubes without anticoagulant and centrifuged at 1050 × *g* at 4 °C for 10 min in order to obtain the serum, which was used for the determination of the urea and creatinine levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. For the δ-ALA-D activity assay, blood was collected in tubes with heparin and hemolyzed in distilled water 1:4 (v/v) by agitation in ice bath for 10 min. The kidneys and liver were quickly removed, placed in ice and respectively homogenized in 5 and 7 volumes of Tris–HCl buffer (10 mM, pH 7.4) with 10 up-and-down strokes at ~1200 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged at 3000 × *g* at 4 °C for 20 min and the supernatant fraction (S1) was used in the enzyme assay and oxidative parameter determination. Furthermore, a portion of the kidney, liver, blood and urine was used in the determination of the Hg and Zn levels.

2.5. Biochemical determinations

2.5.1. δ-aminolevulinic acid dehydratase (δ-ALA-D) activity

The enzymatic activity was assayed according to the method of Sassa [29] by measuring the rate of product formation (porphobilinogen – PBG), as previously described by Peixoto et al. [9]. Incubation was initiated by adding 200 μL of S1 or hemolyzed blood, and was carried out at 39 °C for 60, 30 and 120 min for the kidney, liver and blood, respectively. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 M and the PBG was measured with Ehrlich's reagent at 555 nm, using the molar absorption coefficient of 6.1 × 104 for Ehrlich-PBG salt. The specific enzymatic activity was expressed as the nmol of PBG formed per hour per mg protein.

2.5.2. Determination of metallothionein (MT) levels

Metallothionein content was assayed as described in Peixoto et al. [9] using the colorimetric method with Ellman's reagent at 412 nm [30]. Metallothionein concentration was estimated utilizing GSH as a reference standard and expressed as the μg of SH/g of tissue.

2.5.3. Determination of the total thiol (TSH) and non-protein thiol (NPSH) levels

Thiol levels from the kidney and liver were determined as previously described by Ellman at 412 nm [30]. For non-protein thiol (NPSH) determination, the protein fraction of 200 μL S1 was precipitated with 200 μL of 4% trichloroacetic acid (v/v) followed by centrifugation (1050 × *g*, 10 min) and the supernatant was used for analysis. The colorimetric test was carried out in 1 M phosphate buffer, pH 7.4. A standard curve using glutathione as standard was constructed in order to calculate the SH in the tissue samples.

2.5.4. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity

ALT and AST activities were determined as previously described by Thomas [31] method at 340 nm, using a Kovalent commercial kit in a medium containing Tris–HCl buffer 55.8 mmol/L pH

7.15, L-aLanine 500 mmol/L, 2-oxoglutarate 15 mmol/L and NADH 0.18 mmol/L, with 50 μL of serum or tissue.

2.5.5. Determination of creatinine levels

The determination of creatinine (mg/dL) was carried out by measuring the quantity of creatinine picrate formed at 510 nm. The medium containing picric acid 7.79 mmol/L, NaOH 145.9 mmol/L and 50 μL of serum was incubated at 37 °C for 10 min. Following this period, the first absorbance was determined. The acetic acid 0.4 mmol/L was added and the medium was incubated for another 5 min at room temperature before determining the second absorbance.

2.5.6. Determination of the urea levels

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

2.6. Determination of metal levels

Hg and Zn levels were determined by inductively coupled plasma atomic emission spectrometry (ICPE-9000; Shimadzu Scientific Instruments). The samples of wet tissue (approx. 0.2 g of kidney and liver and 1 mL of blood and urine) were placed in vials and frozen at −20 °C until analysis. Urine used for metal dosage in a pool of 24 h after Hg exposure. Samples were digested as previously described by Ineu et al. [32]. After digestion, samples were diluted with deionized water and metals were determined by ICPE-9000. The analytical mercury and zinc certificate standards (high purity quality) (Merck®) were used to make the curve. The detection limit was considered 0.02 ppb, which is the minimum measurable quantity.

2.7. Protein determination

Protein concentrations were determined by the Coomassie blue method using bovine serum albumin as a standard [33].

2.8. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test when appropriate (at least $p \leq 0.05$). Groups that are statistically equal are accompanied with the same letters, and groups that are statistically different are accompanied with different letters.

3. Results

3.1. δ-ALA-D activity

The δ-ALA-D activity from different tissues is presented in Fig. 1. Animals exhibited a significant decrease on hepatic [$F(7,40) = 2.51$; $p < 0.031$] (Fig. 1A), renal [$F(7,40) = 13.22$; $p < 0.001$] (Fig. 1B) and blood [$F(7,40) = 2.465$; $p < 0.034$] (Fig. 1C) δ-ALA-D activity due to Hg exposure. There was a tendency of the pre-treatment with $ZnCl_2$, NAC, and Zn+NAC combination in preventing the Hg effect on the liver δ-ALA-D activity, as well as a tendency of $ZnCl_2$ pre-treatment in preventing Hg effect on blood enzyme. None of the treatments prevented the harm caused by $HgCl_2$ in the kidneys.

3.2. Metallothionein (MT) levels

MT levels from the liver and kidney are presented in Table 1. Exposure to Zn and Hg or Zn + NAC and Hg (Zn–Hg and ZN + NAC–Hg groups) caused a significant increase in hepatic MT levels when compared with the control group [$F(7,40) = 2.69$; $p < 0.022$]. Kidney metallothionein levels were not altered.

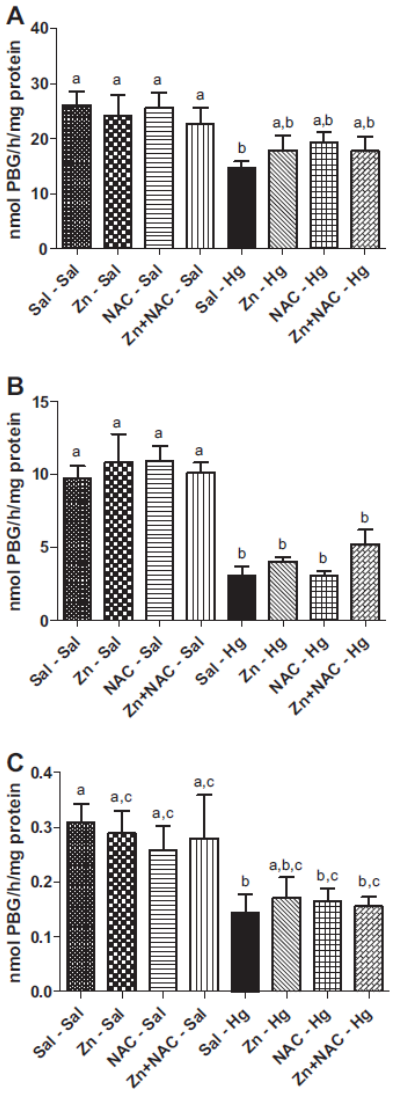

Fig. 1. δ-aminolevulinic acid dehydratase (δ-ALA-D) activity in liver (A), kidney (B) and blood (C). Adult females were exposed (s.c.) to saline, $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) and 24 h after to saline or $HgCl_2$ (5 mg/kg). Results were analyzed by one-way ANOVA followed by Duncan's post hoc test, and are presented as mean ± S.E.M. ($N = 6$). Statistically equal groups are accompanied of the same letters, and statistically different groups are accompanied of the different letters.

3.3. TSH and NPSH levels

Table 1 shows the TSH and NPSH levels from the liver and kidney. Hg exposure caused a significant decrease in TSH levels in the kidney [$F(7,40) = 4.56$; $p < 0.001$]. The pre-treatment with Zn (Zn–Hg group) partially prevented this alteration, whereas the pre-

Table 1
Metallothionein (MT), total SH (TSH) and non-protein SH (NPSH) in liver and kidney of rats exposed (s.c.) to saline (0,9%) or ZnCl2 (27 mg/kg) and/or NAC (5 mg/kg) and 24 h after to saline or HgCl2 (5 mg/kg).

	Liver			Kidney		
	MT	TSH	NPSH	MT	TSH	NPSH
Sal–Sal	50.28 ± 8.53^{a}	3.12 ± 0.28	0.58 ± 0.10	100.3 ± 9.56	1.49 ± 0.12^{a}	0.19 ± 0.04
Zn–Sal	$73.72 \pm 15.72^{a,b,c}$	2.71 ± 0.34	0.51 ± 0.09	88.99 ± 13.08	$1.44 \pm 0.15_{a}$	0.21 ± 0.05
NAC–Sal	51.44 ± 11.21^{a}	2.99 ± 0.51	0.54 ± 0.16	85.60 ± 6.53	1.37 ± 0.11^{a}	0.20 ± 0.03
Zn + NAC–Sal	$62.26 \pm 10.92^{a,b}$	2.67 ± 0.38	0.50 ± 0.16	93.98 ± 10.63	1.31 ± 0.10^{a}	0.19 ± 0.05
Sal–Hg	$64.81 \pm 7.13^{a,b}$	2.41 ± 0.23	0.44 ± 0.07	79.66 ± 13.16	0.92 ± 0.12^{b}	0.13 ± 0.05
Zn–Hg	$90.79 \pm 13.44^{b,c}$	2.80 ± 0.26	0.51 ± 0.11	81.67 ± 7.59	$1.11 \pm 0.16^{a,b}$	0.18 ± 0.04
NAC–Hg	$56.64 \pm 10.92^{a,b}$	2.65 ± 0.43	0.52 ± 0.13	78.60 ± 15.66	0.91 ± 0.11^{b}	0.15 ± 0.05
Zn + NAC–Hg	104.2 ± 13.82^{c}	2.79 ± 0.42	0.48 ± 0.12	80.29 ± 11.98	0.85 ± 0.08^{b}	0.15 ± 0.04

Results were analyzed by one-way ANOVA followed by Duncan's post hoc test, and are presented as mean ± S.E.M. ($N = 6$) and expressed in μg of SH/g of tissue. Letter "a" differ from letters "b" or "c". Letter "b" differ from letter "c".

treatment with NAC or Zn + NAC combination did not prevent the renal TSH level decrease. Hepatic TSH and renal and hepatic NPSH did not present differences between the groups.

3.4. ALT and AST activities

Serum ALT and AST activities are presented in Table 2. Rats exposed to $HgCl_2$ exhibited a significant increase in serum AST activity [$F(7,40) = 6.59$; $p < 0.001$], when compared to control, and the pre-treatments did not prevent this alteration. ALT activity was not altered.

3.5. Creatinine and urea levels

Serum creatinine and urea levels are shown in Table 2. $HgCl_2$ exposure caused an increase in serum creatinine and urea levels [$F(7,40) = 41.91$; $p \leq 0.001$ and $F(7,40) = 24.10$; $p < 0.001$, respectively]. The pre-treatments did not prevent elevation in the creatinine and urea levels caused by $HgCl_2$.

3.6. Metal levels

Hg levels from different tissues are shown in Fig. 2. Animals exposed to $HgCl_2$ presented accumulation of this metal in the liver [$F(7,16) = 82.91$; $p < 0.001$], kidney [$F(7,16) = 45.02$; $p < 0.001$] and blood [$F(7,16) = 19.83$; $p < 0.001$]. Animals pre-treated with Zn (Zn–Hg group) presented lower content of Hg in the liver and blood than those treated only with Hg (Sal-Hg group). NAC pre-treatment decreased the accumulation of Hg in the liver and kidney and totality prevented Hg retention in blood. The combined pre-treatment (Zn + NAC) completely prevented Hg retention in the liver and blood and decreased accumulation in the kidney. All the groups exposed to $HgCl_2$ presented a high content of Hg in urine.

In relation to the Zn levels, animals exposed to $ZnCl_2$ exhibited an increase in the hepatic Zn levels [$F(7,16) = 2.65$; $p < 0.05$]. On the other hand, animals exposed to NAC presented a decrease in the blood Zn levels [$F(7,16) = 4.31$; $p < 0.007$]. The treatments did not alter kidney and urine Zn levels (data not shown).

4. Discussion

This study investigated the effects of Zn and NAC on $HgCl_2$ toxicity by evaluating the oxidative stress and metal tissue levels. Hg may interact and oxidize a great number of micro and macro molecules of the body; however, its greatest affinity is for SH groups [4,34]. The results show that the $HgCl_2$ caused an important inhibition in hepatic, renal and blood δ-ALA-D activity (about 45%, 70% and 55%, respectively). This sulfhydryl enzyme is responsible for the second step of heme biosynthesis. It catalyzes the condensation of two molecules of δ-aminolevulinic acid to form porphobilinogen, thus, it is very important for the aerobic metabolism [35–37]. It is presumed that the δ-ALA-D inhibition is a consequence of high Hg concentration in the tissues. In fact, the highest δ-ALA-D activity inhibition was in the kidneys (approximately 70%) which showed the highest Hg concentration, thereby pointing to this organ as the most affected by the inorganic mercury poisoning. Indeed, a few hours after $HgCl_2$ exposure, 50% of Hg was deposited in the renal system [3]. Zn, NAC and the combination of both partially prevented the inhibition of hepatic δ-ALA-D activity, and Zn partially prevented the inhibition of blood enzyme. This may have happened because Hg was removed from the liver and blood and carried to the kidneys in an attempt to eliminate it. However, none of the pre-treatments protected against the inhibition of the renal δ-ALA-D caused by $HgCl_2$. We believe that this occurred for two reasons: firstly, because of the large renal damage, observed in this work, caused by $HgCl_2$ accumulation and secondly, because we did not observe any difference in Hg excretion between the groups.

