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ENVOLVIMENTO DO SISTEMA DA TIORREDOXINA NAS ALTERAÇÕES INDUZIDAS PELO CHUMBO *IN VITRO* E *IN VIVO*: IMPLICAÇÕES NA TOXICIDADE DO CHUMBO

TESE DE DOUTORADO

Greicy Michelle Marafiga Conterato

Santa Maria, RS, Brasil

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por

Greicy Michelle Marafiga Conterato

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

Orientador: Prof. Dra. Tatiana Emanuelli

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

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Santa Maria, 30 de novembro de 2011

DEDICATÓRIA

Dedico este trabalho aos meus pais, à
minha orientadora e aos amigos e colegas
que diretamente incentivaram e contribuíram
na concretização deste trabalho.

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"If we are going to live so intimately with these chemicals - eating and drinking them, taking them into the very marrow of our bones - we had better know something about their nature and their power."

Rachel Carson

RESUMO

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

ENVOLVIMENTO DO SISTEMA DA TIORREDOXINA NAS ALTERAÇÕES INDUZIDAS PELO CHUMBO *IN VITRO* E *IN VIVO*: IMPLICAÇÕES NA TOXICIDADE DO CHUMBO

Autora: Greicy Michelle Marafiga Conterato
Orientadora: Tatiana Emanuelli
Local e Data: Santa Maria, 30 de novembro de 2011

O estresse oxidativo é um importante mecanismo molecular da toxicidade do chumbo (Pb). O sistema da tiorredoxina (selenoenzima tiorredoxina redutase -TrxR, proteína tiorredoxina -Trx e NADPH) é essencial na defesa antioxidante e no controle redox celular. Em nosso estudo prévio, foi demonstrado que a atividade da enzima TrxR1 (citosólica) renal de ratos aumentou na exposição aguda e prolongada ao Pb, sendo o único parâmetro alterado em ambas exposições a doses baixas de Pb. Assim, foi sugerido que a TrxR1 atuaria precocemente na defesa contra a toxicidade do metal, podendo também ser utilizada como um bioindicador dos efeitos precoces do Pb. Assim, o objetivo geral desta tese foi investigar o papel do sistema da tiorredoxina nas alterações induzidas pelo Pb, avaliando: I) *in vitro* a atividade da TrxR1 purificada, bem como a atividade e expressão protéica da TrxR1 e Trx1 em culturas de células renais HEK 293 expostas ao Pb e II) *in vivo*, os efeitos do Pb em ratos e em humanos ocupacionalmente expostos ao Pb sobre a atividade da TrxR1 renal (somente em ratos) e sanguínea (ratos e humanos), comparando esses efeitos com parâmetros de estresse oxidativo, bem como com indicadores clássicos de efeito e de exposição ao Pb. Os resultados do estudo *in vitro* mostraram que a atividade da enzima TrxR1 purificada foi inibida pelo Pb (IC_{50} = 0.27 µM) de forma menos potente que a sua homóloga estrutural glutationa redutase (IC_{50} = 0.048 µM). Essa inibição foi independente do resíduo de selenocisteína do sítio ativo da TrxR1 e foi revertida pela albumina sérica bovina e pelo quelante EDTA. A inibição da TrxR1 também ocorreu em células HEK 293 expostas à maior concentração de acetato de Pb (60 µM), sem alterações na expressão protéica. Entretanto, quando os níveis celulares de glutationa (GSH) foram depletados por pré incubação das células com L-butionina-[S,R]-sulfoximina (BSO) e posterior exposição ao Pb, a atividade e a expressão da TrxR1 e da Trx1 aumentaram na ausência de citotoxicidade e de alterações nas atividades da GR e glutationa S-transferase, apontando esse sistema como um importante mecanismo contra a toxicidade do Pb em células sob depleção de GSH. Por outro lado, a atividade da TrxR1 sanguínea não alterou na exposição aguda de ratos e prolongada de humanos ao Pb. No entanto, o aumento da atividade da TrxR1 renal em ratos expostos à maior dose de acetato de Pb (25 mg/kg) foi concomitante com o aumento dos níveis sanguíneos e renais de Pb ao longo do tempo (6, 24 e 48 h), enquanto que a inibição da enzima δ-ALA-D eritrocitária, um indicador clássico de efeito do Pb, ocorreu após 6 h de exposição, sendo sua atividade restabelecida posteriormente (24 e 48 h). Além disso, o aumento da atividade da TrxR1 renal ocorreu sem danos histopatológicos renais, confirmando essa alteração como um evento precoce da toxicidade do Pb. Em geral, os resultados do presente estudo apontam o sistema da tiorredoxina como alvo do Pb, mas principalmente como um mecanismo de proteção contra o metal. Entretanto, a ausência de alterações na atividade da TrxR1 sanguínea em animais e humanos expostos ao Pb, indica que essa enzima não é um bioindicador adequado dos efeitos tóxicos do Pb em populações expostas.

Palavras-chave: chumbo; cádmio; exposição ocupacional; estresse oxidativo; antioxidantes; tiorredoxina; tiorredoxina redutase; glutationa; glutationa redutase; glutationa S-transferase

ABSTRACT

Thesis for doctoral degree (Ph.D.)
Federal University of Santa Maria, RS, Brazil

ROLE OF THIOREDOXIN SYSTEM IN LEAD-INDUCED CHANGES *IN VITRO* E *IN VIVO*: IMPLICATIONS FOR LEAD TOXICITY

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Place and Date of the defense: Santa Maria, November 30th, 2011

Oxidative stress is an important molecular mechanism of lead (Pb) toxicity. The thioredoxin system (selenoenzyme thioredoxin reductase-TrxR, thioredoxin protein- Trx and NADPH) is essential for the antioxidant defense and cellular redox control. In our previous study, it was showed that cytosolic renal TrxR1 activity of rats increased after acute and long-term exposure to Pb and this was the only parameter that changed after both exposures to low Pb doses. Then, it was suggested that TrxR1 could operate in the early defense against Pb toxicity and it could also be used as a bioindicator of early effects of Pb. Thus, the main objective of this thesis was to investigate the role of thioredoxin system in Pb-induced changes, evaluating: **I)** *in vitro* the activity of purified TrxR1, as well as the activity and the protein expression of TrxR1 and Trx1 in renal HEK 293 culture cells exposed to Pb; **II)** *in vivo,* the effects of Pb exposure in rats and in occupationally-exposed humans on renal (only in rats) and blood TrxR1 activity (both rats and humans), comparing these effects to oxidative stress parameters, as well as to classical bioindicators of Pb effect and exposure. The results of the *in vitro* study showed that lead is a less potent inhibitor of the purified TrxR1 activity (IC_{50} = 0.27 μM) than its structural homologous glutathione reductase (IC_{50} = 0.048 μM). TrxR1 inhibition was independent on the selenocysteine residue of the active site and was reversible by bovine serum albumin and by the EDTA chelating. TrxR1 inhibition also occurred in HEK 293 cells exposed to the highest Pb acetate concentration (60 μM), without alterations in protein expression. However, under glutathione (GSH) depletion after pre-incubation of cells with L-buthionine-[S,R]-sulfoximine (BSO) and further exposure to Pb, the activity and expression of both TrxR1 and Trx1 increased in the absence of cytotoxicity and of changes in GR and glutathione S-transferase activities, which indicates Trx system as an important protective mechanism against Pb toxic effects in GSH-depleted cells. On the other hand, blood TrxR1 activity did not change either after acute exposure of rats or long-term exposure of humans to Pb. However, the increase of renal TrxR1 activity in rats exposed to the highest dose of Pb acetate (25 mg/kg) occurred concomitantly with the increase of blood and renal Pb levels over time (6, 24 e 48 h), whereas the erythrocyte δ-ALA-D inhibition, which is a classical indicator of Pb effects, occurred after 6 h of exposure and the activity was further recovered (at 24 and 48 h). Moreover, the increase of renal TrxR1 activity occurred without renal histopathologycal damage, which corroborates the increase of this enzyme as an early event of Pb toxicity. Overall, the results of the current study point out the thioredoxin system as a target for Pb, but mainly as a protective mechanism against Pb toxicity. However, the absence of changes in blood TrxR1 activity in Pb-exposed animals and humans indicates that this enzyme is not an appropriate bioindicator of the toxic effects of Pb in exposed populations.

Keywords: lead; cadmium; occupational exposure; oxidative stress; antioxidants; thioredoxin; thioredoxin reductase; glutathione; glutathione reductase

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LISTA DE ABREVIATURAS

ALA – Ácido δ - aminolevulínico

ALA-U - Ácido δ – aminolevulínico urinário

δ -ALA-D – δ-Aminolevulinato desidratase

ALA-S – δ-Aminolevulinato sintase

AP-1 – Proteína ativadora-1

ARE – Elemento de resposta antioxidante

ASK1– Quinase 1 reguladora do sinal da apoptose

BSA – Albumina sérica bovina

CAT – Catalase

CHCM – Concentração de hemoglobina corpuscular média

Cu,ZnSOD – Superóxido dismutase citosólica

DL_{50} – Dose letal 50

DMSA – ácido dimercaptosuccínico

DNA – Ácido desoxirribonucléico

EDTA – Ácido etilenodiamino tetracético

EROs – Espécies reativas de oxigênio

FAD – Flavina adenina dinucleotídio

G6PD – Glicose-6-fosfato desidrogenase

GR – Glutationa redutase

GSH – Glutationa

GSSG – Glutationa oxidada

Grx - Glutarredoxina

GPx – Glutationa peroxidase

GSSG – Glutationa dissulfeto (oxidada)

GST – Glutationa S-transferase

$HgCl_2$ – Cloreto de mercúrio

IBE – Indicador biológico de exposição

ip – Intraperitoneal

MDA – Malondialdeído

MT - Metalotioneínas

MeHg – Metilmercúrio

MiDMSA – ácido monoisoamildimercaptossuccínico
MnSOD – Superóxido dismutase mitocondrial
NADPH – Nicotinamida adenina dinucleotídio fosfato
NF-kB – Fator nuclear κB
Nrf2 – Fator 2 relacionado ao NF-E2
Prx – Peroxirredoxinas
RDW – Amplitude de distribuição dos eritrócitos
RNAm – Ácido ribonucléico mensageiro
Sec – Selenocisteína
SH – Grupos sulfidrílicos
SHNP – Grupos tióis não-protéicos
siRNA - RNA de interferência
SNC – Sistema nervoso central
SOD – Superóxido dismutase
TBARS – Substâncias reativas ao ácido tiobarbitúrico
TrxR – Tiorredoxina redutase
TrxR1 – Tiorredoxina redutase citosólica
TrxR2 – Tiorredoxina redutase mitocondrial
TrxR3 – Tiorredoxina redutase testicular
ZPP - Zinco protoporfirina

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese são apresentados sob a forma de um artigo e dois manuscritos, os quais se encontram no item RESULTADOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam na íntegra este estudo.

Os itens DISCUSSÃO E CONCLUSÃO, dispostos após o item RESULTADOS, contêm interpretações e comentários gerais referentes ao presente estudo e relacionados aos artigos científicos deste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS são relacionadas às citações que aparecem nos itens INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA e DISCUSSÃO desta tese.

1 INTRODUÇÃO

O chumbo (Pb) não é um elemento essencial e não desempenha função benéfica conhecida no organismo. Entre os metais tóxicos, é considerado o mais abundante encontrado no ambiente, sendo derivado de fontes naturais e principalmente de atividades industriais. No ambiente, persiste como uma fonte perigosa e contínua de exposição em longo prazo, representando risco à saúde das populações expostas (Paoliello & Chasin, 2001). Apesar de nas últimas décadas a quantidade de Pb no ambiente ter sido reduzida em função da proibição do uso de Pb na gasolina e em praguicidas, a exposição ocupacional permanece como a principal fonte de exposição ao metal, com destaque para as atividades de mineração, fundição, soldas, fabricação de baterias e produção e utilização de tintas (Needleman, 2004).

O chumbo é conhecido por ter como alvos principais no organismo o sistema nervoso central (SNC) e os sistemas hematológico e renal (Goyer, 1989; ATSDR, 2007). A base da toxicidade do metal nos diferentes órgãos está relacionada à elevada afinidade do chumbo (Pb) por resíduos de cisteína em proteínas (Campagna et al., 1999) assim como à sua semelhança à íons divalentes como o cálcio (Ca^{2+}) e o zinco (Zn^{2+}), podendo substituí-los em processos fisiológicos onde esses íons são requeridos, potencializando ou inibindo as suas ações (Goyer, 1996; Cornelis, 2005). A ligação do Pb ao sítio ativo da enzima δ-aminolevulinato desidratase (δ-ALA-D) eritrocitária promove a inibição dessa enzima e conseqüente inibição da via de biossíntese do heme da hemoglobina (Rocha *et al*., 1995), podendo levar à anemia quando os níveis de Pb sanguíneo forem maiores do que 50 µg/dL (ATSDR, 2007).

A geração de espécies reativas de oxigênio (EROs) e o estresse oxidativo também estão envolvidos nos mecanismos da toxicidade do Pb (Gürer-Orhan *et al*., 2004; Jurczuk *et al*., 2006; Ergurhan-Ilhan *et al*., 2008) em diferentes níveis. A inibição da enzima δ-ALA-D com acúmulo e autoxidação de seu substrato, o ácido δ-aminolevulínico (δ-ALA), a capacidade do Pb em causar peroxidação lipídica *per se* na presença de ferro, a geração de radicais livres durante a metabolização hepática de compostos orgânicos de Pb, bem como a depleção de tióis não-protéicos como a

glutationa (GSH) são apontados como mecanismos da indução do estresse oxidativo decorrente da exposição ao Pb (Ramstoeck *et al*., 1980; Monteiro *et al*., 1989; Adonaylo & Oteiza, 1999; Cornelis, 2005; ATSDR, 2007).

A enzima tiorredoxina redutase (TrxR) é uma selenoflavoproteína, que em conjunto com a tiorredoxina (Trx) e NADPH, forma o sistema da tiorredoxina, o qual desempenha um papel fundamental no reparo de proteínas intracelulares e proteção contra o dano oxidativo (Holmgren & Björnstedt, 1995; Arnér, 2009). Três isoformas da enzima tioredoxina redutase já foram isoladas em mamíferos, incluindo a citosólica (TrxR1), a mitocondrial (TrxR2) e a isolada de testículos de ratos, também mitocondrial (TGR ou TrxR3) (Nordberg & Arnér, 2001). A presença de um resíduo de selenocisteína na porção carboxiterminal das tiorredoxina redutases, torna essas enzimas proteínas com funções versáteis, uma vez que reduzem diferentes substratos além da Trx em diversos compartimentos celulares, sustentando, portanto, uma gama de processos envolvidos na proliferação celular, defesa antioxidante, síntese do DNA e inibição da apoptose (Nordberg & Arnér, 2001; Arnér, 2009). Outros compostos antioxidantes não protéicos que também são substratos da TrxR (principalmente da isoforma citosólica – TrxR1) são o deidroascorbato, o ácido lipóico e a ubiquinona (Nordberg & Arnér, 2001).

Por outro lado, o fácil acesso de diferentes substâncias ao sítio ativo da TrxR1, a torna um alvo fácil para a inibição por drogas e metais, o que pode alterar muitas funções celulares, cujos efeitos dependerão do contexto celular. Em células tumorais, em que a expressão da TrxR1 é significativamente aumentada, a inibição dessa enzima por drogas antitumorais (complexos metálicos de platina) contribui para a inibição do crescimento dessas células e para a apoptose (Witte *et al*., 2005; Ahmadi *et al*., 2006; Marzano *et al*., 2007). Por outro lado, a inibição da TrxR1 de células normais por compostos metálicos ou não metálicos pode causar dano oxidativo severo, em virtude das múltiplas funções do sistema da Trx, culminando por fim, na morte celular (Nordberg & Arnér, 2001; Carvalho *et al*., 2008).

Alguns inibidores da TrxR1 porém, parecem exercer também um efeito ativador sobre a via do Keap1/Nrf2, a qual é a principal via que controla a expressão da TrxR1 e de outras enzimas de fase II, envolvidas na metabolização de xenobióticos e defesa antioxidante (Chen *et al*., 2005; Sakurai *et al*., 2005). A ativação da via Keap1/Nrf2 seguida pelo aumento da expressão da TrxR1 foi responsável por uma resposta adaptativa de células a uma posterior exposição a

alguns agentes tóxicos, como o 4-hidroxinonenal, mercúrio (Hg) e cádmio (Cd) (Chen *et al*., 2005; Sakurai *et al*., 2005; Wataha *et al*., 2008), o que sugere o aumento da expressão da TrxR1 como um mecanismo de proteção celular contra danos causados por esses agentes tóxicos.

Pouco se sabe, porém, do efeito do Pb sobre o sistema da Trx. Em nosso estudo prévio (Conterato *et al*., 2007), observamos que o aumento da atividade da TrxR1 renal em ratos expostos a baixas doses de Pb foi o único parâmetro que se repetiu tanto na exposição aguda (6, 24 e 48h) como na prolongada (30 dias), sendo que essa alteração ocorreu antes da inibição da enzima δ-ALA-D renal, considerada um indicador clássico dos efeitos tóxicos do Pb, e na ausência de alterações nos níveis de indicadores plasmáticos da função renal. Baseado nos resultados desse estudo prévio, nós levantamos as hipóteses de que o aumento da atividade da TrxR1 renal seria um evento precoce da toxicidade do Pb e que atuaria como um mecanismo de defesa contra os efeitos tóxicos do metal. Além disso, sugerimos que a atividade da TrxR1 poderia ser utilizada como um indicador dos efeitos precoces do Pb.

Visando corroborar essas hipóteses, além de verificar um possível efeito direto do Pb sobre a atividade da TxR1, este trabalho visou elucidar o papel do sistema da tiorredoxina nas alterações induzidas pelo Pb.

2 OBJETIVOS

Este trabalho teve como objetivo geral avaliar o envolvimento do sistema da tiorredoxina nas alterações induzidas pelo Pb. Para atingir o objetivo geral proposto, os estudos que se desenvolveram foram baseados nos seguintes objetivos específicos:

- **I)** Avaliar a atividade da TrxR1 purificada, bem como a atividade e a expressão protéica da TrxR1 e Trx1 em culturas de células renais HEK 293 expostas ao Pb, relacionando esses efeitos com a viabilidade celular.
- **II)** Determinar se a atividade da TrxR1 poderia ser utilizada na avaliação da toxicidade do Pb como um indicador de efeito do metal em populações expostas. Para atingir essa finalidade, dois estudos foram desenvolvidos, avaliando-se:
    - A atividade da TrxR1 sanguínea, comparando as alterações nesse parâmetro com outros indicadores de efeito (δ-ALA-D eritrocitária) e de exposição ao Pb (níveis sanguíneos de Pb), além de parâmetros de estresse oxidativo e hematológicos em humanos ocupacionalmente expostos em longo prazo ao Pb;
    - A atividade da TrxR1 sanguínea e renal, comparando as alterações nesse parâmetro com outros indicadores de efeito (δ-ALA-D eritrocitária) e de exposição ao Pb (níveis sanguíneos de Pb), além de parâmetros de estresse oxidativo sanguíneos, análise histopatológica e níveis de Pb no tecido renal em animais expostos ao Pb em curto prazo.

3 REVISÃO BIBLIOGRÁFICA

3.1 Chumbo

3.1.1 Espécies naturais e antropogênicas de chumbo no ambiente

O chumbo (Pb) é um metal de aspecto branco-acinzentado, brilhante, dúctil e maleável. Pertence à família química dos metais do grupo IV A da tabela periódica, juntamente com o carbono, germânio, silício e estanho (Paoliello & Chasin, 2001). Na natureza, o Pb raramente é encontrado na forma elementar, mas aparece frequentemente formando complexos com outros elementos (Merck Index, 2001). Na água, pode ser encontrado nas formas inorgânicas mais simples de hidróxidos de chumbo, tais como $Pb(OH)_3$ e $Pb(OH)_4$, assim como nas formas de hidróxidos de íons poliméricos de Pb tais como $Pb_2(OH)_3$ e $Pb_4(OH)_4$. Compostos insolúveis como o óxido de chumbo (PbO), carbonato de chumbo ($PbCO_3$) e sulfato de chumbo ($PbSO_4$) também podem ser encontrados na água e em sedimentos. No entanto, a maioria dos estudos referem-se às espécies inorgânicas de Pb apenas como o íon divalente Pb^{2+} (Cornelis, 2005). O sulfeto de chumbo ($Pb^{2+}S^{2+}$) é o principal componente do minério galena, de onde grande parte do chumbo é extraído para utilização nos mais diversos processos industriais (Baird, 2002). Apesar das formas orgânicas do Pb também serem encontradas naturalmente no ambiente, a maior parte dessas espécies derivam da produção antropogênica de chumbo orgânico em ambientes altamente oxidantes, onde o Pb^{2+} é convertido a Pb^{4+}, o qual forma os derivados tetralquilados (Cornelis, 2005). Apesar de o uso dos compostos tetralquilados de Pb terem sido banidos da composição da gasolina em muitos países, incluindo o Brasil, ainda representam risco de contaminação ambiental, devido à sua conversão na natureza em formas mais persistentes, como os derivados di e trialquilados (Baird, 2002; Cornelis, 2005).

3.1.2 Fontes de exposição humana

O Pb encontrado na natureza pode ser oriundo de fontes naturais, principalmente das emissões vulcânicas, intemperismo geoquímico e das névoas aquáticas. Estima-se que as taxas de emissão natural do Pb são da ordem de 19.000 toneladas por ano (WHO, 1995). Entretanto, as propriedades de alta densidade, baixo ponto de fusão, elevado ponto de ebulição, alta resistência à corrosão e estabilidade química, entre muitas outras, fazem do Pb um metal amplamente utilizado nas mais diversas profissões em todo o mundo (ATSDR, 2007).

A utilização extensiva desse metal faz com que grande parte do Pb seja lançada no ambiente, causando a contaminação do ar, água e solo de regiões próximas ou mesmo mais distantes das fontes antropogênicas de emissão (Paoliello & Chasin, 2001; Paoliello & De Capitani, 2007). A contaminação ambiental pode levar à absorção de Pb por vegetais, animais e organismos marinhos em geral, culminando na exposição humana em longo prazo ao metal (ATSDR, 2007).

Com a proibição do Pb como componente da gasolina e de agrotóxicos, os níveis de Pb tem diminuído nas últimas décadas no ar e no sangue da população em geral (ATSDR, 2007). Entretanto, a ampla utilização desse metal na fabricação de baterias, produção de equipamentos médicos (materiais de blindagem de radiação), laminados e extrudados (folhas de chumbo, solda e latão), munições, cerâmicas e tintas faz com que os trabalhadores dessas indústrias constituam o principal grupo populacional exposto ao Pb e portanto, mais suscetível à intoxicação por esse xenobiótico (EPA, 1986; Thournton *et al*., 2001). Esses trabalhadores estão expostos principalmente ao Pb metálico e aos compostos inorgânicos de Pb (EPA, 1986; WHO, 1995; Thornton *et al*., 2001; Cornelis, 2005).

Durante a fabricação de baterias de chumbo ácido, os trabalhadores são expostos a altas concentrações de dióxido de chumbo (PbO_2) durante quase todo o processo, principalmente por inalação das partículas (Cornellis et al., 2007). Como o Pb também faz parte da composição de tintas para marcação de ruas, estradas e pintura de automóveis (além de tintura para cabelos), os pintores também são considerados um dos principais grupos expostos ao metal, sendo também concomitantemente expostos ao cádmio presente na composição das tintas em geral (Cornelis, 2005; Bertin & Averbeck, 2006).

3.1.3 Aspectos toxicocinéticos

O Pb é absorvido no organismo principalmente pelas vias gastrointestinal e respiratória, sendo a última predominante na exposição ocupacional. A exposição por via oral ocorre na população em geral, através da ingestão de bebidas e alimentos contaminados, ou mesmo de objetos ou mãos sujas levados à boca, um hábito muito comum entre as crianças (Midio & Matins, 2000; Cornelis, 2005; ATSDR, 2007). Independente da via de absorção, a toxicocinética do Pb no organismo segue o mesmo modelo de três compartimentos. Inicialmente, o Pb distribui-se no compartimento sanguíneo ligado aos eritrócitos, assim como às proteínas plasmáticas e na forma livre no plasma, a qual constitui a fração difusível de Pb para os tecidos. Assim, a fração plasmática livre do Pb é a responsável pela concentração de Pb que entra nos tecidos e pelos efeitos tóxicos do metal (Silva, 1983; Cornelis, 2005). Em condições de equilíbrio, 98% do Pb absorvido circula ligado aos eritrócitos, pois o mesmo possui afinidade elevada às proteínas eritrocitárias, como a δ-aminolevulinato desidratase (δ-ALA-D), uma proteína de 45 kDa (provavelmente a pirimidina 5'-nucleotidase) e uma proteína com peso molecular < 10 kDa (Bergdahl *et al*., 1998). A meia vida (T1/2) do Pb no compartimento sangüíneo é de 36 dias, passando posteriormente para os tecidos moles (T1/2= 40 dias) e finalmente para os ossos (t1/2=20-27 anos), os quais armazenam quase 90% da carga corpórea de Pb (Rabinowitz *et al.*, 1976). Dos ossos, o Pb pode ser mobilizado em situações de desmineralização tais como gravidez, lactação e osteoporose, constituindo-se em uma fonte endógena de Pb para os demais tecidos mesmo anos após o término da exposição (Barry, 1975; Silbergeld *et al*., 1988). Um estudo demonstrou que o Pb depositado no osso contribui para 40 a 70% do Pb circulante em trabalhadores expostos ao Pb inorgânico (Smith *et al*., 1996). Porém, nem todo o Pb depositado nos ossos está disponível para trocas com o sangue, uma vez que a terapia com quelantes é ineficiente em remover quantidades apreciáveis de Pb desse compartimento (Rabinowitz, 1991).

Em relação ao metabolismo, o Pb inorgânico não sofre metabolização, mas é complexado à proteínas como as metalotioneínas, sendo diretamente absorvido, distribuído aos tecidos e posteriormente excretado (Church *et al.,* 1993; Cornelis, 2005). Os compostos orgânicos tetralquilados são biotransformados no fígado a

compostos tri e dialquilados de Pb e Pb inorgânico. Os compostos trialquilados são os responsáveis pela toxicidade da forma orgânica do metal, enquanto os dialquilados parecem não ter relevância toxicológica (Feldman 1999; Cornelis, 2005; ATSDR 2007).

A excreção do Pb ocorre principalmente através da urina (75%) e da bile (15-20%). Parte do Pb também é excretada pelo suor, cabelos, unhas e leite materno (WHO 1995; Paoliello & Chasin, 2001; Cornelis, 2005), sendo que a fração do Pb não absorvida é eliminada pelas fezes. A figura abaixo, demonstra resumidamente a absorção, distribuição e excreção do Pb do organismo.

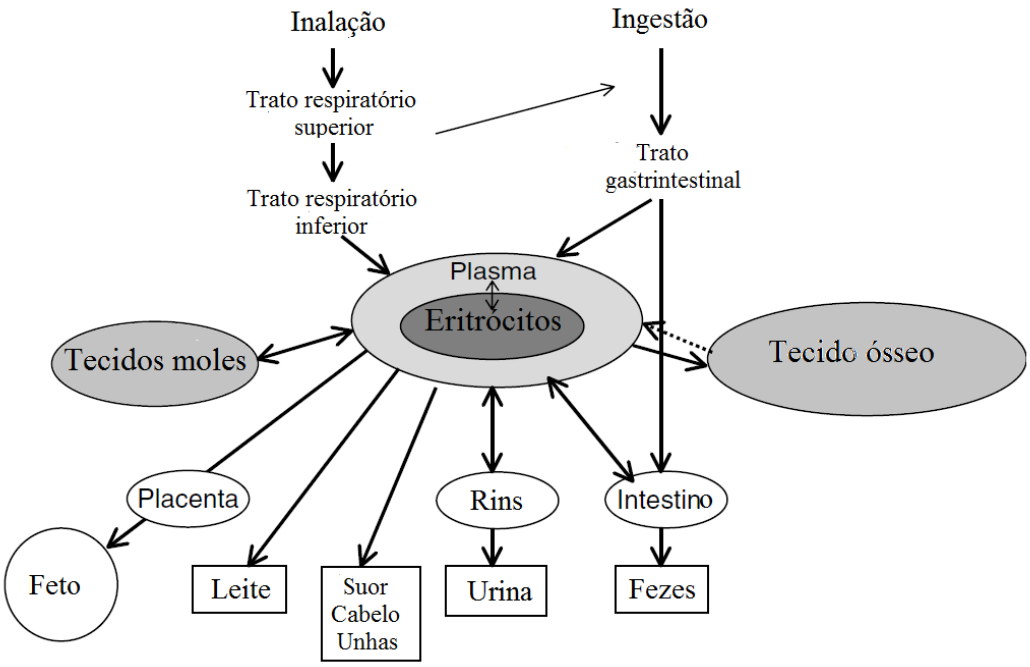

Figura 1- Toxicocinética do chumbo (Pb) (Adaptado de Cornelis, 2005).

3.1.4 Alvos bioquímicos e efeitos tóxicos

O Pb afeta praticamente todos os sistemas do organismo, sendo os sistemas hematológico, renal e nervoso os mais afetados. Estudos também demonstram alterações nos sistemas cardiovascular e reprodutivo em animais e humanos (WHO, 1995; Cornelis, 2005; ATSDR, 2007).

A toxicidade do Pb em diferentes órgãos se deve, basicamente à afinidade do seu íon divalente (Pb^{2+}) pelos grupos sulfidrílicos de proteínas e de compostos não

protéicos de baixo peso molecular, como a glutationa (GSH). A interação do Pb com proteínas sulfidrílicas tais como a enzima δ-ALA-D pode levar ao decréscimo de sua atividade e consequente inibição da biossíntese do grupo heme da hemoglobina (Figura 2) (Warren *et al*., 1998). Como conseqüência da inibição da biossíntese do heme, a anemia pode ser observada em concentrações sanguíneas de Pb acima de 50 µg/dL (ATSDR, 2007), além da hemólise e diminuição da vida útil dos eritrócitos, devido ao aumento da fragilidade da membrana eritrocitária e à diminuição da resistência osmótica dessas células (Cornelis, 2005; ATSDR, 2007).

A depleção de GSH, assim como a inibição das enzimas envolvidas no metabolismo desse tripeptídeo, tais como a glicose-6-fosfato desidrogenase (G6PD) e a glutationa redutase (GR) já foram demonstradas em animais (Sivaprasad *et al.*, 2004; Saxena *et al*., 2005). Além disso, o Pb^{2+} é capaz de aumentar os níveis das metalotioneínas hepáticas, proteínas que atuam como um estoque de zinco e cobre no citoplasma. O aumento dos níveis de metalotioneínas parece atuar como um mecanismo de proteção, uma vez que auxilia na complexação do Pb^{2+}, diminuindo assim, a disponibilidade do íon para atingir seus alvos celulares (Maitani *et al*., 1986; Moffatt & Denizeau, 1997).

Embora os grupos sulfidrílicos sejam o principal sítio de ligação do Pb às proteínas e enzimas, o Pb também interage com outros ligantes nucleofílicos dessas biomoléculas tais como aminas, fosfatos e grupos carboxílicos (Goering, 1993). A afinidade do Pb por proteínas renais ricas em ácido glutâmico e aspártico é responsável pelo depósito de Pb nos rins, sendo 90% do metal encontrado na forma de complexos de Pb-proteínas (Paoliello & Chasin, 2001; Gonick*,* 2008). Alterações nas funções mitocondriais e citomegalia das células epiteliais do túbulo proximal parecem ser responsáveis pelos danos tubulares agudos, os quais podem evoluir para nefropatia crônica irreversível com redução na taxa de filtração glomerular e fibrose intersticial (Goyer, 1989; Nolan & Shaikh, 1992; Paoliello & Chasin 2001).

Além da interação com aminoácidos, a toxicidade do Pb também pode estar relacionada à semelhança do íon Pb^{2+} com minerais essenciais divalentes, tais como o zinco (Zn^{2+}) e o cálcio (Ca^{2+}), podendo, portanto, inibir ou potencializar a ação dos mesmos no organismo (Cornelis, 2005). Assim, alterações na atividade da enzima δ-ALA-D, ferroquelatase e nos níveis de metalotioneínas também podem estar relacionadas à interação direta entre o Pb^{2+} e o Zn^{2+}, uma vez que o Zn^{2+}

encontra-se ligado às cisteínas dos sítios ativos dessas metalo zinco proteínas (Simons, 1997).