Animals exposed to $HgCl_2$ presented high concentration of Hg in all tissues examined. In the liver, $ZnCl_2$ pre-treatment decreased the accumulation of $HgCl_2$ by 25%; the NAC pre-treatment decreased the accumulation of Hg by 80%, and the combination of both treatments (Zn + NAC–Hg group) completely prevented the deposition of this metal. In the kidneys, the pre-treatment with NAC (NAC–Hg and Zn + NAC–Hg groups) decreased approximately 40% of the Hg content; however, this level continues being enough high to inhibit the renal δ-ALA-D activity and to cause the other renal disorders. In the blood, only NAC and the combination of Zn + NAC presented total protection against the deposition of Hg; $ZnCl_2$ pre-treatment (Zn–Hg group) decreased the deposition of Hg in the blood by 33%. Several studies have shown that metals such as copper, zinc, and mercury are important inducers of MT synthesis, especially between 6 and 48 h after exposure [38–43]. Thus, we believe that the lower Hg content from animals that received Zn–Hg may be related to the synthesis of MT, since these proteins are rich in SH groups that may sequester Hg ions helping in its elimination. Moreover, the protective effect of NAC may be associated with the ability of this molecule to chelate Hg since the NAC molecule may form complexes with up to six molecules of Hg [21]. The action of Zn inducing MT synthesis together with the direct effect of NAC chelating Hg bestows promising results. In this way, it was possible to observe a total protection against the retention of Hg in the liver and blood when Zn and NAC are concomitantly administered.

Several studies have shown that inorganic mercury causes serious kidney damage [6,8,38,44]. In our study, even with the exposure of just one dose of $HgCl_2$ renal damage was observed by changes in creatinine and urea levels (up 5 and 4 times, respectively) with only some attenuation by pre-treatment with $ZnCl_2$ combined with NAC (Zn + NAC–Hg) on creatinine levels and by pre-treatment with Zn (Zn–Hg) on urea levels. Hg exposure also

Table 2
ALT and AST activity and creatinine and urea levels in serum of rats exposed (s.c.) to saline (9%), ZnCl2 (27 mg/kg) and/or NAC (5 mg/kg) and 24 h after to saline or HgCl2 (5 mg/kg).

	ALT (U/L)	AST (U/L)	Creatinine (mg/dL)	Urea (mg/dL)
Sal–Sal	66.79 ± 9.08	259.11 ± 52.74[a]	0.61 ± 0.14[a]	77.26 ± 5.62[a]
Zn–Sal	49.76 ± 7.89	250.11 ± 40.71[a]	0.58 ± 0.19[a]	70.54 ± 8.81[a]
NAC–Sal	58.05 ± 7.72	178.97 ± 31.08[a]	0.45 ± 0.18[a]	77.05 ± 11.60[a]
Zn + NAC–Sal	58.36 ± 8.79	192.50 ± 22.30[a]	0.32 ± 0.11[a]	63.17 ± 4.57[a]
Sal–Hg	41.91 ± 6.61	563.96 ± 119.16[b]	3.49 ± 0.14[b]	289.24 ± 13.00[b]
Zn–Hg	58.49 ± 24.90	508.52 ± 78.74[b]	3.05 ± 0.30[b]	222.36 ± 43.95[c]
NAC–Hg	41.47 ± 9.52	547.37 ± 64.45[b]	3.16 ± 0.34[b]	283.05 ± 13.54[b,c]
Zn + NAC–Hg	46.27 ± 15.06	477.97 ± 64.45[b]	2.42 ± 0.20[c]	242.74 ± 30.55[b,c]

Results were analyzed by one-way ANOVA followed by Duncan's post hoc test, and are presented as mean ± S.E.M. ($N = 6$). Letter "a" differ from letters "b" or "c". Letter "b" differ from letter "c".

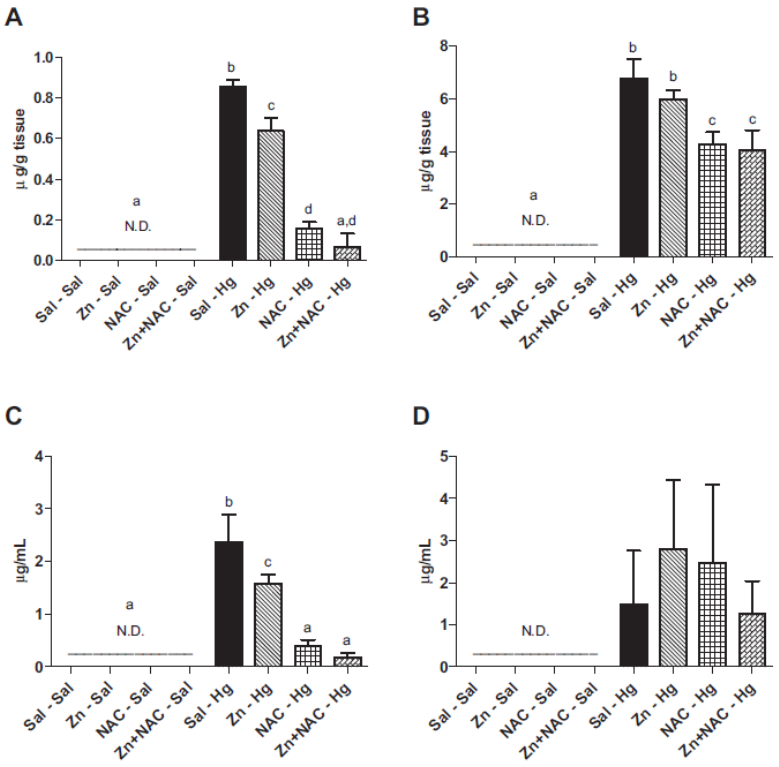

Fig. 2. Mercury levels in liver (A), kidney (B), blood (C) and urine (D) from adult female rats treated as described in the legend of Fig. 1. Results were analyzed by one-way ANOVA followed by Duncan's test and are presented as mean ± S.E.M. ($N = 3$). Statistically equal groups are accompanied by the same letters, and statistically different groups are accompanied by different letters. Samples whose mercury concentrations were below the detectable limit (non-detected, nd) of the technique were considered, for statistical analysis, as containing 0.02 ppb, which is the minimum measurable quantity.
*N.D. – not detected.

caused an increase in the serum AST activity revealing hepatotoxicity without prevention by any pre-treatment applied. Our results differ from those presented by Joshi et al. [19] that found significant NAC protection against hepatic and renal disorders caused by $HgCl_2$. We believe that the protection observed in above mentioned study was due to the administration via (i.p.) which leads to a faster absorption and reaches the highest peak of the compound. In our study, Hg, NAC and Zn were administered by subcutaneous via, which is characterized by slower absorption, leading to a lower but more constant circulating level.

5. Conclusion

In conclusion, these results show that just one dose of Hg causes several alterations in the parameters analyzed. Hg affinity to SH groups caused inhibition of δ-ALA-D activity in liver, kidney and blood and decreased renal TSH levels. We suggest that these changes are related to the Hg retention in liver and kidneys. The pre-treatments with $ZnCl_2$ and/or NAC caused an important redistribution of Hg and presented a tendency in preventing effects of Hg, allowing us to have a glimpse of a complete recuperation of biochemical changes over time. We believe that this occurred due

to at least 2 mechanisms: (i) increase in MT synthesis induced by Zn and (ii) chelating effect of NAC. However, further studies testing the action of these compounds in the long term are needed in order to elucidate the possible alone or combined beneficial effects of these compounds.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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4.2. MANUSCRITO I

Hg toxicity in pregnant and lactating rats: Zn and NAC as alternative of treatment

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Abstract

This study evaluated the toxic effects of mercury in pregnant and lactating rats as well as the possible protective effect of Zn and NAC. Pregnant and lactating rats ($18^{\underline{0}}$ pregnancy day and $7^{\underline{0}}$ lactation day, respectively) received $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) or saline (0.9%) and after 24 h they received $HgCl_2$ (10 mg/kg) or saline (0.9%), subcutaneously. Animals were sacrificed 24 h after Hg exposure and analyses were performed. Hg inhibited renal and blood δ-aminolevulinic acid dehydratase (δ-ALA-D) activity and increased serum creatinine and urea levels from pregnant and lactating rats. Hg exposure decreased placenta δ-ALA-D activity, increased serum AST and liver ALT activity from pregnant rats. All pre-treatments prevented kidney (partiality) and blood δ-ALA-D inhibition from lactating rats and partiality prevented kidney, blood and placenta δ-ALA-D inhibition from pregnant rats. Zn pre-treatments (Zn-Hg and Zn+NAC-Hg groups) totality prevented the increase of hepatic ALT activity in pregnant rats. All pre-treatments partially prevented increase serum AST activity from pregnant rats. Zn, NAC and Hg increased hepatic MT levels from pregnant rats. These results show that this dose of Hg causes biochemistry alterations in pregnant and lactating rats and Zn and NAC present promising results against these damages.

1. Introduction

Mercury (Hg) is a divalent metal in a liquid form at room temperature, without biological functions and anthropogenically released in industrial, agricultural in and the burning of fossil fuels activities. Moreover, it can be released naturally in the biosphere as a result of volcanic and geothermal activity (Berlin et al. 2007, Li et al. 2009). According to the World Health Organization, between 1.5 and 17/1000 children, where fishing is the main source of livelihood, have cognitive alterations due to consumption of food contaminated by Hg. However, the degree of toxicity of the metal may vary depending on the metabolic status of each organism. In this sense, our research group in previous work has showed that lactating rats exposed to 5 mg/kg for 1 or 5 days have fewer biochemical changes than virgin rats exposed to the same doses (Favero et al. 2014, Oliveira C.S. et al. 2014, Oliveira V.A. et al. 2014). We also have noted that rats exposed to mercury throughout pregnancy by via oral do not present biochemical changes (Oliveira et al. 2012).

On the other hand, substances such zinc (Zn) and N-acetylcysteine (NAC) has been tested again mercury toxicity with promissory results (Girardi and Elias 1991, Falluel-Morel 2012). Zn is also a divalent metal, and is the second most abundant trace element in living organisms with different vital functions. This essential metal is involved in the activity and structure of many enzymes, plays an important role in cell division and growth and gene expression (Stehbens 2003, Sandstead and Au 2007, Shah 2011). Moreover, this metal prevented behavioral and biochemical changes in young rats mercury exposured (Franciscato et al. 2009, 2011). Peixoto et al. (2003, 2007) also showed that pre-treatment with zinc protects of the inhibition of liver and kidney

δ-ALA-D activity caused by mercury in young rats, as well as increases in the synthesis of metallothioneins.

NAC is another compound that has shown promising results in the treatment of the intoxication by Hg. NAC administered orally increases the urinary excretion of Hg more effectively than chelators, such as 2,3-dimercapto-1-propanesulfonic acid (DMPS) (Berlin et al. 2007, Jalilehvand et al. 2013). In vitro studies have shown that when added Hg and NAC in a solution occurs the formation of a complex between these two compounds; this fact may suggest, at least in part, a protective effect against mercury (Jalilehvand et al. 2013). The antioxidant action of NAC also contributes significantly to its protective capacity. This antioxidant capacity is linked to the fact that NAC increases the levels of reduced glutathione (GSH) which is an important antioxidant and cell protective against reactive oxygen species (ROS), which are greatly increased in cases of Hg poisoning (Falluel-Morel et al. 2012).

This way, the objective of this study was to evaluated the toxic effects of mercury on different metabolic conditions (pregnancy and lactation), as well as the possible protective effect of Zn and/or NAC.

2. Material and Methods

2.1. *Chemicals*

Mercuric chloride, zinc chloride, sodium chloride, potassium phosphate monobasic and dibasic, absolute ethanol, sodium hydroxide, trichloroacetic acid, nitric acid, sulfuric acid, o-phosphoric acid, perchloric acid and glacial acetic acid were purchased from Merck (Darmstadt, Germany). δ-ALA, N-acetylcysteine, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent), bovine serum albumin, magnesium sulfate, Tris-HCl 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

Pregnant and lactating *Wistar* rats, obtained from the Animal House of the Federal University of Santa Maria, were transferred to our breeding colony and maintained on a 12 h light/dark cycle and at a controlled temperature (22 ± 2ºC). Animals had free access to water and commercial food (GUABI, RS, Brazil) and were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources (096/2011), Federal University of Santa Maria, Brazil.

2.3. Exposure to metals

Pregnant and lactating rats were randomly distributed in five exposure groups (N=5-9). Experiments started in $18^{\underline{0}}$ gestation and $7^{\underline{0}}$ lactation day, respectively. Animals were weighed and subcutaneously (s.c.) injected with 0.9% NaCl (saline solution), $ZnCl_2$ (27 mg/kg), N-acetylcysteine (NAC) (5 mg/kg) or combined treatment with Zn (27 mg/kg) and NAC (5 mg/kg). After 24 hours, the animals received saline or $HgCl_2$ (10 mg/kg) (s.c.). Metals were dissolved in saline solution and injected at a volume of 1 mL/kg body weight (b.w.). The dose of Zn was selected according to Peixoto et al. (2003), NAC dose from Oliveira et al. (2015) and the dose of Hg was based on a pilot study conducted in our laboratory.

This study was conducted using the following experimental design:

Group 1 (Sal–Sal): day 1 – saline, day 2 – saline.

Group 2 (Sal–Hg): day 1 – saline, day 2 – $HgCl_2$.

Group 3 (Zn–Hg): day 1 – $ZnCl_2$, day 2 – $HgCl_2$.

Group 4 (NAC–Hg): day 1 – NAC, day 2 – $HgCl_2$.

Group 5 (Zn + NAC – Hg): day 1 – Zn + NAC, day 2 – $HgCl_2$.

* $ZnCl_2$ 27 mg/kg; NAC 5 mg/kg; $HgCl_2$ 10 mg/kg

* Day 1: $18^{\underline{0}}$ gestational day and $7^{\underline{0}}$ lactation day

*Day 2: $19^{\underline{0}}$ gestational day and $8^{\underline{0}}$ lactation day.