A interferência nos processos de neurotransmissão, nas funções de canais iônicos, funções das proteínas quinase C e calmodulina (proteínas com domínio de ligação para o Ca^{2+}) são descritas como alterações relacionadas à substituição do Ca^{2+} de seus sítios de ligação pelo Pb^{2+}. O Pb^{2+} também substitui o cálcio nos ossos e a sua liberação e deposição nesse tecido segue o metabolismo normal do cálcio no osso (Godwin, 2001).Os efeitos neurotóxicos do Pb são observados principalmente após exposição em longo prazo (níveis sanguíneos de Pb entre 4 e 80 µg/dL), sendo manifestados como déficit cognitivo, redução do quociente de inteligência ou em casos mais graves, encefalopatia associada à convulsões, delírios, paralisia e morte (Feldman, 1999). Na exposição aguda, edema cerebral decorrente da alteração da permeabilidade capilar é uma das principais manifestações clínicas, além da cefaléia, redução na atenção, ansiedade e depressão. Os efeitos neurotóxicos agudos podem ocorrer em níveis sanguíneos de Pb entre 40 e 120 µg/dL (Cornelis, 2005). Ressalta-se, porém, a maior susceptibilidade das crianças aos efeitos neurotóxicos do Pb em relação aos adultos (Mahmoudian *et al*., 2009). A neurotoxicidade do Pb tem sido relacionada tanto a sua ação direta sobre sistemas neurotransmissores quanto aos efeitos do ácido δ-aminolevulínico acumulado devido a inibição da δ-ALA-D pelo Pb (Emanuelli *et al.*, 2000; 2001a; 2001b; 2003; Prauchner *et al.*, 2004).

Os efeitos do Pb sobre outros sistemas, como por exemplo o reprodutivo, também tem sido demonstrados e podem se manifestar em homens como astenospermia, hipospermia, teratospermia e hipogonadismo. No feto são observadas a redução de peso ao nascer, idade gestacional diminuída e déficits neurocomportamentais, uma vez que o metal atravessa a barreira placentária, atingindo o sistema nervoso em desenvolvimento (WHO, 1995; ATSDR, 2007).

3.1.5 Estresse oxidativo como mecanismo da toxicidade do chumbo

Além de depletar e/ou inibir moléculas protéicas e não protéicas e de interferir na função de alguns minerais essenciais, o chumbo também contribui para a produção de espécies reativas de oxigênio (EROs) e para alterações em sistemas antioxidantes em animais e humanos expostos ao Pb, contribuindo para uma

condição denominada de estresse oxidativo (Gürer-Orhan *et al*., 2004; Jurczuk *et al*., 2006; Ergurhan-Ilhan *et al*., 2008). O estresse oxidativo é caracterizado pelo desequilíbrio entre a produção de EROs e a sua remoção por parte dos sistemas antioxidantes celulares (Nordberg & Arnér, 2001). Concentrações fisiológicas das EROs formadas principalmente durante o metabolismo oxidativo mitocondrial (Figura 3) desempenham importante papel como mediadores em vias de sinalização, assim como na defesa contra agentes microbianos, entre outras funções. Entretanto, qualquer condição que favoreça o aumento da produção dessas espécies, sem uma resposta compensatória por parte dos sistemas antioxidantes, implicará em danos oxidativos às biomoléculas celulares, principalmente às proteínas, lipídios de membrana e ao DNA (Nordberg & Arnér, 2001). Dano excessivo às biomoléculas implicará no comprometimento da sobrevivência celular podendo levar à morte da célula por necrose ou apoptose (Nielsen, 2009).

A principal via de geração de EROs na exposição ao Pb tem sido atribuída à inibição da enzima eritrocitária δ-ALA-D, o que leva a um acúmulo do seu substrato, o ácido δ-aminolevulínico (ALA) que se autoxida, gerando EROs que podem oxidar proteínas, lipídios e DNA (Monteiro *et al*., 1989). A figura 2 demonstra esquematicamente a geração de EROs causada pela inibição da via de biossíntese do grupo heme pelo Pb. Na sequência, a figura 3 esquematiza a formação das EROs formadas durante o metabolismo oxidativo mitocondrial (principal via endógena de formação de EROs) a sua concomitante remoção por sistemas antioxidantes endógenos.

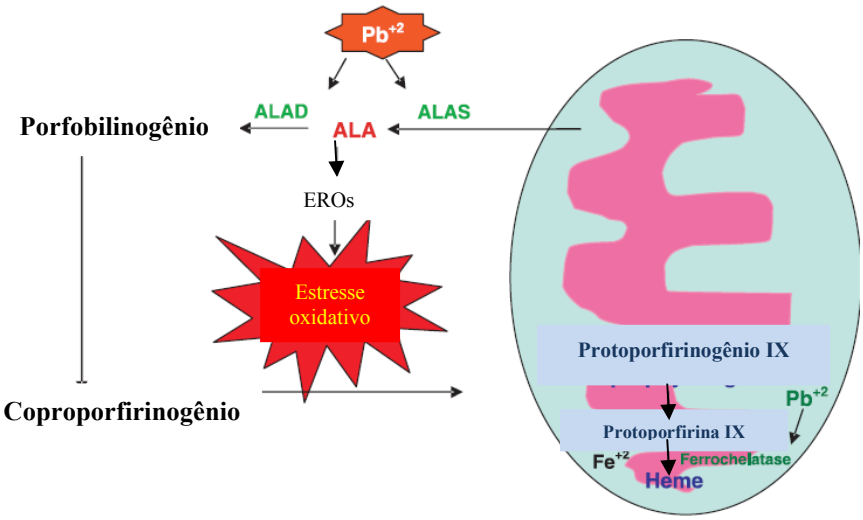

Figura 2 – Efeito do Pb sobre a biossíntese do heme. ALAS (δ-aminolevulinato sintase); EROs (espécies restivas de oxigênio) (Adaptado de Flora *et al.,* 2008).

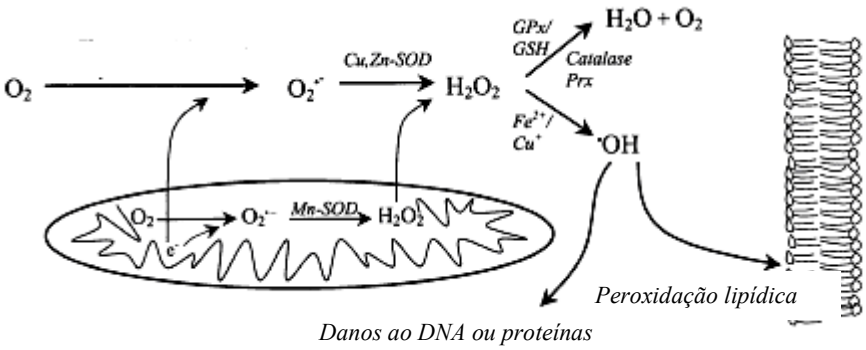

Figura 3 – Sistemas oxidantes e antioxidantes celulares. O radical superóxido ($O_2^{\cdot-}$) é produzido endogenamente por enzimas dependentes de flavinas e na mitocôndria. Duas moléculas de superóxido são enzimaticamente dismutadas a peróxido de hidrogênio (H_2O_2) pela superóxido dismutase mitocondrial (MnSOD) ou citosólica (Cu,ZnSOD). H_2O_2 pode ser metabolizado por diferentes enzimas (GPx/GSH = sistema glutationa, CAT=catalase ou Prx= peroxirredoxinas) ou ser convertido ao radical hidroxil, através de uma reação catalisada por metais de transição (reação de Fenton) (Adaptado de Nordberg & Arnér, 2001).

Além da autoxidação do ALA acumulado, o estresse oxidativo induzido pelo Pb parece também envolver a interação direta desse metal com as cisteínas dos sítios ativos de enzimas antioxidantes e/ou substituição de minerais de seus sítios ativos pelo Pb (Halliwel & Gutteridge, 1989; Quig, 1998; Flora et al., 2008). A produção de EROs também ocorre durante a biotransformação hepática de compostos orgânicos trialquilados de Pb, os quais podem induzir a peroxidação lipídica (Ramstoeck *et al*., 1980). Além disso, foi demonstrado que o Pb^{2+} *per se*

pode estimular a peroxidação lipídica na presença de ferro (Fe^{2+}) (Adonaylo & Oteiza, 1999). A exposição ao Pb também pode alterar as concentrações endógenas de GSH, um tripeptídeo que juntamente com a enzima glutationa S-transferase (GST) está envolvido na quelação e detoxificação de metais pesados em geral, bem como na remoção de EROs geradas por eles (Reed, 1990; Jurczuck *et al*., 2006). Alguns autores observaram depleção de GSH após exposição ao Pb (Reed, 1990; Jurczuck *et al*., 2006). No entanto, o aumento dos níveis de GSH ou de tióis não protéicos (SHNP) também tem sido observado em animais expostos ao Pb, provavelmente como um mecanismo compensatório (Conterato *et al.,* 2007).

3.1.6 Biomonitoramento da exposição e efeito do chumbo

Durante o monitoramento biológico do trabalhador exposto ao Pb, podem ser realizadas avaliações através do uso de indicadores biológicos de exposição (IBE) ou de efeito (Oga, 2008). Os IBEs, também são conhecidos como indicadores de dose interna, e representam a quantidade efetiva do metal absorvida pelo organismo. Além disso, os níveis do IBE devem correlacionar com os níveis do metal no ambiente onde o indivíduo trabalha. Os indicadores de efeito, por sua vez, refletem uma alteração bioquímica precoce e reversível, causada pelo Pb absorvido no organismo. Essa alteração revela o estado de saúde do indivíduo e pode ser aplicada na prevenção de danos mais graves e irreversíveis (ACGIH, 2000; IPCS, 2001).

Atualmente, o indicador biológico de exposição ao chumbo mais utilizado é o nível de Pb no sangue. O Pb sanguíneo reflete principalmente a concentração do Pb inorgânico, embora o Pb orgânico também contribua para o aumento dos níveis sanguíneos do metal após a sua desalquilação hepática (McGrail *et al*., 1995). Com relação ao tempo de exposição, o Pb sanguíneo reflete uma exposição relativamente recente, uma vez que sua meia vida sanguínea é de 36 dias (Rabinowitz *et al.*, 1976; Cornelis, 2005). No entanto, quando o Pb é mobilizado dos seus reservatórios teciduais, principalmente o tecido ósseo, ele entra novamente na circulação sanguínea, contribuindo para o aumento dos níveis do metal nesse compartimento (Schütz *et al*., 1996). No Brasil, a Norma Regulamentadora n◦7 (NR 7 de 30 de dezembro de 1994) do Ministério do Trabalho estabelece como Índice Biológico Máximo Permitido (IBMP) para o Pb sanguíneo o valor de 60 µg/dL, o que

significa que em níveis maiores do que esses, o trabalhador deverá ser imediatamente removido do local de exposição (Oga, 2008).

Quanto aos indicadores de efeito, a enzima δ-ALA-D eritrocitária é aceita como o indicador mais sensível dos efeitos do Pb, sendo inibida à partir de concentrações sanguíneas de Pb entre 3,2 e 4,8 µg/dL ou maiores. Por isso, essa enzima é considerada um indicador muito útil para avaliar a exposição recente (Cornelis, 2005). No entanto, tem sido sugerido que a avaliação concomitante dos níveis sanguíneos de Pb e da atividade da δ-ALA-D eritrocitária é mais adequada para avaliar a exposição cumulativa em longo prazo ao metal (Alessio *et al*., 1981). Outros indicadores de efeito utilizados são os níveis de ALA urinário (ALA-U) e da zinco protoporfirina (ZPP) sanguínea que alteram em concentrações sanguíneas de Pb maiores do que as concentrações mínimas que inibem a δ-ALA-D como 35 µg/dL e 25 a 35 µg/dL, respectivamente (Cornelis, 2005).

3.1.7 Tratamento da intoxicação por chumbo

O tratamento mais efetivo nas intoxicações por metais consiste na utilização de agentes quelantes. Esses são definidos como compostos orgânicos capazes de ligarem-se aos íons metálicos, formando um complexo denominado quelato que é posteriormente excretado (Kalia & Flora, 2005).

O ácido etilenodiaminotetracético (EDTA) pertence ao grupo dos quelantes poliaminocarboxílicos, cujos grupos aminos e carboxílicos são os responsáveis pela ligação e eliminação do metal do organismo (Kalia & Flora, 2005). O EDTA, na forma do seu derivado, o edetato dissódico de cálcio (Ca_2Na_2EDTA) é utilizado por via intravenosa como tratamento de escolha na intoxicação pelo Pb em pacientes com níveis sanguíneos elevados de Pb (>70 µg/dL) e/ou que apresentam sintomas clínicos característicos da intoxicação pelo metal (AAP, 1998; Lowry, 2010). O EDTA quela o Pb circulante, mas também remove consideravelmente o Pb do osso e contribui para a remoção do metal presente no fígado e no rim (Bredberry & Vale, 2009).

No entanto, a administração do Ca_2Na_2EDTA sozinho pode agravar os sintomas da intoxicação por Pb em pacientes com níveis de Pb muito elevados (Lowry, 2010), além de apresentar outros efeitos adversos, tais como a redistribuição do Pb em tecidos moles, nefrotoxicidade e depleção de minerais essenciais (Flora &

Tandon, 1990; Powell *et al*., 1990). Devido a esses efeitos indesejáveis, tem sido avaliada a eficácia do tratamento em longo prazo com agentes quelantes contendo grupos sulfidrílicos na redução dos níveis sanguíneos de Pb em animais. Nesses estudos, os quelantes foram administrados individualmente e/ou em combinação com o EDTA (Flora *et al*., 1995; Kostial *et al*., 1999; Meldrum & Ko, 2003). Os resultados revelaram que a terapia combinada do ácido meso-2,3 dimercaptosuccínico (DMSA) com o EDTA parece não oferecer qualquer vantagem no tratamento da intoxicação pelo Pb em relação ao EDTA administrado individualmente em bezerros (Meldrum & Ko, 2003), nem em ratos em amamentação (Kostial *et al*., 1999). No entanto, a terapia somente com o DMSA foi efetiva na remoção do Pb sanguíneo e dos tecidos moles, além de melhorar os sintomas e algumas alterações bioquímicas induzidas pelo Pb em ratos e humanos (Graziano *et al*., 1985; Ercal *et al*., 1996). O EDTA por sua vez, tem sido mais utilizado em humanos em combinação com o quelante sulfidrílico dimercaprol, também conhecido como BAL (*British Antilewisite*), pois essa associação parece ser efetiva na remoção do Pb sanguíneo em pacientes com encefalopatia e com níveis sanguíneos de Pb muito elevados (Lowry, 2010).

Embora os estudos tenham como principal foco a remoção do Pb do organismo por agentes quelantes que apresentem pouco ou nenhum efeito adverso, as informações acerca de seus possíveis efeitos benéficos na reversão de alterações bioquímicas causadas pelo Pb são escassas. Flora *et al*. (2003) demonstraram que a administração de monoisoamildimercaptossuccínico (MiDMSA) e de DMSA recuperou os níveis de GSH reduzida e oxidada (GSSG) em ratos expostos ao Pb e a terapia combinada desses dois quelantes com a vitamina E foi mais adequada na recuperação dos parâmetros de estresse oxidativo nesses animais. Outro estudo demonstrou que o Ca_2Na_2EDTA reverteu moderadamente as alterações nos parâmetros de estresse oxidativo em ratos submetidos à exposição aguda, enquanto que a sua administração combinada com o MiDMSA foi o tratamento mais efetivo para recuperar esses parâmetros (Saxena & Flora, 2004).

Uma vez que o EDTA é o principal quelante preconizado na terapia da intoxicação pelo Pb, mais estudos acerca do seu potencial em reverter as alterações bioquímicas induzidas pelo Pb devem ser realizados. O conhecimento dessas informações é importante para determinar se o restabelecimento dessas alterações

bioquímicas estaria associado à melhora dos sintomas clínicos ou mesmo à prevenção de um dano irreversível associado à exposição ao Pb.

3.2 Sistema da tiorredoxina

O sistema da tiorredoxina (Trx), o qual atua em paralelo com o sistema da GSH, está envolvido na redução de dissulfetos de proteínas intracelulares, mantendo-as, portanto, em seu estado reduzido (Holmgren, 1989; Lillig & Holmgren, 2007). Nesse sistema, a selenoenzima tiorredoxina redutase (TrxR) utiliza os elétrons fornecidos pelo NADPH para reduzir os dissulfetos da proteína tiorredoxina (Trx). Essa por sua vez, é responsável por reduzir proteínas envolvidas em inúmeros processos celulares que requerem seus resíduos sulfidrílicos no estado reduzido (-SH_2) (Holmgren, 1989; Lillig & Holmgren, 2007). A manutenção de um ambiente celular reduzido é essencial para a prevenção do estresse oxidativo, o que torna o sistema da tiorredoxina indispensável na proteção contra danos celulares induzidos por EROs (Nordberg & Arnér, 2001). A essencialidade desse sistema para a sobrevivência celular foi corroborada pelos estudos de Nonn *et al*. (2003) e de Matsui *et al*. (1996) que demonstraram que a deleção dos genes tanto das isoformas citosólicas do sistema (Trx1 e TrxR1) quanto das mitocondriais (Trx2 e TrxR2) é embriologicamente letal em camundongos.

Funções adicionais àquelas de prevenção, intervenção e reparo de danos às proteínas causados pelo estresse oxidativo (Arnér & Holmgren, 2000), são as que envolvem a participação do sistema da tiorredoxina na regulação direta de vários fatores de transcrição, inibição da apoptose e em muitas vias metabólicas, como a síntese do DNA, metabolismo da glicose, metabolismo do selênio, e reciclagem da vitamina C (Nordberg & Arnér, 2001). A multiplicidade de funções atribuídas a esse sistema se deve ao fato de que a enzima TrxR atua sobre outros substratos além da Trx, podendo portanto desempenhar funções distintas daquelas desempenhadas pela Trx, mas que somadas, contribuem na defesa contra os danos oxidativos causados por insultos endógenos e exógenos (Arnér, 2009). A figura 4 evidencia a semelhança entre as reações catalisadas pela TrxR no sistema da Trx e pela glutationa redutase (GR) no sistema da GSH. Note que ambos os sistemas são dependentes de NADPH, e atuam juntos na regulação redox intracelular. Entretanto, a atividade redox do sistema da Trx é essencial em nível nuclear (regulação da

atividade de fatores de transcrição de genes inflamatórios e relacionados à proteção contra o estresse oxidativo, além da síntese do DNA), enquanto o sistema da GSH atua predominantemente em nível citosólico e extracelular.

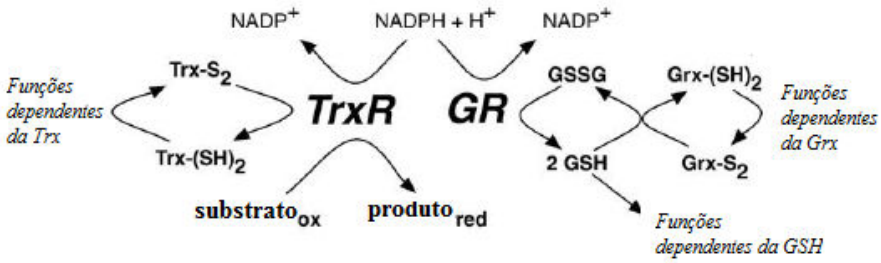

Figura 4 - Comparação entre as reações catalizadas pelo sistema da Trx (à esquerda) e o sistema da GSH (à direita). Tiorredoxina oxidada (Trx-S_2); tiorredoxina reduzida (Trx-(SH_2); glutationa oxidada (GSSG), glutationa reduzida (GSH), glutarredoxina oxidada (Grx-S_2) e glutarredoxina reduzida (Grx-$(SH)_2$) (Adaptado de Arnér, 2009).

3.2.1 Tiorredoxina redutase

A enzima TrxR de mamíferos (em todas as suas isoformas) (E.C.1.6.4.5) é uma selenoflavoproteína homodimérica e membro da família oxidorredutase de dissulfetos de nucleotídios de piridina, a qual também inclui a sua homóloga estrutural, a GR (Zhong *et al.*, 1998). Entretanto, diferencia-se estruturalmente da GR por possuir um resíduo de selenocisteína (Sec) na porção C-terminal (-Gly-Cys^{497}-Sec^{498}-Gly).

O resíduo de Sec na porção C-terminal é essencial para a atividade catalítica e juntamente com a cisteína adjacente (Cys^{497}) formam o sítio ativo selenilsulfeto/selenotiol que recebe elétrons da outra região do sítio ativo (-Cys-Val-Asn-Val-Gly-Cys-) situada na porção N-terminal da outra subunidade dessa enzima dimérica (Sandalova *et al*., 2001). O sítio ativo na porção N-terminal também está presente na estrutura da GR e de outras enzimas dessa família e recebe os elétrons do grupo prostético flavina (FAD) que por sua vez é reduzido pelo NADPH (Zhong et al., 2000; Sandalova *et al.,* 2001). A comparação entre as estruturas das flavoproteínas TrxR e GR pode ser observada na figura 5.

O baixo valor de pKa (5,3) do resíduo de Sec, associado à sua localização facilmente acessível no sítio ativo da enzima, confere à TrxR grande reatividade. Por isso, a TrxR pode atuar na redução de muitos outros compostos além da Trx, seu principal substrato (Arner & Holmgren, 2000; Lillig & Holmgren, 2007).

Além das isoformas citosólica (TrxR-1) e mitocondrial (TrxR-2), uma terceira isoforma foi isolada dos testículos de ratos (TrxR3/TGR) e tem como alvo proteínas que formam componentes estruturais do esperma (Su *et al.,* 2005).

A função antioxidante da TrxR está associada à sua propriedade de reduzir diretamente o peróxido de hidrogênio e hidroperóxidos lipídicos e indiretamente por fornecer Trx reduzida como doador de elétrons para as enzimas peroxirredoxinas (Prxs), as quais atuam na remoção de peróxidos. Além disso, a TrxR regenera diretamente outros compostos antioxidantes como o deidroascorbato, o ácido lipóico, a ubiquinona (Nordberg & Arnér, 2001) e compostos de selênio (Arnér & Holmgren, 2000). A redução do selenito (SeO_3^{2-}) a seleneto de hidrogênio (HSe^-) pela TrxR é importante durante a incorporação co-traducional de selênio à própria TrxR e à estrutura de outras selenoproteínas (Nordberg & Arnér, 2000). Através da redução da proteína Trx, esse sistema também atua na redução de fatores de transcrição que regulam a atividade de genes inflamatórios e apoptóticos, controlando, portanto, vias relacionadas à proliferação e sobrevivência celular (Nordberg & Arnér, 2000; Powis & Montfort, 2001).

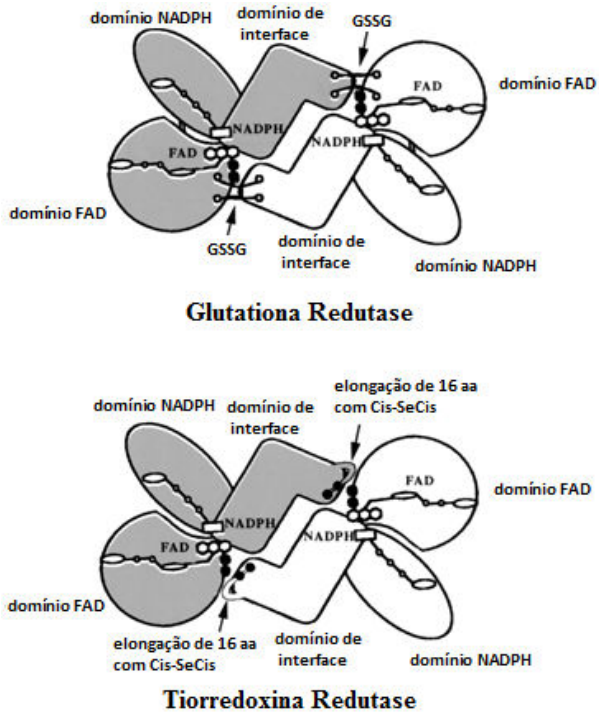

Figura 5: Comparação entre os modelos de estruturas das enzimas TrxR e GR. A GR (mais acima) é representada com os domínios FAD, NADPH e de interface. A GSSG e as cisteínas do sítio ativo (círculos pretos) são também indicadas. A TrxR (abaixo) aparece com uma sequência adicional de 16 aminoácidos que contém os resíduos $SeCis^{498}$ (Sec) e Cis^{497} adjacente à Cis^{59} de uma subunidade e à Cis^{64} de outra subunidade (Adaptado de Zhong *et al*., 2000).

3.2.2 Tiorredoxina

A proteína Trx foi inicialmente descrita em *Escherichia coli* como um cofator da enzima ribonucleotídeo redutase na reação que converte os ribonucleotídeos a desoxiribonucleotídeos durante a síntese do DNA (Laurent *et al.,* 1964). Atualmente, muitos outros substratos são conhecidos tais como as peroxirredoxinas (Prx), a enzima metionina sulfóxido redutase, a proteína quinase-1 reguladora da apoptose (ASK-1), a proteína quinase dependente de AMPc, o receptor de glicocorticóides**,** bem como de fatores de transcrição de genes inflamatórios e supressores de tumor, tais como o fator nuclear κB (NF-kB) e o p53 (Nielsen, 2009), respectivamente. Entre os substratos não protéicos da Trx, estão o H_2O_2, a GSSG e a insulina (Nielsen, 1999).

A Trx humana possui 12kDa e pertence a uma família de proteínas que possuem um motivo estrutural comum – a folha de tiorredoxina - que consiste de uma porção central de quatro ou cinco folhas β-pregueadas circundadas por três ou quatro α-hélices. Apesar de não ser uma enzima, a maioria dos trabalhos

envolvendo a Trx utilizam o termo atividade catalítica para se referir à capacidade dessa proteína reduzir diversas moléculas protéicas e não protéicas, as quais, por sua vez, são consideradas “substratos” da Trx. A região estrutural da tiorredoxina responsável por esta redução é denominada sítio ativo. Ela possui no seu sítio ativo um ditiol conservado mesmo em diferentes espécies, Cys32-Gly-Pro-Cys35, além de três resíduos de cisteína estruturais, Cys62, Cys69 e Cys73. Esses resíduos adicionais tornam a Trx suscetível à oxidação pela geração de um segundo dissulfeto (Cys62-Cys69), causando a perda de sua atividade (Holmgren, 1985; Lillig & Holmgren, 2007). Outras proteínas que fazem parte da mesma família da Trx, são as Grx, GST e GPx. A estrutura tridimensional da Trx-1 humana é mostrada em detalhe na figura 6.

Em condições de estresse oxidativo, a isoforma citosólica (Trx1) pode sofrer translocação nuclear, onde ativa genes de proteínas inflamatórias, ou pode ser exportada das células para o meio extracelular (Rubartelli *et al*., 1992; Hirota *et al*., 1999), onde desempenha funções de citocinas e/ou quimiocinas, estimulando a resposta inflamatória (Arnér, 1999). Uma vez que as TrxRs são a única classe de enzimas conhecidas capazes de reduzir a Trx oxidada, acredita-se que sejam essenciais na regulação da atividade do sistema da tiorredoxina (Mustacich & Powis, 2000).

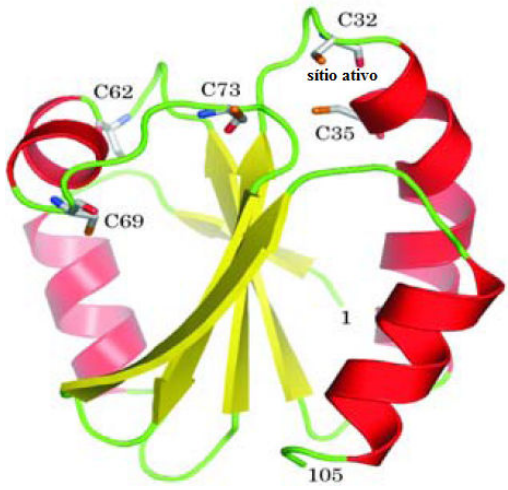

Figura 6- Estrutura tridimensional da tiorredoxina citosólica humana (Trx-1). Em evidência, aparecem os resíduos de cisteína do sítio ativo (C32 e C35) e as cisteínas estruturais (C62, C69 e C73) (Adaptado de Hashemy, 2011).

3.2.3 Vias envolvidas na ativação do sistema da tiorredoxina

Embora o estresse oxidativo seja definido como um desequilíbrio entre os níveis de antioxidantes e pró-oxidantes, em favor dos últimos, um nível mínimo de estresse pode estimular uma cascata de eventos antioxidantes, fazendo com que os mecanismos de defesa celulares respondam independentemente do nível de estresse (Chen *et al.,* 2005).

O fator de transcrição denominado fator 2 relacionado ao NF-E2 (Nrf2), parece ser a principal proteína envolvida na ativação constitutiva ou ativada por estresse oxidativo dos genes situados na região do elemento de resposta antioxidante (ARE) (Chan & Kan, 1999). Sob condições de homeostase, a proteína Keap-1 permanece ligada ao Nrf2 no citosol, facilitando a sua degradação pelo sistema de proteossomos (Dinkova-Kostova *et al.,* 2002). Por outro lado, sob o mínimo estímulo oxidativo, o Nrf2 dissocia-se do Keap-1, é translocado para o núcleo e por heterodimerização com uma proteína Maf, ativa a expressão de genes dependentes de ARE (Alam *et al*., 1999; Ramos-Gomez *et al.,* 2001; Itoh *et al*., 2004). A ativação da via Keap1/Nrf2 é demonstrada na figura 7.

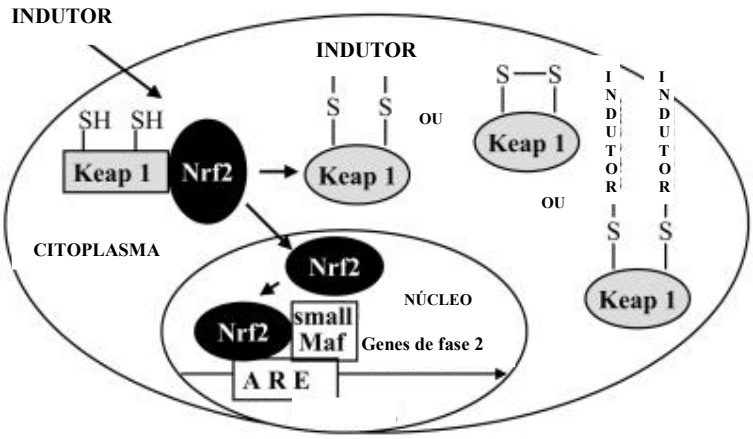

Figura 7- Mecanismo de regulação da resposta de proteínas de fase 2: ativação da via Keap-1/Nrf2. Diversos tipos de modificações do Keap1 por indutores são mostrados (Dinkova-Kostova *et al*., 2002).

Apesar dos mecanismos responsáveis pela ativação da expressão do sistema da Trx não terem sido totalmente esclarecidos, a via do Keap-1/Nrf2 é a mais

conhecida como ativadora desse sistema (Sakurai *et al.,* 2005). Isso porque muitos estudos demonstraram o aumento da expressão da enzima TrxR-1 pela ativação da via Keap-1/Nrf2 por diferentes fatores pró-oxidantes, o que desempenhou importante papel na resposta adaptativa de células a esses insultos oxidativos, protegendo da morte celular (Park *et al.,* 2004; Chen *et al.,* 2005; Sakurai *et al*., 2005).

Além da TrxR-1 e da Trx-1, a expressão de outras proteínas com atividade antioxidante e de destoxificação de xenobióticos também são reguladas pela via Keap-1/Nrf2, como a GR, a glutationa S-transferase (GST) e a heme oxigenase-1 (HO-1) (Jones *et al*., 2007).

3.2.4 O sistema da tiorredoxina como alvo da toxicidade de metais e efeitos celulares relacionados

Apesar de conferir funções tão versáteis à enzima TrxR, principalmente à isoforma citosólica TrxR1, o resíduo de Sec é alvo de muitos compostos eletrofílicos que levam à inibição da enzima e conseqüentemente ao desequilíbrio de todas as funções reguladas por esse sistema. A inibição desse sistema pode desencadear uma série de eventos que culminam com a morte celular (Lillig & Holmgren, 2007). Para a maioria dos inibidores, a inibição é irreversível e permanece até que nova enzima seja sintetizada (Nordberg & Arnér, 2001). Ressalta-se que compostos que inibem a TrxR1, normalmente não inibem ou inibem em extensão bem menor sua homóloga estrutural GR, pois a mesma não possui o resíduo de Sec (Witte *et al*., 2005; Nordberg & Arnér, 2001).

Muitos inibidores da TrxR1 são drogas ou compostos usados no tratamento da artrite reumatóide e câncer, além de outras doenças (Arnér, 2009), uma vez que a TrxR1 está envolvida em processos que garantem a sobrevivência e a proliferação celular, além de estimular processos inflamatórios. Entre os principais inibidores metálicos com essa ação terapêutica, destacam-se os compostos de platina (Witte *et al.*, 2005) e de ouro (Gromer *et al.,* 1998). Da mesma forma, a inibição da TrxR por compostos de rutênio correlaciona-se fortemente com a atividade antitumoral desses compostos (Casini *et al.,* 2008).

Por outro lado, em condições que não envolvam patologias, a inibição da TrxR1 pode aumentar significativamente o estresse oxidativo, dada a importância do

sistema na defesa antioxidante e prevenção da morte celular (Nordberg & Arnér, 2001). Recentemente, Carvalho *et al.* (2008) mostraram que tanto a TrxR1 purificada, como a de cultura de células HeLa foram fortemente inibidas por compostos de mercúrio (concentrações na ordem de nanomolar *in vitro* e de micromolar em células), o que foi imediatamente seguido por morte celular. Posteriormente, a ligação do mercúrio ao resíduo de Sec do sítio ativo da enzima foi confirmada por análise estrutural (Carvalho *et al*., 2011). Por outro lado, o mercúrio estimulou a via Keap1/Nrf2 em monócitos humanos e consequentemente aumentou a expressão da enzima TrxR1 nessas células .(Wataha *et al*., 2008).

A utilização de RNA de interferência (siRNA) da isoforma citosólica TrxR1 aumentou a sensibilidade de células HeLa ao cádmio (Cd) quando expostas a concentrações baixas desse metal (<10 μM), enquanto diminuiu a sensibilidade em concentrações maiores (Nishimoto *et al*., 2006). Esses resultados levaram à conclusão de que a TrxR1 poderia recuperar as células de um dano moderado causado pelo Cd, mas promoveria a morte celular em células com danos mais severos. De fato, apesar da ausência de estudos que demonstrem o efeito direto do Cd sobre a atividade e estrutura da TrxR1, formas dessa enzima com o selênio comprometido por agentes alquilantes metálicos (ex: cisplatina) e não metálicos induzem rapidamente a apoptose, enquanto a enzima nativa não possui essa propriedade (Anestal & Arnér, 2003). Um estudo observou que o Cd também ativou a via do Nrf2, com conseqüente aumento da expressão da TrxR1 em células endoteliais de bovinos (Sakurai *et al*., 2005), o que sugere fortemente um mecanismo de proteção celular contra o estresse oxidativo causado pelo Cd.

Por ser uma proteína sulfidrílica, a Trx também se torna alvo da inibição e/ou oxidação causada por metais. Hansen *et al*. (2006) demonstraram que a oxidação da Trx citosólica (Trx1) e mitocondrial (Trx2) pelo Cd, mercúrio e arsênio desencadeou a apoptose em cultura de células HeLa ao mesmo tempo em que foi observado pouco efeito oxidativo sobre a glutationa (GSH). Posteriormente, Carvalho *et al.,,* (2008) demonstraram que a Trx1 de células HeLa é oxidada por formas orgânicas e inorgânicas de mercúrio. A ligação do mercúrio aos dissulfetos estruturais e do sítio ativo da Trx foi confirmada pela análise de espectro de massas, a qual revelou a presença de complexos muito estáveis entre íon divalente do mercúrio (Hg^{2+}) e a proteína Trx1.

Entretanto, embora a atividade desse sistema antioxidante seja afetada por muitos metais, os efeitos do Pb têm sido pouco estudados. Algum efeito do Pb sobre a TrxR1 foi previamente demonstrado por nosso grupo de pesquisa (Conterato *et al*., 2007), onde a atividade da isoforma citosólica TrxR1 renal aumentou precocemente em ratos expostos ao acetato de chumbo, provavelmente como um mecanismo compensatório à uma suposta inibição inicial da TrxR1, ou mesmo pela ativação da via Keap1/Nrf2, conforme já demonstrado para o mercúrio e outros agentes oxidantes não-metálicos (Park *et al*., 2004; Chen *et al*., 2005; Wataha *et al.,* 2008).

Assim, existem poucas informações sobre o envolvimento do sistema da Trx nas alterações causadas pelo Pb, sobre as quais ainda não se sabe exatamente o seu significado. Baseado nos resultados obtidos em nosso estudo prévio (Conterato *et al.*, 2007) acredita-se que o sistema da Trx possa ser um alvo da toxicidade do Pb e/ou um sistema que atua na defesa contra esse metal.

A realização de estudos que avaliem o efeito do Pb sobre a TrxR1 *in vitro* poderia esclarecer qual efeito esse metal exerceria diretamente sobre a enzima. Além disso, a comparação do efeito do Pb sobre a TrxR1 e sobre sua homóloga estrutural GR, traria informações importantes com relação ao mecanismo e à sensibilidade de ambos os sistemas da Trx e da GSH ao Pb. Posteriormente, a identificação de substâncias endógenas (moléculas sulfidrílicas) e exógenas (quelantes) que protegessem esses sistemas dos efeitos do Pb, poderia fornecer informações importantes sobre os efeitos protetores dessas substâncias no tratamento da intoxicação por Pb em humanos, bem como na prevenção de seus efeitos tóxicos ao organismo humano.

Além disso, uma vez que esse sistema parece responder precocemente à exposição ao Pb, a avaliação da atividade da TrxR em animais e em populações expostas ao metal contribuiria para elucidar a utilidade da enzima como biomarcador de efeito do Pb tanto na exposição aguda como na exposição prolongada. Ressalta-se que os biomarcadores de efeitos são parâmetros medidos em amostras biológicas que devem alterar antes de qualquer outro efeito mais grave atribuído ao agente tóxico ser observado, para que se possam instituir medidas de intervenção visando a proteção da saúde humana, evitando assim, o estabelecimento de dano irreversível. A precocidade do aumento da atividade da TrxR renal, levou-nos também a avaliar o possível uso dessa enzima como biomarcador de efeito em animais e humanos.

4 RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo científico e dois manuscritos, os quais se encontram aqui organizados. Os itens Material e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. As apresentações dos artigos estão baseadas na versão final de impressão (**Manuscritos**) e na versão publicada (**Artigo**). Em anexo (**Anexo 1 e 2**) encontram-se os roteiros para a edição dos manuscritos, que serão submetidos para publicação nas revistas Chemical Research and Toxicology e Basic and Clinical Pharmacology and Toxicology, respectivamente.

4.1 Manuscrito 1

LEAD INHIBITS MAMMALIAN THIOREDOXIN REDUCTASE *IN VITRO* AND INDUCES ITS OVEREXPRESSION IN HEK 293 CELLS: IMPLICATIONS FOR THE TREATMENT OF LEAD TOXICITY

Manuscrito a ser submetido à revista *Chemical Research and Toxicology*

Lead Inhibits Mammalian Thioredoxin Reductase *in vitro* and Induces its Overexpression in HEK 293 Cells: Implications for the Treatment of Lead Toxicity

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Running title: LEAD AFFECTS MAMMALIAN TrxR

Depletion of sulfhydryl-containing molecules and resultant oxidative stress have been implicated in lead toxicity. The thioredoxin system composed of NADPH, thioredoxin reductase (TrxR), thioredoxin (Trx) and glutathione-glutaredoxin system composed of NADPH, glutathione (GSH), glutathione reductase (GR), and glutaredoxin (Grx) are the two major disulfide-dithiol reductases and essential for antioxidant defense and cell redox regulation. Here we show that lead inhibits TrxR and GR *in vitro*, and the inhibitions could be reversible by the endogenous sulfhydryl-containing molecule bovine serum albumin (BSA) and GSH respectively, and by ethylenediamine tetraacetic acid (EDTA). HEK 293 cell-based experiments revealed that lead inhibited TrxR activity only at the highest concentration (60 μM), while it increased GR and glutathione-S-transferase (GST) activity. However, under GSH depletion by L-buthionine-[S,R]-sulfoximine, lead increased TrxR and Trx activity, whereas it did not cause any change in GR or GST activity. The increase of GR and GST activity suggests an early activation of detoxification mechanisms that protect cells against the toxic effects of lead. Meanwhile, the reversibility on the inhibition of thiol-dependent disulfide reductases may provide the basis for the treatment of lead toxicity by EDTA, BSA and GSH.

Keywords: metal toxicity; antioxidant; glutathione; chelating therapy; thioredoxin

Lead is a common environmental and industrial pollutant that still remains a significant public health concern in many countries (*1*). Routes of lead exposure include ingestion of contaminated food and water, soil and inhalation of lead-contaminated dust, but occupational settings are still the main forms of lead exposure (*2*). Lead is a highly neurotoxic agent and also impairs the haemopoietic system, kidneys and liver (*3*).

Two endogenous antioxidant systems include the thioredoxin and glutathione-glutaredoxin system operates as powerful protein disulfide reductases (*4, 5*). Thioredoxin reductase (TrxR) (E.C. 1.6.4.5) is a member of the pyridine nucleotide disulfide oxidoreductase family and possesses high homology to glutathione reductase (GR) (*6*). In addition to the NADPH-dependent reduction of the protein Trx, which is its main substrate, the mammalian TrxR reduces selenium compounds, dehydroascorbate, lipoic acid, and ubiquinone. In addition, mammalian TrxR also acts as an electron donor for peroxiredoxins, which scavenge peroxides (*7*). Mammalian TrxR contains one Sec residue in its C-terminus redox-active site (-Gly-Cys^{497}-Sec^{498}-Gly), which is not found in its homolog GR (*5, 7*). The easily accessible Sec residue may provide a strong affinity for metallic complexes and ions, which makes TrxR a potential target for the inhibition by drugs and metals (*8*).

Glutathione (GSH) depletion and the inhibition of sulfhydryl-containing enzymes with consequent reactive oxygen species (ROS) generation are implicated in lead toxicity (*9, 10*). GSH is an important antioxidant that accounts for up to 90% of the total low molecular weight thiols in cells and its intracellular concentration ranges from 0.5 to 10 mM (*11, 12*). It protects cells towards xenobiotics, such as heavy metals through mechanisms that include metal chelation and detoxification as well as direct interaction of its sulfhydryl group with ROS generated by the metal (*12, 13*).

Although it is well established that GSH system is involved in the cellular mechanisms of lead detoxification, little is known about the relationship of Trx system with lead toxicity in

cells (*14*). Some effect of lead on TrxR activity was recently reported, where rat kidney TrxR activity increased after *in vivo* exposure to the metal (*14*), presumably as a protective mechanism. TrxR is widely expressed and up-regulated by several stimuli via activation of Keap1/Nrf2 pathway, as previously demonstrated for cadmium (*15*) and it is recognized to play a significant role in the cell defense against oxidative stress (*16*). However, the role of Trx system in lead-induced changes in cells was still not established.

Ethylenediamine tetraacetic acid (EDTA) chelation therapy is approved by the U.S. Food and Drug Administration as a choice treatment for lead poisoning. Its effectiveness is based on rapid mobilization and excretion of lead, but its efficacy to recover lead-changed biochemical parameters is still a topic that requires further investigations. Based on the evidence that lead can interact with thiol groups of proteins and interferes in cellular antioxidant response, we hypothesized that Trx system could be involved in the cellular biochemical changes induced by lead. Then, *in vitro* studies with both purified and cellular TrxR and Trx were carried out to investigate how is the Trx system affected by lead and what is its importance in protection against lead toxicity. Our data revealed that the inhibition of purified TrxR and GR activities were recovered by endogenous sulfhydryl compounds, and exogenous EDTA was more effective to recover TrxR than GR activity. The studies revealed that these thiol dependent systems are involved in the mechanisms of defense against lead toxicity and give the implications for the treatment of lead toxicity.

Experimental Procedures

Materials. Recombinant rat TrxR and double mutant C62/C73S human Trx were prepared as previously described (*17, 18*). Yeast GR, glutathione disulfide (GSSG), NADPH, GSH, bovine serum albumin (BSA), DL-dithiothreitol (DTT), 5,5’ – dithiobis-(2-nitrobenzoic acid) (DTNB), juglone (5-OH-1,4-naphthoquinone), 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT), cysteine, L-buthionine-[S,R]-sulfoximine (BSO), lead acetate ($PbAc_2$), lead nitrate and cadmium chloride ($CdCl_2$) were from Sigma. Stock solutions of heavy metals were prepared as 100 mM stocks in MilliQ water. Tris base 99.2% purity titration and <0.0005% heavy metal was supplied by Calbiochem.

DTNB Reduction Assay of TrxR Activity. To measure the NADPH-dependent TrxR-catalyzed DTNB reduction activity, 50 nM recombinant rat TrxR (EDTA-free) was first reduced with 200 μM NADPH in 50 mM Tris-HCl buffer, pH 7.5, using 96-well plates. Subsequently, $PbAc_2$ and $CdCl_2$ of various concentrations were added for different time points at room temperature. Then, TrxR activity was determined as described elsewhere (*19*) upon adding 100 μl of Tris-HCl buffer containing 200 μM NADPH and 5 mM DTNB (final concentration). The DTNB reduction was immediately followed at 412 nm and determined as the linear increase in absorbance over the initial 2 min using a VersaMax microplate reader (Molecular Devices). For studies on the protection of TrxR inhibition by BSA, the NADPH-reduced TrxR was preincubated with lead or cadmium for 10 min at room temperature. Then, 1 mg/ml of BSA was added along with the Tris-HCl buffer containing NADPH and DTNB (as described above) and readings were immediately taken at 412 nm. BSA, which is an extracellular protein, was used instead of GSH, because the latter reacts with DTNB substrate and interferes in the color reaction.

To assess the EDTA effect on lead inhibited TrxR, 100 μl of TE buffer (Tris-HCl 50 mM, 1 mM EDTA, pH 7.5) containing NADPH and DTNB was added instead of Tris-HCl buffer, after 10 min of preincubation with different concentrations of organic ($PbAc_2$) or inorganic (lead nitrate) lead compounds. Then, the absorbance was followed at 412 nm.

Juglone Reduction Assay of TrxR Activity. Recombinant rat TrxR (EDTA-free) was reduced by 250 μM NADPH in 50 mM Tris-HCl buffer (pH 7.5) and preincubated with different concentrations of $PbAc_2$ for 5 minutes at room temperature. Juglone (50 μM) was

added to the preincubation mixture (final volume 500 µl) and NADPH-dependent TrxR-catalyzed juglone reduction activity was determined at 340 nm as the linear change in absorbance over the initial 2 min in an Ultrospec 3000 spectrophotometer.

Yeast GR Activity Determination. $PbAc_2$ of different concentrations was added to the 2 nM GR and 200 µM NADPH and preincubated for 5 minutes. Then, 50 mM Tris-HCl, pH 7.5, containing GSSG (final concentration 1 mM) was added to the preincubation mixture to a final volume of 500 µl and the absorbance was followed at 340 nm in an Ultrospec 3000 spectrophotometer. To evaluate the possible protective effect of GSH and EDTA on the GR inhibition by lead, 1 mM GSH or 1 mM EDTA was added along with Tris-HCl buffer and GSSG after GR preincubation with $PbAc_2$ and at the time of activity measurement. The GR activity was calculated based on the linear decrease of absorbance over the initial 5 min.

Calculation of the Inhibitory Concentration 50 (IC_{50}). IC_{50} for enzymes inhibition by metals (concentration inhibiting 50% of enzyme activity) was determined by non-linear regression analyses using GraphPad Prism software version 4.0.

Cell Culture. Embryonic kidney (HEK 293) cells were grown in RPMI medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37ºC in humidified atmosphere of 5% CO_2.

Cell Viability Assay. Cells were seeded into 96-well plates at a density of 2.5 x 10^3/well and allowed to grow for 24h. Then, cells were incubated in the growing medium with different concentrations of $PbAc_2$ for 24, 48 and 72 h and cell viability was determined by the MTT assay. After 4 h of incubation with MTT (final concentration 400 µg/ml) at 37ºC, MTT was metabolized to formazan salt by viable cells and dissolved by the addition of dimethyl sulfoxide/glycine buffer (pH 10.5) (ratio 4:1). Cell viability, which was proportional to the amount of formazan formed, was determined by measuring the absorbance at 550 nm.

To evaluate the effect of GSH depletion on cell viability, cells were preincubated with 100 μM BSO 24 h before lead treatment. Then, medium was changed and cells were treated with different concentrations of $PbAc_2$ and incubation was conducted for further 24, 48 and 72 h followed by MTT addition.

Determination of Non-protein Thiol (NPSH) Groups in HEK 293. The content of non-protein thiols (NPSH) after lead treatment was determined according to Ellman (*20*) with modifications for using in 96-well plates. After incubation with different concentrations of $PbAc_2$ for 24 and 48 h, cell pellets were obtained by washing with potassium phosphate buffer and centrifugation at 1,000 rpm for 5 min. Then, cell pellets were deproteinized with 2% TCA in 0.5 M potassium phosphate buffer (final concentration), pH 7.4 and centrifuged during 10 min at 3,000 rpm. Supernatants were collected and added to each well containing 1 M potassium phosphate buffer pH 7.4 and DTNB (final concentration 0.5 mM) in a total volume of 200 μl. The absorbance was read at 412 nm against a blank that contained the same amount of DTNB, but without sample. Results were calculated using a standard curve of cysteine and expressed as the percentage of non-treated control.

Preparation of Cell Lysates. Cells (1×10^6) were plated onto 100-mm plates, and when cell confluence reached ~70%, cells were incubated with a fresh volume of growing medium (described in the cell culture subsection) containing different concentrations of $PbAc_2$ for 24 and 48 h. Whole cell lysates were obtained by lysing cell pellets (composed of attached and floating cells) in lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM NaF, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.5% Triton X-100 containing freshly added protease inhibitor mixture) (Roche Applied Science). Before using, lysates were precleared by centrifugation at 13,000 g for 6 min, followed by protein content determination using a modified Lowry assay (Bio-Rad) according to the manufacturer's manual.

Enzyme Activity Determination in Cell Lysates. TrxR activity was determined using a previously published end-point Trx-dependent insulin reduction method, applied to microtiter plates (*19*). Protein cell lysate (25 µg) was incubated with the reaction solution containing 0.3 mM insulin, 660 µM NADPH, 2.5 mM EDTA and 5 µM human Trx (C62S/C73S) in 85 mM HEPES buffer, pH 7.6, for 40 min at 37ºC, in a final volume of 50 µl. Control wells containing all components except Trx were incubated and treated in the same manner. The reaction was then stopped by addition of 250 µl of 1 mM DTNB and 240 µM NADPH in 6 M guanidine hydrochloride, 200 mM Tris-HCl, pH 8, and absorbance was recorded at 412 nm using a VersaMax microplate reader. TrxR activity was expressed as percent of control absorbance. To determine Trx activity, the same procedure for TrxR activity assay was followed, except that the reaction solutions contained 600 nM recombinant rat TrxR instead of 5 µM human Trx.

GR activity in cell lysates was determined in cuvettes according to a published method (*21*) with modifications. In short, 100 µg of protein cell lysate was mixed to master mixture containing 1 mM GSSG in 50 mM Tris-HCl pH 7.5 buffer (total volume 450 µl). Then, 50 µl NADPH (final concentration 200 µM) was added and the decrease of absorbance was followed at 340 nm using an Ultrospec 3000 spectrophotometer. The GR activity was calculated based on the linear change of absorbance during the initial 10 min.

Glutathione S-transferase (GST) activity was assessed according to the reference (*22*) using 96-well plates. Firstly, 100 µg of protein cell lysates was added to each well that previously contained 2.5 mM GSH in 100 mM potassium phosphate buffer, pH 7. Then, 1-chloro-2, 4-dinitrobenzene (CDNB, final concentration 1 mM in a total volume of 200 µl) was added and the absorbance change was determined during the initial 10 min at 340 nm using a VersaMax microplate reader (Molecular Devices).

Western blot. $PbAc_2$ and/or BSO-treated HEK 293 were washed with potassium phosphate buffer twice, lysed and precleared by centrifugation followed by protein content determination as described above. The cell lysates (25 µg) were incubated with SDS-loading buffer and 50 mM DTT at 95ºC for 5 min and separated on a 12% [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) gel with MES running buffer. TrxR was detected with rabbit anti-TrxR1 antibody at 1:1,000 dilutions and Trx was detected by goat anti human Trx at 1:500 dilutions, followed by the detection with Western Lightning Cheluminescence Reagent (PerkinElmer) according to the manufacturer's instructions.

Statistical analysis. Data were analyzed using analysis of variance (ANOVA) followed by Duncan's post hoc test when necessary. Activity of TrxR and Trx were analyzed by Student`s t test. Differences were considered significant when $p<0.05$. Results were expressed as mean ± SD.

Results

Both Lead and Cadmium Inhibit TrxR Activity *In Vitro*. The inhibitory effects of lead and cadmium on TrxR activity were time-independent (Figure 1A, B) and were observed as early as within 10 seconds (data not shown). On the other hand, the inhibition of TrxR by lead and cadmium was dose-dependent (Figure 1A, B) and was observed from 0.2 µM $PbAc_2$ and from 0.05 µM $CdCl_2$ onwards. Despite this difference, the IC_{50} values for these compounds were similar: 0.27 µM (confidence interval 0.25-0.29) for $PbAc_2$ and 0.20 µM (0.17-0.26) for $CdCl_2$. Since no difference was observed in the inhibitory effect among the different incubation times, these IC_{50} values were calculated as the average of all incubation times presented in Figure 1 for lead and cadmium.

Inhibition of TrxR by lead is not dependent on the reaction with Sec of TrxR. Besides of the DTNB reduction, the effect of $PbAc_2$ on TrxR activity was also evaluated using

juglone as a Sec-independent substrate (*23*). In this assay we evaluated only the preincubation with $PbAc_2$ for 5 min, once TrxR activity inhibition by lead observed in the DTNB assay was rapid and time-independent (Figure 1A). As shown in Figure 2, the TrxR inhibition by $PbAc_2$ was also observed in the juglone reduction assay, although the IC_{50} (0.37 μM) was a slightly higher than the IC_{50} value detected in the DTNB reduction assay (0.27 μM). It suggests that the Sec of TrxR did not play predominant role in the reaction with lead, but other active site thiols of TrxR seem to be involved.

TrxR Inhibition by Lead and Cadmium Is Reversible by BSA and EDTA Chelating Agent *In Vitro*. Recovery of metal-inhibited TrxR activity by BSA or EDTA was assessed by the DTNB reduction assay, upon adding 1 mg/ml BSA solution or TE buffer (Tris-HCl 50 mM containing 1 mM EDTA, pH 7.5), after preincubation with lead or cadmium. BSA was able to recover both lead- and cadmium-inhibited TrxR to a similar extent up to 0.5 μM metal concentration (82% recovery for lead inhibited TrxR and 68% for cadmium-inhibited TrxR) (Figure 3A, B). However, BSA recovered TrxR inhibition by 1 μM $CdCl_2$, but not by 1 μM $PbAc_2$. On the other hand, EDTA completely recovered TrxR inhibition up to 4 μM $PbAc_2$ (Figure 4A), and partially recovered enzyme inhibition by 5-10 μM $PbAc_2$ (Figure 4B).

To determine if TrxR activity could also be inhibited by an inorganic form of lead we evaluated the effect of lead nitrate on the enzyme activity. Lead nitrate inhibited TrxR with a potency (IC_{50}= 0.32 μM, confidence interval 0.28-0.38, Figure 5) similar to that of the organic compound $PbAc_2$ (Figure 1A). In addition, both EDTA and BSA completely recovered TrxR inhibition up to 2 μM lead nitrate (Figure 5).

GR Inhibition by Lead Is Reversible by GSH and EDTA Chelating Agent *In Vitro*. Moreover, we evaluated the effect of $PbAc_2$ on the activity of GR, which is a structural homolog of mammalian TrxR (Figure 6). The inhibitory effect of $PbAc_2$ on GR activity was dose-dependent and stronger than that on TrxR activity, once GR inhibition was observed at

0.06 μM $PbAc_2$ (44% remaining activity), whereas TrxR inhibition occurred just from a $PbAc_2$ dose 4 times higher, i.e., 0.20 μM (84% activity, Figure 1A). The IC_{50} value for yeast GR inhibition by lead (0.048 μM) was 5.6 times lower than for TrxR activity inhibition. GSH was more effective than EDTA in recovering lead inhibited GR, since it was observed that GSH recovered from 23 to 45% GR activity, while EDTA recovered from 0 to 25%. In addition, lead-inhibited TrxR activity is more easily recovered by EDTA as compared to GR, so that the percent recovery was higher for TrxR (Figure 4A, 4B and 5).

Lead Acetate Does Not Affect HEK 293 Cell Viability. After incubation of HEK 293 cells with different concentrations of $PbAc_2$ (with or without previous incubation with 100 μM BSO) for 24, 48 and 72 h, cell viability was determined by the MTT assay. BSO (100 μM) caused a decrease of 60% in NPSH levels in HEK 293 cells, without any *per se* effect on cell viability (data not shown). As shown in Figure 7A, $PbAc_2$ did not affect cell viability of HEK 293 cells even at concentration as high as 40 μM. In addition, the pre-incubation with 100 μM BSO for 24 h before adding $PbAc_2$ caused a slight, but statistically significant decrease in cell viability (~67% viable cells remained) after 48 h incubation with 40 μM $PbAc_2$. However, cell viability was recovered after 72 h of incubation with this same $PbAc_2$ concentration (88% viable cells remained) (Figure 7B). Lead cytotoxicity was also assessed by the trypan blue and the neutral red uptake assays following the same incubation protocol for $PbAc_2$ and BSO as used in the MTT assay. In agreement, lead did not affect cell viability in these assays even at 60 μM $PbAc_2$ (data not shown).

Lead Causes a Biphasic Effect on NPSH Levels in HEK 293 Cells. NPSH is mostly composed of GSH in the kidney and its levels were measured with DTNB. NPSH levels increased 24 h after exposure to 10-60 μM $PbAc_2$, but no change was observed 48 h after exposure at any concentration evaluated (Figure 8). These results seem to indicate an early defense response against lead, which disappeared at 48 h after lead exposure, probably

because of GSH consumption to detoxify lead. The role of GSH in the detoxification of lead has been previously reported and the enhancement of its levels appears to occur independently on oxidative stress and anticipates renal histopathological changes caused by lead (*24*).

Lead Increases TrxR and Trx activity and expression in HEK 293 Cells under GSH Depletion. TrxR and Trx activity was measured in cell lysates with the endpoint insulin reduction assay. TrxR activity was strongly inhibited by 60 μM $PbAc_2$ after 24 h of incubation, but no effect was observed at lower lead concentrations (Figure 9A). Interestingly, TrxR activity was increased by 10 μM $PbAc_2$ onwards when GSH levels were previously depleted by 100 μM BSO (Figure 9A). Similarly, Trx protein activity was not affected when cells were exposed only to lead, but a significant increase occurred under GSH depletion (Figure 9B). As showed in Figure 9C, although TrxR activity was inhibited by 60 μM of $PbAc_2$ (Figure 9A) the protein expression level remained unchanged compared to the untreated control. In agreement with Trx activity results, Trx expression did not change after lead treatment (Figure 9C). Consistent with activity assay, TrxR and Trx expression increased upon the treatment of lead under the condition of GSH depletion by BSO for 24 h before (Figure 9D).

Lead Increases GR and GST Activity in HEK 293 Cells. $PbAc_2$ at concentrations of 40 and 60 μM increased GST activity 24 and 48 h after exposure (Figure 10A), but GR activity was increased only 48 h after exposure (Figure 10B) at the same lead concentrations. Interestingly, GSH depletion (pre-incubation with BSO) abolished the increase of GST and GR (Figures 10A and B) activity by lead.

Discussion

This study investigated the effects of lead and cadmium on purified TrxR and the related flavoprotein GR, as well as the protection of EDTA chelating agent and physiological thiols like GSH and BSA against enzyme inhibition. The experiments were extended to cell

cultures, to better elucidate and compare the role of both GSH and Trx systems in lead poisoning. Cadmium toxicity in cells and its relationship with Trx system was not assessed in the current study once it was already reported that cadmium increases TrxR expression in cell culture and that gene silencing of TrxR increases cell susceptibility towards cadmium (*25, 26*). However, no previous study investigated if cadmium exerts a direct effect on purified mammalian TrxR activity.

Here we demonstrated that TrxR inhibition by lead was detected both in the DTNB and in the juglone reduction assays. Since juglone is a Sec-independent substrate, we suggest that lead does not target the Sec residue in the active site (*27*), but other site(s) in the enzyme (*28, 29*) probably involving Cys residues. The strong inhibitory effect of lead on GR, which is structurally and functionally close to TrxR, but lacking Sec residue in its active site (*6, 27*) strongly supports this hypothesis. In fact, lead is widely recognized by irreversibly binding to the sulfhydryl-dependent δ-aminolevulinate dehydratase enzyme (*9*) and may affect the antioxidant functions by inhibiting antioxidant enzyme activity in humans (*25, 26*).

The influence of sulfhydryl-containing molecules on TrxR and GR inhibition was evaluated to verify if the inhibitory effect of lead or cadmium on these enzymes could be reversible in physiological conditions. The protective effects observed for both BSA and GSH against enzymes inhibition by metals indicate that physiological levels of thiol-containing molecules are important in the protection of TrxR and GR against toxicologically relevant cadmium and lead levels.

The studies on the protection against TrxR and GR inhibition by lead were also extended to the exogenous EDTA chelating agent, once lead poisoning treatment in humans is primarily based upon chelation therapy. Despite its side effects (*30, 31*), EDTA is still considered the most effective chelating agent for lead intoxication. In this study, 1 mM EDTA recovered TrxR activity much more than GR activity at all lead concentrations, even though

this protection was not complete at concentrations higher than 4 μM lead (Figure 4). Moreover, EDTA concentrations as low as 1-10 μM also recovered TrxR activity (data not shown). Intravenous EDTA dosage recommended for chelation therapy ranges from 700-3,500 mg for an average-sized adult. This dosage would yield 0.5-2.4 mM EDTA in blood, which is similar to the concentration used in this study. The ability of EDTA to remove lead already bound to TrxR and GR is a very relevant finding, once chelating agents generally are able to chelate only free metal, but not metal bound to molecular targets. EDTA protection against TrxR inhibition by lead may be involved in the benefits of this chelation therapy in humans.

Cell experiments were conducted to verify whether the results obtained *in vitro* with purified enzymes could also be observed in intact cells. Although lead toxicity to living systems is well known (*32, 33*), lead did not effectively affect HEK 293 cell viability. Similarly, no toxicity was observed in HeLa or MCF-7 cells exposed to the same $PbAc_2$ concentrations (data not shown). Indeed, previous data regarding lead toxicity in cell culture are contrasting, once cells exposure to the same concentrations of lead during the same time period revealed different degrees of cytotoxicity in distinct studies (*34, 35*). Furthermore, cellular susceptibility to lead generally seems to be lower than other toxic metals (*36*), including mercury (*37*).

The absence of cytotoxicity in this study was accompanied by the lack of inhibition of GR and by the inhibition of TrxR activity only after cell exposure to the highest lead concentration, which contrasts with the strong inhibition of purified TrxR and GR *in vitro*. This may be explained by the free Pb^{2+} ions being chelated or complexed in the cell culture medium, which may reduce the intracellular lead concentration available for reactions. Cell culture medium had fetal bovine serum that is rich in proteins, mainly BSA that could reduce lead availability and/or recover lead-inhibited enzymes. As shown here *in vitro*, GR and TrxR

inhibition by lead can be rapidly reversed by GSH and BSA, respectively. In addition, increased NPSH levels after 24 hours of lead exposure may have protected the enzymes from lead inhibition and helped to avoid its cytotoxic effects in HEK 293 cells.

GR, GST and TrxR are phase II proteins involved in the detoxification of xenobiotics and antioxidant defense in cells, which are regulated at least in part by Keap1/Nrf2 antioxidant response pathway (*38*). TrxR overexpression is induced by oxidative stress-preceded activation of Keap1/Nrf2 pathway (*16*), even though this activation was reported even without an exogenous oxidative insult (*39*). It was recently reported that lead increases the expression of mouse cytochrome P450 2A5 in primary hepatocytes through the activation of Nrf2 pathway, but the mechanisms involved were not clearly demonstrated (*40*). As lead may exert its toxic effects by binding to the sulfhydryl moieties of proteins and cellular GSH (*41*), lead might interact with thiol residues in Keap1 and, consequently, activate target genes such as antioxidant response elements (ARE) in nucleus.

The induction of GR and GST but not TrxR and Trx upon the treatment of lead alone (Figure 9 and 10) indicates that glutathione system is critical for the preliminary lead detoxification. However, in more critical oxidative stress conditions like those involving GSH depletion, the Trx system appears to be more important to protect against lead toxicity. GR protects cells from lead toxicity by providing reduced GSH, whereas GST detoxifies lead through its conjugation with GSH and also reduce lipid hydroperoxides through its Se-independent glutathione peroxidase activity (*42*). Suzuki *et al.* (*43*) showed that gene silencing of cytosolic TrxR increased HeLa cells susceptibility towards low cadmium levels suggesting a protective role of TrxR against cadmium cytotoxicity. Although the mechanism involved in the cytoprotection provide by TrxR against heavy metals has not been established yet, as an antioxidant enzyme, TrxR may scavenge ROS produced by lead via Trx and peroxiredoxins or even directly (*44*).

Very interestingly, under the condition of GSH depletion by BSO, TrxR and Trx activity and expression increased after lead exposure, whereas GR and GST activity remained unchanged. The different behavior of TrxR and Trx compared to GR and GST towards lead might implicate that protective mechanisms other than the activation of Keap1/Nrf2 pathway regulate GR and GST (*43, 45, 46*). Another explanation for the suppression of the increase of GST and GR activity upon BSO treatment may be due to the GSH depletion influence on GR on GST stability.

In general, our results showed that TrxR is inhibited by lead and cadmium *in vitro*, but it is less sensitive to lead inhibition than GR. Lead inhibition of TrxR is independent on the Sec in the C-terminal active site and is reversible by the endogenous sulfhydryl-containing molecule BSA and by EDTA chelating agent. Induction of GR and GST enzymes without cytotoxicity point them as an early event in lead poisoning, which protects cells against the toxic effects of lead. However, this protection seems to be predominantly provided by the Trx system when GSH is depleted.

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Figure legends

Figure 1. Inhibition of TrxR activity by (A) $PbAc_2$ and (B) $CdCl_2$ *in vitro*: time-independent effect. Recombinant rat TrxR was preincubated with the indicated concentrations of both compounds for different time points. Then, TrxR activity was measured by the DTNB reduction assay (n=3). Results are percent of activity without lead or cadmium addition. All data points are means ± SD. *Bars under the line are different ($p<0.05$) from control (without metal) at the same time point.