Groups treated only with Zn, NAC or Zn+NAC were not conducted once this compound did not have effect *per se* on parameters analyzed (pilot study). . This reduced considerately the animal's numbers.

2.4. Tissue preparation

Twenty-four hours after the administration of saline or $HgCl_2$, rats were weighed and killed by decapitation. Blood samples were collected in tubes without anticoagulant and centrifuged at 1,050 *g* for 10 min at 4°C to obtain the serum, which was used for the determination of urea and creatinine levels and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity. A portion of liver was retired and homogenized in Tris-HCl buffer (10 mM, pH 7.4) containing 1 mM of magnesium sulfate ($MgSO_4$) for determination of the ALT and AST activity in tissue. For the δ-ALA-D activity assay, blood was collected in tubes with heparin and hemolyzed in distilled water 1:4 (v/v) by agitation on ice bath for 10 min. Liver, kidney and placenta were quickly removed, placed on ice and respectively homogenized in 7, 5 and 10 volumes of Tris-HCl buffer (10 mM, pH 7.4). The homogenate was centrifuged at 3,000 *g* for 20 min at 4°C and the supernatant fraction (S1) was used in the enzyme assay. For metallothionein assays, liver and kidney were homogenized in 4

volumes of 20 mM Tris–HCl buffer, pH 8.6, containing 0.5 mM PMSF as agent antiproteolytic and 0.01% β-mercaptoethanol as a reducing agent. The homogenate was then centrifuged at 16,000 *g* for 30 min to obtain a supernatant containing metallothioneins.

2.5. δ-aminolevulinic acid dehydratase activity

The enzymatic activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen - PBG) formation, as previously described by Peixoto et al. (2003). The incubation was initiated by adding 200 µL of S1 or hemolyzed blood, and was carried out for 60, 30 and 120 min for kidney, liver and blood, respectively, at 39 ºC. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 M and the PBG was measured with Ehrlich's reagent, using the molar absorption coefficient of 6.1 x 10^4 for Ehrlich-PBG salt. The specific enzymatic activity was expressed as nmol of PBG formed per hour per mg protein.

2.6. Metallothionein levels

Metallothionein content was assayed as described in Peixoto et al. (2003) using the colorimetric method with Ellman's reagent (Ellman 1959). Metallothionein concentration was estimated utilizing GSH as a reference standard and expressed as µg of SH/g of tissue.

2.7. Alanine aminotransferase and aspartate aminotransferase activity

Serum and liver ALT and AST activities was determined by the Thomas (1998) method at 240 nm, using a Kovalent commercial kit in a medium containing Tris–HCl buffer 55.8 mM pH 7.15, L-alanine 500 mM, 2-oxoglutarate 15 mM and NADH 0.18 mM, with 50 µL of serum or tissue.

2.8. Determination of creatinine levels

The determination of creatinine (mg/dL) was carried out by measuring the quantity of creatinine picrate formed at 510 nm. The medium containing picric acid 7.79 mM, NaOH 145.9 mM and 50 µL of serum was incubated at 37 °C for 10 min. After this period of incubation, the first absorbance was determined. The acetic acid 0.4 mM was added and the medium was incubated for another 5 min at room temperature before determining the second absorbance.

2.9. Determination of urea levels

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

2.9. Protein determination

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

2.10. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test when appropriate (at least $p \leq 0.05$). Comparisons among all groups were made. Groups statistically equals are accompanied of the same letters, and groups statistically different are accompanied of the different letters. Pregnant and lactating rats were independently analyzed. Due to discrepancies of values among the samples of the same group, δ-ALA-D activity and MT levels data were logarithmically transformed and were submitted to ANOVA similarly to the other results (Sheats and Pankratz 2002); the results were shown as real values (not transformed).

3. Results

3.1. *δ-aminolevulinic acid dehydratase activity*

The δ-ALA-D activity from different tissues of the pregnant and lactating rats is presented in Table 1. None treatment altered hepatic δ-ALA-D activity from both pregnant and lactating rats. Hg exposure significantly decreased δ-ALA-D activity in kidney [$F(4,38)=3.357$; $p\leq0.01$], blood [$F(4,36)=3.207$; $p\leq0.02$] and placenta [$F(4,37)=3.986$; $p\leq0.01$] from pregnant rats, and in kidney [$F(4,23)=3.335$; $p\leq0,02$] and blood [$F(4,19)=7.800$; $p\leq0,01$] from lactating rats. The pre-treatments with Zn and/or NAC totality prevented inhibition on the δ-ALA-D activity in blood lactating rats. Still, Zn and/or NAC partially prevented inhibition on the δ-ALA-D activity in kidney, blood and placenta from pregnant rats and kidney from lactating rats.

3.2. *Metallothionein levels*

The MT levels from liver and kidney are presented in table 2. Exposure to Zn and/or NAC more Hg (Zn-Hg, NAC-Hg and Zn+NAC-Hg groups) caused an increase in hepatic MT levels from pregnant rats when compared with the control group [$F(4,35)=3.354$; $p\leq0.02$]. Still in these animals, Zn and NAC more Hg (Zn+NAC-Hg group) caused an increase in renal MT levels [$F(4,25)=2.909$; $p\leq0.04$]. Liver and kidney MT levels were not altered significantly in lactating rats.

3.3. *Serum Alanine aminotransferase and aspartate aminotransferase activity*

Serum ALT and AST activity are represented in table 3. Pregnant rats exposed to $HgCl_2$ exhibited a significant increase in serum AST activity when compared to control; all pre-treatment partially prevented this alteration

[F(4,37)=2.773; p≤0.04]. ALT activity was not altered by treatments. Lactating rats exposed to $HgCl_2$ presented a significant decreased in serum ALT activity [F(4,19)=8.609; p≤0.01] and the pre-treatments did not prevent this alterations. AST activity was not altered by treatments.

3.4. *Liver Alanine aminotransferase and aspartate aminotransferase activity*

Liver ALT and AST activity are represented in table 4. Pregnant rats exposed to $HgCl_2$ exhibited a significant increase in liver ALT activity when compared to control; Zn pre-treatment (Zn-Hg and Zn+NAC-Hg groups) totality prevented this alteration [F(4,36)=3.681;p≤0.01]. AST activity was not significantly altered by treatments. Lactating rats did not present alterations in liver ALT and AST activity.

3.5. *Creatinine and urea levels*

Serum creatinine and urea levels are shown in table 3. Mercury exposure caused an increase in serum creatinine and urea levels from pregnant and lactating rats [F(4,37)=4.659; p≤0.01, F(4,37)=5.094; p≤0.01, F(4,31)=4.023; p≤0.01 and F(4,31)=3.985; p≤0.01, respectively]. Pre-treatments did not prevent this alteration.

4. Discussion

This study investigated the toxic effect the $HgCl_2$ in pregnant and lactating rats and the capacity of the $ZnCl_2$ and NAC or combinations of these two compounds in prevention of this toxicity.

Numerous metabolic adaptations occur in pregnancy and lactation period, so the animal may respond differently to drugs, toxic agents and other chemicals

substances (Hanwell and Linzell 1973, Suzuki et al. 1993, Kensinger 1998, Suzuki et al. 2000, Solaiman et al. 2001, Picciano 2003, Guyton 2006, Carvalho and Tavarez 2010). In this work, one dose of 10 mg/kg of $HgCl_2$ caused metabolic alterations in different tissues from pregnant and lactating rats. On the other hand, pre-treatments showed satisfactory preventive effect of these alterations.

To our knowledge, the protection observed by pre-treatment with NAC of the Hg toxicity happened because of its chelating ability. In fact, Jalilehvand et al. 2013 showed that just one NAC molecule can bind up to Hg. Moreover, Trumpler et al. 2009 concluded that NAC chelates Hg more efficient than 2,3-dimercapto-1-propanesulfonic acid (DMPS), which is commonly used in clinic. Thus, we believe that the NAC may form a complex with Hg (NAC-Hg) and even the Hg continues in body, this complex reduces its toxic effects until it to be eliminated.

The results observed in our study suggest also protective effects of Zn, which can due to synthesis of the MT induced by Zn , since this protein also chelates Hg decreasing its the toxic effects. The liver is one of the main organs of synthesis of MT (Panemangalore et al. 1983, Cherian et al. 2003) and in our study pregnant rats Zn and Hg exposed presented increase of hepatic MT levels. This explains, at least in part, the protective action of Zn.

This dose of $HgCl_2$ (10 mg/kg) did not cause changes in hepatic δ-ALA-D activity from pregnant and lactating rats. This is a surprising, but very interesting result, because recently we observed that 5 mg/kg $HgCl_2$ inhibits about 45% of the activity of δ-ALA-D liver in virgin rats (Oliveira et al. 2015). As mentioned, several metabolic changes occur on pregnancy and lactation period, which may

have favored the lower toxicity of Hg. In fact, if we compare the basal levels of liver MT (control groups) of virgin rats (Oliveira et al. 2015) and the present study, pregnant and lactating rats have three times more MT in liver. Thus, we suggest that the hormonal adaptations of the perinatal period with higher levels of liver MT may decrease the toxic effects of Hg.

In this study, $HgCl_2$ exposure significantly increased ALT activity in the liver of pregnant rats and zinc treatment (Zn-Hg and Zn+NAC-Hg) prevented this change. Moraes-Silva et al. (2012) showed that young rats exposed $HgCl_2$ (5 mg/kg/day) for 5 consecutive days also presented increase in liver ALT activity, suggesting, that this increase may be is related to hepatic metabolism adaptive effect, for intance, gluconeogenesis. Furthermore, in the present study we observed that exposure to Hg decreased approximately 40% and 50% of serum ALT activity from pregnant and lactating rats, respectively. This result differs from Kumar et al. (2005) that found elevation in serum ALT activity of adult mice exposed intraperitoneally to $HgCl_2$, revealing hepatotoxicity. However, it has been observed frequently in other studies carried out by our research group (Peixoto et al. 2003, Franciscato et al. 2011, Oliveira V.A. et al. 2014). Possibly, this result was due to binding of Hg to SH groups present in the ALT structure, culminating in chemical modification of the enzyme and consequently its inhibition (Vedavathi et al. 2004, Moraes-Silva et al. 2012).

The inorganic form of Hg is known for its high toxicity to the renal system (Berlin et al. 2007, Rooney 2007, Li et al. 2009). In the present study the effect was not different. The Hg exposure increased serum creatinine and urea levels

on both pregnant and lactating rats, featuring renal injury and pre-treatments did not prevent this alteration.

5. Conclusion

The objective of this study was to evaluate the toxic effects of mercury on different metabolic conditions (pregnancy and lactation). Besides, evaluate the protection of Zn and/or NAC against the toxic effects of $HgCl_2$. In general, we observed that a dose of $HgCl_2$ (10 mg/kg) causes various biochemical changes from both pregnant and lactating rats. However, in a comparison with work published by our research group recently (Oliveira et al. 2015) we realize that this dose of Hg causes less damage from pregnant and lactating rats than half the dose (5 mg/kg) caused in virgin rats. Thus we conclude that pregnant and lactating rats are less sensitive to mercury than virgin rats. Furthermore, the pre-treatment with Zn and/or NAC showed interesting results, decreasing the toxic effects of Hg. We believe that this may have occurred due to the increase in MT levels caused by Zn along with a chelating effect of NAC. Both MT and NAC can capture Hg, forming complexes inert, which explains the protective effect. Nevertheless, additional studies are need to better understand the effect of Hg during pregnancy and lactation, well as and the possible action protective of Zn and/or NAC.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1: δ-ALA-D activity in liver, kidney, blood and placenta of rats exposed (s.c.) to saline (0,9%) or $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) and 24 hours after to saline or $HgCl_2$ (10 mg/kg).

	Pregnant				Lactating		
	Liver	Kidney	blood	Placenta	Liver	Kidney	blood
Sal-Sal	16.20±3.63	15.68±1.59[a]	1.52±0.32[a]	35.63±6.49[a]	10.90±0.48	11.72±1.73[a]	1.02±0.14[a]
Sal-Hg	13.18±1.72	07.54±0.83[b]	0.56±0.10[b]	16.95±1.46[b]	11.01±0.86	4.97±0.83[b]	0.12±0.02[b]
Zn-Hg	12.81±1.55	10.26±1.05[a,b]	0.93±0.25[a,b]	20.52±2.65[a,b]	11.51±0.99	6.69±0.87[a,b]	0.96±0.30[a]
NAC-Hg	12.77±1.66	10.32±1.93[a,b]	0.83±0.14[a,b]	23.74±2.35[a,b]	10.76±0.34	5.62±1.48[a,b]	0.92±0.32[a]
Zn/NAC-Hg	11.59±1.43	10.78±1.60[a,b]	0.96±0.20[a,b]	25.39±3.54[a,b]	13.41±1.28	6.85±1.43[a,b]	0.86±0.25[a]

Results were analyzed by one-way ANOVA followed by Tukey's post hoc test, and are presented as mean ± S.E.M. (N=6-9) and expressed in nmolPBG/h/mg of protein. Groups statistically equals are accompanied of the same letters, and groups statistically different are accompanied of the different letters.

Table 2: Metallothionein levels in liver and kidney of pregnant and lactating rats exposed (s.c.) to saline (0.9%) or $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) and 24 hours after to saline or $HgCl_2$ (10 mg/kg).