Figure 2. Similar inhibition of TrxR activity by $PbAc_2$ *in vitro* in the juglone reduction assay. Recombinant rat TrxR was preincubated with different concentrations of $PbAc_2$ for 5 min and activity was measured upon adding juglone. Results are percent of TrxR activity without $PbAc_2$ addition.

Figure 3. Effect of BSA on the inhibition of TrxR by (A) $PbAc_2$ or (B) $CdCl_2$ *in vitro*. BSA (1 mg/ml) was added after 10 min of preincubation with lead or cadmium. TrxR activity was assessed by the DTNB reduction assay (n=3). Results are expressed as % of activity without lead, cadmium or BSA addition (means ± SD). *Different from TrxR activity without BSA at the same metal concentration ($p<0.05$).

Figure 4. Effect of EDTA on TrxR inhibition by lead *in vitro*. EDTA (1 mM final concentration) was added after 10 min of preincubation with (A) 0, 2 and 4 μM or (B) 0, 5 and 10 μM lead acetate. TrxR activity was measured by the DTNB reduction assay (n=3). Results are percent of activity without lead or EDTA. *Different from activity without EDTA at the same metal concentration ($p<0.05$).

Figure 5. Inhibition of TrxR activity in the presence of lead nitrate *in vitro*. BSA (1 mg/ml) and EDTA (1 mM) were added after 10 min of preincubation with lead. TrxR activity was measured by the DTNB reduction assay (n=3). Results are expressed as percent of activity without lead (means ± SD). *Lead+BSA and Lead+EDTA were different from Lead at the same lead concentration ($p<0.05$).

Figure 6. Inhibition of NADPH-reduced GR activity in the presence of $PbAc_2$ *in vitro*. Results (means ± SD) are % of activity without lead, EDTA or GSH (n=3). *Lead+GSH and Lead+EDTA were different from Lead at the same lead concentration ($p<0.05$). #Lead+GSH was different from Lead+EDTA and Lead at the same lead concentration ($p<0.05$).

Figure 7. Effect of lead on HEK 293 cell viability. HEK 293 cells were exposed to $PbAc_2$ (A) in the absence or (B) in the presence of a previous incubation with BSO 24 h before the addition of different concentrations of $PbAc_2$. After 24, 48 and 72 h of lead exposure, cell viability was assessed by the MTT assay. Assays were performed on three separate experiments and bars represent means ± SD. *Different from control without lead ($p<0.05$).

Figure 8. Effect of lead on NPSH levels in HEK 293 cells. HEK 293 cells were exposed to different concentrations of $PbAc_2$ during 24 and 48 h. Then, NPSH levels were determined by titration with DTNB after deproteinization with 2% TCA (final concentration).

Figure 9. Effect of lead on TrxR and Trx activity and expression in HEK 293 cells. Cells were treated with $PbAc_2$ or with 100 μM BSO + $PbAc_2$ at the indicated concentrations for 24 h. Cell lysates were collected for determination of TrxR (A) and Trx activity (B), which were expressed as % of untreated control activity. Data are means ± SD of three independent

experiments. *Different from control (without lead and BSO) ($p<0.05$). (C) HEK 293 cells were incubated for 24 h with $PbAc_2$. (D) HEK 293 cells were preincubated for 24 h with 100 μM BSO and then treated with $PbAc_2$ at different concentrations for additional 24 h. After 24 h of lead treatment, HEK 293 cells were washed with PBS buffer, lysed and precleared by centrifugation. Then, protein samples (25 μg) were separated on 12% bis-Tris gel with MES running buffer and TrxR and Trx were detected with rabbit anti-TrxR and goat anti-Trx antibodies, respectively.

Figure 10. Effect of lead and/or GSH depletion on GST and GR activity in HEK 293 cells. Cells were treated with $PbAc_2$ or with 100 μM BSO + $PbAc_2$ at the indicated concentrations for 24h and 48h. Cell lysates were collected for determination of GST (A) and GR activity (B), which were expressed as % of untreated control activity. Data are means ± SD of three independent experiments. *Different from control (without lead and BSO) ($p<0.05$). §Different from 60 μM lead at 24h ($p<0.05$).

Fig. 1

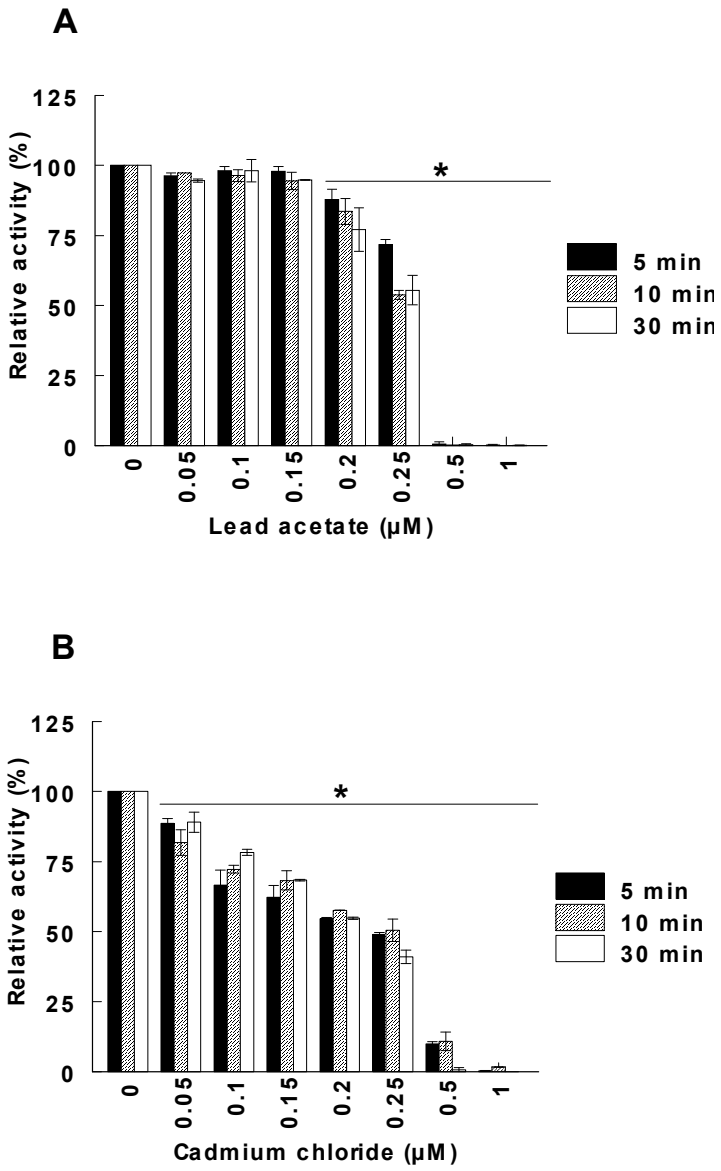

A
125
100
75
50
25
0
Relative activity (%)
*
5 min
10 min
30 min
0
0.05
0.1
0.15
0.2
0.25
0.5
1
Lead acetate (μM)
B
125
100
75
50
25
0
Relative activity (%)
*
5 min
10 min
30 min
0
0.05
0.1
0.15
0.2
0.25
0.5
1
Cadmium chloride (μM)

Fig. 2

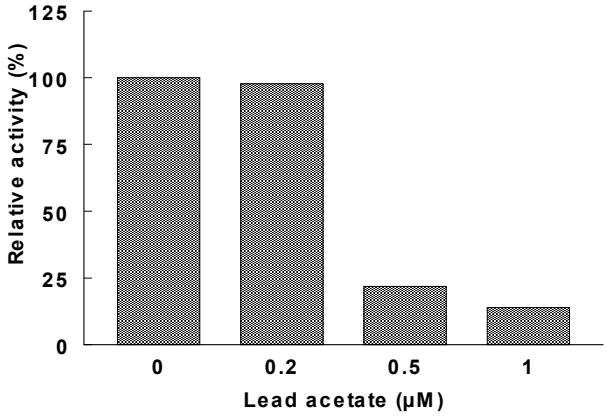


Fig. 3

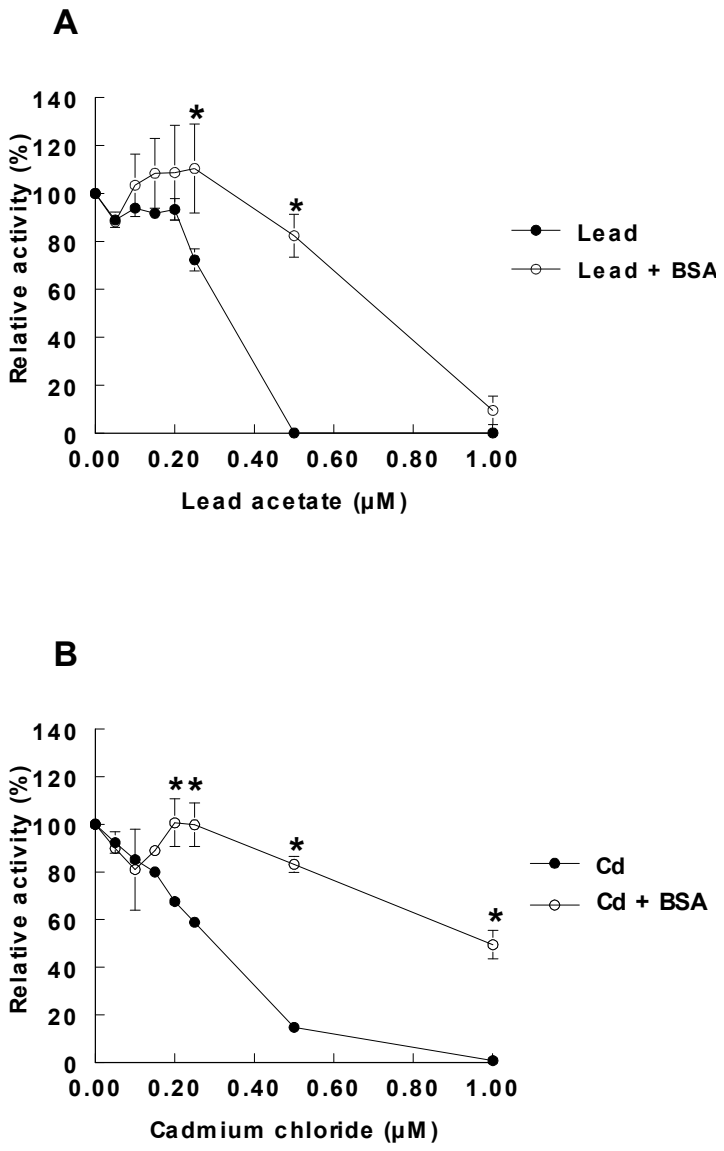

A
Relative activity (%)
140
120
100
80
60
40
20
0
0.00
0.20
0.40
0.60
0.80
1.00
Lead acetate (μM)
Lead
Lead + BSA
*
*
B
Relative activity (%)
Cadmium chloride (μM)
Cd
Cd + BSA
**
*
*

Fig. 4

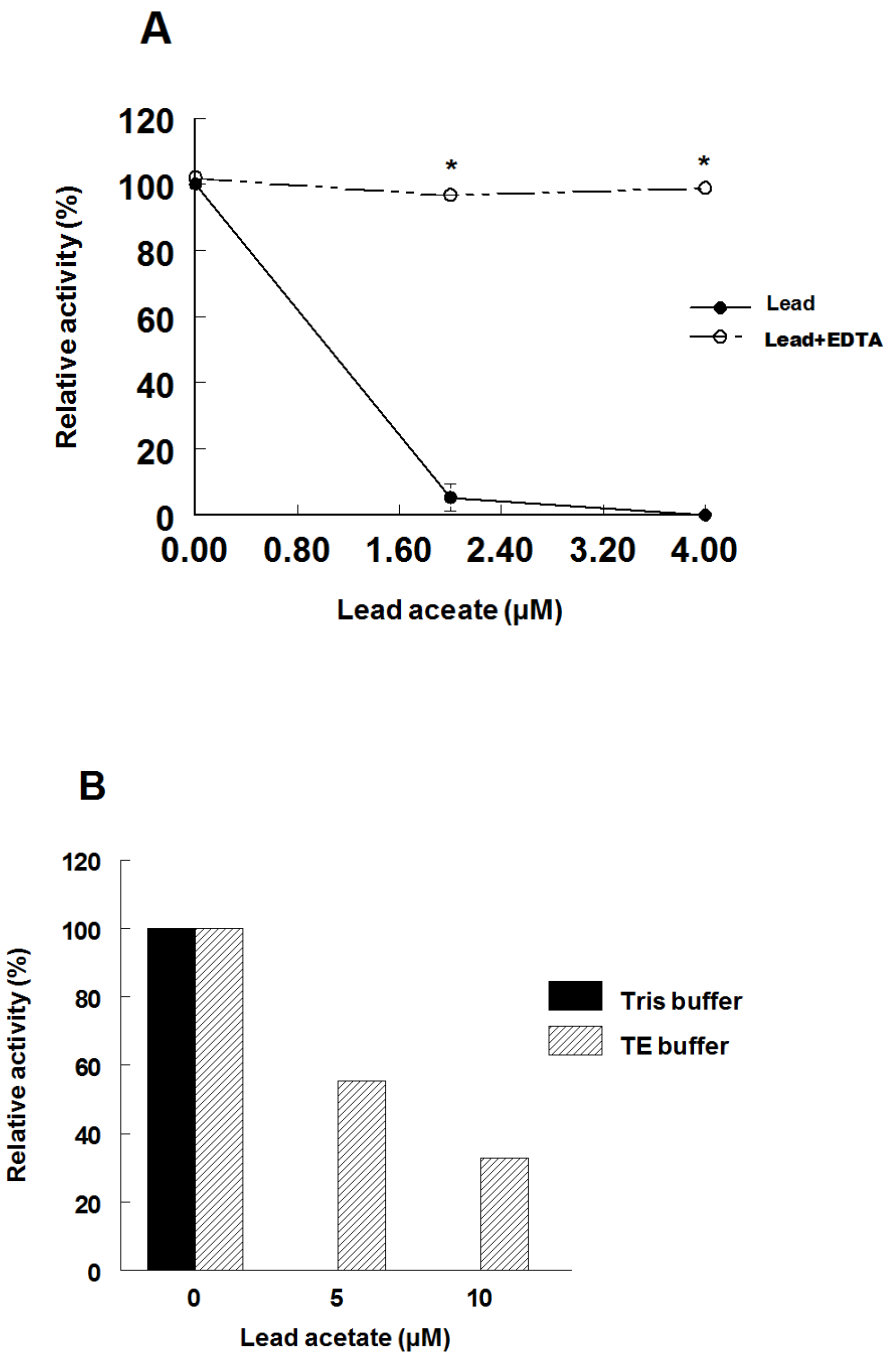

A
120
100
80
60
40
20
0
Relative activity (%)
*
*
Lead
Lead+EDTA
0.00
0.80
1.60
2.40
3.20
4.00
Lead aceate (μM)
B
120
100
80
60
40
20
0
Relative activity (%)
Tris buffer
TE buffer
0
5
10
Lead acetate (μM)

Fig. 5

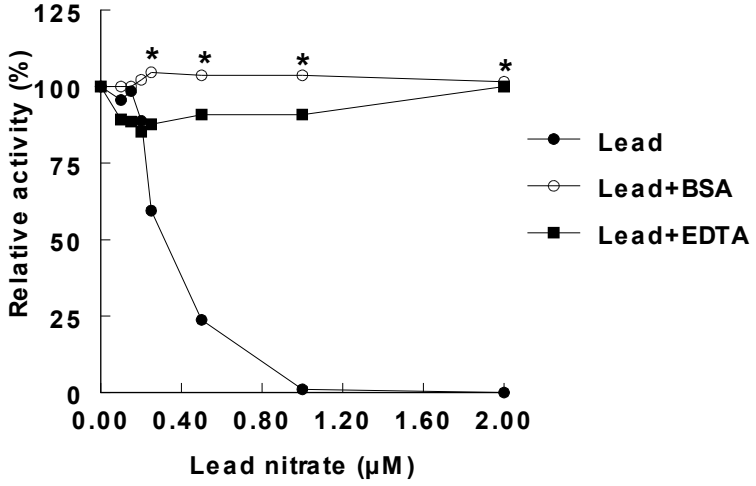

125
100
75
50
25
0
Relative activity (%)
0.00
0.40
0.80
1.20
1.60
2.00
Lead nitrate (μM)
Lead
Lead+BSA
Lead+EDTA
*
*
*
*

Fig. 6

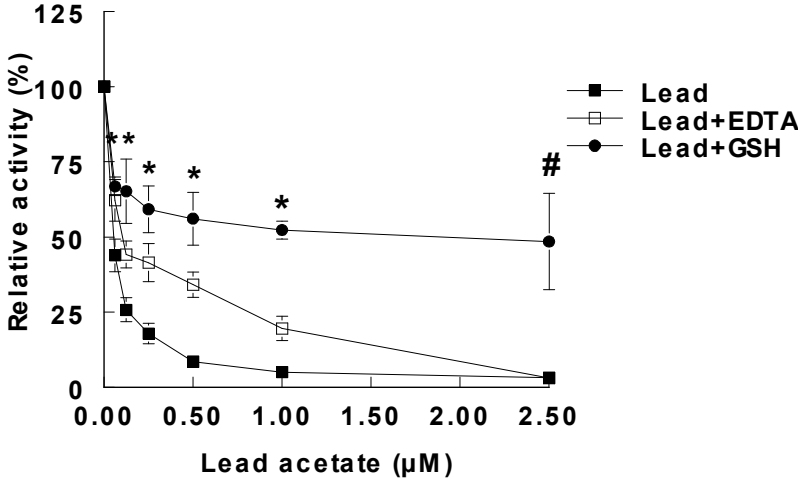

125
100
75
50
25
0
Relative activity (%)
0.00
0.50
1.00
1.50
2.00
2.50
Lead acetate (μM)
Lead
Lead+EDTA
Lead+GSH
**
*
*
*
#

Fig. 7

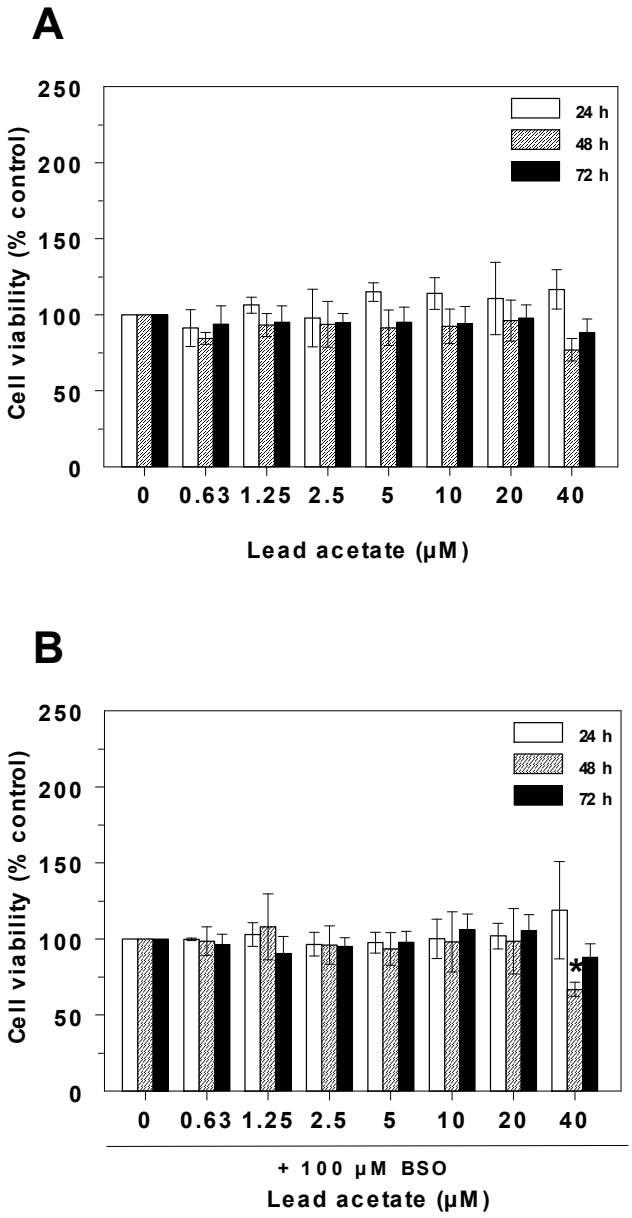

A
Cell viability (% control)
250
200
150
100
50
0
24 h
48 h
72 h
0 0.63 1.25 2.5 5 10 20 40
Lead acetate (µM)
B
Cell viability (% control)
250
200
150
100
50
0
24 h
48 h
72 h
*
0 0.63 1.25 2.5 5 10 20 40
+ 100 µM BSO
Lead acetate (µM)

Fig. 8

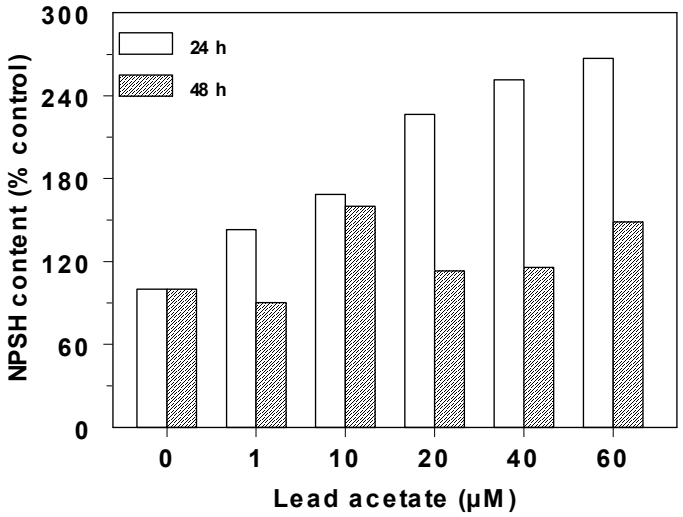

300
240
180
120
60
0
NPSH content (% control)
24 h
48 h
0
1
10
20
40
60
Lead acetate (µM)

Fig. 9

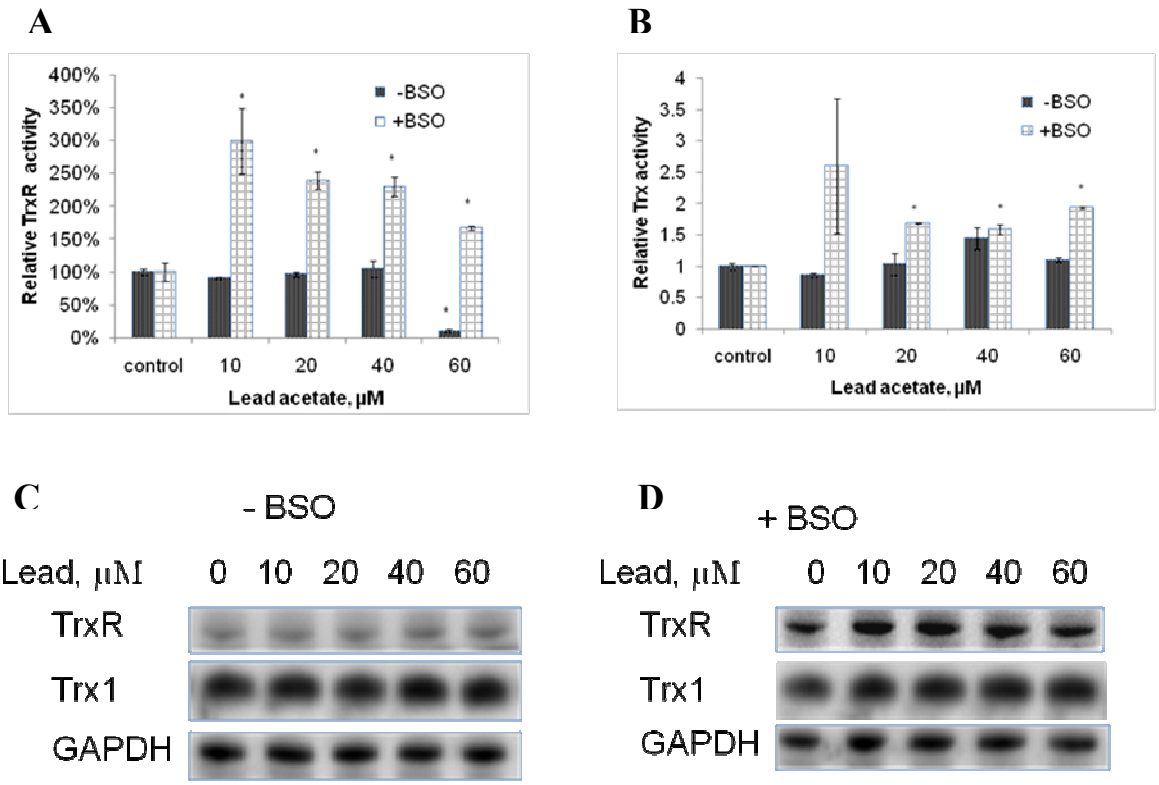
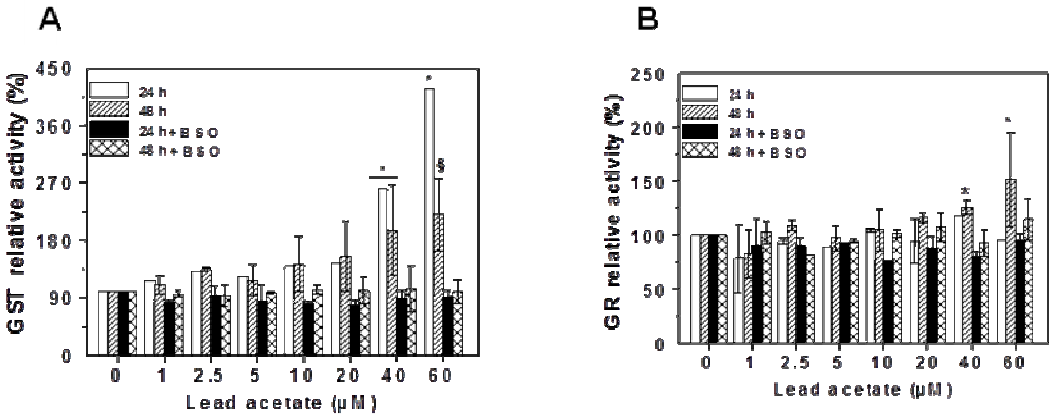

A
Relative TrxR activity
400%
350%
300%
250%
200%
150%
100%
50%
0%
-BSO
+BSO
control
10
20
40
60
Lead acetate, μM
B
Relative Trx activity
4
3.5
3
2.5
2
1.5
1
0.5
0
-BSO
+BSO
control
10
20
40
60
Lead acetate, μM
C
- BSO
Lead, μM
0 10 20 40 60
TrxR
Trx1
GAPDH
D
+ BSO
Lead, μM
0 10 20 40 60
TrxR
Trx1
GAPDH

Fig. 10


A
GST relative activity (%)
450
360
270
180
90
0
24 h
48 h
24 h+BSO
48 h+BSO
0 1 2.5 5 10 20 40 60
Lead acetate (μM)
B
GR relative activity (%)
250
200
150
100
50
0
24 h
48 h
24 h+BSO
48 h+BSO
0 1 2.5 5 10 20 40 60
Lead acetate (μM)

4.2 Artigo 1

BLOOD THIOREDOXIN REDUCTASE ACTIVITY, OXIDATIVE STRESS AND HEMATOLOGICAL PARAMETERS IN PAINTERS AND BATTERY WORKERS: RELATIONSHIP WITH LEAD AND CADMIUM LEVELS IN BLOOD

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Blood thioredoxin reductase activity, oxidative stress and hematological parameters in painters and battery workers: relationship with lead and cadmium levels in blood

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ABSTRACT: Oxidative stress has been shown to be involved in lead and cadmium toxicity. We recently showed that the activity of the antioxidant enzyme thioredoxin reductase (TrxR) is increased in the kidneys of lead-exposed rats. The present study evaluated the blood cadmium and blood lead levels (BLLs) and their relationship with hematological and oxidative stress parameters, including blood TrxR activity in 50 painters, 23 battery workers and 36 control subjects. Erythrocyte δ-aminolevulinate dehydratase (δ-ALA-D) activity and its reactivation index were measured as biomarkers of lead effects. BLLs increased in painters, but were even higher in the battery workers group. In turn, blood cadmium levels increased only in the painters group, whose levels were higher than the recommended limit. δ-ALA-D activity was inhibited only in battery workers, whereas the δ-ALA-D reactivation index increased in both exposed groups; both parameters were correlated to BLLs ($r = -0.59$ and 0.84, $P < 0.05$), whereas the reactivation index was also correlated to blood cadmium levels ($r = 0.27$, $P < 0.05$). The changes in oxidative stress and hematological parameters were distinctively associated with either BLLs or blood cadmium levels, except glutathione-*S*-transferase activity, which was correlated with both lead ($r = 0.34$) and cadmium ($r = 0.47$; $P < 0.05$). However, TrxR activity did not correlate with any of the metals evaluated. In conclusion, blood TrxR activity does not seem to be a good parameter to evaluate oxidative stress in lead- and cadmium-exposed populations. However, lead-associated changes in biochemical and hematological parameters at low BLLs underlie the necessity of re-evaluating the recommended health-based limits in occupational exposure to this metal. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: lead; cadmium; oxidative stress; hematological parameters; thioredoxin reductase

INTRODUCTION

Lead is one of the most common heavy metals that persist in the environment. Although the general population is exposed to lead through contaminated food and water, occupational exposure is considered the major exposure source (Needleman, 2004). Batteries, paints and pigments, plastics, ceramics, secondary foundries and welding are the most significant occupational settings (Needleman, 2004). Therefore, lead occupational exposure still remains a significant public health concern, especially in developing countries such as Brazil, where the control and prevention of lead exposure is still negligible (Paoliello and De Capitani, 2007).

Lead impairs the hematological, hepatic, renal and nervous systems (ATSDR, 2007). The hematological effects of lead involving the inhibition of heme synthesis and the shortening of erythrocyte lifespan have long been known (ATSDR, 2007). However, studies on the effects of lead exposure on white blood cells and platelets are scarcer.

Owing to its high affinity for the sulfhydryl groups of proteins, oxidative stress has been recognized as an important mechanism

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of lead toxicity (Adonaylo and Oteiza, 1999a; Jurczuk *et al.*, 2006). Lead irreversibly inhibits the sulfhydryl enzyme δ-aminolevulinate dehydratase (δ-ALA-D) (Gürer et *al.*, 1998; Rocha *et al.*, 1995), which results in the inhibition of hemoglobin synthesis (Monteiro *et al.*, 1989). δ-Aminolevulinic acid that accumulates owing to δ-ALA-D inhibition suffers autoxidation, which yields reactive species and induces lipid peroxidation (Hermes-Lima *et al.*, 1999). Glutathione (GSH) depletion (Jurczuk *et al.*, 2006) as well as the increase of glutathione *S*-transferase activity and expression also seem to be involved in lead toxicity (Daggett *et al.*, 1998). In this context, GSH levels and antioxidant enzyme activities such as glutathione peroxidase, catalase and superoxide dismutase have been used to evaluate the oxidative status of animals and humans exposed to lead (Conterato *et al.*, 2007; Ergurhan-Ilhan *et al.*, 2008).

Cadmium is a highly toxic heavy metal and is present in paints and batteries (Bertin and Averbeck, 2006). Like lead, cadmium stimulates the production of reactive oxygen species, causing changes in antioxidant enzymes and the loss of membrane function in erythrocytes and in other tissues (Kostic *et al.*, 1993). Decreased hemoglobin concentrations, hematocrit values and red blood cell counts were previously reported in cadmium-exposed rats (Hamada *et al.*, 1998; Kostic *et al.*, 1993). Therefore, it could be important to consider that painters and battery workers are co-exposed to cadmium and lead and are subjected to the concomitant toxic effects of these xenobiotics.

Thioredoxin reductase (TrxR) is a selenoflavoprotein that, along with its substrate thioredoxin and nicotinamide adenine dinucleotide (NADPH), forms an essential system for cellular redox regulation, cell proliferation and antioxidant defense (Nordberg and Arnér, 2001). Through its highly reactive C-terminal selenocysteine residue, TrxR can reduce a number of different substrates in addition to thioredoxin in different cellular compartments, thereby supporting crucial functions for cell survival (Arnér, 2009). A wide range of toxicants have been shown to increase its expression and activity in cell cultures (Arnér, 2009). On the other hand, the easy access of different compounds to the selenocysteine residue makes TrxR a potential target for inhibition by metals and electrophilic agents, which disrupt its function and promote oxidative events (Nordberg and Arnér, 2001).

Despite the knowledge that oxidative stress is involved in lead and cadmium toxicity, there are no reports on TrxR activity in humans exposed to these metals. Renal TrxR activity was shown to increase in rats as an early event in lead poisoning (Conterato *et al.*, 2007) suggesting a protective response. In turn, TrxR expression is increased in cadmium-exposed culture cells (Sakurai *et al.*, 2005), and gene silencing of TrxR increases cell susceptibility to cadmium (Nishimoto *et al.*, 2006). However, no studies have evaluated if TrxR activity could be a useful parameter in the evaluation of human occupational exposure to lead or cadmium. Therefore, it is important to investigate the involvement of TrxR in lead- and cadmium-induced oxidative stress in humans.