	Pregnant		Lactating	
	Liver	Kidney	Liver	Kidney
Sal-Sal	140.4±32.2[a]	110.7±21.2[a]	140.9±47.8	181.5±41.6
Sal-Hg	239.4±35.5[a,b]	180.4±37.1[a,b]	132.1±34.7	193.5±49.6
Zn-Hg	316.7±25.3[b]	236.3±44.6[a,b]	166.9±53.2	196.6±41.5
NAC-Hg	245.1±38.9[a,b]	218.2±51.7[a,b]	128.8±37.0	220.0±60.6
Zn/NAC-Hg	302.4±37.8[b]	325.2±58.4[b]	164.5±32.0	188.1±31.9

Results were analyzed by one-way ANOVA followed by Tukey's post hoc test and are presented as mean ± S.E.M. (N=5-8) and expressed in µg of SH/g of tissue. Groups statistically equals are accompanied of the same letters and groups statistically different are accompanied of the different letters.

Table 3: ALT and AST activity and creatinine and urea levels in serum of pregnant and lactating rats exposed (s.c.) to saline (9%), $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) and 24 hours after to saline or $HgCl_2$ (10 mg/kg).

	Pregnant				Lactating			
	ALT	AST	Creatinine	Urea	ALT	AST	Creatinine	Urea
Sal-Sal	69.09±6.69	134.01±16.00[a]	0.61±0.07[a]	59.27±7.00[a]	92.05±12.03[a]	296.2±68.04	0.72±0.49[a]	65.45±3.21[a]
Sal-Hg	42.65±7.08	292.36±36.01[b]	2.60±0.44[b]	179.42±20.00[b]	44.54±6.35[b]	322.7±88.03	2.08±0.35[b]	111.20±7.94[b]
Zn-Hg	41.90±4.58	191.81±2.85[a,b]	1.45±0.26[a,b]	147.79±24.21[b]	39.91±3.24[b]	255.6±91.44	1.49±0.52[a,b]	104.41±6.41[a,b]
NAC-Hg	47.14±5.82	190.97±37.47[a,b]	1.77±0.49[a,b]	174.48±36.16[b]	32.96±3.67[b]	300.9±118.5	1.74±0.22[b]	130.72±4.78[b]
Zn/NAC-Hg	48.69±7.84	222.27±40.62[a,b]	1.48±0.22[a,b]	121.13±11.08[b]	43.54±12.72[b]	253.3±77.65	1.77±0.50[a,b]	120.41±9.94[b]

Results were analyzed by one-way ANOVA followed by Tukey's post hoc test and are presented as mean ± S.E.M. (N=5-9) and expressed in U/L (ALT and AST) or mg/dL (creatinine and urea). Groups statistically equals are accompanied of the same letters, and groups statistically different are accompanied of the different letters.

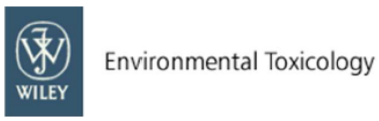
Table 4: ALT and AST activity in liver of pregnant and lactating rats exposed (s.c.) to saline (9%), $ZnCl_2$ (27 mg/kg) and/or NAC (5 mg/kg) and 24 hours after to saline or $HgCl_2$ (10 mg/kg).

	Pregnant		Lactating	
	ALT	AST	ALT	AST
Sal-Sal	143.01±16.80[a,c]	64.66±15.46	250.70±52.16	78.35±20.15
Sal-Hg	245.98±36.39[b]	91.45±29.73	290.80±11.48	92.79±5.46
Zn-Hg	117.33±29.48[a]	58.48±5.32	297.92±9.790	92.68±8.26
NAC-Hg	214.48±30.04[b,c]	57.94±11.96	264.00±12.83	97.29±8.59
Zn/NAC-Hg	155.51±20.56[a,c]	66.90±8.68	246.20±17.30	98.52±9.09

Results were analyzed by one-way ANOVA followed by Tukey's post hoc test and are presented as mean ± S.E.M. (N=6-9) and expressed in U/mg of protein. Groups statistically equals are accompanied of the same letters, and groups statistically different are accompanied of the different letters.

4.3. MANUSCRITO II

Environmental Toxicology



Acute mercury exposition of virgin, pregnant and lactating rats: histopathological kidney and liver evaluations.

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Keywords:	Kidney, Liver, Lactating rats, Mercury, Pregnant rats

Acute mercury exposition of virgin, pregnant and lactating rats: histopathological kidney and liver evaluations

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Short running title: "*Acute mercury exposition of virgin, pregnant and lactating rats*"

Abstract

This work investigated the effects of mercury chloride ($HgCl_2$) acute exposure on virgin, pregnant and lactating rats by determination of renal and hepaticmorphological and ultrastructural parameters, the expression of oxidative stress and stress tolerance markers and confirmed. Adult Wistar rats virgin (90 days old), pregnant (18^{th} gestation day) and lactating (7^{th} lactation day) were injected once with $HgCl_2$ (5 mg/kg) or saline (controls). The kidney and liver are the organs that more accumulate inorganic mercury. $HgCl_2$ exposure of virgin rats caused significant inflammatory infiltration and severe morphological alterations, like glomeruli atrophy, dilatation of Bowman's capsule, tubular degeneration and degenerated hepatocytes. Moreover, virgin rats presented mitochondrial alterations, important oxidative stress and increase in stress tolerance proteins at both kidney and liver level, compared with virgin controls. Interestingly, pregnant and lactating rats exposed to $HgCl_2$ presented weak renal and liver alterations, although, both control and $HgCl_2$-exposed rats, showed renal glomeruli greater in diameter respect virgin rats. In conclusion, we believe that virgin rats are more sensitive to $HgCl_2$ respect pregnant and lactating rats.

Keywords: Kidney, liver, lactating rats, mercury, pregnant rats, virgin rats.

Introduction

Mercury (Hg) is a divalent metal, liquid at room temperature, without biological functions and released in industrial, agricultural and during fossil fuels activities. Moreover, it can be discharge naturally in the biosphere as a result of volcanic and geothermal activity (Berlin et al. 2007; Li et al. 2009). Hg is found in different forms including elemental Hg, inorganic salts and organic compounds (Berlin et al. 2007; Martín-Yerga et al. 2013). According to the Agency for Toxic Substance and Disease Registry (ATSDR), Hg is the third most dangerous heavy metal after arsenic and lead (Emsley 2001; Othman et al. 2014), exposure to this metal is impossible to avoid, especially in regions with high levels of pollution in air, water, food or soil (Othman et al. 2014) and includes food, like fish, but also dental amalgam restorations (Goodrich et al. 2016). The three most affected organs by Hg exposure are kidneys, liver and brain (Boroushaki et al. 2015; Flora et al. 2008; Magos and Clarkson 2006; Othman et al. 2014). Notwithstanding that Hg neurotoxicity has been well reported in both humans and mammalian models, it is well documented that organic Hg easily crosses the blood brain barrier, whereas inorganic Hg salts, that are lipid insoluble, are not able to readily penetrate the blood brain barrier (Abdel Moneim 2015; Lohren et al. 2015; Niehoff et al. 2015). Hg exposure causes increased production of free radicals and so oxidative stress which are implicated in the pathogenesis of acute renal and hepatic disorders (Othman et al. 2014). Toxicity of inorganic Hg can lead to disturb the pro-oxidant or anti-oxidant balance thereby inducing oxidative stress in kidney and liver of the rats and mice following parenteral administration of Hg (Ansar and Iqbal 2016; Antune et al. 2001; Mahboob et al. 2001; Othman et al. 2014). The principal inorganic form (chloride mercury - $HgCl_2$) is mainly recognized to cause nephrotoxicity (Franciscato et al. 2011; Moraes-Silva et al. 2012; Oliveira VA et al. 2014; Roza et al. 2005) but also hepatotoxicity (Ansar and Iqbal 2016; Deng et al. 2014; Karapehlivan et al. 2014).

These toxic effects are related to the fact that Hg interfere in many different biochemical processes in the body due to its great affinity for sulfhydryl (SH), carboxyl and phorphoryl groups which are abundantly present in proteins and polypeptides (Rooney 2007) and this interaction lead to connection with cysteines forming the dicisteinilmercury, which easily passes the cell membrane and thereby causes severe damage (Zalups et al. 2014). Other researchers have demonstrated that inorganic Hg causes hepatotoxicity, since adult mice treated with one dose of $HgCl_2$ (5 mg/kg) showed significant elevation in serum alanine (ALT) and aspartate (AST) aminotransferase activities (Kumar et al. 2005), important markers of hepatocellular

function (Franciscato et al. 2011). The mechanisms of Hg-induced nephrotoxicity and/or hepatotoxicity are actually not completely understood, but many studies have found that the Hg-alterations affect mainly the renal proximal tubules (Zalups 2000; Zalups et al. 2014) and that the $HgCl_2$-induced hepatotoxicity is commonly associated with oxidative stress (Ansar and Iqbal 2016; Deng et al. 2014; Karapehlivan et al. 2014). In fact, Hg favors oxidative stress and inflammation, changes the expression of stress proteins, like glucose regulated proteins 75 or 78 (GRP75 or GRP78) and alters the morphology and activity of cellular organelles, such as mitochondria and endoplasmic reticulum. In particular, mitochondria are an important target of Hg poisoning, consequently affecting several cell functions (Reyes-Vivas et al. 1996).

Oliveira C.S. et al. (2012) demonstrated that, during pregnancy, the exposition to low doses of $HgCl_2$ in drinking water causes a decrease in food intake and body weight gain in rats dams. Recently, it was suggested that virgin female rats are different from lactating dams in relation to $HgCl_2$ sensitivity (Favero A.M. et al. 2014; Oliveira C.S. et al. 2014). In fact, the perinatal period, is characterized by several important physiological and metabolical changes in all the body of mammals and this makes the pregnant and lactating dams respond differently when exposed to chemical substances, whether drugs or toxic agents (Cheung and Lafayette 2013; Cummings et al. 2010; Houpert et al. 1997; Oliveira C.S. et al. 2014; Sundberg et al. 1998; Tan and Tan 2013). In addition to hormonal changes involved during pregnancy and lactation period, also some organs and systems, that are not directly involved in the development of the fetus, like urinary and digestive apparatus, are significantly altered. In fact, kidneys show significantly increase in size, glomerular filtration rate, creatinine clearance and uric acid excretion and reduction of proximal tubular reabsorption and blood pressure (Cheung and Lafayette 2013; Tan and Tan 2013). Thus, the kidneys need a strict control to support these changes in mothers and her offspring during pregnancy. Another organ that suffers important adaptations during pregnancy is the liver, but its changes are not so intense as observed in kidney. At the liver level, there is a decrease in the production of some metabolites, like bilirubin, a reduction of gamma-glutamyl transferase activities, that plays a key role in the synthesis and metabolism of the glutathione; and an increase in alkaline phosphatase activity that is a hydrolase enzyme responsible for removing phosphate groups from proteins and alkaloids. However, there are no alterations in ALT and AST or in the synthesis of bile acids (Bulusu and Sharma 2015).

Moreover, many other metabolic and morphological changes occur also in lactation period. Greenwood et al. (1978) and Prester et al. (1994) showed that during lactation the biological half-life of Hg, both in

humans and in animals, decreased of 40%-50% depending on the dose which they were exposed. In this context, also Franco et al. (2007) found a possible resistance of lactating rats to oxidative parameters when exposed to $HgCl_2$.

The aim of the present study was to evaluate in virgin, pregnant and lactating rats the effects of $HgCl_2$ exposure and the responses against inorganic Hg. Since it is known that the kidney is the primary site of accumulation of inorganic Hg in dams (Bridges and Zalups 2010; Favero A.M. et al. 2014; Oliveira C.S. et al. 2015; Zalups 2000), we investigated kidney morphology, ultrastructural characterization and expression of markers related to oxidative stress and cellular organelle alterations. Furthermore, due to the rapid uptake and accumulation of Hg by liver, besides kidneys, in the present study were evaluated also Hg hepatic alterations and oxidative stress induction.

Material and Methods

Experimental design

Virgin, pregnant and lactating Wistar rats, obtained from the Animal House of the Federal University of Santa Maria, RS, Brazil, were transferred to our breeding colony and maintained on a 12h light/dark cycle and at a controlled temperature (22 ± 2°C). Animals had free access to water and commercial food (GUABI, RS, Brazil) and were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, RS, Brazil.

Twelve virgin rats at 90 days old, twelve pregnant rats at the 18th gestation day and twelve lactating rats at the 7th lactation day were randomly distributed in six experimental groups: 1) virgin rats treated with saline; 2) virgin rats treated with $HgCl_2$; 3) pregnant rats treated with saline; 4) pregnant rats treated with $HgCl_2$; 5) lactating rats treated with saline and 6) lactating rats treated with $HgCl_2$.

Animals were weighed and subcutaneously injected with saline or $HgCl_2$ (5 mg/kg body weight) once. In particular, the $HgCl_2$ was dissolved in saline solution and injected at a volume of 1 mL/kg body weight. The dose of $HgCl_2$ was selected according to Oliveira V.A. (2014, 2015).

Tissue preparation

Twenty-four hours after the injection of saline or $HgCl_2$, virgin, pregnant or lactating rats were euthanized. Liver and one kidney were rapidly extracted and fixed in 4% paraformaldehyde for 24 hours and then paraffin embedded for histopathological and immunohistochemical analyses (Favero G. et al., 2015; Rezzani et al. 2013). The other kidney was divided in two parts and one of these parts was fixed in fresh 2.5% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.4) for 3 hours at 4°C and post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 hour at 4°C for ultrastructural analyses (Favero G. et al. 2015; Rezzani et al. 2013; Stacchiotti et al. 2014). Furthermore, brain samples were quickly removed, placed in ice and then processed for the oxidative stress parameters determination.