Therefore, in this study we evaluated blood lead and cadmium levels and their relationship with hematological and oxidative stress parameters, including blood TrxR activity in subjects occupationally exposed to these metals. This approach is important because most studies of occupational lead exposure do not consider the co-exposure to other toxic substances like cadmium in the work environment, which could also affect the evaluated parameters.

MATERIALS AND METHODS

Chemicals

δ-Aminolevulinic acid, ascorbic acid ≥99.0%, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), DL-dithiothreitol (DTT), epinephrine, GSH ≥ 98.0%, glutathione disulfide (GSSG), NADPH, 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid and tris(2-caboxyethyl) phosphine were purchased from Sigma (St Louis, MO, USA). Cadmium, lead and rhodium standard solutions for metal quantification were obtained from PerkinElmer (Shelton, CT, USA). Glacial acetic acid, 30% hydrogen peroxide, 85% *ortho*-phosphoric acid, 70% perchloric acid and Suprapur 65% nitric acid were obtained from Merck (Darmstadt, Germany). Laboratory kits for γ-glutamyltransferase and aspartate aminotransferase activity were obtained from Doles (Goiânia, GO, Brazil). All other chemicals used were of reagent grade.

Subjects

This study was approved by the Ethics Committee of the Federal University of Santa Maria (no. 23081.000310/2008-32) and is in accordance with the Declaration of Helsinki. The control group included healthy male workers who were nonoccupationally exposed to lead ($n = 36$), while the lead-exposed groups consisted of automotive battery-manufacturing workers ($n = 50$) and painters from an automotive industry ($n = 23$) from Rio Grande do Sul State (Brazil). Written informed consent was obtained from all subjects prior to their inclusion in the study. The inclusion criteria were at least one year of work in the battery factory or automotive industry, no history of acute or chronic disease and no use of medications. The duration of exposure was recorded as the number of years working in the battery factory or automotive industry. Information about smoking and alcohol consumption was recorded. None of the subjects had consumed alcohol during the 24 h prior to blood collection.

Sample Collection

Venous blood samples were collected in heparinized tubes for carrying out all biochemical analyses and collected in trace-metal-free tubes containing ethylenediamine tetraacetic acid for metal blood level measurements and hemograms. Aliquots from heparinized blood were immediately used for the determination of δ-ALA-D activity and centrifuged to collect the erythrocytes used for measuring glutathione levels and the plasma for measuring malondialdehyde, protein carbonyl content and vitamin C levels. The remaining heparinized blood was then frozen at −20 °C for up to 2 weeks before measuring antioxidant enzymes, aspartate aminotransferase and γ-glutamyltransferase activities.

Determination of Blood Metals and δ-ALA-D Activity

Blood metals were assessed as described previously (Batista *et al.*, 2008) using a quadrupole inductively coupled plasma mass spectrometer (ICP-MS Elan DRCII, PerkinElmer, Sciex, Norwalk, CT, USA). All reagents used were of analytical grade. Internal standard rhodium (1000 mg l^{-1}) and the multielement (10 mg l^{-1}) solution were obtained from PerkinElmer (Shelton, CT, USA). The calibration protocol for matrix matching and sample preparation were performed by homogenizing whole blood and then diluting

it 50 times with a solution containing 0.01% (v/v) Triton® X-100 and 0.5% (v/v) sub-distilled nitric acid. The concentration range of the curves was 1 to 20 $\mu g\,l^{-1}$ for cadmium and lead determination. The samples were analyzed against a reagent blank. Each curve calibration point (blank, reagent blank and sample) was analyzed with 10 $\mu g\,l^{-1}$ of internal standard rhodium.

δ-ALA-D activity was assessed in lysed erythrocytes, in the absence or presence of 5.8 mM zinc and 6.2 mM DTT for evaluating the percent reactivation of δ-ALA-D (Berlin and Schaller, 1974). Modified Ehrlich's reagent was used to react with the porphobilinogen (PBG) final product to yield a pink colored compound, which is measured at 555 nm. The activity is shown as µmol PBG $min^{-1}\,l^{-1}$ erythrocytes. The reactivation index is based on the replacement by zinc and DTT of lead bound to the enzyme, restoring activity to a maximum value, and was calculated as follows:

$$[(\text{Zn-DTT-}\delta\text{-ALAD activity} - \delta\text{-ALAD activity})/\delta\text{-ALAD activity}] \times 100$$

Determination of Oxidative Stress Parameters

Antioxidant enzyme activities were assessed using spectrophotometric methods. Glutathione-*S*-transferase was measured at 340 nm using 1-chloro-2,4-dinitrobenzene and GSH as substrates (Habig and Jakoby, 1981). Determination of glutathione peroxidase activity was based on NADPH oxidation at 340 nm (Paglia and Valentine, 1967). Superoxide dismutase activity was assessed by its ability to prevent the oxidation of epinephrine to adrenochrome, which was recorded at 480 nm (Misra and Fridovich, 1972). One unit of superoxide dismutase is defined as the amount of enzyme necessary to inhibit 50% of epinephrine autoxidation. Catalase activity was assessed as the pseudo-first-order reaction constant (k) of the decrease in hydrogen peroxide absorption at 240 nm and 25 °C (Aebi, 1984). To measure TrxR activity, total blood was hemolyzed with four volumes of cold Milli-Q water and centrifuged at 9000 *g* for 15 min at 4 °C. The supernatant was then diluted and used for an enzyme assay based on the reduction of DTNB to 5′-thionitrobenzoic acid (TNB^-) at 412 nm (Holmgren and Björnstedt, 1995). One unit of TrxR is defined as the amount of enzyme that reduces 1 nmol of DTNB to TNB^- per min. Quantification of erythrocyte GSH and plasma malondialdehyde levels was performed as previously described (Grotto *et al.*, 2006; Schott *et al.*, 2007) by high-performance liquid chromatography using an Eurospher-100 C_{18} reversed-phase column (150 × 4 mm, 5 µm) and visible detection. Plasma vitamin C levels were also measured by high-performance liquid chromatography. In short, plasma samples were vortex-mixed with 10 µl 9% tris(2-carboxyethyl)phosphine for 10 s, stored on ice for 15 min and diluted (1:1) with 10% perchloric acid, and then vortex-mixed. After centrifugation at 6000 *g* for 3 min, the supernatant was injected directly into the chromatography system. A C_{18} column (250 × 4.6 mm, 5 µm) with a guard column was used. The mobile phase consisted of 25 mM NaH_2PO_4 and 0.00125% NaN_3 with the pH adjusted to 2.5 with *ortho*-phosphoric acid. The measurements were performed at room temperature at a flow-rate of 1 ml min^{-1} with detection at 245 nm. Plasma protein carbonyl levels were determined using dinitrophenylhydrazine at 370 nm (Levine *et al.*, 1990). Blood methemoglobin levels were determined at 630 nm as previously described (Hegesh *et al.*, 1970).

Gamma Glutamyltransferase and Aspartate Aminotransferase Levels

Plasma γ-glutamyltransferase and aspartate aminotransferase were measured spectrophotometrically using a Doles kit (GO, Brazil) according to the manufacturer's instructions.

Hematological Parameters

Analyses were carried out using a Coulter STKS hematology flow cytometer (Beckman Coulter, CA, USA). Red blood cell parameters such as the erythrogram (number of red blood cells, hematocrit and hemoglobin) and the red blood cell indices (mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and red cell distribution width) were assessed. White blood cell parameters (the total number of leukocytes and the total number and percentage of neutrophils, lymphocytes, eosinophils, monocytes and basophils) and platelet parameters (mean platelet volume, platelet distribution width and plateletcrit) were also determined.

Statistical Analyses

Data were analyzed by one-way analyses of variance (ANOVA), followed by Duncan's test when appropriate. Data that did not exhibit a normal distribution were transformed (log or square root transformation) before analyses in order to meet ANOVA assumptions. Variables that did not show a normal distribution even after the transformation were analyzed by nonparametric Kruskal–Wallis ANOVA followed by a multiple comparison test when required. The frequencies of smokers in the studied groups were compared using the χ^2 test. The associations between variables were evaluated by Spearman's rank order correlation. Data were analyzed using the Statistica® 7.0 software system (Statsoft Inc., 2001). Results were expressed as means ± SEM, and differences were considered significant when $P < 0.05$.

RESULTS

Demographic Characteristics of the Study Groups

The average age of battery workers was higher than the control group, but the average age of painters was similar to that of the battery workers and control groups (Table 1). There was no difference in the duration of occupational exposure between the two exposed groups (Table 1). The number of smokers was higher in the exposed groups than in the control group (Table 1). T1

Blood Metals Levels

Both exposed groups had higher blood lead levels (BLLs) than the control group, but battery workers had much higher level (Table 1). All subjects in the control group had BLLs lower than 10 $\mu g\,dl^{-1}$. Although the BLLs of the painters were higher than those of the control group, only one subject of the painters group had a BLL between 10 and 20 $\mu g\,dl^{-1}$, whereas the other subjects had BLLs lower than 10 $\mu g\,dl^{-1}$. In contrast, in the battery workers group only one subject had a BLL lower than 10 µg dl^{-1}, five subjects were between 10–40 µg dl^{-1}, 12 subjects were between 40–60 µg dl^{-1} and five subjects were higher than 60 µg dl^{-1}. Interestingly, painters had higher blood cadmium levels than control and battery worker groups (Table 1). The painter

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Table 1. Characteristics and blood metal levels of the study groups

	Control ($n = 36$)	Painters ($n = 50$)	Battery workers ($n = 23$)
Age (years)			
Mean ± SEM	33.5 ± 1.2 [b]	33.5 ± 1.2 [a,b]	37.3 ± 2.1 [a]
(minimum–maximum)	(21–49)	(21–54)	(15–61)
Duration of exposure (months)			
Mean ± SEM	0	133.9 ± 14.5 [a]	117.3 ± 26.1 [a]
(minimum–maximum)		(24–444)	(6–372)
Number of smokers	1 [b]	12 [b]	5 [b]
(%)	(2.8%)	(24.0%)	(21.7%)
Lead ($\mu g\,dl^{-1}$)			
Mean ± SEM	1.5 ± 0.1 [c]	5.4 ± 0.4 [b]	49.8 ± 4.0 [a]
(minimum–maximum)	(0.4–3.0)	(1.4–14.0)	(5.3–89.7)
Cadmium ($\mu g\,dl^{-1}$)			
Mean ± SEM	0.003 ± 0.001 [b]	1.606 ± 0.074 [a]	0.022 ± 0.005 [b]
(minimum–maximum)	(n.d.–0.020)	(0.900–3.120)	(0.001–0.135)

[a–c] Values that have no common superscript letter within the line are statistically different from each other ($P < 0.05$). n.d., not detected.

who had the BLL between 10 and 20 $\mu g\,dl^{-1}$ had blood cadmium levels within the lowest quartile range of the group, i.e. 0.94 $\mu g\,dl^{-1}$, but this value is still much higher than the maximum cadmium level found in the other groups. The highest cadmium level of the painters group was not related to the number of smokers in this group, since blood cadmium levels were not different between smokers and nonsmokers and no correlation was found between smoking habit and blood cadmium levels (data not shown). Both lead and cadmium blood levels were positively correlated to the duration of the occupational exposure (Table 2). T2

Oxidative Stress Parameters and their Relationship with Blood Metal Levels and with the Duration of Exposure

The erythrocyte δ-ALAD activity, which is a clinical lead toxicity parameter, was only inhibited in battery workers, but not in painters when compared with the control group (Fig. 1A). On the other hand, the δ-ALAD reactivation index increased in both exposed groups when compared with the control (Fig. 1A). To assess if changes in the indicators of lead toxicity could take place even at BLLs lower than the health-based biological exposure limit for workers (WHO, 1980), we also assessed the battery workers group without 17 subjects that had BLLs higher than 40 $\mu g\,dl^{-1}$ (battery workers BLL < 40 $\mu g\,dl^{-1}$). This group still had changes in the indicators of lead toxicity (lower ALA-D activity and higher reactivation index than the control group, P 0.05; Fig. 1A). Although the δ-ALAD activity did not change in painters (whose BLLs were lower than battery workers), this parameter had an inverse correlation with BLLs and the duration of exposure (Table 2). In turn, blood lead and cadmium levels as well as the duration of exposure were directly correlated with the δ-ALAD reactivation index, akthough BLL seems to be the major factor to influence this parameter (Table 2). F1

Methemoglobin, which is an oxidized form of hemoglobin, was higher in battery workers, but not in painters when compared with the control group (Fig. 1B). However, all groups were within the reference value for humans, i.e. up to approximately 1.5% (Fischbach and Dunning, 2009). Methemoglobin concentration had a positive correlation with BLL (Table 2).

Regarding oxidative damage indicators, malondialdehyde levels, which are an indicator of lipid oxidation, did not change among groups (Fig. 1B), but they had a positive correlation with blood cadmium levels and with the duration of exposure (Table 2). In addition, malondialdehyde levels were also directly correlated with plasma protein carbonyl content ($r = 0.22$; $P < 0.05$), which is an indicator of protein oxidation. Protein carbonyl content was decreased in the plasma of battery workers, but not in painters, when compared with the control group (Fig. 1B). In addition, protein carbonyl content was inversely correlated with BLLs (Table 2).

Table 2. Spearman correlation coefficients (R) among blood metal levels, biochemical parameters and the duration of exposure.

	Lead	Cadmium	Duration of exposure
Duration of exposure	0.67*	0.69*	—
δ-ALA-D	−0.59*	0.08	−0.21*
δ-ALA-D reactivation index	0.84*	0.27*	0.56*
Methemoglobin	0.46*	0.17	0.18
Malondialdehyde	0.01	0.28*	0.28*
Protein carbonyl	−0.28*	−0.06	−0.06
Vitamin C	−0.14	0.14	0.03
Glutathione	−0.03	0.47*	0.19
Glutathione-*S*-transferase	0.34*	0.47*	0.31*
Glutathione peroxidase	0.38*	−0.16	0.02
Catalase	−0.11	−0.58*	−0.44*
Superoxide dismutase	0.32*	−0.01	0.10
Thioredoxin reductase	0.16	0.16	0.13
Aspartate aminotransferase	0.26*	0.18	0.17
Gamma glutamyltransferase	−0.16	−0.08	−0.10

* Significant correlation between variables ($P < 0.05$).

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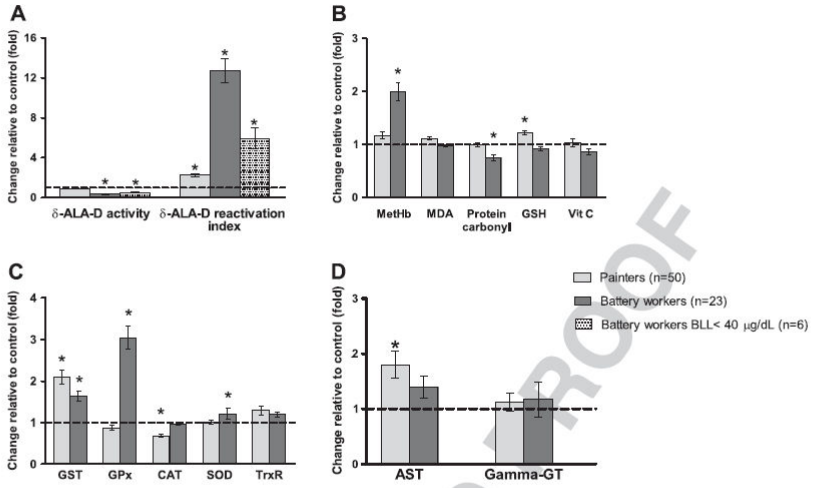

Figure 1. Changes of biochemical parameters in the exposed workers as compared with the control group ($n = 36$). Subcritical indicators of lead effects (δ-ALA-D activity and δ-ALA-D reactivation index) (A); oxidative stress parameters (B,C) and indicators of hepatic function (AST and γ-GT) (D). Results are means ± SEM of the change relative to control (fold). The horizontal dotted line indicates the control group, which had the following averages: 26.5 μmol porphobilinogen $min^{-1} l^{-1}$ erythrocytes for δ-ALA-D activity; 21.1% for δ-ALA-D reactivation index; 0.6% MetHb; 6.8 mM MDA; 0.8 nmol protein carbonyl mg^{-1} protein; 5.8 mg vitamin C l^{-1}; 5.4 μmol GSH g^{-1} Hb; 6.0 nmol CDNB $min^{-1} mg^{-1}$ Hb for GST; 10.7 μmol NADPH $min^{-1} g^{-1}$ Hb for GPx; 93.7 *k* g^{-1} Hb for CAT; 0.95 U mg^{-1} Hb for SOD; 8.4 U mg^{-1} Hb for TrxR; 9.8 U l^{-1} for AST; and 21.6 U l^{-1} for γ-GT. *Different from control group ($P < 0.05$). MetHb, methemoglobin; MDA, malondialdehyde; GSH, glutathione; GST, glutathione-*S*-transferase; GPx, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase; TrxR, thioredoxin reductase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase; BLL, blood lead level.

There were no differences in the nonenzymatic antioxidant, vitamin C, among the studied groups, but the erythrocyte GSH level was higher in painters, but not in battery workers, when compared with the control group (Fig. 1B), and it was directly correlated with blood cadmium levels (Table 2).

Concerning enzymatic antioxidants, glutathione-*S*-transferase activity was significantly increased in painters and in battery workers (Fig. 1C), but glutathione peroxidase and superoxide dismutase activities increased only in the latter group when compared with control subjects (Fig. 1C). The increase of glutathione-*S*-transferase in both exposed groups was in agreement with the positive correlation of glutathione-*S*-transferase activity with blood lead and cadmium levels and with the duration of exposure ($P < 0.05$, Table 2). On the other hand, glutathione peroxidase and superoxide dismutase activities were only correlated with BLLs (Table 2). Catalase activity, in turn, decreased only in painters when compared with the control group (Fig. 1C). Accordingly, it was negatively correlated with the duration of exposure and with blood cadmium levels (Table 2), which were higher in this group than in others (Table 1). TrxR activity was similar among all studied groups (Fig. 1C).

The plasma indicator of hepatic function, aspartate aminotransferase, was increased in painters, but not in the battery workers when compared with the control group ($P < 0.05$, Fig. 1D). Aspartate aminotransferase activity was directly correlated with BLLs (Table 2). The activity of plasma γ-glutamyltransferase activity, which is usually associated with hepatic and biliary injuries, was not changed among the studied groups (Fig. 1D).

Interestingly, the only painter who, in addition to high blood cadmium level, also had a BLL higher than the other subjects in the same group (14 μg dl^{-1}), showed distinct changes in some parameters compared with the other painters. Oxidative stress parameters such as malondialdehyde and protein carbonyl levels seemed to be increased (1.35- and 1.42-fold, respectively), while vitamin C and thioredoxin reductase activity seemed to be lower than the control (0.34- and 0.63-fold, respectively); the behavior of these parameters in the painters group was not different from the control group. The δ-ALA-D reactivation index (0.77-fold) and glutathione-*S*-transferase activity (0.73-fold) of this particular painter did not follow the same tendency of increase of the painters group compared with the control. In addition, the glutathione levels (0.81-fold) and superoxide dismutase activity (1.24-fold) of this particular painter were more similar to the battery workers group, and did not follow the behavior of the painters group (increased and unchanged values compared with control group, respectively).

Hematological Parameters and their Relationship with Blood Metals Levels and with the Duration of Exposure

The hematocrit and hemoglobin concentration and the number of red blood cells decreased only in painters when compared with the other groups (Table 3), and these values were negatively correlated to blood cadmium levels (Table 4). Interestingly, the only painter who, in addition to high blood cadmium level, also had BLL higher than the other subjects in the same group (14 μg dl^{-1}), had no decrease in these parameters (47% hematocrit, 16.3 g hemoglobin dl^{-1} and 5.2 million red blood cells mm^{-3}) when compared with the control group or the battery workers group. Mean corpuscular volume and mean corpuscular

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Table 3. Hematological parameters (means ± SEM) of the study groups

	Control ($n = 36$)	Painters ($n = 50$)	Battery workers ($n = 23$)
Htc (%)	45.3 ± 0.5 [a]	43.8 ± 0.4 [b]	45.2 ± 0.7 [a]
Hb (g dl^{-1})	15.4 ± 0.2 [a]	15.0 ± 0.1 [b]	15.6 ± 0.3 [a]
RBC (million mm^{-3})	5.2 ± 0.7 [a]	5.0 ± 0.5 [b]	5.2 ± 0.8 [a]
MCV (fl)	86.9 ± 0.6	87.9 ± 0.5	86.6 ± 0.8
MCH (pg)	29.6 ± 0.2	30.0 ± 0.2	29.9 ± 0.3
MCHC (g dl^{-1})	34.1 ± 0.1 [b]	34.2 ± 0.1 [b]	34.5 ± 0.1 [a]
RDW (%)	13.1 ± 0.2 [b]	13.1 ± 0.1 [b]	14.3 ± 0.1 [a]
Leukocytes (10^3 mm^{-3})	6.88 ± 0.30 [a]	6.06 ± 0.16 [b]	5.91 ± 0.30 [b]
Neutrophils (10^3 mm^{-3})	3.75 ± 2.49 [a]	3.07 ± 0.13 [b]	2.87 ± 0.27 [c]
Neutrophils (%)	52.6 ± 2.0	50.2 ± 1.2	46.9 ± 2.2
Lymphocytes (10^3 mm^{-3})	2.27 ± 0.11	2.28 ± 0.08	2.26 ± 0.11
Lymphocytes (%)	34.6 ± 1.7	38.0 ± 1.1	38.9 ± 2.1
Eosinophils (10^3 mm^{-3})	0.24 ± 0.03	0.19 ± 0.12	0.25 ± 0.05
Eosinophils (%)	3.7 ± 0.5	3.2 ± 0.3	3.6 ± 0.6
Monocytes (10^3 mm^{-3})	0.52 ± 0.03	0.49 ± 0.02	0.49 ± 0.03
Monocytes (%)	7.5 ± 0.4	8.2 ± 0.3	7.7 ± 0.6
Basophils (10^3 mm^{-3})	0.09 ± 0.02 [a]	0.03 ± 0.00 [c]	0.05 ± 0.07 [b]
Basophils (%)	1.1 ± 0.2 [b]	0.5 ± 0.0 [c]	2.3 ± 0.5 [a]
Platelets (10^3 mm^{-3})	244.3 ± 8.3 [a]	203.7 ± 6.5 [b]	190.7 ± 7.7 [b]
MPV (μm^3)	8.6 ± 0.1 [b]	9.1 ± 0.1 [a]	9.4 ± 0.2 [a]
PDW (%)	14.3 ± 0.5 [b]	15.3 ± 0.3 [a,b]	16.6 ± 0.5 [a]
PCT (%)	0.21 ± 0.01 [a]	0.18 ± 0.00 [b]	0.18 ± 0.01 [b]

[a–c] Values that have no common superscript letter within the line are statistically different from each other ($P < 0.05$). Htc, hematocrit; Hb, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit.

hemoglobin values were similar among the studied groups (Table 3). On the other hand, the mean corpuscular hemoglobin concentration, which indicates the concentration of hemoglobin in a given volume of packed red blood cells, and red blood cell distribution width, which indicates the variability in red blood cell size, were increased in battery workers when compared with the other groups (Table 3). These parameters were directly correlated to BLLs (Table 4).

The total number of leukocytes decreased in both painters and battery workers, which may be attributed to the decrease in the total number of neutrophils and basophils (Table 3). Despite these changes, the percentage of neutrophils did not change among groups. However, the percentage of basophils was decreased in the painters, but increased in the battery workers compared with the control group (Table 3). No change was observed in the total number or the percentage of lymphocytes, eosinophils or monocytes among the studied groups (Table 3). The number of leukocytes and neutrophils and the percentage of neutrophils were negatively correlated with BLLs (Table 4). The number of basophils was negatively correlated with blood cadmium levels (Table 4). The number or percentage of lymphocytes, eosinophils or monocytes had no significant correlation with blood metal levels or with the duration of exposure (data not shown). The mean platelet volume is a biological variable that is related to platelet function and is inversely related to the number of platelets. As expected, the number of platelets decreased, whereas the mean platelet volume increased in both exposed groups (Table 3). The number of platelets was inversely correlated to lead and cadmium blood levels and to the duration of exposure, whereas the mean platelet volume was positively correlated to these factors (Table 4). These results indicate that the changes in these parameters were partially related to lead and cadmium exposure. Platelet distribution width, which indicates the variability in platelet size, was significantly enhanced in battery workers, whereas the plateletcrit decreased in both exposed groups (Table 3). Platelet distribution width was positively correlated with BLL and with the duration of exposure, whereas the plateletcrit was negatively correlated with BLL and with the duration of exposure. Among all hematological parameters evaluated, the platelet parameters were the sole parameters to correlate with the duration of occupational exposure (Table 4).

DISCUSSION

The correlation between the clinical indicators of lead poisoning and some oxidative stress parameters in lead-exposed battery workers has already been published elsewhere (Gürer-Orhan *et al.*, 2004). However, our study included workers exposed to different lead levels (painters and battery workers). In addition to evaluating hematological parameters, we also evaluated for the first time the behavior of the antioxidant enzyme TrxR after lead exposure in humans. In addition, as most studies on lead occupational exposure do not consider the co-exposure to other toxic substances in the work environment, we also investigated the contribution of cadmium to changes in the exposed workers. The average BLL of battery workers is in agreement with other studies (Kašuba *et al.*, 2010). In addition, the average BLLs of both exposed groups are within the limits of the Regulatory Act (NR7) from the Brazilian Ministry of Labor (30 December

Table 4. Spearman correlation coefficients (*R*) between hematological parameters and blood metal levels and the duration of exposure

	Lead	Cadmium	Duration of exposure
Htc	0.01	−0.25*	−0.12
Hb	0.07	−0.23*	−0.10
RBC	0.01	−0.28*	−0.15
MCV	0.07	0.11	0.10
MCH	0.16	0.13	0.14
MCHC	0.23*	0.11	0.10
RDW	0.44*	−0.04	0.12
Number of leucocytes	−0.22*	−0.15	−0.18
Number of neutrophils	−0.25*	0.16	−0.10
Percentage of neutrophils	−0.20*	−0.14	−0.14
Number of basophils	0.09	−0.21*	−0.11
Percentage of basophils	−0.02	−0.18	−0.16
Number of platelets	−0.38*	−0.25*	−0.38*
MPV	0.32*	0.23*	0.34*
PDW	0.26*	0.01	0.21*
PCT	−0.27*	−0.12	−0.22*

* Significant correlation between variables ($P < 0.05$). Htc, hematocrit; Hb, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit.

1994), which establishes 40 μg dl^{-1} as the reference value for adults and 60 μg dl^{-1} as the maximum allowed biological value for workers. However, the WHO established 40 μg dl^{-1} BLL as the health-based biological exposure limit for workers (WHO, 1980). Only five subjects from the battery workers group had BLLs higher than 60 μg dl^{-1}, but most subjects (17) from this group had BLLs higher than the WHO limit. Even when all of these subjects were excluded from the battery workers group, this group still had changes in the indicators of lead toxicity (lower ALA-D activity and higher reactivation index than the control group). Thus, our data suggest that this limit value cannot be considered safe. In addition, painters, who had much lower BLLs, still showed a significant increase in the δ-ALA-D reactivation index. Accordingly, we found that the δ-ALA-D reactivation index was strongly correlated to the blood lead levels. In addition, neurological disturbances and cognitive deficit were found in workers whose BLLs ranged from 40 to 80 μg dl^{-1} (Stollery *et al.*, 1989).

Plasma aspartate aminotransferase activity is an indicator of hepatic function (Koyuturk *et al.*, 2007). Although activity was positively correlated with blood lead levels, it was increased only in the painters group, who had lower BLLs than battery workers. Thus, we propose that, in addition to lead, other toxic agent(s) found in paints, but not in batteries, could be the major agent (s) responsible for this hepatic dysfunction. Cadmium blood level, which was also increased in the painters group, does not appear to contribute to this hepatic dysfunction, because it did not correlate to aspartate aminotransferase activity.

Erythrocyte δ-ALA-D activity has been recognized as a sensitive clinical indicator of subcritical effects of lead, because it correlates to BLLs (Gürer-Orhan *et al.*, 2004). Moreover, while BLL mainly reflects recent lead exposure levels, the concomitant evaluation of BLL and erythrocyte δ-ALA-D activity is believed to better evaluate the long-term cumulative exposure to lead (Alessio *et al.*, 1981). However, in our study δ-ALA-D activity inhibition was observed only in the battery workers who had BLLs around 50 μg dl^{-1}, whereas the δ-ALA-D reactivation index was increased even in the painters, whose average BLL was 5.3 μg dl^{-1}. Thus, the δ-ALA-D reactivation index seems to be more reliable as an early biochemical index of lead toxicity than δ-ALA-D activity. In fact, the δ-ALA-D reactivation index was more strongly correlated to BLLs than δ-ALA-D activity. Although the use of the δ-ALA-D reactivation index as an indicator of the effects of lead was previously suggested (Yagminas and Villeneuve, 1987), most studies on lead-exposed workers have only used δ-ALA-D activity as a subclinical indicator of lead effects (Gürer-Orhan *et al.*, 2004). δ-ALA-D inhibition could contribute to the toxic effects of lead, since the accumulation of its substrate, δ-aminolevulinic acid, may generate reactive oxygen species leading to oxidative damage in proteins and DNA (Daggett *et al.*, 1998; Monteiro *et al.*, 1989).

Methemoglobin is the oxidized form of hemoglobin that is not able to transport the oxygen molecule. Similar to our results, the increase of methemoglobin levels in lead-exposed workers was previously reported (Costa *et al.*, 1997). Although the mechanisms responsible for this increase are not yet well established, we cannot rule out a direct effect of lead on oxyhemoglobin oxidation, as suggested elsewhere (Costa *et al.*, 1997). In addition, there is evidence regarding the cooxidation between oxyhemoglobin and the δ-aminolevulinic acid that has accumulated owing to δ-ALA-D inhibition (Monteiro *et al.*, 1986). There is also a strong correlation between methemoglobin formation and plasma concentrations of δ-aminolevulinic acid, as found by Costa *et al.* (1997).

In addition to reactive oxygen species generation by accumulated δ-aminolevulinic acid (Monteiro *et al.*, 1989), lead itself may stimulate lipid peroxidation in the presence of ferrous iron (Adonaylo and Oteiza, 1999b). Although the malondialdehyde levels were not increased in the exposed groups in the present study, there was an increase in the activity of antioxidant enzymes such as glutathione-*S*-transferase, glutathione peroxidase and superoxide dismutase when compared with the control group. Glutathione-*S*-transferase is a phase II enzyme involved in the metabolism and detoxification of xenobiotics, including heavy metals (Daggett *et al.*, 1998), and in the removal of lipid hydroperoxides (Yang *et al.*, 2001). In the current study, the activity of this enzyme was increased in both exposed groups. Whereas the battery workers group only had increased BLLs, the painters had increased lead and cadmium levels. Both lead and cadmium can stimulate the overexpression of glutathione-*S*-transferase in different tissues by gene regulation (Daggett *et al.*, 1998; Casalino *et al.*, 2005). In the present study, correlation results suggest that lead and cadmium make a similar contribution to the increase of glutathione-*S*-transferase activity, probably by inducing glutathione-*S*-transferase expression. In turn, the increased glutathione peroxidase and superoxide dismutase activities were observed only in the battery workers group and were only correlated to BLL. This finding is in agreement with other studies showing elevated glutathione peroxidase (Ergurhan-Ilhan *et al.*, 2008) and superoxide dismutase (Conterato *et al.*, 2007; Ye *et al.*, 1999). Because superoxide dismutase scavenges superoxide anion radicals, generating hydrogen peroxide, whereas glutathione peroxidase scavenges hydrogen peroxide, the enhancement of their activities may be a defense response against these reactive oxygen species, thereby protecting

cells against lipid and protein oxidation. Thus, the negative correlation of glutathione peroxidase with both malondialdehyde and protein carbonyl content ($r = -0.21$ and -0.35, respectively; P 0.05) might explain the absence of changes in malondialdehyde levels and the unexpected decrease in the protein carbonyl content in the battery workers, whose glutathione peroxidase activity was remarkably increased. Unlike glutathione-*S*-transferase, catalase activity changed only in the painters group. The observed reduction of catalase activity seems to be attributable to the increased blood cadmium levels found in painters relative to the other groups because catalase activity was inversely correlated to blood cadmium levels. In fact, Casalino *et al.* (2002) provided preliminary evidence that cadmium directly inhibits catalase in the pH range of 6.0–8.0, probably by binding to the imidazole residue of His-74, which is essential for the decomposition of hydrogen peroxide.

Several toxicants that induce oxidative stress, including cadmium, were found to increase the synthesis and expression of TrxR *in vitro* through gene activation (Arnér, 2009; Sakurai *et al.*, 2005). In addition, we have previously shown that low lead doses increase renal TrxR activity in rats upon both short- and long-term exposure (Conterato *et al.*, 2007). Thus, the unchanged blood TrxR activity in the exposed groups in the present study suggests that lead is more effective at altering the thioredoxin system in the kidneys than in the blood. Therefore, blood might not be an appropriate biological sample for evaluating the effects of lead and cadmium on the thioredoxin system. In fact, the thioredoxin system has lower expression in red blood cells than in other cells types and tissues (Söderberg *et al.*, 2000).

Occupational exposure to metals caused small changes in the red blood cell parameters. The negative association between blood cadmium levels and red blood cell counts, hematocrit and hemoglobin might reflect some effect of this metal on the destruction of erythrocytes or a decrease in their lifespan owing to increased membrane fragility (Hamada *et al.*, 1998). It may even be the case that cadmium reduces blood iron levels and consequently lowers hemoglobin levels (Kostic *et al.*, 1993). On the other hand, the positive association between some red blood cell indices (mean corpuscular hemoglobin concentration and red cell distribution width) and BLLs indicates the presence of lead-induced disturbances in erythrocyte hemoglobin concentrations and erythrocyte size, respectively. These findings are in line with the previously reported effects that lead has on hemoglobin synthesis and lifespan (ATSDR, 2007). However, no anemia was observed in the exposed groups, possibly because most subjects had BLLs lower than the threshold known to reduce hemoglobin levels (50 $\mu g\,dl^{-1}$; ATSDR, 2007).

The negative correlation between BLLs and the number of leukocytes, neutrophils, platelets and plateletcrit values is in agreement with a previous study that found lower total leukocytes and platelet counts in lead-exposed rats (Kostic *et al.*, 1993). In addition to lead, we showed that cadmium may also be associated with alterations observed in white blood cells (basophils) and platelet counts. López *et al.* (1992) showed that cadmium and lead inhibit the aggregation of rat and human platelets *in vitro*, but our study reported for the first time that changes in platelet parameters found in occupationally exposed subjects were mostly related to BLLs, with a minor contribution of blood cadmium levels.

It is notable that, in addition to BLLs, blood cadmium levels also influenced many of the alterations in oxidative stress and hematological parameters seen in occupationally exposed subjects. Moreover, cadmium seemed to be the major factor affecting catalase activity and erythrocyte glutathione levels, which only changed in painters, whose blood cadmium levels were higher than the control and battery workers. In fact, all subjects from the painters group had blood cadmium levels higher than the maximum allowed biological value for workers according to the Regulatory Act (NR7) from the Brazilian Ministry of Labor (0.5 $\mu g\,dl^{-1}$; 30 December 1994), and 94% of subjects had cadmium levels higher than the WHO individual critical level for regular and long-term exposure (1 $\mu g\,dl^{-1}$; WHO, 1980). The concomitant exposure to cadmium and lead may have influenced some parameters (thioredoxin reductase, vitamin C and malondialdehyde levels) that were not changed in painters or battery workers, but only in the specific painter who had high blood cadmium level and a BLL higher than 10 $\mu g\,dl^{-1}$. In addition, this subject also had high protein carbonyl levels, in contrast to the other painters who had no change in this parameter and battery workers who had decreased levels. Alternatively, these parameters could have been influenced by factors other than cadmium and lead, because vitamin C levels and thioredoxin reductase activity did not correlate to either cadmium or lead levels in blood. In addition, as shown here, malondialdehyde levels were correlated in part to blood cadmium levels, but other substances present in paints can also induce oxidative damage (Costa *et al.*, 2005). Surprisingly, this subject had glutathione, hematocrit, hemoglobin and red blood cell values similar to the control group, and they did not change as in the other painters. The relatively lower cadmium level of this subject (0.94 $\mu g\,dl^{-1}$) as compared with the whole painters group (0.90 to 3.12 $\mu g\,dl^{-1}$) may be responsible for this result, because all of these parameters were significantly correlated to cadmium levels.

In summary, our data showed alterations in oxidative stress and hematological parameters in exposed workers, which were distinctively associated with either blood lead or cadmium levels, except in the case of glutathione-*S*-transferase activity, which correlated with both metals to a similar degree. Although our previous study clearly showed the early increase of kidney TrxR activity in lead-exposed rats, the lack of a change in blood TrxR in exposed workers suggests that it may not be a good oxidative stress parameter in lead- and cadmium-exposed populations. The ALA-D reactivation index, which was closely correlated to BLLs, was a good index of the subcritical effects of lead and should be used along with δ-ALA-D activity to evaluate lead toxicity in exposed populations. Finally, the lead and cadmium-related alterations observed at low blood lead and high cadmium levels underline the importance of monitoring blood levels of toxic metals in exposed workers and reevaluating the recommended health-based limit for BLL in occupational exposure.

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4.3 Manuscrito 2

Lead exposure stimulates the activity of thioredoxin reductase in kidney but not in blood: implications for lead toxicity.

Manuscrito a ser submetido à revista Basic and Clinical Pharmacology and Toxicology

Lead exposure stimulates the activity of thioredoxin reductase in kidney, but not in blood: implications for the lead toxicity

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Running title: Thioredoxin reductase-1 and lead exposure

We recently demonstrated that renal thioredoxin reductase-1 activity is stimulated at lead doses lower than that necessary to inhibit δ-aminolevulinate dehydratase activity (δ-ALA-D), which is a classical early biomarker of lead effects. Thus, we hypothesized that the activity of thioredoxin reductase could be an earlier indicator of lead effects more sensitive than δ-ALA-D. To evaluate this hypothesis we assessed blood and renal thioredoxin reductase-1 activity, oxidative stress parameters such as lipid peroxidation, protein carbonyl and antioxidant enzymes and lead exposure biomarkers in rats acutely exposed to lead. Histopathological analysis was performed to verify the occurrence of renal damage. The increase in renal thioredoxin reductase-1 activity was concomitant to the increase in blood and renal lead level at 25 mg/kg lead acetate after 6, 24 and 48 h of exposure ($P<0.05$) and these effects were not accompanied by oxidative or tissue damage in kidneys. Erythrocyte δ-ALA-D activity, which is considered the most sensitive lead effect biomarker was inhibited at 25 mg/kg lead acetate after 6 h of exposure ($P<0.05$), but it was recovered after 24 and 48 h of exposure. There were no changes in any parameters at lead acetate doses lower than 25 mg/kg (5 and 10 mg/kg). Our results indicate that blood thioredoxin reductase-1 activity, cannot be used as an indicator of lead effects. In contrast, renal thioredoxin reductase-1 is implicated in the early effects induced by lead and it is probably a protective cellular mechanism against lead-induced oxidative stress.

Lead is not an essential element and plays no useful role in the organism. Although lead toxicity has been widely known over years, harmful lead exposure is still widespread, especially in workplaces [1]. In addition, significant amount of lead is released to the environment from industrial settings, where it persists as a hazardous and continuous long-term source for human contamination [2].

Renal and hematological systems are among the main systems affected by lead [2-3]. In this sense, novel biomarkers of the toxic effects of lead are desirable to detect early biochemical alterations that precede tissue damage [4]. Currently, erythrocyte δ-aminolevulinate dehydratase (δ-ALA-D) activity is considered the most sensitive indicator of subcritical effects of lead [2; 5]. Due to its high affinity for sulfhydryl groups, lead is usually attached to the sulfhydryl moieties of proteins like δ-ALA-D and lead-binding proteins in blood, kidneys and other tissues [5-6]. δ-ALA-D inhibition causes the accumulation of its substrate, δ-aminolevulinic acid, which undergoes autoxidation, generating reactive oxygen species [7]. In fact, the oxidative stress triggered by the overproduction of reactive oxygen species has been involved in lead-induced tissue damage in different organs, including hematological and renal systems [8-9].

Changes in antioxidant enzymes activity in lead-exposed humans and animals have been widely reported [8-10], which can be attributed to the direct effect of lead on enzyme molecule or to a defense response against lead-induced overproduction of reactive oxygen species. We have shown that renal antioxidant enzymes activity such as catalase, superoxide dismutase, glutathione S-transferase and cytosolic thioredoxin reductase-1 increase after short- or long-term lead exposure [10]. However, only thioredoxin reductase-1 activity was increased after both short- and long-term exposure to low doses of lead, presumably as an early protection mechanism [10]. In addition, in short-term exposure, the renal thioredoxin reductase-1 activity is stimulated at lead doses lower than that necessary to inhibit δ-ALA-D

activity [10]. Thus, we hypothesized that thioredoxin reductase-1 activity could be an earlier indicator of lead effects than δ- ALA-D activity after short-term exposure.

Mammalian thioredoxin reductases (E.C. 1.6.4.5) along with their main substrates thioredoxins and NADPH comprise the thioredoxin system, which sustain many vital cellular processes including antioxidant defense, cell-proliferation, redox-regulated signaling cascades and the production of deoxyribonucleotides for DNA synthesis [11]. Thioredoxin reductase-1, the cytosolic isoform of these enzymes, is overexpressed in cells exposed to different toxicants such as mercury, cadmium, 4-hydroxynonenal and acrolein [12-15]. Once renal thioredoxin reductase-1 activity also increased in rats exposed to low lead doses [10] we hypothesized that this alteration could be involved in the protection against lead renal toxicity. However, it is not known if this increase precedes tissue damage.

Thereby, this study was aimed at evaluating whether thioredoxin reductase-1 activity could be an earlier indicator of lead effects than erythrocyte δ-ALA-D activity after short-term exposure. We have also assessed the blood and renal lead concentrations associated to changes in thioredoxin reductase-1 activity. As thioredoxin reductase-1 is a selenium-dependent enzyme, we measured blood and renal selenium levels to verify if changes in the activity of this enzyme could be related to altered selenium levels during lead exposure. Histopathological analysis of renal tissue as well as renal and plasmatic oxidative stress parameters were assessed to determine whether changes in thioredoxin reductase-1 precede oxidative and tissue damage in lead-exposed rats.

Materials and Methods

Animals. This study was approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria, Brazil (no. 23081.000310/2008-32). Eight weeks-old

male Wistar rats from our breeding colony (190 ± 15 g body weight) were maintained in an air conditioned room (22-25 °C) under natural lighting conditions, with free access to water and standard laboratory chow (Guabi, Campinas, SP, Brazil).

Exposure and sample collection. To assess the short-term exposure, the animals received one intraperitoneal injection (1 ml/kg body weight) of vehicle (120 mM NaCl, 10 mM phosphate buffer, pH 7.4) or three different doses of lead acetate (5, 10 or 25 mg/kg). Lead acetate suspensions were prepared in 120 mM NaCl, 10 mM phosphate buffer, pH 7.4. Six, twenty-four or forty-eight hours after the injection, rats were anaesthetized with sodium thiopental and exsanguinated by cardiac punction. The doses of lead acetate and time of exposure were based on our preliminary study [10] in which 25 mg/kg lead acetate enhanced renal thioredoxin reductase-1 activity at 6, 24 and 48 h after exposure. Here, we have used 25 mg/kg lead acetate or less to assess the lowest dose that changes thioredoxin reductase-1 activity.

Blood was separated into heparinized tubes for determination of the erythrocyte δ-aminolevulinate dehydratase, thioredoxin reductase and superoxide dismutase activities and into tubes with EDTA for measurement of blood lead and selenium levels. An aliquot of heparinized blood was centrifuged to yield the plasma fraction that was used immediately to determine thiobarbituric acid reactive substances and protein carbonyl levels.

After blood collection, animals were killed by decapitation and kidneys were collected. The right kidney was homogenized in 3 volumes of 150 mM NaCl. The homogenate was centrifuged at 3000 g at 4 °C for 10 minutes to yield a low-speed supernatant that was used to determine thiobarbituric acid reactive substances, protein carbonyl levels and superoxide dismutase activity. The left kidney was stored at -20 ºC to determine lead and selenium levels.

Another set of animals were treated with lead acetate as described above to provide kidneys for measurement of thioredoxin reductase. The kidneys were used to measure enzyme activity.

Blood and renal lead and selenium levels. All reagents used were of analytical grade and dilutions were made using Milli-Q® purified water (resistivity: 18.2 MΩ cm-1). Nitric acid (65%, Merck, Darmstadt, Germany) was doubly destilled (duoPUR, Subboiling Distillation System, Milestone, Sorisole, Italy).

Kidneys and blood samples were digested with concentrated HNO_3 and 30% hydrogen peroxide (Synth, São Paulo, Brazil) using a Model Multiwave 3000® equipped with high-pressure quartz vessels (Anton Paar, Graz, Austria). After digestion, samples were diluted with water to 25 ml and transferred to polypropylene flasks.

Lead and selenium levels were assessed using inductively coupled plasma mass spectrometer (ICP-MS PerkinElmer SCIEX, Elan DRC II model, Thornhill, Canada) equipped with a cyclonic spray chamber and concentric nebulizer. Argon (99.999% of purity) was used for plasma generation. Operational conditions were selected according to the manufacturer`s instructions. The calibration solutions were prepared from a multielementar solution (PlasmaCal Calibration Solution, SCP33MS, 10 mg l^{-1}, 5% HNO_3, Quebec, Canada) by diluting them in HNO_3 5% (v/v). Digested samples were appropriately diluted with 5% HNO_3 (v/v) to reach the same concentration range of the calibration curve. Spike recovery tests and analysis of a biological certified reference material (SRM NIST 1577, bovine liver) were carried out to validate the results. Blanks were run after each 10 measurements in order to check eventual memory effects.

δ-Aminolevulinate dehydratase activity. δ-Aminolevulinate dehydratase activity was assessed in lysed erythrocytes as described by Berlin and Schaller [16]. Modified Ehrlich`s reagent was used to react with the porphobilinogen (PBG) final product to yield a pink colored compound, which was measured at 555 nm. The activity is shown as nmol PBG min^{-1} l^{-1} erythrocytes.

Plasma and renal lipid peroxidation. After the addition of 7.2 mM of butylated hydroxytoluene to prevent further oxidation, the kidney homogenate supernatant and plasma were used for determination of thiobarbituric acid reactive substances [17]. Samples were extracted with *n*-butanol and the absorbance of reaction product was determined at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane.

Blood and renal protein carbonyl. Protein oxidation was determined as the amount of protein carbonyl groups, which were quantified at 370 nm after reaction with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone [19]. The carbonyl content was calculated using the molar absorption coefficient for aliphatic hydrazones (22,000 M^{-1} cm^{-1}) and expressed as nmol carbonyl mg^{-1} protein.

Blood and renal antioxidant enzymes. For measuring blood thioredoxin reductase-1 activity, whole blood was hemolyzed by adding 4 volumes of cold Milli-Q water. Then, the hemolyzed sample was centrifuged at 9000 g for 15 minutes at 4 ºC and the supernatant was harvested and diluted with 1 M potassium phosphate buffer for the enzyme assay. Kidney was homogenized in 9 volumes of buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_2, 1.4 mM KH_2PO_4, pH 7.3), centrifuged and prepared as described by Hill et al. [19] to measure enzyme activity. Both in kidney homogenate and in blood samples thioredoxin

reductase-1 activity was measured as the increase in absorbance at 412 nm due to the NADPH-dependent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reduction to 5'-thionitrobenzoic acid (TNB^-) [20]. Thioredoxin reductase-1-independent DTNB reduction was also assessed by adding gold (III) chloride trihydrate (500 nM) to inhibit thioredoxin reductase-1 activity [21]. This reduction was subtracted from the total DTNB reduction in order to obtain thioredoxin reductase-1 activity. The amount of DTNB reduced was calculated using an absorption coefficient of $13.6\times10^3 M^{-1}\ cm^{-1}$.

Superoxide dismutase activity was determined in blood and in the low-speed renal supernatant. The reaction was based on its ability to inhibit the autoxidation of adrenaline to colorful adrenochrome product at an alkaline pH [22].

Hemoglobin quantification. Hemoglobin was measured by using routine laboratory kit (Doles, Goiânia, GO, Brazil).

Protein quantification. Protein was measured according to Lowry et al. [23], using bovine serum albumin as standard.

Hystopathological analysis. Kidneys were fixed with 10% formalin, embedded in paraffin, seccionated at 5 μm with a standard microtome and stained with hematoxylin and eosin. The sections were analyzed by a pathologist who did not have knowledge of the experimental groups.

Statistical analysis. Because data did not meet the assumptions of parametric ANOVA, the nonparametric Kruskal-Wallis ANOVA was used for assessing the effect of

lead at each time point, followed by a multiple comparison test when required. Differences were considered statistically significant when $P < 0.05$.

Results

As showed in fig. 1A and B, the short term exposure to the highest lead acetate dose (25 mg/kg) increased blood and renal lead levels at the three time points evaluated ($P<0.05$). Lead acetate doses lower than 25 mg/kg had no such an effect, even though it was possible to observe a tendency of increasing blood lead levels at 5 and 10 mg/kg overtime when compared to the control group (fig. 1A). On the other hand, selenium levels, which are essential for thioredoxin reductase-1 activity beyond other selenoproteins, were not influenced by any dose of lead acetate either in rat blood or kidney up to 48 h after exposure (fig. 1C and D). Once erythrocyte δ-ALA-D activity is recognized as the most sensitive indicator of lead effects, we measured its activity to verify if alterations in a lead exposure indicator, i.e, blood lead levels would be accompanied by changes in δ-ALA-D activity. In fact, short-term lead exposure decreased erythrocyte δ-ALA-D activity at the highest lead acetate dose (25 mg/kg; fig. 2; $P<0.05$), but unlike to blood and renal lead levels, this change occurred only 6 h after lead exposure and was recovered to the control levels at 24 and 48 h after exposure. To test the hypothesis that thioredoxin reductase-1 could be used as an early indicator of lead effects, its activity was measured in rat blood and kidneys and compared to the alterations in lead levels, erythrocyte δ-ALA-D activity and oxidative stress parameters. As we can see in fig. 3 A, blood thioredoxin reductase-1 was not changed by any dose of lead acetate up to 25 mg/kg, which was the dose that increased blood and renal lead levels from 6 to 48 h. On the other hand, the exposure to 25 mg/kg of lead acetate increased renal thioredoxin reductase-1 activity ($P<0.05$; fig. 3B), which unlike erythrocyte δ-ALA-D activity, paralleled the increase of the blood and renal lead levels that occurred overtime after the exposure to this same dose

(fig. 1A and B). In addition, the increase of renal thioredoxin reductase-1 activity was not accompanied by any change in blood or renal superoxide dismutase activity (fig. 3 C and D) or in markers of oxidative damage such as lipid peroxidation and protein carbonyl levels in rat plasma and kidneys (data not shown). In addition to the lack of alterations in the markers of oxidative damage, histopathological analysis revealed no renal tubular damage, as shown by the preserved architecture of the tubules and regular epithelial cells, which was accompanied by the absence of necrotic cells, even after exposure to the highest lead acetate dose (25 mg/kg) at the three time points assessed (fig. 4 A-D).

Discussion

The current study was aimed at evaluating whether thioredoxin reductase-1 activity could be an earlier indicator of lead effects than erythrocyte δ-ALA-D activity. To achieve this objective, both blood and renal thioredoxin reductase-1 activity were evaluated along with oxidative damage parameters, lead exposure and effect indicators, beyond histopathological analysis of renal tissue.

The absence of effect of short-term exposure to 10 mg/kg or lower lead acetate dose on the renal or blood biochemical parameters could be due to the low blood lead levels that were around 2 µg/dL. Although there is no blood lead level considered safe, the World Health Organization (WHO) considers that should be concern at levels of 10 µg/dL or higher [24]. On the other hand, the increase in blood and renal lead levels following acute exposure to 25 mg/kg lead acetate was accompanied by the inhibition of erythrocyte δ-ALA-D activity at 6 h after lead exposure, along with the increase of renal thioredoxin reductase-1 activity that persisted over 48 h after exposure. In fact, erythrocyte δ-ALA-D activity is considered the most sensitive lead effect biomarker in humans, being mainly indicated to evaluate recent exposure to metal [25]. Erythrocyte δ-ALA-D activity can be inhibited from blood lead levels

of 3.2 μg/dL onwards [5], which is in agreement with the inhibition found for this enzyme at 5.98 μg/dL blood lead that was the concentration reached 6 h after exposure to 25 mg/kg lead acetate. The unexpected disappearance of erythrocyte δ-ALA-D activity inhibition at 24 and 48 h after lead (25 mg/kg) exposure even without change in blood lead levels compared to 6 h after exposure, could be associated to lead complexation by metallothionein-like intra-erythrocyte proteins, which were demonstrated to protect against lead toxicity [26]. In fact, metallothioneins are zinc-dependent proteins that donate zinc to δ-aminolevulinate dehydratase, which is an essential mineral for the activation of this enzyme. In addition, metallothioneins also chelate the lead already bound to the active site of the enzyme [27]. Although some studies have attributed the increase of erythrocyte δ-ALA-D activity caused by short-time lead exposure to the increased synthesis of this enzyme [28-29], this effect would be unlikely to occur within 24 h, once synthesis of new erythrocytes is required by bone narrow.

Unlike erythrocyte δ-ALA-D, the increase of renal thioredoxin reductase-1 activity persisted for 48 h after exposure to 25 mg/kg lead acetate, being parallel to the increase in blood and renal lead levels. This occurred despite blood lead levels (5-7.2 μg/dL) were still lower than the limit values recommended for humans. Blood levels greater than 30 μg/dL are generally considered elevated in adults [2], whereas values greater than 10 μg/dL are excessive for children [30]. Thus, renal thioredoxin reductase-1 activity is more sensitive to lead effects than erythrocyte δ-aminolevulinate dehydratase. The difference of sensitivity between these enzymes was not related to the higher lead levels found in kidney compared to blood (5-7 μg/dL would correspond to 0.05-0.07 μg/g), because when we previously compared both enzymes in the kidney we also found the highest lead sensitivity for thioredoxin reductase-1 [10]. In that study, renal thioredoxin reductase-1 activity increased

after exposure to a lower lead dose (25 mg/kg) than that required to inhibit renal δ-ALA-D activity (50 mg/kg) [10].

The enhancement of renal thioredoxin reductase-1 activity seems to be an early effect of lead exposure because it was not accompanied by any change in plasma or renal oxidative stress parameters or by histological damage. However, the unchanged blood thioredoxin reductase-1 suggests that blood enzyme is less sensitive to the increase of lead levels than its renal isoform. Accordingly, blood thioredoxin reductase-1 also did not change in long-term lead exposed workers, whose blood lead levels amounted to 50 μg/dL [31]. This lowest sensitivity of the blood enzyme may be related to the lower blood lead levels compared to renal lead levels or to the low expression of the thioredoxin system in red blood cells [32]. In this sense, the lack of changes in blood thioredoxin reductase activity after long-term lead exposure of humans [31] and after short-term lead exposure of rats indicate that this enzyme is not an appropriate indicator of lead effects.

Thioredoxin reductase-1 is part of phase-II enzymes, which are involved in xenobiotics metabolism [33]. The expression of phase-II enzymes involves, at least in part, the activation of Keap1/Nrf2 antioxidant response pathway, which target genes located in antioxidant response elements (ARE) in nucleus. Several metal and non-metal toxicants have been found to activate Keap1/Nrf2 pathway either directly or through of reactive oxygen species generation, and consequently induce thioredoxin reductase-1 expression in cell cultures [12-14]. Increased TrxR-1 expression may trigger an adaptive response in cells against further exposure to these toxicants [14-15]. Accordingly, it was recently demonstrated that lead increased mouse cytochrome P450 expression via activation of Keap1/Nrf2 pathway in primary hepatocytes [34], but there is no study showing thioredoxin reductase-1 overexpression following lead exposure, neither the mechanism behind up to now.

The protection supplied by the renal thioredoxin reductase-1 against lead toxicity, could involve a chelating action of this enzyme on lead and its further detoxification from cells, as previously suggested for other metals [35]. Furthermore, thioredoxin reductase-1 is the unique enzyme capable to reduce thioredoxin-1 protein, which serves as a co-factor for peroxiredoxins in a reaction that scavenges hydrogen peroxide [36], beyond all other functions displayed by this protein such as cell redox control and antioxidant defense [11].

Thioredoxin reductase-1 can also directly remove hydrogen peroxide and lipid hydroperoxides [37], protecting cells against oxidative stress. Thus, the increase of renal thioredoxin reductase-1 activity without change in superoxide dismutase activity, which generates hydrogen peroxide, could indicate an enhanced capacity to remove hydrogen peroxide. These protective actions of thioredoxin reductase-1 could have counteracted the oxidant effects of lead in kidneys, once no damage was observed in lipids, protein or in renal tissue cells morphology. On the other hand, the lack of changes in all oxidative stress parameters in blood and plasma suggests that either oxidative stress did not occur, because of the low blood lead levels attained after short-time exposure, or protection were provided by antioxidant enzymes other than superoxide dismutase and thioredoxin reductase-1, which are not evaluated here [38-39].

In summary, our results show that the increase of renal thioredoxin reductase-1 activity occurred after short-term exposure to low lead levels without evidence of oxidative damage in blood and kidneys, which suggests that thioredoxin reductase-1 stimulation could be an early event in the response of renal cells against lead toxicity. This enhancement paralleled the increase of blood and renal lead levels over time, which was not observed for the lead-sensitive erythrocyte δ-aminolevulinate dehydratase. Thus, we demonstrated that renal thioredoxin reductase-1 is more sensitive to lead effects after short-term exposure than

erythrocyte δ-ALA-Dactivity. However, the unchanged blood thioredoxin reductase-1 activity could limit its use to monitor the effects of short-term exposure to lead.

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Figure Legends

Fig. 1**:** Evaluation of lead (Pb) and selenium (Se) levels in rats after lead exposure. (A) blood Pb, (B) renal Pb levels, (C) blood Se and (D) renal Se levels. Data are expressed as mean ± S.E.M (n=6-8).*Different from control of the same time point ($P<0.05$).

Fig.2: Erytrocyte δ-aminulevulinate dehydratase (-ALA-D) activity in rats after lead exposure. Data are expressed as mean ± S.E.M (n=6-8).*Different from control at the same time point ($p<0.05$).

Fig. 3: Antioxidant enzymes activities in rats after lead exposure. (A) blood thiroedoxin reductase-1 (TrxR-1), (B) renal TrxR-1, (C) blood superoxide dismutase (SOD) and (D) renal SOD activity. Data are expressed as mean ± S.E.M (n=6-8). *Different from control at the same time point ($p<0.05$).

Fig. 4: Representative histology analyses of renal tissue from rats after lead exposure (25 mg/kg). Control (A), 6 hours after lead exposure (B), 24 hours after lead exposure (C) and 48 hours after lead exposure (D). Renal tissue was fixed in 10% formalin and embedded in paraffin. Sections of 5 μm were obtained with a standard microtome and were stained with hematoxylin and eosin. The magnification was 200x. Observe the preserved architecture of the tubules, regular epithelial cells and absence of necrotic cells (A). In lead-exposed rats (B, C and D), the characteristics were identical to that of the control group. n= 5 for each group.

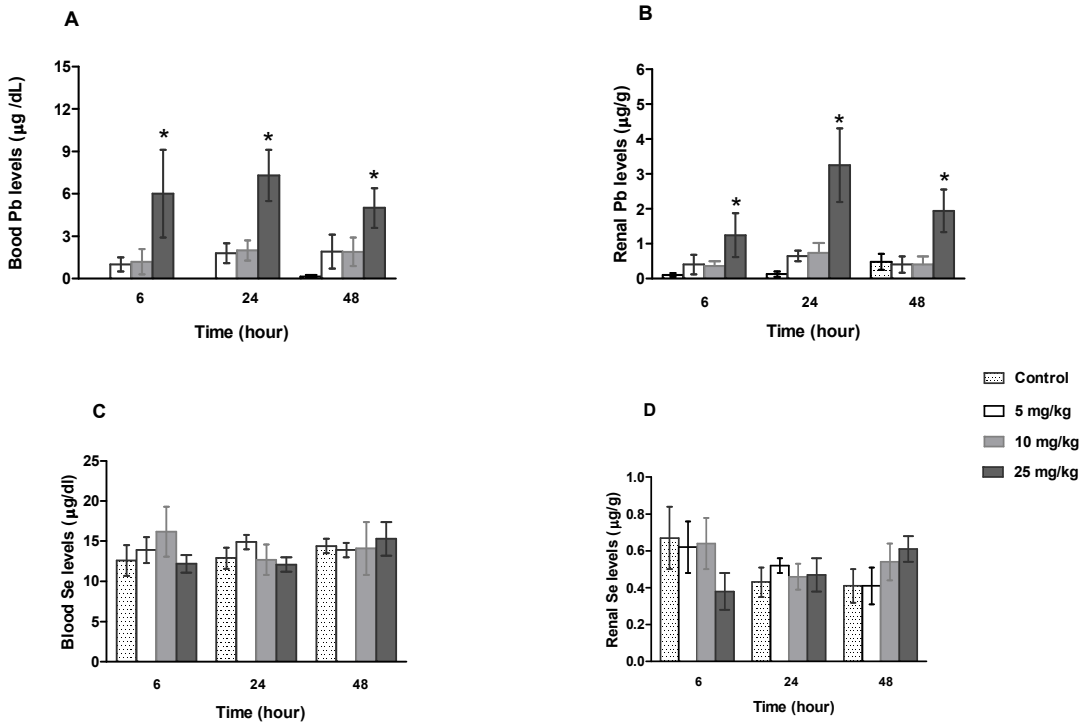

A
Bood Pb levels (µg /dL)
15
12
9
6
3
0
*
*
*
6
24
48
Time (hour)
B
Renal Pb levels (µg/g)
6
5
4
3
2
1
0
*
*
*
6
24
48
Time (hour)
C
Blood Se levels (µg/dl)
25
20
15
10
5
0
6
24
48
Time (hour)
D
Renal Se levels (µg/g)
1.0
0.8
0.6
0.4
0.2
0.0
6
24
48
Time (hour)
Control
5 mg/kg
10 mg/kg
25 mg/kg

Fig. 1

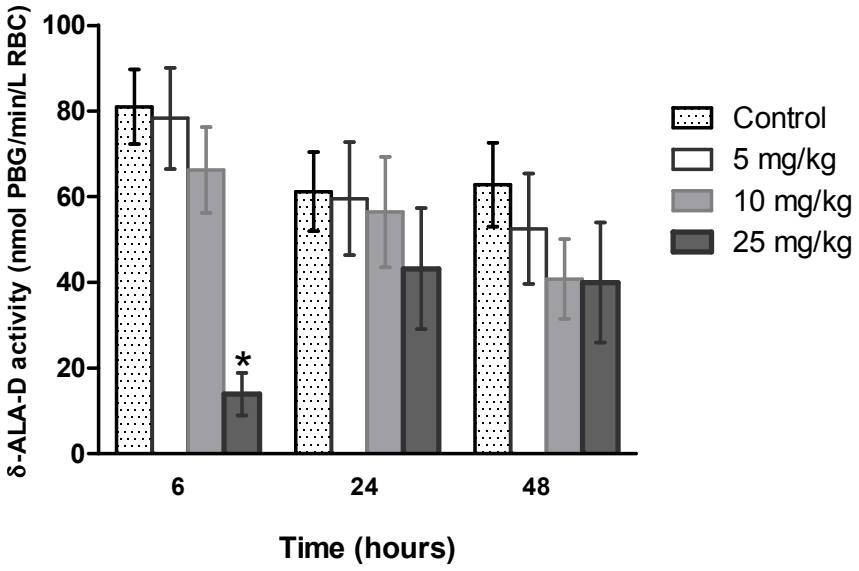
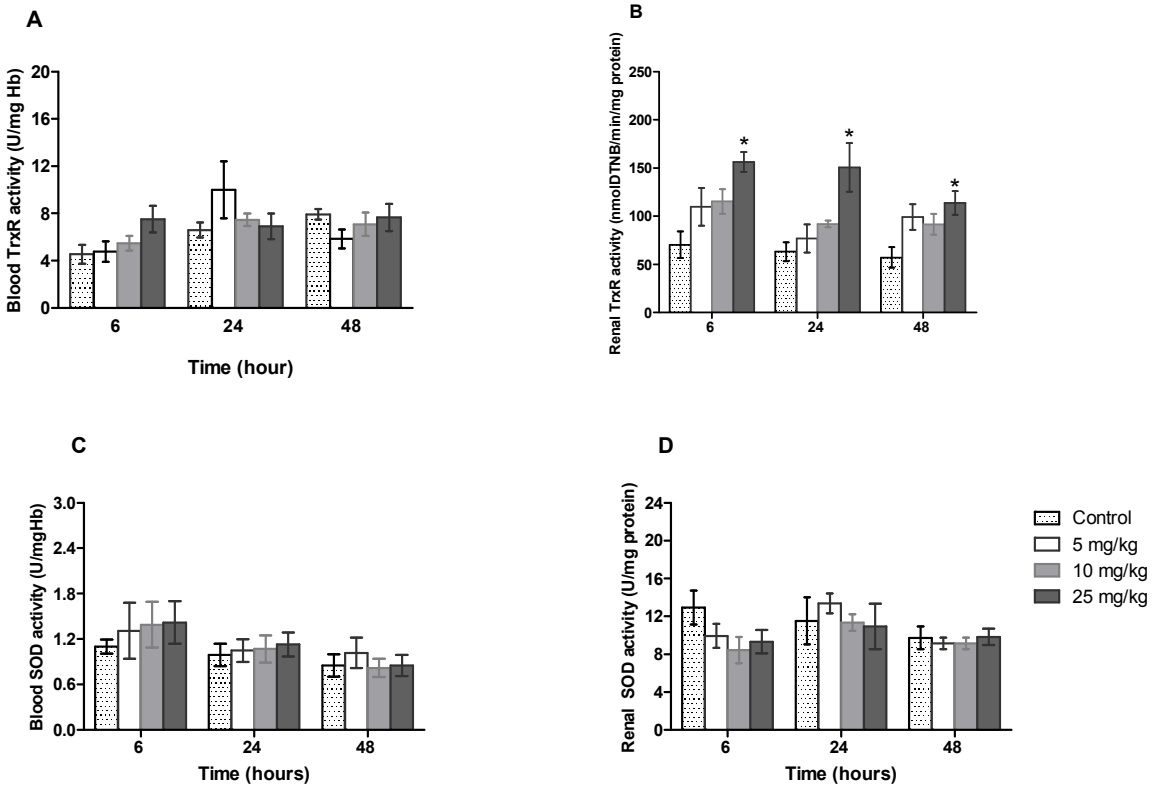