Kidney and liver histopathological and immunohistochemical analyses

The samples were embedded in paraffin and sectioned using a microtome (7 µm of section thick). For histopathological evaluation, alternate sections were deparaffined, rehydrated and finally stained with haematoxylin-eosin according to standard procedures (Stacchiotti et al. 2014). The sections were then observed with a light microscopy at the final magnification of 400x (Olympus, Germany) and the percentage of renal inflammatory infiltrate and the glomerular diameter (expressed in µm) were calculated using an image analyzer (Image Pro Premier 9.1, MediaCybernetics Inc., Rockville USA). In particular, were observed 100 random kidney areas, only at cortical level, and 100 glomeruli.

Furthermore, for immunohistochemical analyses, alternate paraffin sections of both kidney and liver were deparaffined, rehydrated and then incubated with the following primary antibodies: polyclonal rabbit anti-inducible nitric oxide synthase (iNOS) (diluted 1:400; Santa Cruz Biotechnology, Inc., USA), monoclonal mouse anti-mitofusion2 (Mfn2) (diluted 1:600; Abnova, Taiwan) (only for kidney sections), polyclonal rabbit anti-heat shock protein 27 (HSP27) (diluted 1:400; Santa Cruz Biotechnology, Inc., USA) and polyclonal rabbit anti-glucose regulated proteins 75 (GRP75) (diluted 1:200; Santa Cruz Biotechnology, Inc., USA). After washing, the sections were labeled with 488 anti-rabbit or 543 anti-rabbit or 543 anti-mouse Alexa Fluor conjugated secondary antibodies (diluted 1:200; Invitrogen, UK). Finally, the sections were counter-stained with 4'-6-diamidino-2-phenylindole (DAPI), mounted and observed with fluorescent microscopy (i50 Eclipse, Nikon, Germany) at final magnification of 400x. The sections incubated in phosphate buffer saline (PBS) without primary antibody served as negative controls.

The immunopositivity for each primary antibody wase quantified in different random zones using an image analyzer (Image Pro Premier 9.1, MediaCybernetics Inc., Rockville USA). All the quantitative analyses were performed in blinded fashion.

Kidney transmission electron microscopy

Kidney samples processed for electron microscopy were dehydrated in ethanol-propylene oxide at room temperature, embedded in epoxy resin and polymerized at 60°C for 72 hours (Araldite 502, Electron Microscopy Sciences, USA). Ultrathin sections (70–80 nm) were obtained using an ultramicrotome Ultracut E (Leica Instruments, Italy) equipped with diamond blades. All sections were collected on copper grids, stained with uranyl acetate for 10 minutes, then with lead citrate for few minutes and finally observed with a Philips CM10 electron microscope at 80 kV.

Brain oxidative stress parameters evaluation

The brain samples of virgin and lactating rats, either controls or $HgCl_2$-exposed, were firstly homogenized in tris- hydrochloric acid buffer (10 mM, pH 7.4) with 10 up-and-down strokes at ~1200 rpm in a Teflon-glass homogenizer. Then the homogenates were centrifuged at 3000×g at 4°C for 20 minutes and so the supernatant fractions were used for the oxidative stress parameters evaluations.

The brain total thiol (TSH) levels were determined as previously described by Ellman (1959) and meantime for the non-protein thiol (NPSH) evaluation, 200 µL of the protein supernatant fraction were precipitated with 200 µL of 4% trichloroacetic acid (v/v) by centrifugation (1050×g for 10 minutes), as previously described by Oliveira V.A. et al. (2014). The colorimetric analyses were carried out in 1M phosphate buffer (pH 7.4). A curve using glutathione as standard was constructed in order to calculate the SH in the tissue samples. The data were expressed for TSH and NPSH respectively as mmol SH/g brain tissue and µmol SH/g brain tissue.

Furthermore, brain ascorbic acid level was also determined as previously described by Roe (1954), but with some modifications to the standard protocol. In detail, the supernatant fractions of brain were precipitated in 10 volumes of cold 4% trichloroacetic acid (v/v) and then incubated at 37°C for 3 hours. Then 500 µL of sulfuric acid 65% (v/v) were added to each experimental sample solutions. Finally, the reaction products

were observed using a color-reagent containing dinitro-phenyl hydrazine (4.5 mg/mL) and copper sulphate (0.075 mg/mL). The data were expressed as µg ascorbic acid/g brain tissue.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA corrected Bonferroni test). $p \leq 0,05$ was considered significant for all statistical analysis in this study.

Results

Histopathological analyses

The histopathological appearance of glomeruli, proximal and distal renal tubules was "normal", without alterations, in the control groups (virgin, pregnant and lactating) (Figs 1a, b, c). In contrast, virgin rats $HgCl_2$-treated presented strong inflammation, prominent alteration of glomeruli with dilatation of Bowman's capsule, tubular degeneration characterized by the swelling and thinning of the epithelial cells lining the renal tubules and protein and/or mononuclear cell deposition in and narrowing the tubules lumen (Fig. 1d). Interestingly, pregnant and lactating rats $HgCl_2$-treated showed weak, but not significant, inflammation and rarely presented alteration at glomerular and tubular level (Figs 1e, f). Furthermore, the renal inflammatory infiltrate was calculated for each experimental group and the $HgCl_2$-treated virgin rats were characterized by a significant inflammatory cell accumulation respect the other groups treated and not with $HgCl_2$ (Fig. 1g). The glomerular diameter were also evaluated in the different experimental groups and the results are shows in Figure 1h. In particular, $HgCl_2$ exposure caused a significant decrease in the glomerular diameter of virgin rats when compared with respective controls. Another interesting result is that pregnant and lactating rats, both control and $HgCl_2$ treated groups, presented glomeruli greater in diameter respect virgin rats treated and untreated. The inner renal stripe of the outer medulla and the inner medulla were also examined and no significative evidence of pathological alterations was observed in any experimental groups (data not showed).

Liver, as kidney, exhibits histological changes after $HgCl_2$ exposition. In detail, virgin, pregnant and lactating control rats showed "normal" architecture of liver lobules and hepatic parenchyma with clear

central veins (Figs 1i, l, m). Whereas, $HgCl_2$-exposition of virgin rats denoted degenerated hepatocytes, alteration of central vein and severe/moderate inflammatory cell infiltrations both at central vein level and portal region (Fig. 1n). Interestingly, the liver of pregnant and lactating $HgCl_2$-treated rats showed almost normal parenchyma with a clear central vein, underlining slow hepatic histopathological alterations, as in kidneys of dams exposed to $HgCl_2$, respect virgin (Figs 1o, p).

Kidney ultrastructural assay and organelle damages analyses

Considering the above results, we focused on both glomerular and proximal tubular changes in different experimental groups.

Firstly, virgin rats $HgCl_2$-exposed showed a striking renal damage compared with relative control group. Both glomerular and proximal tubular organization was deeply changed. In particular, inside glomerulus, podocytes pedicels were thin and irregular, often fused together, and basal membrane amorphous, but these alterations were absent in virgin controls. Furthermore, in virgin $HgCl_2$ treated rats, proximal tubules were often necrotic, devoid of brush border and detached epithelial cells filled the lumen (Figs 2a, b). At higher magnification, myelin figures (data not showed) and many irregular mitochondria with scarce cristae were often observed. Whereas, pregnant rat kidneys treated with $HgCl_2$ appeared relatively well preserved, without glomerular alterations and showed proximal tubules eccentric nucleoli and scarce brush border detachment. Many round mitochondria, with a dense matrix and regular cristae, scattered around nuclear area were visualized. Glomerular and tubular ultrastructural organization was well preserved in both $HgCl_2$-treated and controls (Figs 2c, d). Finally, lactating rats $HgCl_2$-exposed presented focal and weak proximal tubular alterations together with abundant, elongated and round mitochondria with a dense matrix and regular cristae. Glomerular and tubular ultrastructural feature in lactating-$HgCl_2$ group were similar to lactating control rats (Figs 2e, f). Interestingly, in both pregnant and lactating dams, the nuclei of epithelial cells maintained a normal feature and nucleoli were often segregated, as index of active metabolic activity.

Furthermore, to better analyzed the mitochondrial damage we investigated also the Mfn2 expression (identified in red) at tubular level. The virgin control rats showed a very weak expression of this structural mitochondrial transmembrane protein respect virgin $HgCl_2$-exposed (Figs 2g, l). However, pregnant and lactating dams showed a moderate expression of Mfn2 both in control and in $HgCl_2$-exposed groups (Figs

2h, i, m, n), comparable with virgin controls. In particular, the Mfn2 expression was evident at cytoplasm level of renal tubules and very weakly at the glomerular level.

Kidney and liver immunohistochemical assay of oxidative stress and stress tolerance

iNOS immunofluorescence (identified in green) of virgin, pregnant and lactating kidney rats is presented in Figure 3. $HgCl_2$ treatment caused a significant increase in iNOS expression, mainly at tubular level, in virgin rats when compared with respective controls (Figs 3a, d). In contrast, pregnant (Figs 3b, e) and lactating (Figs 3c, f) rats exposed to $HgCl_2$ not presented difference in kidney iNOS expression with relationship to respective controls and the immunopositivity observed was very weak. Moreover, pregnant and lactating rats (both control and $HgCl_2$-treated) not presented significant difference in iNOS expression respect the control virgin rats. All these results were confirmed also by the quantitative measurement of iNOS immunopositivity, summarized in Figures 3g.

Kidney expression of HSP27, identified in red, is summarized in figure 4. $HgCl_2$ treatment increases HSP27 expression at kidney tubular level of virgin rats (weak/moderate immunopositivity) compared with control virgin rats (absent/very weak signal) (Figs 4 a, d). Interestingly, pregnant (Figs 4b, e) and lactating (Figs 4c, f) rats not presented a significant increase in expression of this protein, even when received $HgCl_2$, compared with respective controls. Moreover, the low increase in HSP27 immunopositivity in pregnant and lactating rats treated with $HgCl_2$ (weak/very weak expression) is not significant respect to the immunopositivity observed in virgin rats exposed to $HgCl_2$. Finally, we investigated the GRP75 expression, identified in red, and we observed that the $HgCl_2$ exposition caused the same profile of alterations observed in iNOS and HSP27 analyses, previously showed. In other words, just virgin $HgCl_2$-treated rats presented a weak/moderate GRP78 expression, mainly at tubular level and the others group showed a very weak/absent signal (Figs 4g-n). All these results were confirmed also by the quantitative measurement of the relative immunopositivities, summarized in Figure 4o and 4p.

Furthermore, both oxidative stress and stress tolerance markers evaluated for kidney were investigated also at liver level showing the same trends of expression: higher level of iNOS, GRP78 and HSP27 (strong/moderate signals) in virgin rats treated with $HgCl_2$ in comparison with the other controls and $HgCl_2$ treated rats (weak/very weak expression). Interestingly, also at liver level pregnant and lactating $HgCl_2$-treated dams showed a slight increased expression of the investigated markers compared to the respective

controls (data not showed). The liver quantitative analyses of oxidative stress and stress tolerance markers (iNOS, GRP78 and HSP27) were summarized in Figure 5.

Brain oxidative stress parameters evaluation

In the present study, we evaluated also the potential neurotoxic effect of $HgCl_2$ in virgin or lactating rats based on oxidative stress parameters determination, analysing the total thiol (TSH), non-protein thiol (NPSH) and ascorbic acid levels. Interestingly, no significative difference were observed in the experimental group analyzed for each oxidative parameter evaluated (Fig. 6); so the HgCl2 treatment did not change the brain biochemical parameters analyzed both in virgin rats and in lactating dams.

Discussion

This work investigated the effect of $HgCl_2$ on kidney and liver of virgin, pregnant and lactating rats through morphological analysis, ultrastructural characterization and expression of markers related to oxidative stress, cellular organelle alterations and stress tolerance.

Interestingly, we observed in this study that a single dose of $HgCl_2$ (5 mg/kg body weight) caused serious alterations at kidney and liver level almost only in virgin rats; whereas no significant alterations were observed at brain level of no one experimental groups. Despite its low liposolubility, inorganic Hg has been previously detected in the brain, disrupting neuronal homeostasis, by Clarkson and Magos (2006). The exact mechanism that underlies its accumulation in the nervous system as well as its effects after chronic exposure are poorly understood. The possible mechanism involved in $HgCl_2$ transport through the blood brain barrier implies an indirect effect resulting from interference with the activities of cerebrovascular enzymes involved in blood brain barrier transport, providing evidence that inorganic mercury can also promote neurotoxic effects, even in the mature brain (Texeira et al. 2014). In the present study, instead of Texeira et al. (2014), we not observed oxidative stress alteration at brain level, because the inorganic Hg penetrate the blood brain barrier difficulty and in our study the $HgCl_2$ was administered in a single injection and not in a multiple/chronic treatment. It is noteworthy that, Franco et al. (2007) observed that dams directly exposed to inorganic Hg during the lactation period showed no significant neurotoxicity, confirming our hypothesis that during gestational and/or lactation periods the dams are, at least in part, protect against Hg-toxicity.

Virgin animals presented significant inflammatory infiltration and severe morphological alterations, like glomeruli atrophy, dilatation of Bowman's capsule, tubular degeneration and disarrangement of hepatic parenchyma. These observations corroborate with Berlin (2007) that described that few hours after exposure to inorganic Hg the 50% of this metal accumulated at kidney level. Furthermore, He indicates that a largest mercury pool in the body is the liver with the highest concentration in the periportal areas; also because, a part from the excretion through saliva, Hg is excreted by the liver through the bile. Similar observation were described also by Enli et al. (2010) studying cadmium toxic effects on pregnant dams. The Authors observed that the higher cadmium level accumulated at kidneys level, followed by liver of both dams and fetuses. This finding, together with the Hg previous observations, suggested that metal damages occur in kidneys much more than in livers, probably because metals excretion is through kidneys.

Furthermore, all these observations confirmed the hard morphological damages caused by Hg almost only in virgin animals observed in the present study. In particular, we observed in virgin rats $HgCl_2$-exposed that proximal tubules present necrosis, lack of brush border and detachment of epithelial cells. These nephropathological changes hit primarily the proximal tubules (Boroushaki et al. 2015; Zalups et al. 2014). Actually, there are strong evidences indicating that the primary potential mechanism of Hg excretion, in this renal area, is thought conjugation of this metal with cysteine, forming the dicysteinylmercury. This molecule can mimic the amino acid cysteine and so crosses easily the luminal membrane of proximal tubules, causing tissue alterations (Zalups 2000). In the present study, the proximal tubular alterations were confirmed as well by the ultrastructural analyses, that revealed also important mitochondrial irregularities in virgin rats exposure to $HgCl_2$. These observations were confirmed also by the analyses of Mfn2, important transmembrane protein linked to the structural bridge between mitochondria and endoplasmic reticulum (de Brito and Scorrano 2008) that showed a marked diminution of Mfn2 expression only in virgin rats $HgCl_2$-exposed, confirming the structural organelle alteration.

The main mechanism of Hg toxicity is associated with oxidative stress, as reported by numerous studies (Ansar 2015; Ansar and Iqbal 2016; Berlin et al. 2007; Li et al. 2009). The oxidative action after Hg exposition may be linked to the fact that this metal increases lipid peroxidation (Ansar 2015) reduces non-enzymatic and enzymatic antioxidants (Agarwal et al. 2010; Pal and Ghosh 2012; Su et al. 2008) and total thiol and non-protein thiol defenses (Farina et al. 2003; Oliveira V.A. 2014). Furthermore, the high affinity of Hg with the SH group contributes to its toxicity, because induces the formation of a stable complex

which changes several proteins fundamental for the correct function of the cells and tissues (Berlin et al. 2007; Farina et al. 2011; Rooney 2007). Given that many antioxidants present SH group in their structures, so the formation of this complex promotes indirectly the cell oxidative damage. In this work, it was not different, in fact virgin rats $HgCl_2$-exposed presented an increase in iNOS expression at renal tubular level and hepatocytes. Under physiological conditions this enzyme is detected only in small quantities (Esmaelizadeh et al. 2015). However, the oxidative stress, caused by many different routes, like heavy metal poisoning as in the case of Hg, induces a significant iNOS expression that in turn increases the amount of nitric oxide and consequently alters renal and hepatic functions (Othman et al. 2014; Todorović et al. 2016). Fortunately, throughout of evolution, living organisms have developed endogenous defense mechanisms, which can be activated after stress. In fact, the damage caused by $HgCl_2$ in virgin rats has induced the HSP27 and GRP75 expression. HSP27 is a chaperone with an important role in stress tolerance at cellular level, maintains protein homeostasis (Ziemann et al. 2013) and its expression may be induced in larger quantities in several diseases, including kidney and liver damage related to oxidative stress. In summary, the main protective activity of HSP27 is related to its ability to reduce the amount of free radicals, modulating endogenous antioxidant systems, recovering, partially, denatured proteins and protecting the cytoskeleton (Mymrikov et al. 2011; O'Reilly et al. 2010). GRP75 was another protein that we observed moderately expressed after $HgCl_2$-exposition in virgin rats. This protein plays several functions that help to maintain cell homeostasis and it is very common observed its increased expression in cases of oxidative stress (Rezzani et al. 2013; Stacchiotti et al. 2004) which explains our results. All these results are in agreement with other previous study which showed a large sensitivity of kidney and liver oxidative stress and stress tolerance enzymes to Hg (Franciscato et al. 2011; Oliveira V.A. et al. 2014; Peixoto et al. 2003)

Interestingly, in this study, the same dose of $HgCl_2$ which caused important damages in kidney and liver of virgin rats, caused slight injuries in pregnant and lactating rats. This interestingly observation is in line with the previous data of Oliveira V.A. et al. (2014). In this previous work, we can observe that all animals exposed to $HgCl_2$ presented kidney and liver Hg accumulation; however, virgin rats accumulates twice more Hg respect lactating dams that, in contrast, excrete a fourfold higher amount of Hg through urine respect virgin rats.

We believe also that because there was little renal impairment in these animals their defense mechanisms were not activated explaining the lower expression of HSP27 and GRP75 after $HgCl_2$ exposition. This study and previous works of our research group suggested greater resistances due to the several metabolic adaptations that occur in pregnant and lactating periods, that allows the pregnant and lactating dams to a more efficient excretion of xenobiotics, such as Hg (Favero A.M. et al. 2014; Oliveira C.S. et al. 2014; Oliveira V.A. et al. 2014). Indeed, in the present study we observed that pregnant and lactating dams, both control and $HgCl_2$-treated, showed glomerulus diameter greater than virgin rats; this may indicate a better renal filtration that helps in the elimination of Hg and so decreases alterations. These results should also suggest that the fetus may act as a "sink" for the $HgCl_2$, as Aschner and Clarkson (1987) suggested for methylmercury in near-term pregnant and virgin Long-Evans female rats.

Conclusion

In summary, we believe that in virgins rats the $HgCl_2$ entries into the tubular cells and/or hepatocytes through the cysteine conjugation, resulting in important oxidative and inflammatory damages. In an attempt to prevent further damage and cell death, there is a significant increase of stress tolerance proteins expression, such as HSP27 and GRP75 (Fig. 7). We also believe, that pregnant and lactating rats not presented evident renal and hepatic alterations because the metabolism of these animals is accelerated and may excrete Hg more easily, thereby preventing severe renal and liver damages including morphological alterations, oxidative stress and inflammation. Further studies are needed to better explain the causes of protection against Hg exposition observed in pregnant and lactating dams.

Conflict of interest statement

The Authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. Photomicrographs indicative of renal (a-f) and hepatic (i-p) haematoxylin-eosin staining of virgin (a, i), pregnant (b, l) and lactating (c, m) control rats and of virgin (d, n), pregnant (e, o) and lactating (f, p) mercury ($HgCl_2$)-exposed rats. Bar equals 20 µm (g) identifies glomeruli, (dt) the cortical distal tubules, (pt) the cortical proximal tubules and (cv) central vein. The graphs summarize the percentage of inflammatory infiltrate (g) and the glomerular diameter expressed as micrometers (h). *p<0.05 *vs* mercury ($HgCl_2$)-exposed virgin rats and # p<0.05 *vs* control virgin rats.

Fig. 2. Photomicrographs showing cortical proximal tubules and in the zoom the mitochondria of virgin (a), pregnant (c) and lactating (e) control rats and of virgin (b), pregnant (d) and lactating (f) mercury ($HgCl_2$)-exposed rats. Bar equals 5 µm (a-c), 10 µm (d, f, h) and 1 µm (zooms). (N) identifies the nucleus, (m) the mitochondrial and (BB) the brush border.

Photomicrographs indicative of renal mitofusin 2 (Mfn2) immunostaining, identified in red, of virgin (g), pregnant (h) and lactating (i) control rats and of virgin (l), pregnant (m) and lactating (n) mercury ($HgCl_2$)-exposed rats. Bar equals 20 µm.

Fig. 3. Photomicrographs showing kidney inducible nitric oxide synthase (iNOS) immunostaining, identified in green, of virgin (a), pregnant (b) and lactating (c) control rats and of virgin (d), pregnant (e) and lactating (f) mercury ($HgCl_2$)-exposed rats. Bar equals 20 µm. The graph summarizes the immunopositivity for iNOS, expressed as percentage of area. *p<0.05 *vs* mercury ($HgCl_2$)-exposed virgin rats.

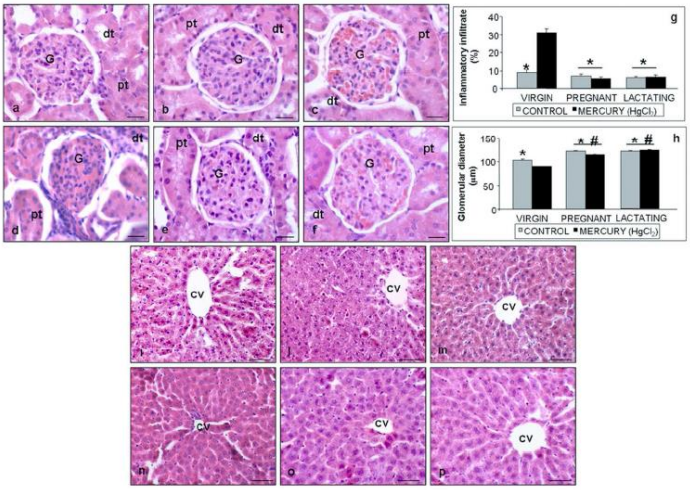
Fig. 4. Photomicrographs indicative of kidney heat shock protein 27 (HSP27) (a-f) and glucose regulated proteins (GRP78) (g-n) immunostainings, both identified in red, of virgin (a, g), pregnant (b, h) and lactating (c, i) control rats and of virgin (d, l), pregnant (e, m) and lactating (f, n) mercury ($HgCl_2$)-exposed rats. Bar equals 20 µm. The graphs summarize the immunopositivity for HSP27 (O) and GRP78 (P), both expressed as percentage of area. *p<0.05 *vs* mercury ($HgCl_2$)-exposed virgin rats.

Fig. 5. The graphs summarize the histomorphometrical analyses, expressed as arbitrary units (AU), of liver iNOS (a), GRP75 (b) and HSP70 (c) immunopositivity of all the experimental groups. *p<0.05 *vs* mercury ($HgCl_2$)-exposed virgin rats.

Fig. 6. The graphs summarize the biochemical analyses of total thiol (TSH) (a), non-protein thiol (NPSH) (b) and ascorbic acid (c) levels evaluated at brain level of virgin and lactating rats treated and not with mercury ($HgCl_2$).

Fig. 7. Schematic representation of the prominent toxic effects, observed mainly at renal cortical proximal tubules level, of virgin rats treated with mercury chloride once.

Figure 1



Photomicrographs indicative of renal (a-f) and hepatic (i-p) haematoxylin-eosin staining of virgin (a, i), pregnant (b, l) and lactating (c, m) control rats and of virgin (d, n), pregnant (e, o) and lactating (f, p) mercury (HgCl2)-exposed rats. Bar equals 20 µm (g) identifies glomeruli, (dt) the cortical distal tubules, (pt) the cortical proximal tubules and (cv) central vein. The graphs summarize the percentage of inflammatory infiltrate (g) and the glomerular diameter expressed as micrometers (h). *p<0.05 vs mercury (HgCl2)-exposed virgin rats and # p<0.05 vs control virgin rats.
98x69mm (300 x 300 DPI)

Figure 1

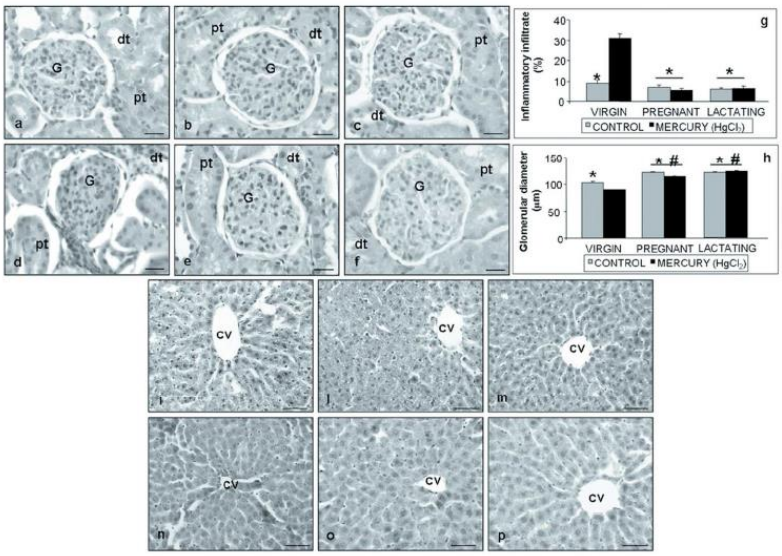


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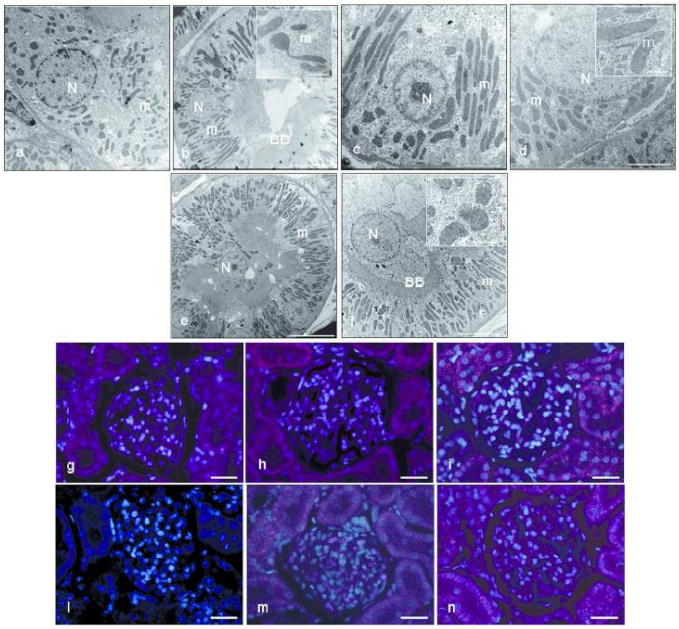
Figure 2