δ-ALA-D activity (nmol PBG/min/L RBC)
100
80
60
40
20
0
*
6
24
48
Time (hours)
Control
5 mg/kg
10 mg/kg
25 mg/kg

Fig. 2

Fig. 3


A
Blood TrxR activity (U/mg Hb)
20
16
12
8
4
0
6
24
48
Time (hour)
B
Renal TrxR activity (nmolDTNB/min/mg protein)
250
200
150
100
50
0
*
*
*
6
24
48
C
Blood SOD activity (U/mgHb)
3.0
2.4
1.8
1.2
0.6
0.0
6
24
48
Time (hours)
D
Renal SOD activity (U/mg protein)
24
20
16
12
8
4
0
6
24
48
Time (hours)
Control
5 mg/kg
10 mg/kg
25 mg/kg

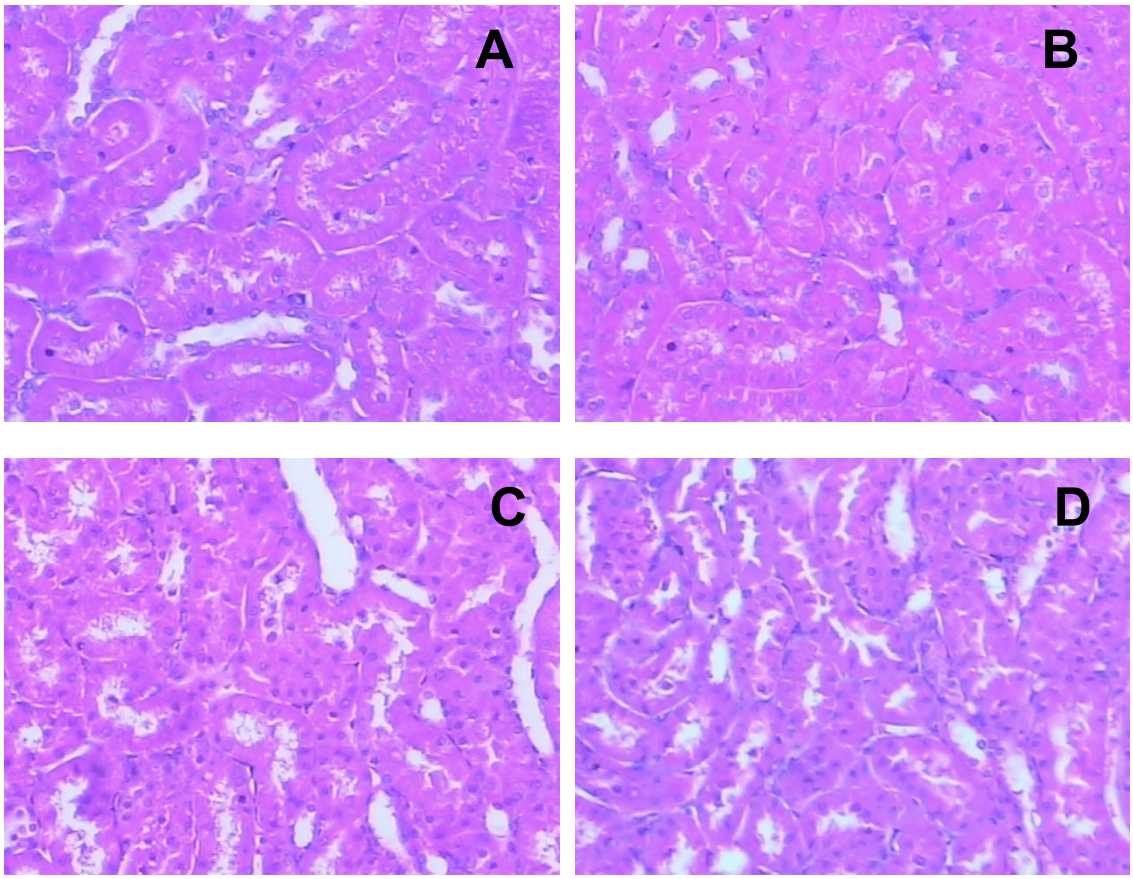


Fig. 4

5 DISCUSSÃO

A depleção de GSH e inibição da enzima sulfidrílica δ-ALA-D com conseqüente geração de EROs estão envolvidas na toxicidade do Pb (Monteiro *et al*., 1989; Warren *et al*., 1998). Além disso, a exposição ao Pb pode diminuir a atividade de enzimas antioxidantes, promovendo o dano oxidativo (Ito *et al.*, 1985; Reed, 1990; Chiba *et al*., 1996; Sivaprasad *et al*., 2004).

No manuscrito 1, nós demonstramos que o Pb tanto na forma orgânica (acetato de Pb) como na forma inorgânica (nitrato de Pb) inibe a atividade da enzima TrxR1 purificada e esse efeito se repetiu em cultura de células renais HEK 293, o que caracteriza a enzima TrxR1 como um alvo do Pb. No entanto, diferente da maioria dos inibidores da TrxR1 relatados até agora, o Pb não inibe a enzima por interagir com o resíduo de Sec no sítio ativo, uma vez que a inibição também ocorreu quando a atividade enzimática foi avaliada usando o método de redução da juglona, um substrato que ao contrário do ácido 5,5-ditiobis-2-nitrobenzóico (DTNB), é reduzido apenas pelo sítio ativo da porção N- terminal (-Cys-Val-Asn-Val-Gly-Cys-) (Cenas *et al*., 2004,). Essa conclusão também está baseada na inibição mais potente de sua homóloga GR pelo Pb, o que sugere que o Pb teria como alvo as cisteínas da porção N-terminal, que são comuns a ambas as enzimas. Isso não exclui, entretanto, que o Pb também possa se ligar a outros aminoácidos importantes para a estrutura dessas enzimas e consequentemente, interferir nas suas atividades. Por outro lado, a reversão da inibição da TrxR1 pela albumina sérica (BSA) e da GR pela GSH, caracteriza as inibições da TrxR1 e da GR como reversíveis, o que pode ser atribuído à complexação do Pb pelas cisteínas presentes nessas moléculas. De fato, sabe-se que a GSH atua na complexação e eliminação de metais pesados (Reed., 1990; Dagget et al., 1998) e a BSA liga-se a metais, incluindo o cálcio (Ca^{2+}), bem como metais pesados (Grant *et al*., 1987). Assim, o fato da inibição da GR pelo Pb não ter ocorrido em cultura de células HEK 293 e da inibição da TrxR1 celular ter ocorrido somente na maior concentração de acetato de Pb (60 μM) pode ser explicado pela complexação do metal pela GSH celular, o que é sustentado pelo aumento dos níveis de grupos SHNP após 24h de exposição ao Pb nas concentrações de 1 a 60 μM. Ressalta-se também, que o meio de cultura para as células HEK 293 é suplementado com 10 % de soro fetal bovino (contém albumina),

o que pode ter diminuído a quantidade de Pb disponível para ligar às suas moléculas alvo nas células.

A proteção por moléculas sulfidrílicas também explicaria o fato de não ter sido observada inibição da TrxR1 renal em ratos expostos ao Pb tanto no manuscrito 2 desta tese, como em nosso estudo anteriormente publicado (Conterato *et al*., 2007), em que os níveis de SHNP renais aumentaram de uma maneira dose-dependente na exposição prolongada. Ressalta-se, entretanto, que o rim possui proteínas ricas em ácido aspártico e glutâmico com poucas cisteínas, denominadas proteínas ligadoras de Pb (PbBPs), assim como as metalotioneínas (MT) que complexam o Pb, tornando-o indisponível para ligar-se a outros alvos moleculares, atenuando, inclusive, a inibição da enzima δ-aminolevulinato desidratase (δ-ALA-D) (Goering, 1993; Fowler, 1998).

Além da proteção fisiológica fornecida pelas moléculas sulfidrílicas endógenas, a inibição *in vitro* das atividades da TrxR1 e GR purificadas também foi reversível na presença do EDTA, o quelante de escolha para o tratamento da intoxicação pelo Pb. Esses achados são relevantes, uma vez que os quelantes são conhecidos por ligarem-se aos metais livres, mas não àqueles que já se encontram ligados aos seus alvos moleculares. Nesse sentido, a terapia de quelação do Pb com EDTA além de auxiliar na eliminação mais rápida do metal, reverteria alterações bioquímicas importantes como o restabelecimento da atividade da GR e principalmente da TrxR1, cuja magnitude da reversão da inibição pelo EDTA foi maior do que aquela observada para a GR *in vitro*. Assim, durante o tratamento com EDTA, essas enzimas antioxidantes poderiam conferir maior proteção ao organismo contra a geração e a ação de EROs produzidas como conseqüência da exposição ao Pb. Ao contrário dos nossos resultados, o EDTA interferiu muito pouco na inibição da TrxR1 purificada pelo cloreto de mercúrio ($HgCl_2$) e pelo metilmercúrio (MeHg) (Carvalho *et al*., 2008), sendo observadas IC_{50} na presença de EDTA de 14,2 nM para o $HgCl_2$ e de 25,6 nM para o MeHg contra as IC_{50} de 7,2nM e 19,7nM, respectivamente, na ausência de EDTA. Assim, o EDTA parece ser mais eficaz em reverter as alterações causadas pelo Pb na atividade da TrxR1 do aquelas causadas pelo Hg.

Com relação aos efeitos do Pb sobre a Trx1 purificada, não foi possível medir a atividade dessa proteína *in vitro* pelo método de redução da insulina. Uma vez que tanto a TrxR1 como a Trx1 são suscetíveis à oxidação por metais,

preconiza-se preparar soluções de TrxR1 e Trx1 purificadas em tampões com EDTA antes de estocá-las (Arnér, 2009). No entanto, quando se deseja avaliar o efeito de um metal específico sobre a atividade dessas proteínas, o EDTA deve ser removido passando a solução estoque das proteínas através de uma coluna. Infelizmente, não foi possível detectar atividade da Trx1 e TrxR1 pelo método de redução da insulina (Luthman & Holmgren, 1982) quando o EDTA foi removido, mesmo na ausência de Pb. De qualquer forma, para nossa surpresa, a oxidação significativa dos grupos sulfidrílicos (40%) da Trx1 purificada ocorreu somente quando o acetato de Pb estava presente na proporção molar de 20:1 em relação à essa proteína (dados do manuscrito 1 não mostrados), o que sugere fortemente que a Trx1 não é um alvo direto do Pb. Nosso resultado contrasta com a oxidação da Trx1 pelo $HgCl_2$ em uma proporção molar em relação à Trx1 bem menor, ou seja, 2,5:1 (Carvalho *et al.*, 2008).

Os experimentos realizados em células HEK 293 apontam o sistema da GSH como defesa primária dessas células contra a toxicidade do Pb, uma vez que os níveis de SHNP (os quais representam a maior parte da GSH celular) aumentaram em 24 h, assim como a atividade da GST em 24 e 48 h e da GR em 48 h, enquanto que a atividade da TrxR1 foi inibida e da Trx1 não foi afetada após 24 h de preincubação com acetato de Pb, confirmando os resultados obtidos no estudo *in vitro* com as TrxR1 e Trx1 purificadas. Não pode ser descartada a hipótese de que essa resposta compensatória do sistema da GSH tenha protegido as células contra o dano oxidativo e de uma conseqüente morte celular causada pelo excesso de EROs. Por outro lado, o aumento da atividade e da expressão da TrxR1 e Trx1 pelo Pb em células com níveis depletados de GSH (100 µM de BSO depletou 40 % dos níveis de GSH, dados não mostrados) e na ausência de alterações nos componentes do sistema da GSH aponta o sistema da Trx como o principal responsável pela defesa contra o estresse oxidativo e morte celular nessas condições. Assim, o decréscimo de 20 % na viabilidade celular observado em 48 h nas células incubadas previamente com BSO foi recuperado em 72 h, o que poderia ser atribuído à estimulação da expressão da TrxR1 que também ocorreu em 48 h sob condições de depleção de GSH (dados não mostrados). É importante lembrar, entretanto, que a TrxR1 quando inibida por compostos que tem como alvo o resíduo de Sec induz a apoptose *per se*, sendo referida como um “assassino pró-oxidante de células” (Anestal *et al*., 2003; Anestal *et al*., 2008). A condição fundamental para

essa função da TrxR1 com Sec derivatizada por compostos oxidantes é a de que os resíduos de cisteínas na porção N-terminal estejam livres. Pelos dados de inibição obtidos em nosso estudo com a TrxR1 purificada, o Pb não tem como alvo o Sec da região C-terminal mas muito provavelmente se liga à porção N-terminal ativa da enzima, o que explicaria a maior dificuldade de induzir apoptose pelo Pb em relação a outros oxidantes que têm como alvo a Sec da enzima (Anestal *et al*., 2003; Carvalho *et al*., 2008; Anestal *et al*., 2008)

Tanto a expressão das enzimas GST, GR, TrxR1 como da proteína Trx1 são ativadas pela via antioxidante Keap1/Nrf2 (Arnér, 2009; Nielsen, 2009). Porém outras vias, as quais também são ativadas pelo Pb, podem promover o aumento da expressão das enzimas GR e GST (Rushmore *et al.,* 1990; Friling *et al.,* 1992; Suzuki *et al.,* 1996), enquanto que a via Keap1/Nrf2 é apontada como a principal via ativadora do aumento da expressão do sistema da Trx. Isso explicaria o aumento da atividade da GR e GST pelo Pb sem aumento da atividade e expressão do sistema da Trx. Condições que envolvem a depleção de GSH (Arnér, 2009), a qual age com os demais componentes do sistema da glutationa no equilíbrio redox celular podem oxidar o complexo citosólico Keap1/Nrf2 e promover o aumento da expressão do sistema da Trx e de proteínas envolvidas na defesa antioxidante. Entretanto, a ausência de resposta ao Pb por parte da GR e GST em células pré incubadas com BSO, poderia estar relacionada a alguma influência dos níveis drasticamente diminuídos de GSH sobre a estabilidade dessas enzimas envolvidas no metabolismo da GSH.

Não se pode descartar, entretanto, a ativação direta da via Keap1/Nrf2 pelo Pb, uma vez que esse metal pode ter como alvo os resíduos de cisteínas do Keap1. De fato, o Pb aumentou a expressão do citocromo P450 2A5 de hepatócitos através da ativação dessa via (Lämsä *et al*., 2010), embora o mecanismo dessa indução não tenha sido demonstrado.

Diferente dos resultados obtidos em culturas de células, o aumento da atividade da TrxR1 renal em ratos foi uma alteração bioquímica do Pb que ocorreu sem evidências de um estatus oxidativo aumentado (como aquele caracterizado pela depleção de GSH por BSO nas culturas de células). Embora os níveis de SHNP e as atividades da GST e GR não tenham sido avaliados aqui, nosso estudo prévio (Conterato *et al.,* 2007) mostrou que não houve alterações nos níveis de SHNP, nem na atividade da GST renal na dose de 25 mg/kg, que foi a dose necessária para

estimular a atividade da TrxR1 renal em ambos os estudos, além de ser a dose que causou aumento nos níveis sanguíneos e renais de Pb e inibição da δ-ALA-D. Assim, no rim de ratos, a TrxR1 parece ser a principal proteína envolvida na defesa imediata contra a toxicidade do Pb, sendo alterada mesmo sem evidências de dano oxidativo de proteínas e lipídios celulares ou de dano tecidual, conforme evidenciado pela análise histopatológica do tecido renal. A falta de evidências de que o estresse oxidativo ocorreu no tecido renal, pode ser resultado da proteção fornecida pela TrxR1 através de mecanismos como a ação quelante da enzima sobre íons metálicos (Nishimoto *et al*., 2006), redução direta do H_2O_2 e de hidroperóxidos lipídicos (Bjornstedt *et al*., 1995) e redução da Trx1 a qual além de ser cofator das peroxirredoxinas (enzimas responsáveis pela remoção do H_2O_2) (Chae *et al*., 1994), exerce funções múltiplas no controle redox e defesa antioxidante celular (Norberg & Arnér, 2001).

O efeito estimulatório da TrxR1 renal de ratos também pode envolver a ativação da via Keap1/Nrf2, uma vez que essa via responde mesmo em um nível mínimo de estresse oxidativo, ou mesmo, na ausência desse (Singh *et al*., 2008). Assim, conforme mencionado anteriormente, a ativação da via Keap1/Nrf2 poderia estar mais relacionada a uma interação direta do Pb com o complexo Keap1/Nrf2, do que a oxidação do complexo pelas EROs produzidas pela exposição ao Pb.

Uma vez que a atividade da TrxR1 sanguínea, assim como a atividade da SOD, não alterou após a exposição ao Pb, a ausência de estresse oxidativo evidenciado pelos níveis plasmáticos inalterados de proteínas carboniladas e de TBARS (substâncias reativas ao ácido tiobarbitúricos) pode ser atribuído aos níveis baixos de Pb no sangue encontrado em todas as doses de acetato de Pb, as quais foram menores do que aquelas encontradas no rim, conforme foi demonstrado no manuscrito 2. Além disso, o aumento na atividade de outras enzimas antioxidantes que não foram avaliadas no estudo pode também ter ocorrido, conforme foi observado no sangue de humanos ocupacionalmente expostos ao Pb para a GPx e GST (artigo 1).

O aumento da atividade da TrxR1 renal, o qual diferente da inibição da atividade da δ-ALA-D eritrocitária, acompanhou o aumento dos níveis do Pb ao longo do tempo (6, 24 e 48 h) e não foi precedido de estresse oxidativo, sugere fortemente que esse efeito além de ser uma resposta de defesa é também uma alteração precoce da toxicidade do Pb. No entanto, a falta de alteração da TrxR1

sanguínea tanto em ratos como em humanos expostos a diferentes níveis de Pb (Conterato *et al*., 2011) exclui a possibilidade dessa enzima ser usada no monitoramento dos efeitos do Pb em populações expostas ao metal. A falta de alteração na TrxR1 sanguínea pode ser atribuída aos níveis sanguíneos baixos de Pb ou ao fato de que a enzima é bem menos expressa em eritrócitos do que nos demais tecidos (Södeberg *et al*., 2000).

Uma vez que a δ-ALA-D eritrocitária foi inibida tanto na exposição aguda de ratos, como na exposição prolongada de humanos ocupacionalmente expostos ao Pb, os resultados sugerem que essa enzima ainda é um indicador sensível de efeito do Pb. No entanto, conforme observado no estudo em trabalhadores expostos (artigo 1), o índice de reativação da δ-ALA-D parece ser mais adequada do que a atividade da δ-ALA-D no monitoramento dos efeitos do Pb, uma vez que aumentou no grupo pintores, com níveis sanguíneos baixos de Pb, enquanto que a atividade da δ-ALA-D foi inibida apenas nos fabricantes de baterias, com níveis sanguíneos de Pb mais elevados. Além disso, o índice de reativação de δ-ALA-D correlacionou-se muito mais com o Pb do que com o Cd sanguíneo (0,84 *vs* 0,27, $p<0,05$). Assim, a avaliação da índice de reativação da δ-ALA-D deveria ser realizada paralelamente à determinação da atividade da δ-ALA-D eritrocitária no monitoramento dos efeitos do Pb.

A hipótese proposta no artigo 1 de que o Cd poderia influenciar em alguns parâmetros avaliados nos trabalhadores, em vista da sua presença em tintas foi confirmada. Isso porque os parâmetros de estresse oxidativo e hematológicos correlacionaram-se ou com o Pb ou com o Cd sangüíneo, com exceção da GST que correlacionou-se com os dois metais com força similar e da TrxR1, a qual não correlacionou-se com nenhum dos metais. Esses resultados contrastam com os efeitos do Cd sobre a TrxR1 em culturas celulares (Sakurai *et al*., 2005; Nishimoto *et al*., 2006) e também com a inibição da TrxR1 purificada pelo Cd (IC_{50} = 0,20 μM) observada no manuscrito 1, a qual assim como a inibição pelo Pb, também foi revertida pela BSA. Apesar da TrxR1 ser importante na proteção contra a morte celular induzida por Cd em células HeLa (Nishimoto *et al*., 2006) e do Cd induzir a expressão da TrxR1 pela via Keap1/Nrf2 em cultura de células endoteliais (Sakurai *et al*., 2005), esta é a primeira vez em que se avaliou o efeito direto do Cd sobre a atividade da TrxR1 (manuscrito 1).

A resposta de defesa antioxidante parece ter sido efetiva contra a toxicidade do Pb e do Cd (mesmo com a inibição da catalase em pintores), uma vez que o aumento da atividade das enzimas antioxidantes GPx, SOD (fabricantes de baterias) e GST (fabricantes de baterias e pintores), bem como dos níveis de GSH eritrocitária (pintores) ocorreu sem aumento dos níveis plasmáticos de MDA ou de proteínas carboniladas nos dois grupos expostos. De fato, os níveis de proteínas carboniladas, que estranhamente diminuíram no grupo com maiores níveis de Pb (fabricantes de baterias) foram inversamente correlacionados à atividade da GPx sanguínea, o que pode explicar ao menos em parte, esse efeito. Por outro lado, efeito oxidativo foi observado com relação à hemoglobina, uma vez que os níveis de metemoglobina aumentaram nos fabricantes de baterias e correlacionaram com os níveis de Pb. Esse aumento pode ser atribuído a um efeito direto do Pb (Costa *et al*., 1997), bem como ao efeito pró oxidante do ALA acumulado em decorrência da inibição da δ-ALA-D eritrocitária (Monteiro *et al*., 1986), e não pôde ser evitado pelo aumento das enzimas antioxidantes.

As alterações na atividade das enzimas antioxidantes, nos parâmetros hematológicos (CHCM, RDW, leucócitos, neutrófilos, plaquetas e plaquetócrito) assim como a inibição da atividade da δ-ALA-D eritrocitária mesmo em níveis sanguíneos de Pb dentro do limite máximo estabelecido pela legislação de 60 µg/dl (Norma regulamentadora n◦ 7 de 1994), indicam que esses níveis considerados seguros para a saúde dos trabalhadores expostos devem ser reavaliados.

De uma maneira geral, os resultados do presente estudo apontam o sistema da tiorredoxina como alvo do Pb, mas principalmente como um mecanismo de proteção contra o metal. Entretanto, a ausência de alterações na atividade da TrxR1 sanguínea em animais e humanos expostos ao Pb, indica que essa enzima não é um bioindicador adequado dos efeitos tóxicos do Pb em populações expostas.

6 CONCLUSÕES

Os resultados obtidos nesse estudo indicam que:

- A enzima TrxR1, mas não a proteína Trx1 é um alvo do Pb, conforme evidenciado pela inibição da enzima purificada e de cultura de células HEK 293;
- A inibição da atividade da TrxR1 pelo Pb não tem como alvo o resíduo reativo de Sec no sítio ativo da enzima, conforme evidenciado pela maior inibição de sua homóloga estrutural não dependente de Sec, a enzima GR e pela inibição da TrxR1 também observada no método de redução da juglona, um substrato que não é reduzido pelo resíduo de Sec da enzima;
- A reversão pelo quelante EDTA das inibições das enzimas TrxR e GR pelo Pb implicaria em um efeito benéfico dessa terapia no restabelecimento das funções do sistema da Trx e do sistema da GSH, protegendo contra o estresse oxidativo induzido pela intoxicação por Pb. Estudos posteriores deverão ser conduzidos para a confirmação desse efeito *in vivo*.
- O sistema da Trx está envolvido na proteção contra a toxicidade do Pb, conforme observado pelo aumento da atividade e expressão da TrxR1 e Trx1 in *vitro* (cultura de células renais HEK 293) e pelo aumento precoce da atividade da TrxR1 renal *in vivo*, o qual ocorreu sem evidências de dano oxidativo e tecidual;
- A atividade da TrxR1 sanguínea não é um indicador adequado dos efeitos tóxicos do Pb, uma vez que não alterou em ratos e em humanos ocupacionalmente expostos ao Pb.

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8 ANEXOS

8.1 ANEXO A - Roteiro para autores / Guia para a redação e edição de artigo científico a ser submetido à Chemical Research in Toxicology

Scope, Editorial Policy, and Preparation of Manuscripts

Scope and Editorial Policy

Chemical Research in Toxicology publishes Articles, Rapid Reports, Chemical Profiles, Reviews, and Perspectives on structural, mechanistic, and technological advances in research related to the toxic effects of chemical agents. In addition, features entitled Forum and Letters to the Editor are published periodically. The Journal is intended to provide a venue for presentation of research relevant to all aspects of the chemical basis of toxic responses. It emphasizes rigorous chemical standards and encourages application of modern techniques of chemical analysis to mechanisms of toxicity. It publishes papers devoted to: (1) Studies on the molecular mechanisms by which toxic agents (chemical and biological) interact with living systems. "Toxic agent" is broadly defined as any molecule or aggregate of molecules that causes cellular damage. "Interactions" are broadly defined to include metabolism, chemical alteration of cellular constituents, adduct formation with cellular constituents, and specific or nonspecific interactions with cellular receptors or enzymes, leading to activation or inactivation. (2) Studies of the molecular mechanisms of the cellular responses to toxic agents. These may include, but are not necessarily limited to, alterations in global cellular constituents (DNA, proteins, lipids, carbohydrates), repair of damaged cellular constituents, alterations in gene transcription/translation, induction of genetic mutations, activation/inactivation of stress responses, cell cycle arrest, apoptosis, or necrosis. (3) Studies of the mechanisms by which the cellular responses to toxic agents affect the intact organism, leading to the pathogenesis of disease. (4) Studies of the development and application of new methodologies for the investigation of the interaction of toxic agents with living systems. Authors are encouraged to consult recent issues for examples of the scope of work published in the Journal (http://pubs.acs.org/journal/crtoec). In the case of uncertainty regarding the suitability of a manuscript, authors may send an abstract to the Editor by e-mail (crt@vanderbilt.edu) to request an opinion prior to submission.

Editorial Organization and Reviewing Process

The Editor is appointed by the American Chemical Society and has the final responsibility for all editorial decisions. Associate Editors, together with the Editor, process all manuscripts received. Members of the Editorial Advisory Board are usually appointed for a three-year term and may be reappointed.

Contents (click on the topic)

When a manuscript is received, Editors first judge whether its content falls within the scope of *Chemical Research in Toxicology. Manuscripts that are primarily descriptive, confirmative of previous work, or do not address fundamental aspects of the chemical nature of toxicologic processes or those that in the judgement of the Editors are not of sufficient interest to the general readers of Chemical Research in Toxicology will be returned to the author without review.* These decisions are reached with the participation of at least two Editors or an Editor and an Editorial Advisory Board member and should be considered final. Following this initial evaluation, manuscripts are assigned to an Editor (the Editor-in-Chief or an Associate Editor) for review. The author will receive an e-mail notification indicating the Editor who will be processing the manuscript.

Manuscripts are evaluated for scientific content and significance by independent reviewers and the Editors. Reviewers are selected for their competence in specialized areas of toxicology from a database of qualified specialists. In addition, authors are encouraged to provide the names of *three to five* individuals who are competent to review the work. Authors should take care not to recommend reviewers that have a real or perceived conflict of interest (e.g., a collaborator or someone who has recently published with one of the authors), and all reviewers are expected to disqualify themselves if they have a conflict of interest. Authors may also request that certain (but not more than three) reviewers not be chosen. Members of the Editorial Advisory Board cannot be disqualified from participating in the final disposition of a manuscript. The reviewers are advisory to the Editor, and their reports are used to reach the editorial decision. If the reviewers disagree or, if in the judgment of the Editor, the manuscript has not received adequate consideration, the manuscript and the reviewers' opinions are submitted to a member of the Editorial Advisory Board for arbitration. Editorial decisions that result from this process are usually final. Reviews will normally be sent to authors by e-mail unless the authors request otherwise.
When a manuscript is returned to the author for revision, the author should reply to the specific recommendations of the reviewers and, in an accompanying letter, indicate those recommendations that have been incorporated into the revision and the reasons any have been disregarded. Usually only one major revision will be considered. Manuscripts requiring a minor revision should be returned within 30 days. Manuscripts requiring a major revision should be returned within 60 days. Authors requiring longer periods may request an extension, which will be granted at the discretion of the Editor. *In general, a revised manuscript received beyond the specified deadline will be considered a new submission, will receive a new manuscript number, and will usually undergo a new review process.*

ASAP Publication: Accepted manuscripts will be published on the "Articles ASAP" page on the Journal Web site as soon as page proofs are corrected and all author concerns are resolved. Publication on the Web usually occurs within 4 working days of receipt of page proof corrections, and this can be anywhere from 3 to 6 weeks in advance of the cover date of the issue. Manuscripts assigned to a special issue often remain published ASAP for several months. Authors should take this schedule into account when planning intellectual and patent activities related to a manuscript. The actual date on which an accepted paper is published on the Web is recorded on the Web version of the manuscript and on the first page of the PDF version.

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Manuscripts must be submitted via the ACS Paragon Plus Environment (http://paragonplus.acs.org/login). Complete instructions and an overview of the electronic online (Web) submission process are available through the secure ACS Paragon Plus Web site.

Authors must also submit all revisions of manuscripts via the ACS Paragon Plus Environment. The web submission site employs state-of-the-art security mechanisms to ensure that all electronically submitted papers are secure. These same security mechanisms are also utilized throughout the peer-review process, permitting access only to editors and reviewers who are assigned to a particular paper.

Authors will view the PDF version of their manuscripts prior to formal submission to the Editor. In order to use Web submission, authors must be able to provide electronic versions of text and graphics. Any Supporting Information should be submitted electronically. Complete instructions, including information on which platforms and word processing packages are supported, are available on the submission site. **Manuscripts submitted as e-mail attachments will not be accepted.**

Authors should review the Journal's Preparation of Manuscripts (below) prior to submission of a manuscript. Close attention to all of the required details discussed in the Preparation of Manuscripts will expedite review and reduce the time to publication. An optional electronic manuscript template is available in several word processing versions (go to http://pubs.acs.org/page/crtoec/submission/authors.html). Use of the template helps to ensure the correct manuscript format. Please note: If the author chooses not to use the template, the text of the manuscript should be double-spaced.

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Chemical Research in Toxicology considers for publication only original work that has not been previously published and is not under consideration for publication elsewhere. When submitting a manuscript, an author should inform the Editor of any prior dissemination of the content in print or electronic format. This includes electronic posting of conference presentations, posters, and preprints on institutional repositories and other Web sites. Any content that has been made publicly available, in either print or electronic format, and that contains a significant amount of new information, if made part of a submitted manuscript, may jeopardize the originality of the submission and may preclude consideration for publication. Duplication of already published data eliminates the paper from consideration in most circumstances. Web posting of content (including posting of conference presentations, posters, and preprints on institutional repositories and other Web sites), whether made openly available to the public or under restricted access, must be disclosed to the Editor. Such postings may preclude consideration for publication but will be considered by the Editor on a case-by-case basis. Regular exceptions will be made for posting of a conference presentation for a period of no longer than one month on the ACS Division of Chemical Toxicology website with members-only access. For further information, contact crt@vanderbilt.edu.

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For policy regarding conflicts of interest, authors are referred to the American Chemical Society Journal's ethical guidelines statement, which can be found on the web at http://pubs.acs.org/ethics. All conflicts of interest must be fully disclosed to the Editor at the time of submission in the letter accompanying the manuscript.

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Authors are strongly encouraged to use the electronic Journal Publishing Agreement. If the PDF version is used, **all pages of the signed PDF Agreement must be submitted.** If the Corresponding Author cannot or should not complete either the electronic or PDF version for any reason, another Author should complete and sign the PDF version of the form. Forms and complete instructions are available at http://pubs.acs.org/page/copyright/journals/index.html.

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General information on the preparation of manuscripts for ACS Journals may be found in The ACS Style Guide, 3rd ed. (2006), available from Oxford University Press, Order Department, 201 Evans Road, Cary, NC 27513. Information about The ACS Style Guide can also be found at http://pubs.acs.org/4authors (select "Publishing Tools" and select "ACS Style Guide").

Authors should write in clear, concise English. Authors for whom English is a second language are encouraged to use a language editing service prior to submission. A list of editing services is available at http://pubs.acs.org/4authors (select "Publishing Tools" and select "Language Editing Services"). Editors, reviewers, and readers may tend to be biased against results reported in complex and excessively verbose language. The responsibility for all aspects of manuscript preparation rests with the authors. Extensive changes or rewriting of the manuscript will not be undertaken by the Editors.