Photomicrographs showing cortical proximal tubules and in the zoom the mitochondria of virgin (a), pregnant (c) and lactating (e) control rats and of virgin (b), pregnant (d) and lactating (f) mercury ($HgCl_2$)-exposed rats. Bar equals 5 µm (a-c), 10 µm (d, f, h) and 1 µm (zooms). (N) identifies the nucleus, (m) the mitochondrial and (BB) the brush border.
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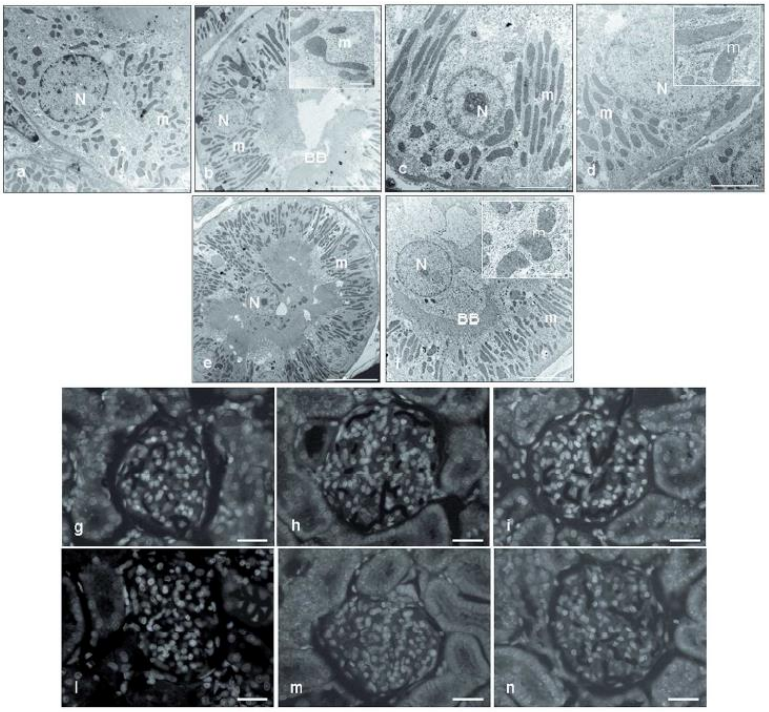
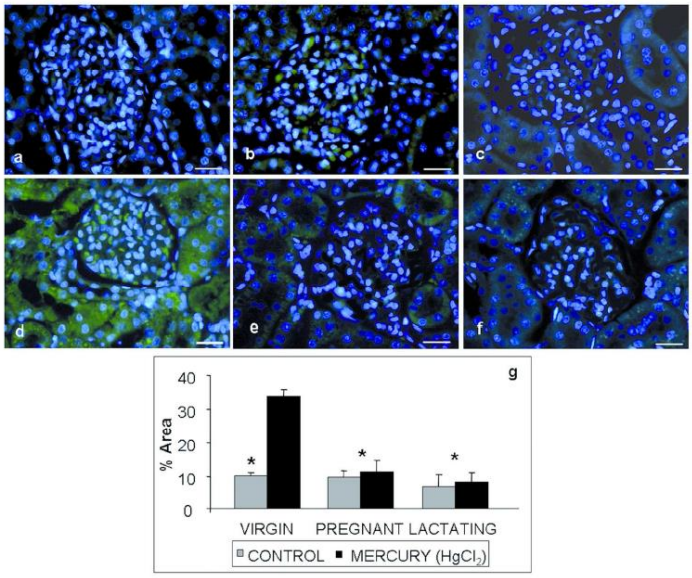
172x156mm (300 x 300 DPI)

Figure 2



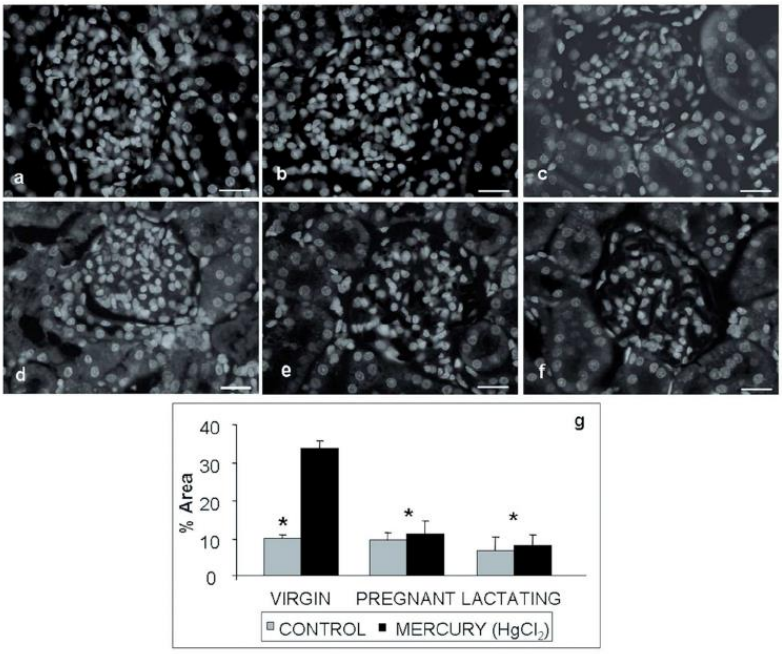
172x156mm (300 x 300 DPI)

Figure 3



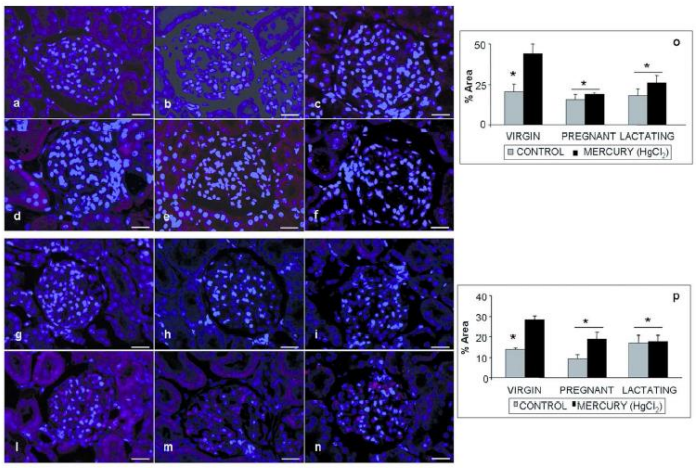
Photomicrographs showing kidney inducible nitric oxide synthase (iNOS) immunostaining, identified in green, of virgin (a), pregnant (b) and lactating (c) control rats and of virgin (d), pregnant (e) and lactating (f) mercury (HgCl2)-exposed rats. Bar equals 20 µm. The graph summarizes the immunopositivity for iNOS, expressed as percentage of area. *p<0.05 vs mercury (HgCl2)-exposed virgin rats.
115x95mm (300 x 300 DPI)

Figure 3



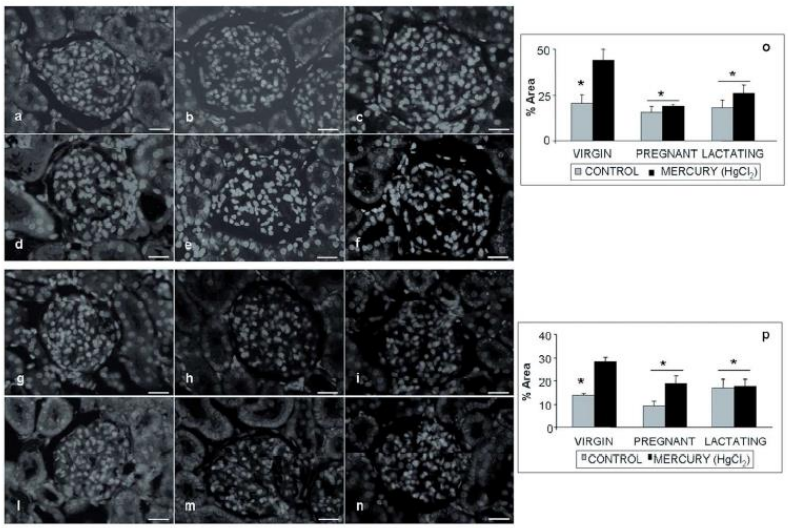
106x88mm (300 x 300 DPI)

Figure 4



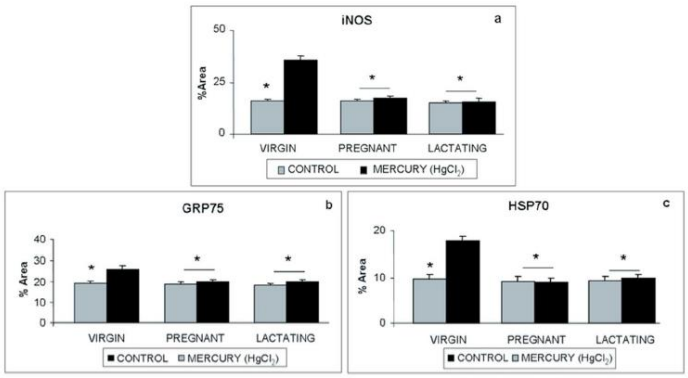
Photomicrographs indicative of kidney heat shock protein 27 (HSP27) (a-f) and glucose regulated proteins (GRP78) (g-n) immunostainings, both identified in red, of virgin (a, g), pregnant (b, h) and lactating (c, i) control rats and of virgin (d, l), pregnant (e, m) and lactating (f, n) mercury (HgCl2)-exposed rats. Bar equals 20 μm. The graphs summarize the immunopositivity for HSP27 (O) and GRP78 (P), both expressed as percentage of area. *p<0.05 vs mercury (HgCl2)-exposed virgin rats.
128x86mm (300 x 300 DPI)

Figure 4



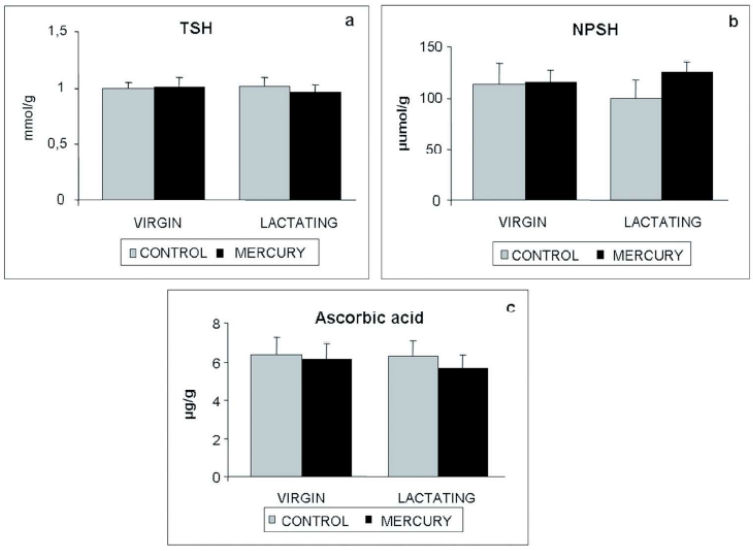
128x86mm (300 x 300 DPI)

Figure 5



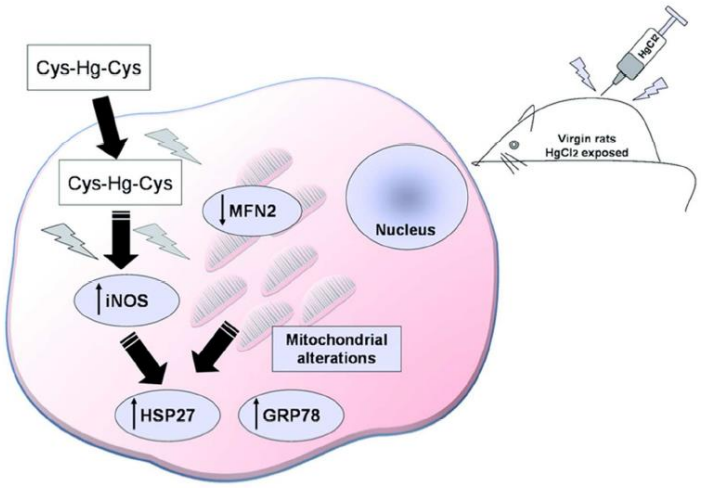
The graphs summarize the histomorphometrical analyses, expressed as arbitrary units (AU), of liver iNOS (a), GRP75 (b) and HSP70 (c) immunopositivity of all the experimental groups. *p<0.05 vs mercury (HgCl2)-exposed virgin rats.
77x42mm (300 x 300 DPI)

Figure 6



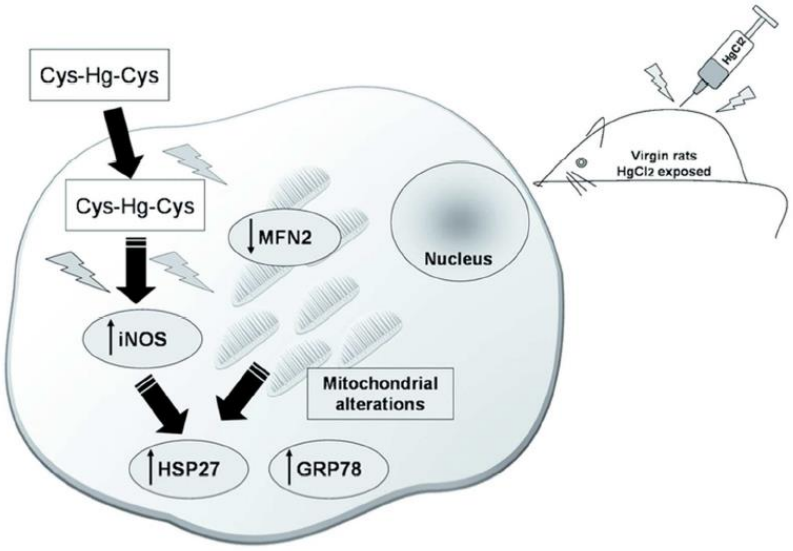
The graphs summarize the biochemical analyses of total thiol (TSH) (a), non-protein thiol (NPSH) (b) and ascorbic acid (c) levels evaluated at brain level of virgin and lactating rats treated and not with mercury (HgCl2).
135x96mm (300 x 300 DPI)

Abstract Graph



Schematic representation of the prominent toxic effects, observed mainly at renal cortical proximal tubules level, of virgin rats treated with mercury chloride once.
62x43mm (300 x 300 DPI)

Abstract Graph



62x43mm (300 x 300 DPI)

5. DISCUSSÃO

Atualmente é quase impossível para os seres humanos evitar completamente o contato com o mercúrio, devido a sua utilização em diversos setores do cotidiano (ZALUPS 2000). Com isso, esse estudo busca contribuir no entendimento dos mecanismos de toxicidade do Hg, especialmente no período gestacional e lactacional. Além disso, a pesquisa almeja encontrar alternativas de prevenção contra a intoxicação por Hg.