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Nomenclature

It is the responsibility of the authors to provide correct nomenclature. All nomenclature must be consistent and unambiguous and should conform with current American usage. Insofar as possible, authors should use systematic names similar to those recommended by the International Union of Pure and Applied Chemistry, the International Union of Biochemistry and Molecular Biology, and Chemical Abstracts Service.

Chemical Abstracts (CA) nomenclature rules are described in Appendix IV of the Chemical Abstracts Index Guide. For CA nomenclature advice, consult the Manager of Nomenclature Services, Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210-0012. A name generation service is available for a fee through CAS Client Services, 2540 Olentangy River Road, P.O. Box 3343, Columbus, OH 43210-0334; Telephone: (614)447-3870; Fax: (614)447-3747; or e-mail: answers@cas.org.

For IUPAC rules, see the following: IUPAC Nomenclature of Organic Chemistry, Sections A, B, C, D, E, F, and H; Rigaudy, J., Klesney, S. P., Eds.; Pergamon Press: Elmsford, NY, 1979. A Guide to IUPAC Nomenclature of Organic Compounds, Recommendations 1993; Panico, R., Powell, W. H., Richer, J.-C., Eds.; Blackwell Scientific Publications: Oxford, U.K., 1993 (includes revisions, both published and hitherto unpublished, of the preceding). Nomenclature of Inorganic Chemistry, Recommendations 1990; Blackwell Scientific Publications: Oxford, U.K., 1990. Biochemical Nomenclature and Related Documents, 2nd ed.; Portland Press, Ltd.: London, England, 1992. Enzyme Nomenclature, 1992; Academic Press: Orlando, FL, 1992. Compendium of Macromolecular Nomenclature; Blackwell Scientific Publications: Oxford, U.K., 1991. Selected IUPAC recommendations can also be found on the Web at http://www.chem.qmw.ac.uk/iupac/iupac.html.

Manuscript Organization

Articles, Communications, and Chemical Profiles should be assembled in the following order: title page (including full title, byline, and running title), Table of Contents (TOC) graphic, abstract, footnotes, introduction, experimental procedures (materials and methods), results, discussion, funding support, acknowledgements, references, tables, figure legends, figures, scheme legends, and schemes. Reviews and Perspectives should be assembled in the following order: title page, TOC graphic, abstract, footnotes, main body of the paper (including introduction, the text subdivided by major topics, and conclusions), funding support, acknowledgements, references, tables, figure legends, figures, scheme legends, and schemes. In all cases, individual sections should begin on a separate page, and all pages should be numbered consecutively starting with the title page. Reviews, Perspectives, and Forum articles discuss published research and may employ a narrative style. Descriptions of experimental procedures should be made throughout the text to an extent judged appropriate by the author.

Title Page. A brief and informative title will aid in the classification and indexing of the paper. Do not use trade names of drugs or abbreviations. List full names and institutional affiliations of all authors, and if differentiation is necessary, indicate the affiliations of each author by the superscript symbols, †, ‡, §, ¦, -, etc. These symbols should also be used to indicate author affiliations different from those stated on the title page and present address information. The author to whom correspondence should be addressed is indicated by an asterisk. It is implicit in listing a person as an author that this individual has agreed to appear as an author of the manuscript. Provide a brief and informative running title that is easy to index and does not exceed 50 letters and spaces.

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manuscript's title, should give the reader a quick visual impression of the essence of the paper without providing specific results. The TOC graphic must be included upon manuscript submission and should be in the form of a structure, graph, drawing, SEM/TEM photograph, or reaction scheme. Color is acceptable and will be free of charge upon approval of the Editor. The author must submit a graphic in the actual size to be used for the TOC that will fit in an area 8.2 cm wide (3.25 in. wide). Larger images will be reduced to fit within those dimensions. Type size of labels, formulas, or numbers within the graphic must be legible at the specified size. Tables or spectra are not acceptable. Place the TOC graphic after the title page and before the abstract page of the manuscript.

Abstract. An abstract should be included with all Articles, Communications, Chemical Profiles, Reviews, and Perspectives. For Articles, Communications, and Chemical Profiles, the abstract should briefly (300 word maximum) present, in one paragraph, the problem and experimental approach and state the findings and conclusions. For Reviews and Perspectives, the abstract should introduce the topic, summarize key points, and state the major conclusions. In all cases, the abstract should be self-explanatory and suitable for reproduction without rewriting. Footnotes or undefined abbreviations may not be used. If a reference must be cited, complete publication data must be given.

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Abbreviations. Abbreviations are used in ACS Journals without periods. Standard abbreviations should be used throughout the manuscript. Table 1 lists the abbreviations considered standard for Chemical Research in Toxicology.

All nonstandard abbreviations should be kept to a minimum and must be defined in the text following their first use. In addition, nonstandard abbreviations must be defined in an "abbreviations" footnote. This footnote should only be cited once after the first use of a nonstandard abbreviation. There are some abbreviations that are considered standard in certain fields that have been determined to be nonstandard for Chemical Research in Toxicology. These abbreviations are listed in Table 2.

In the interest of standardizing abbreviation use, there are several abbreviations that are not acceptable and should, therefore, not be used. These are listed in Table 3.

Introduction. The introduction should state the purpose of the investigation and its relation to other work in the field. Background material should be brief and relevant to the research described. Detailed or lengthy reviews of the literature should be avoided.

Experimental Procedures. The experimental procedures should be described in sufficient detail to enable others to repeat the experiments. Names of products and manufacturers (with city, state address) should be included only if alternate sources are deemed unsatisfactory or if the product is of limited availability. Novel experimental procedures should be described in detail, but published procedures should merely be referred to by literature citation of both the original and any published modifications. The purity of key compounds and description(s) of the method(s) used to determine purity should be included in this section. For buffers, use terminology such as "2 mM potassium phosphate buffer (pH 7.7) containing...". Also state w/v or v/v for defining solution composition when appropriate.

Analyses. Adequate evidence to establish identity and purity should be provided for new compounds. When possible, this should include elemental analysis. The purity of compounds used for biological testing should be stated with a description of the method used to evaluate it.

Spectral Data. It may be desirable to include such data for representative compounds in a series, for novel classes of compounds, and in structural determinations. Usually, it is not desirable to include routine spectral data for every compound in the manuscript. Papers in which interpretations of spectra are critical to structural elucidation and those in which band shape or fine structure needs to be illustrated may be published with spectra included. When such presentations are deemed essential, only pertinent sections should be reproduced.

Biological Methods. Biological test methods must be referenced or described in sufficient detail to permit the experiments to be repeated by others. Detailed descriptions of biological methods should be placed in the experimental procedures section. Data may be presented as numerical expressions or in graphical form. Statistical limits (statistical significance) for the biological data are usually required. If statistical limits cannot be provided, the number of determinations and some indication of the variability and reliability of the results should be given. References to statistical methods of calculation should be included. Doses and concentrations should be expressed as molar quantities (e.g., μmol/kg, mM, etc.) when comparisons of potencies are made on compounds having large differences in molecular weights. The routes of administration of test compounds and vehicles should be indicated.

Hazardous Materials. All hazardous chemicals should be clearly identified as such. Precautions for handling dangerous materials or for performing hazardous procedures should be explicitly stated and referenced.

Identification of and precautions for handling hazardous chemicals and dangerous procedures should be placed at the beginning of this section. An example would be: “Caution: The following chemicals are hazardous and should be handled carefully: (list of chemicals and handling procedures or references)”.

Results. The results should be presented concisely. Tables and figures should be designed to maximize the presentation and comprehension of the experimental data. The same data should not be presented in more than one figure or in both a figure and a table. As a rule, interpretation of the results should be reserved for the discussion section of an Article, but under some circumstances, it may be desirable to combine results and discussion in a single section. In the interest of economy of space, it is sometimes desirable to place supplementary data (also subject to review; see section below on “Supporting Information”) in a separate section for inclusion in the online edition of the Journal.

Data Deposition. Authors of manuscripts containing protein or DNA sequence or structural data must make those data publically available.

Protein Sequence Data: These data should be deposited with the Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC 20007. For the documentation of the sequence analysis of proteins, see Biochemistry (1983) 22, 2595. Authors of accepted papers containing nucleotide sequences should submit the sequence data, preferably in computer-readable form, plus information for annotation of the data and a copy of the paper to GenBank Submissions, National Center for Biotechnology Information (NCBI), Building 38A, Room 8N-805, 8600 Rockville Pike, Bethesda, Maryland 20894 [telephone (301) 496-2475; fax (301) 480-9241; e-mail (submissions) gbsub@ncbi.nlm.nih.gov, (information) info@ncbi.nlm.nih.gov]. Submission to GenBank ensures entry also into the EMBL Nucleotide Sequence Library. A footnote should appear on the title page indicating that such a deposit has been made.

Structural Data: Atomic coordinates and structure factors for structures of proteins determined by X-ray and atomic coordinates for all macromolecules determined by NMR should be deposited with the Protein Data Bank (PDB; http://www.rcsb.org/pdb/; info@rcsb.org) at the Research Collaboratory for Structural Bioinformatics (RCSB; http://pdb.rutgers.edu/) or at the European Bioinformatics Institute [EBI; http://autodep.ebi.ac.uk/; telephone (732)445-0103]. It is the responsibility of the author to obtain a File Name for the macromolecule; the File

Name must appear in a footnote on the title page. A manuscript will be accepted only after receipt from the submitting author of a written statement that the coordinates have been deposited. Coordinates must be released immediately upon publication. Manuscripts that report X-ray crystallographic structures should include a table of data statistics that contains the number of reflections, data cutoff (e.g., F > 0), Rwork/Rfree, I/s(I), percent completeness, redundancy, Rmerge, number of atoms per asymmetric unit, and B-factors for protein, waters, and ligands/ions. Please address all inquiries about deposition to the Protein Data Bank. For papers that involve NMR studies in which complete or nearly complete resonance assignments of biopolymers have been carried out, authors are required to deposit relevant NMR assignments and related experimental data at the BioMagResBank (BMRB; http://www.bmrb.wisc.edu). These data may include assigned chemical shifts, coupling constants, relaxation parameters (T1, T2, and NOE values), dipolar couplings, or other data accepted by BMRB. The author is responsible for obtaining a BMRB entry accession number (e.g., 4238), which should appear in a footnote on the title page. The data must be released upon publication. Crystal structures of nucleic acids should be deposited with the Nucleic Acid Database (NDB; http://ndbserver.rutgers.edu/; ndbadmin@ndbserver.rutgers.edu). A preprint of the related manuscript should be mailed or faxed to The Nucleic Acid Database, Department of Chemistry, Rutgers, The State University of New Jersey, 610 Taylor Road, Piscataway, New Jersey 08854-8087 [fax (732) 445-4320]. Manuscripts dealing with the development of structures from sequence homology are generally not considered unless significant experimental tests of the model also are presented.

Other Databases: Authors may wish to submit binding constants and associated information to the public database BindingDB (www.bindingdb.org).

Discussion. The purpose of the discussion is to interpret the results and to relate them to existing knowledge in the field in as clear and brief a fashion as possible. Information given elsewhere in the manuscript should not be repeated in the discussion. Extensive reviews of the literature should be avoided.

Funding Sources. This section acknowledges financial support. All other acknowledgments should be listed separately (see below).

Acknowledgment. This section should acknowledge technical assistance, advice from colleagues, gifts, etc. Permission should be sought from persons whose contribution to the work is acknowledged in the manuscript.

Supporting Information. Occasionally manuscripts are submitted with extensive tabular, graphical, or spectral data. Such “Supporting Information” should be relegated to the Web edition of the Journal. Authors are encouraged to use this resource in the interests of shorter articles which not only save journal space but also result in clearer and more readable presentations. However, “dumping” of materials to this section is to be discouraged, and the Editors reserve the right to delete material they consider superfluous. All Supporting Information will be subject to the same review process as the submitted manuscript. Refer to The ACS Style Guide for more specific information. Supporting Information must be submitted at the same time as the manuscript and uploaded separately to the ACS Paragon Plus Environment. A list of acceptable file types is available on the Web. All Supporting Information files of the same type should be prepared as a single file (rather than submitting a series of files containing individual images or structures). For example, all Supporting Information available as PDF files should be contained in one PDF file.

DO NOT UPLOAD FIGURES AND TABLES THAT ARE TO BE PUBLISHED IN THE ARTICLE INTO THE SUPPORTING INFORMATION FILE.

A statement of the availability of Supporting Information should be placed after the Acknowledgment section, using the following format: “Supporting Information Available: Give description of material. This material is available free of charge via the Internet at http://pubs.acs.org.”

References. References to the literature should be numbered in one consecutive series in the text. Each literature reference should be assigned one number and placed in the text as an italicized Arabic numeral in parentheses. The complete list of references should be typed double-spaced beginning on a separate page after the acknowledgements section and following the format shown: Keller, G. M., Turner, C. R., and Jefcoate, C. R. (1982) Kinetic determinants of benzo[a]pyrene metabolism to dihydrodiol epoxides by 3-methylcholanthrene-induced rat liver microsomes. Mol. Pharmacol. 22, 451-458. The volume numbers in the references should be italicized, not bolded. Titles of journals are abbreviated according to Chemical Abstracts Service Source Index. Serial publications such as Methods in Enzymology and CRC Critical Reviews in Toxicology should be listed in the same form as journals. References to chapters and monographs are listed as follows: Koop, D. R., Morgan, E. T., and Coon, M. J. (1982) Purification of multiple forms of rabbit hepatic cytochrome P-450. In Microsomes, Drug Oxidations, and Drug Toxicity (Sato, R., and Kato, R., Eds.) pp 85-86, Wiley-Interscience, New York. Submitted manuscripts should be designated as “in press” only if formally accepted for publication; otherwise “unpublished results” should be placed after the names of authors as a footnote in the text. The accuracy of the references is the responsibility of the author. As subscribers are now able to click on the “CAS” or PubMed hyperlink following each reference to retrieve the corresponding CAS abstract, reference accuracy is critical.

Tables. Tabulation of experimental results is encouraged when this leads to more effective presentation or to more economical use of space. Tables may be created using a word-processor’s text mode or table format feature. The table format feature is preferred. Ensure each data entry is in its own cell. If the text mode is used, separate columns with a single tab and use a line feed (return) at the end of each row.

Tables should be numbered consecutively with Arabic numerals. Provide a brief title with each table and a brief heading for each column. Clearly indicate the units of measure (preferably SI). Data should be rounded to the nearest significant figure. Explanatory material referring to the whole table is to be included as a footnote to the title (a). Footnotes in tables should be given lower case letter designations and cited in the tables as italicized superscripts. All tables should be cited in the text in consecutive order.

Figures and Figure Legends. Line drawings, graphs, stereograms, histograms, and black and white (or color) photographs are all classified as figures and should be numbered consecutively with Arabic numerals in order of citation. Figure legends should be placed after the tables as a single list with the figures following. The figures must be embedded in the same document as the article and not submitted separately as Supporting Information. See below for guidelines for preparing publication quality illustrations.

Schemes and Scheme Legends. Schemes show a sequence or group of reactions taking place together. They should be numbered consecutively with Arabic numerals in order of citation. Scheme legends should be placed after the figures as a single list with the schemes following. See below for guidelines for preparing publication quality chemical structures.

8.2 ANEXO B – Roteiro para autores / Guia para a redação e edição de artigo científico a ser submetido à Basic and Clinical Pharmacology and Toxicology

Basic & Clinical Pharmacology & Toxicology
Formerly Pharmacology & Toxicology

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Kim Brøsen

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Impact Factor: 2.371

TopAuthor Guidelines

Aim and Scope
BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY is an independent journal, publishing original scientific research in all fields of toxicology, basic and clinical pharmacology. This includes experimental animal pharmacology and toxicology and molecular (-genetic), biochemical and cellular pharmacology and toxicology. It also includes all aspects of clinical pharmacology: pharmacokinetics, pharmacodynamics, therapeutic drug monitoring, drug/drug interactions, pharmacogenetics/-genomics, pharmacoepidemiology, pharmacovigilance, pharmacoeconomics, randomized controlled clinical trials and rational pharmacotherapy. For all compounds used in the studies, the chemical constitution and composition should be known, also for natural compounds.

Submission of manuscripts
Manuscripts written in the English language should be submitted online to http://www.manuscriptmanager.com/bcpt.

All submitted manuscripts must be accompanied by a completed Exclusive Licence Form.

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OnlineOpen is available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley **Online Library**, as well as deposited in the funding agency's preferred archive. For the full list of terms and conditions, see **http://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms**.

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Reports on studies with natural products will only be considered for publication if the exact compound composition and chemical structure of active entities are fully characterized.

Manuscripts must be accompanied by a covering letter, which should include information about prior or duplicate publication or submission elsewhere of any part of the work. It should also include a statement that the manuscript has been read and approved by all authors. In addition, **the submission letter must include the names of 5 unbiased reviewers who are active researchers in the relevant field. These unbiased reviewers should not be picked from the authors' local environment and preferably from other countries.** Finally, the submission letter should contain any other information that may be helpful to the Editor.

Manuscripts not following the present instructions are returned without editorial evaluation. Authors are advised to check the consistency between the present instructions and their manuscript as to style and language, and to consult a current issue of the journal for general and specific manners of presentation.

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Ethical guidelines

Studies involving human beings must be conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice.

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Conflict of interest and sources of funding

The politics of BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY regarding conflict of interest and sources of funding complies with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (http://www.icmje.org/). Thus, a conflict of interest exists if an author, reviewer or an editor handling any given manuscript has ties that could influence his or her judgement in an inappropriate manner. This could typically be financial ties with industry either directly or indirectly via family in the form of research funding, employment, consultancies, stock ownership, honoraria or expert testimony. Other sources of conflict of interest could be personal relationships, academic competition and intellectual passion. Authors submitting a manuscript to BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY are entitled to disclose any conflict of interest, and in the event the manuscript will be published, these will be published as well. This includes disclosure of all sources of funding. The Editor and MiniReview Editors will abstain from participation in the peer review process and editorial handling of a manuscript if a conflict of interest exists, and the same applies to the reviewers.

Organisation of manuscripts

Manuscripts should be submitted in the English language (see the Language paragraph below), double-spaced with a wide margin and pages should be numbered consecutively. They should be submitted as only one Word file including all text, and with tables and figures at the end.

Title page

This should contain a concise title, institution(s) or laboratory(ies) where the work was done (in English), names of all authors with first names spelled out, and an abbreviated form of the title (running title). Full mailing address in English for the corresponding author should also be given including telefax number and e-mail address.

Page 2

This should contain an abstract of up to 250 words. The abstract should contain a summary of what was done, the results obtained, and valid conclusions drawn therefrom.

Main text

The following pages should contain Introduction and background (long historical introductions should be avoided, a reference to bibliographies in handbooks or the like will suffice), followed by Materials and Methods, Results, Discussion which should incorporate the conclusion(s) drawn from the study, Acknowledgements and References. Numbered tables with legends, illustrations or graphs in high quality with legends should be on separate pages at the end of the manuscript document. Abbreviations should be kept to an absolute minimum.

Abbreviations in tables and figures should be explained in the legends. Text and footnotes must contain all the information necessary to understand and interpret the table without reference to the text. Illustrations must be clear enough to permit readable reproduction. Symbols should be large enough to be readable also after reduction of the illustration.

The experimental results should on the whole be published only in the form of graphs or tables, which must contain all the information necessary to understand the table or illustration without reference to the text.

Illustrations
Tables. Each table should have a brief, specific, descriptive title, giving sufficient explanation to make the data intelligible without reference to the text. Number all tables and cite in numerical order in the text, using Arabic numerals.

Preparation of figures. Cite figures in the text in numerical order using Arabic numerals. For peer-review submission, follow the online uploading instructions. Please save vector graphics (e.g. line artwork) in Encapsulated Postscript Format (EPS) and bitmap files (e.g. half-tones) in Tagged Image File Format (TIFF). Ideally, vector graphics that have been saved in metafile (.WMF) or pict (.PCT) format should be embedded within the body of the text file. More information on electronic artwork can be found at http://authorservices.wiley.com/bauthor/faq.asp.

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Figure legends. All legends must begin with a short descriptive sentence that sums up the intent and content of the data contained in the figure. This sentence should be in boldfont. A more detailed explanation of the data contained in the figure and/or its parts should follow. The detailed description should be in Roman type (ie, not in boldfont).

Colour charges. Colour charges apply to all articles (except MiniReviews) printed in *Basic & Clinical Pharmacology and Toxicology.* Therefore, please note that if there is colour artwork in your manuscript when accepted for publication, which you would like to appear in print, you are required to complete and return a colour work agreement (CWA) form before your paper can be published. This form can be downloaded from http://www.blackwellpublishing.com/pdf/SN_Sub2000_F_CoW.pdf. (Please note that we are pleased to provide colour online only free of charge. If you would like to take advantage of this, please ensure that your image is suitable for both colour and black-and-white publication. For example, please do not refer to the 'red dots' or 'blue lines', as these will NOT be apparent in the print version.) Please correspond with the Editor-in-chief on acceptance of the manuscript.

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BCPT Production Editor
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1 Fusionopolis Walk, #07-01 Solaris South Tower,
Singapore 138628

References

As of 1 January 2007, references must be cited according to the Vancouver system (http://www.icmje.org).
Basic & Clinical Pharmacology & Toxicology deviates from icmje in the following respects:

- No full stop after the abbreviated name of the journal
- As journals paginate consecutively, issue number is not used; only volume and page number.

Journal names should be abbreviated according to the system used in Index Medicus (Pubmed Service "Journals Database").
Number the references consecutively in the order they appear for the first time in the text. References that are cited in table or figure texts should be numbered in accordance with the first appearance of the table or figure in question. References in the text must be cited with the appropriate number in square bracket. In case of more than one reference in one square bracket, the numbers must be separated by a comma, e.g. [3, 4, 8]; In case of more than two consecutive reference numbers, use hyphen, e.g. [6-9];

Avoid abstracts and meeting proceedings as references; unpublished observations and personal communications must not be used as references. Accepted manuscripts that have not yet been published may appear in the reference list. Indicate journal name and year in question followed by "in press" in brackets. Articles published online are identified by their DOI (Digital Object Identifier System) reference (http://dx.doi.org/). Electronic material is cited like other literature.

In general, references must be written as follows

Surname followed by initial(s) without comma or full stop; then comma before the next author's surname followed by his/her initial(s): Larsen JT, Brøsen K.
If there are more than six authors, et al after the sixth author's initial(s) should be added: Smith PJ, Byron AM, Jones E, Andersson C, Tucker AD, Rowland P et al.
Put a full stop after the last author's initials (or after et al), and before the article/monography title etc.
The title should be written with initial capital while all other words are in small letters, unless it is a matter of nationalities (in a Swedish population) or names of e.g. commissions and the like. As a rule, continue with small letters after colon. Do not include sub-title.
The journal name should be written with ordinary font (no italics); do not put full stops between the individual parts of the abbreviation or between the journal name and year: Basic Clin Pharmacol Toxicol 2005.
Year, volume number and pages should be written without space.
Put a semicolon between year and volume number and colon between volume number and page number: BMJ 2004;329:1233-6.

Examples of correctly written references

Journal article
Brøsen K, Skjelbo E, Rasmussen BB, Poulsen HE, Loft S. Fluvoxamine is a potent inhibitor of cytochrome P4501A2. Biochem Pharmacol 1993;45:1211-4.
Rasmussen SG, Gether U. Purification and fluorescent labelling of the human serotonin transporter Biochemistry 2005;44:3494-505.

Book chapter
Zanger UM, Eichelbaum M. CYP2D6. In: Levy RH, Thummel KE, Trager WF, Hansten PD, Eichelbaum M (eds). Metabolic Drug Interactions. Lippincott Williams & Willis Philadelphia PA 2002;87-94.

DOI *reference*
Masmanian SK, Thon-That H, Schneewind O. Sortase catalysed anchoring of surface proteins to the cell wall of Stapylococcus aureus. Mol Microbiol 2001;40:1049-1057.

Doi: 10.1046/j. 1365-2958.2001.02411.x
Internet reference
http://www.imm.ki.se/CYPalleles /March 2006

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. EndNote reference styles can be searched for here: http://www.endnote.com/support/enstyles.asp. Reference Manager reference styles can be searched for here: http://www.refman.com/support/rmstyles.asp.

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The manuscript should be written in a concise and clear British English language. Some linguistic in-house correction is performed, however, a manuscript may be returned to the authors for major linguistic revision and rewriting. The authors have the full responsibility for the English language and the style of the manuscript. The manuscript will be edited according to the style of the journal, and the proofs must be read carefully by the author.

Nomenclature
The international nomenclature should be used. Chemical formulae should as far as possible be written on one line. If proprietary names are used, the chemical constitution or, if this is not known, the outline of the preparation must appear clearly in the text. When available, INN-names should be used.
Only officially accepted abbreviations and abbreviations of long chemical names etc. should be used. Unnecessary abbreviations impede the reading of a paper; therefore the number of abbreviations should be kept at an absolute minimum. Necessary abbreviations should be used with consistency and defined at first mention. Abbreviations should not be used in the title of the paper and in the running title.

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Authors will be charged GBP 150 for each page in the final published version exceeding 6 printed pages, although for "Short Communications" GBP 150 for each page exceeding 2 printed pages. Page charge does not apply to MiniReviews. As a rule of

thumb, an average text page in the printed issue (without figures and tables) is approximately 900 words.

8.3 ANEXO C - Trabalhos desenvolvidos durante o doutorado que não fazem parte desta tese

1. AUGUSTI, P.R.; QUATRIN, A.; SOMACAL, S.; **CONTERATO, G.M.M.** ; SOBIESKI, R.; RUVIARO, A.; MAURER, L.; DUARTE, M.M.F.; ROEHRS, M.; EMANUELLI, T. Astaxanthin prevents changes in the activities of thioredoxin reductase and paraoxonase in hypercholesterolemic rabbits. **Journal of Clinical Biochemistry and Nutrition**, 2011 (Artigo aceito).

2. AUGUSTI, P. R. ; **CONTERATO, G.M.M**. ; SOMACAL, S. ; SOBIESKI, R. ; QUATRIN, A. ; MAURER, L. ; ROCHA, M.P. ; DENARDIN, I.T. ; EMANUELLI, T. Astaxanthin Reduces Oxidative Stress, but not Aortic Damage in Atherosclerotic Rabbits. **Journal of Cardiovascular Pharmacology and Therapeutics**, v. 14, p. 314-322, 2009.

3. HAHN, M ; **CONTERATO, G.M.M**. ; FRIZZO, C. ; AUGUSTI, P. ; DA SILVA, J. ; UNFER, T. ; EMANUELLI, T . Effects of bone disease and calcium supplementation on antioxidant enzymes in postmenopausal women. **Clinical Biochemistry,** v. 41, p. 69-74, 2008.

4. AUGUSTI, P.R; **CONTERATO, G.M.M.** ; SOMACAL, S. ; SOBIESKI, R.; SPOHR, P. ; TORRES, J.; CHARAO, M. ; MORO, A.; ROCHA, M.; GARCIA, S . Effect of astaxanthin on kidney function impairment and oxidative stress induced by mercuric chloride in rats. **Food and Chemical Toxicology**, v. 46, p. 212-219, 2008.

5. AUGUSTI, P. R. ; **CONTERATO, G. M. M.** ; SOMACAL, S. ; EINSFELD, L. ; RAMOS, A. T. ; HOSOMI, F.Y.M.; GRAÇA, D. L. ; EMANUELLI, T . Effect of Lycopene on Nephrotoxicity Induced by Mercuric Chloride in Rats. **Basic & Clinical Pharmacology & Toxicology,** v. 100, p. 398-402, 2007.

9. APÊNDICES

9.1 APÊNDICE A - Termo de consentimento livre e esclarecido

Você está sendo convidado a participar voluntariamente de uma pesquisa intitulada “SISTEMA TIORREDOXINA E SUA RELAÇÃO COM O ESTRESSE OXIDATIVO INDUZIDO PELO CHUMBO”, que tem como objetivo geral avaliar e relacionar os indicadores de estresse oxidativo e de exposição ao chumbo no sangue de humanos.

O estudo será desenvolvido no Núcleo Integrado de Desenvolvimento em Análises Laboratoriais (NIDAL) (Departamento de Tecnologia e Ciência dos Alimentos) e no Laboratório de Toxicologia (LATOX) (Departamento de Análises Clínicas e Toxicológicas) da Universidade Federal de Santa Maria, e envolverá trabalhadores do Estado do Rio Grande do Sul. Os pesquisadores responsáveis pelo estudo são a Profa. Tatiana Emanuelli e a Msc. Greicy M.M. Conterato, aluna do Programa de Pós Graduação em Bioquímica Toxicológica da UFSM. Em qualquer etapa do estudo você terá acesso aos pesquisadores responsáveis pelo estudo para esclarecimento de eventuais dúvidas.

Este estudo obteve aprovação junto ao Comitê de Ética em Pesquisa da Universidade Federal de Santa Maria, com protocolo n° XXXXXX.

Procedimentos a serem realizados

Você deverá realizar apenas uma coleta de sangue, sem qualquer custo e responder as questões da ficha de avaliação clínica. O sangue será armazenado por um período de no máximo 24 meses, para a realização de todas as análises previstas. Você receberá os resultados dos exames laboratoriais de creatinina, fosfatos, ácido úrico e atividade das enzimas aspartato aminotransferase e alanina aminotransferase, que são indicadores das funções renal e hepática, recebendo orientação em caso de resultados fora dos padrões de referência.

Riscos individuais, possibilidade de exclusão e benefícios

A coleta de sangue é um procedimento de baixo risco e desconforto, no entanto, em alguns casos pode ocorrer formação de hematoma local, que será tratado, sem nenhum custo.

Fica claro que você não é obrigado a participar do projeto. No caso de recusa você não terá nenhum prejuízo no seu atendimento rotineiro, nem represálias. A qualquer momento da pesquisa você é livre para retirar-se da mesma.

No caso de aceite, fica claro que não haverá benefício financeiro pela sua participação, nem prejuízos ou riscos a sua saúde.

Confidencialidade

Os dados obtidos com esta pesquisa se constituirão em publicações em revistas médico-científicas. Os seus dados serão analisados em conjunto com outros pacientes, e será garantida a confidencialidade dos dados que lhe identifiquem.

Utilização dos dados obtidos

O material coletado e os seus dados serão utilizados somente para esta pesquisa.

Telefones para contato com os pesquisadores

Prof. Dra. Tatiana Emanuelli – Departamento de Tecnologia e Ciência dos Alimentos – CCR
(55) 3220 8547
email: tatiemanuelli@smail.ufsm.br

Msc. Greicy Michelle Marafiga Conterato – Programa de Pós Graduação em Bioquímica Toxicológica – CCNE – UFSM
Email:greicymmc@hotmail.com
(55) 3220 8547, (55) 3221 7313, (55) 91079739

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo" SISTEMA TIORREDOXINA E SUA RELAÇÃO COM O ESTRESSE OXIDATIVO INDUZIDO PELO CHUMBO". Eu discuti com a Profa. Dra. Tatiana Emanuelli sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Identificação do sujeito da pesquisa:

Nome: _____RG (número da identidade):_____
Assinatura:
_____
Santa Maria, _____ de _____de 2007.

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste sujeito de pesquisa ou representante legal para a participação neste estudo.

Santa Maria, _____ de _____de 2007.

_____

Assinatura do responsável pelo estudo

9.2 APÊNDICE B- Ficha clínica de avaliação dos trabalhadores

FICHA CLÍNICA DE AVALIAÇÃO

Data: ___/___/___

1. IDENTIFICAÇÃO

Nome: _____

Fone 1: _____ Fone 2: _____ Sexo: □ 1- M □ 2- F

Data de nascimento: ___/___/___ Idade: _____ Estado civil: _____

Endereço: _____ Bairro: _____

Cidade: _____ Estado: ____ CEP: _____

Entrevistador: _____

LOCAL DE TRABALHO:

_____

ATIVIDADE QUE DESENVOLVE _____

TEMPO DE SERVIÇO NA ATIVIDADE ATUAL? _____

JÁ TRABALHOU ANTES EM OUTRO TIPO DE ATIVIDADE? QUAL?

_____

QUANTO TEMPO?

_____

2. DADOS DE DIAGNÓSTICO

MEDICAMENTOS (quais, tempo de uso e freqüência de uso):

_____

_____

DOENÇAS:

Possui algum tipo de doença? Qual? _____

_____

Depressão?_____

Dor de cabeça?_____

Tonturas? _____

Mal estar? _____

CIGARRO:

Sr(a) fuma atualmente? 1- sim 2- não

Se **sim**, quantos cigarros por dia? ___

Com que idade começou a fumar? _____ anos

Tipo de fumo: 1- cigarro 2- charuto 3- cachimbo

Se **não** fuma, o Sr(a) já fumou? (fumo passado) 1- sim 2- não

Se **sim**, quantos cigarros por dia? _____ Com que idade começou a fumar? _____ anos

Há quanto tempo parou de fumar? _____ anos _____ meses

Tipo de fumo: 1- cigarro 2- charuto 3- cachimbo

FUMO **1- Nunca fumou** **2- Ex-fumante** **3- Fumante**

ÁLCOOL:

Faz uso de bebidas alcoólicas? 1- sim 2- não

Em caso positivo, qual a freqüência, a quantidade e o tipo de bebida (por semana)? _____

Fez uso de bebidas alcoólicas nos últimos 2 dias? Qual?_____