Os resultados mostram que a ratas virgens são mais sensíveis ao $HgCl_2$ do que ratas gestantes e lactantes. Com apenas uma dose de Hg (5 mg/kg de $HgCl_2$) ratas virgens apresentaram inibição da atividade da δ-ALA-D em rins, fígado e sangue (aproximadamente 70%, 45% e 55%, respectivamente), decréscimo nos níveis de SH total renais, aumento na concentração de MT hepática e atividade AST sérica, bem como aumento nos níveis de creatinina e ureia no soro. Todos esses resultados podem ser em decorrência do aumento acentuado de Hg observado em rins, fígado e sangue (**artigo I**). Surpreendentemente, o dobro da dose de $HgCl_2$ utilizada em ratas virgens (10 mg/kg) causa menos efeitos tóxicos em ratas gestantes e lactantes. Esses animais apresentaram inibição da atividade da δ-ALA-D em rins, sangue e placenta (gestantes), sem alteração da atividade da enzima hepática. Ratas gestantes e lactantes expostas ao Hg também apresentaram aumento na atividade sérica de AST e níveis de creatinina e ureia (**manuscrito I**).

De fato, todos os animais expostos ao $HgCl_2$ apresentaram alterações bioquímicas, mesmo que em diferentes intensidades. A inibição da δ-ALA-D em rins foi uma constante. Essa enzima é responsável pelo segundo passo na biossíntese do heme, catalisando a condensação de duas moléculas de δ-

aminolevulínico para formar o porfobilinogênio, portanto, indispensável no metabolismo aeróbico (GIBSON et al. 1955, SASSA 1982, JAFFE 1995). Além disso, é um importante marcador de efeito tóxico (ROCHA et al. 1995, PEIXOTO et al. 2003, FRANSCISCATO et al. 2009, 2011). É provável que a inibição mais grave nos rins ocorre porque este é o principal órgão alvo do Hg inorgânico.

Vários estudos sugerem que essa interação Hg-SH favorece o transporte do metal pelo organismo (ROCHA et al. 1995, MAGOS et al. 2006, BERLIN et al. 2007, ROONEY 2007, LI et al. 2009, OLIVEIRA VA et al. 2014, 2015). Zalups (2000) afirma ainda que a grande afinidade entre esses dois compostos possibilita a formação de complexos de Hg com a cisteína (aminoácido que contém SH em sua estrutura), que resultam em uma molécula chamada dicisteinilmercúrio. Essa molécula transpõem mais facilmente as membranas citoplasmáticas dos túbulos proximais renais através do sistema de transporte que envolve a absorção de cisteína e assim pode causar alterações graves. Assim, o Hg estaria chegando aos túbulos proximais através do mecanismo supracitado, e após entrar nas células tubulares, se ligaria a δ-ALA-D, que é uma enzima sulfidrílica, inibindo-a.

Ratas gestantes e lactantes mostram-se menos sensíveis a toxicidade do $HgCl_2$. No decorrer do período perinatal, muitas alterações morfológicas, estruturais e principalmente metabólicas ocorrem no organismo (ver itens 1.1 e 1.2 desse estudo) e podem contribuir para a maior resistência frente à toxicidade do Hg. Alguns estudos tem constatado uma relação positiva da gestação e lactação com o aumento nos níveis de MT (CHAN et al. 1993, SALAIMAN et al. 2001). Essa proteína atenua os efeitos tóxicos de várias

substâncias, inclusive o Hg, e assim aumenta a resistência desses animais (MARET 2000, SAKAMOTO et al. 2000, PEIXOTO e PEREIRA 2007, PEIXOTO et al. 2007, OLIVEIRA et al. 2015). No presente estudo podemos constatar que ratas gestantes e lactantes apresentam 2,5 vezes mais MT hepática em comparação a ratas virgens (comparação entre os grupos controle). MT é uma proteína de baixo peso molecular, sintetizada principalmente no fígado e distribuída aos diferentes tecidos, e desempenha importante função “scavenger” nos organismo vivos (MARET 2000, TANDON et al. 2001, MINAMI et al. 2007, KIMURA e ITOH 2008, HWANG et al. 2013). Com essas características, as MTs sequestrariam moléculas de Hg, principalmente no fígado, formando complexos inertes, diminuindo os efeitos tóxicos desse metal até a sua eliminação. Essa hipótese explica, pelo menos em parte, o menor grau de toxicidade apresentado pelas ratas gestantes e lactantes, inclusive sem inibição da atividade de δ-ALA-D hepática (**manuscrito I**). Corroborando com esses resultados, observamos recentemente que a exposição ao mercúrio inorgânico por via oral durante todo o período gestacional não causa alterações bioquímicas nas ratas gestantes expostas ao metal (OLIVEIRA et al. 2012). Também constatamos que ratas lactantes expostas ao $HgCl_2$ durante 5 dias consecutivos apresentam diferentes respostas bioquímicas comparadas a ratas virgens expostas a mesma dose (FAVERO et al. 2014, OLIVEIRA CS et al. 2014). E que ratas virgens expostas subcutaneamente a uma única dose de $HgCl_2$, são mais sensíveis que ratas lactantes expostas de igual maneira (OLIVEIRA VA et al. 2014). Resultados esses que nos levam a pensar que ratas gestantes e lactantes são mais resistentes aos Hg que ratas virgens.

O rim e o fígado são os órgãos que mais sofrem com a exposição ao Hg inorgânico. Por esse motivo, este estudo aprofundou as investigações da toxicidade renal e hepática do $HgCl_2$ em ratas virgens gestantes e lactantes, através de análises morfológicas, ultra-estruturais e expressão de marcadores relacionados ao estresse oxidativo e inflamação (**manuscrito II**). Ratas virgens expostas a uma dose de 5 mg/kg de $HgCl_2$ apresentam dilatação da cápsula de Bowman, degeneração dos túbulos proximais com dano mitocondrial, diminuição do diâmetro de glomérulos, bem como, aumento de infiltrado inflamatório, degeneração dos hepatócitos e maior expressão de iNOS, HSP27 e GRP75 em ambos os tecidos. Esses resultados corroboram com as análises bioquímicas (**artigo I**) e reforçam a hipótese de interação entre Hg-SH; esse complexo atinge os túbulos renais mais facilmente e causa todos os danos constatados. Em contra partida, ratas gestantes e lactantes expostas a mesma dose de $HgCl_2$ apresentaram poucas alterações morfológicas nos rins e fígado. Um resultado interessante observado foi o aumento no diâmetro glomerular em consequência da gestação e lactação. Essa adaptação está diretamente relacionada a maior capacidade de filtração renal o que juntamente com outras alterações metabólicas, como aumento nos níveis de MT no fígado, podem ajudar na diminuição dos efeitos tóxicos do Hg e explica, pelo menos em parte, o menor dano bioquímico (**manuscrito I**) e estrutural (**manuscrito II**) nesses animais.

A chegada com maior facilidade do Hg aos túbulos proximais das ratas virgens pode ter desencadeado um aumento na expressão de iNOS. Essa enzima produz grandes quantidades de óxido nítrico (ON) o qual é um radical livre produzido a partir da L-arginina e pode reagir com o oxigênio formando

moléculas como o ânion peroxinitrito ($ONOO^-$). O $ONOO^-$ tem relação direta com o aumento do estresse oxidativo e indução de apoptose. Confirmando essa hipótese, constatamos uma diminuição de mitofusina 2, uma proteína mitocondrial que se encontra diminuída, provavelmente pelo estresse oxidativo induzido pelo $ONOO^-$. Possivelmente esses danos iniciais tenham desencadeado mecanismos de proteção celular que explicam o aumento renal de HSP27 e GRP75 em ratas virgens expostas ao Hg. HSP27 é uma chaperona, responsável por manter a homeostase proteica de várias células, principalmente no citosol e pode ser encontrada em grandes quantidades em diferentes tecidos lesados, incluindo rins e fígado danificados por EO. Em resumo, a proteção dessa proteína está relacionada com a sua habilidade em reduzir EROs por prevenir agregação proteica, recuperar desnaturações parciais e proteger o citoesqueleto (O'REILLY et al. 2010, MYMRIKOV et al. 2011). Outra proteína mais expressa após a exposição ao Hg em ratas virgens foi a GRP75. Essa proteína realiza diversas funções que ajudam a manter a homeostase celular e é comum ver o aumento de sua expressão em casos de EO, o que explica os resultados desse estudo (STACCHIOTTI et al. 2004, 2014). O Hg e/ou o dano oxidativo causado por esse metal podem perturbar a estabilidade de certas proteínas mitocondriais, desestabilizando essa organela, aumentando a liberação de citocromo c e consequentemente ativando a cascata pro apoptótica ligada a caspase 9. Entretanto o aumento de GRP75 pode impedir essa alteração em proteínas mitocondriais e assim evitar a morte celular antecipada (TAURINS et al. 2002, KAUL et al. 2007, YANG et al. 2008, STACCHIOTTI et al. 2014, ZHANG et al. 2015). Assim sendo, o aumento da

GRP75 em ratas virgens expostas ao $HgCl_2$ pode ser uma tentativa de bloquear os danos causados pelo Hg.

Além das defesas endógenas do organismo contra os ataques que as células estão expostas corriqueiramente, nos últimos anos vários estudos testam substâncias que possam proteger e ampliar a longevidade celular. Como a intoxicação por Hg é um fator importante no que diz respeito a envenenamento, dano a saúde, tem estreita relação com estresse oxidativo e não são raros episódios envolvendo a contaminação por esse metal, nesse trabalho foi testado o Zn e a NAC contra a toxicidade do Hg (**Artigo I e manuscrito I**). Os tratamentos com Zn, NAC ou a combinação de ambos os compostos preveniram, mesmo que em alguns momentos de forma parcial, a inibição da δ-ALA-D, alterações em marcadores hepáticos e diminuíram os níveis de Hg nos diferentes tecidos analisados. Outro resultado que nos chamou a atenção foi o aumento hepático dos níveis de MT em todos os grupos de animais tratados com Zn. Analisando esses resultados, o mecanismo de proteção do Zn pode estar relacionado com a sua capacidade de induzir a síntese de MT principalmente no fígado. Como mencionado anteriormente, a MT é uma proteína de baixo peso molecular, possui seis grupos tióis em sua estrutura e tem função “scavenger” (TANDON et al. 2001, HWANG et al. 2013). Devido as suas características, juntamente com a afinidade do Hg por grupos SH provavelmente haja uma interação entre essas duas moléculas, de forma que o metal seja neutralizado até que possa ser eliminado e consequentemente diminuído seus efeitos tóxicos. Por outro lado, a ação protetora envolvendo a NAC nos sugere um efeito direto desse composto. Segundo Jalilehvand et al. (2013), a NAC tem capacidade quelante

melhor que algumas substâncias já conhecidas e usadas na clínica como o DPMS, isso porque a NAC liga-se facilmente ao Hg. Com isso o principal efeito protetor da NAC seria pela sua ação quelante em relação ao Hg, neutralizando os efeitos tóxicos do metal até que o mesmo possa ser eliminado (**artigo I e manuscrito I**). Os resultados com o Zn e da NAC foram bastante promissores, embora não foram efetivos sobre alguns parâmetros analisados. Isto sugere que mais estudos são necessários entender melhor o mecanismo envolvendo a proteção de ambos e dessa forma tirar o melhor proveito dos efeitos benéficos dos mesmos.

6. CONCLUSÕES

Analisando os resultados podemos concluir que:

1. Ratas virgens são mais sensíveis ao Hg que ratas gestantes e lactantes: a exposição ao Hg causa danos morfológicos e alteração na expressão de proteínas relacionadas ao estresse oxidativo mais severos em ratas virgens que ratas gestantes e lactantes.
2. O Zn induziu a síntese hepática de MT e assim foi efetivo em diminuir a toxicidade do Hg em ratas virgens, gestantes e lactantes.

3. A NAC apresentou resultados promissores contra os danos tóxicos causados pelo Hg em ratas virgens, gestantes e lactantes.

7. PERSPECTIVAS

- Nas ratas gestantes e lactantes expostas a 10 mg/kg de $HgCl_2$ pretendemos avaliar a quantidade de Hg nos tecidos, bem como a excreção do metal nos grupos pré tratados com Zn e/ou NAC.
- Faremos também uma avaliação hepática mais aprofundada em ratas virgens gestantes e lactantes expostas a 5 mg/kg $HgCl_2$, através de análise ultra-estrutural e expressão de antioxidantes (MT-1, MT-3, Nrf2), e proteínas relacionadas a apoptose (BAX, Mfn2, Citocromo c e Caspase 9).
- Pretendemos ainda, através de análise por Western Blot, investigar esses mesmos marcadores em fígado e rins.

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ANEXO 1

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