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Avaliação dos efeitos promovidos pelo chumbo, selênio e/ou sacarose em parâmetros oxidativos em roedores

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

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Santa Maria, RS, Brasil

AGRADECIMENTOS

À todos aqueles
Pessoas ou animais
Que voluntariamente - ou não -
Colaboraram com este trabalho

Muito obrigado!

Depois da chuva, vem o sol amarelo...

Santa Maria, 16 de março de 2006

RESUMO

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

Avaliação dos efeitos promovidos pelo chumbo, selênio e/ou sacarose em parâmetros oxidativos em roedores

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DATA E LOCAL DA DEFESA: Santa Maria, 16 de março de 2006.

O chumbo apresenta um risco ocupacional e ambiental, e sabe-se que pode ser tóxico para diversas espécies de animais. Diversos estudos experimentais confirmam a teoria de dano oxidativo na toxicidade promovida pelo chumbo. Dados da literatura tem mostrado que dietas ricas em sacarose podem incrementar o estresse oxidativo. Estresse oxidativo está relacionado com diversas patologias, desde inibições enzimáticas até distúrbios comportamentais. Compostos orgânicos de selênio, ebselen e disseleneto de difenila tem uma descrita atividade antioxidante, semelhante à atividade tiol-peroxidase. Em um protocolo de intoxicação sub- crônica (ARTIGO 1), determinou-se se estes compostos seriam efetivos para reverter a toxicidade promovida pelo chumbo em camundongos. Injeções de acetato de chumbo, com subseqüentes injeções de ebselen reduziram apenas os níveis hepáticos de grupos tiólicos não protéicos (NPSH). O tratamento com ebselen também reduziu os níveis de TBARS no rim. Enquanto o chumbo inibiu a atividade da δ-ALA-D em todos os tecidos, o ebselen reverteu a inibição enzimática no cérebro. Também foi observado que o metal ou os organocompostos de selênio não modificaram a captação de glutamato, enquanto o ebselen promoveu um aumento neste parâmetro. Os resultados deste estudo indicam que a inibição da atividade da δ-ALA-D antecede a produção de espécies reativas de oxigênio em um protocolo sub- crônico de intoxicação em camundongos. Em outro estudo (MANUSCRITO 2), ratas foram tratadas por 12 meses com acetato de chumbo e/ou dieta rica em sacarose, para avaliar se a exposição simultânea a estes agentes poderia produzir o aparecimento de discinesia orofacia, ou modificar o comportamento locomotor. Os ratos demonstraram um incremento na discinesia orofacial, enquanto a ingestão de sacarose não esteve associada à este parâmetro. A associação entre chumbo e

sacarose causou uma diminuição na discinesia orofacial, o que pode estar relcionado a uma adaptação após uma prolongada exposição a agentes pró-oxidantes. No MANUSCRITO 3, foi conduzido um estudo por 24 meses com uma dieta rica em sacarose e exposição ao chumbo, onde foram observados uma especificidade dos efeitos nos tecidos. Outro resultado obtido neste estudo foi um aumento na atividade da δ-ALA-D de baço, o que é um resultado contraditório, uma vez que esta enzima é um clássico marcador de toxicidade por chumbo. Estes resultados podem indicar que a enzima δ-ALA-D é um indicador de toxicidade aguda ou sub- crônica, e que pode ocorrer alguma adaptação à toxicidade promovida pela sacarose e/ou pelo chumbo após exposição prolongada a estas substâncias.

Palavras-chave: acetato de chumbo, sacarose, discinesia orofacial, estresse oxidtivo, atividade da δ-ALA-D.

ABSTRACT

Thesis of Doctor's Degree
Federal University of Santa Maria, RS, Brazil

Evaluation of lead, selenium and/or sucrose- effects on oxidative parameters in rodentes

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DATE AND PLACE OF THE DEFENSE: Santa Maria, 2006

Lead is a common occupational and environmental hazard, and it is known that can be toxic to several species of animals, and experimental studies support the theory of oxidative damage in lead toxicity. Literature data have shown that high- sucrose diet can increase oxidative stress. Oxidative stress is related with several pathologies, such as enzyme inhibitions to behavioral disorders. Organoselenium compounds, Ebselen and diphenyl diselenide have described antioxidant properties, such as thiol- peroxidase activity. In a sub-chronic protocol of intoxication (ARTICLE 1), was determined whether these selenocompounds were effective to restore the lead-toxicity in mice. Lead acetate injection with subsequent injection of Ebselen only reduce the hepatic levels of non- protein thiol groups (NPSH). The treatment with Ebselen also reduced TBARS levels in kidney. Whereas lead inhibited δ-ALA-D activity in all tissues, Ebselen performed a recovery brain enzyme inhibition. It was also observed that metal or selenocompounds did not change glutamate uptake, whereas lead plus Ebselen showed an increase on this parameter. The results of this study indicate that δ-ALA-D inhibition antecedes the overproduction of reactive oxygen species in a short- term protocol of mice intoxication. In other study (MANUSCRIPT 2), where treated female rats for 12 months with lead acetate and/or sucrose- diet, to evaluate whether simultaneous exposure to these agents could enhance the appearance of orofacial dyskinesia, or could disturb the locomotor behavior. The aged rats demonstrated an increased orofacial dyskinesia, whereas sucrose ingestion was not associated with this parameter. The association between lead and sucrose caused a reduction on orofacial dyskinesia, and can be related to an adaptation after long-term exposure to pro-oxidant agents. In MANUSCRIP 3, a study carried out for 24 months of sucrose diet and lead exposure, where observed that the effects were tissue- specific. Another result obtained in this study was an increased spleen ALA-D activity, it is a

contraditory result, because this enzyme is a classical marker of lead toxicity. These results may indicate that ALA-D is an indicator of acute or sub-chronic lead exposure and that some adaptations to lead and/or sucrose toxicity occur after long-term exposure to these substances.

Keywords: Lead acetate, sucrose, orofacial dyskinesia, oxidative stress, ALA-D activity

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APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de manuscritos, no item **PUBLICAÇÕES**. A metodologia utilizada, resultados, discussão e referências bibliográficas, encontram-se nos próprios manuscritos.

Os itens **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre os manuscritos científicos contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **DISCUSSÃO e CONCLUSÕES** desta tese.

1. INTRODUÇÃO

Os metais pesados assim são chamados por possuírem grande massa específica e a característica de apresentar toxicidade em uma gama de atividades biológicas. Por isso há, teoricamente, tantas respostas a esses metais quantos forem as atividades biológicas afetadas. Todavia, o acesso variado aos componentes biológicos faz com que certos tipos de respostas predominem. Por exemplo, os sistemas enzimáticos são potencialmente suscetíveis aos metais pesados.

Por outro lado, nos organismos vivos, o acesso dos metais pesados pode ser limitado pelas estruturas anatômicas; além disso, os sítios ligantes inertes podem competir pelo íon metálico. Por essas razões, freqüentemente existem consideráveis diferenças de sensibilidade entre diferentes órgãos e tecidos, entre espécies e entre respostas típicas de envenenamento.

1.1. Chumbo

1.1.1. Histórico

Dentre os metais, o chumbo foi dos primeiros a ser utilizado pelo homem. Há evidências que já era utilizado na Ásia Menor em 4000 a.C. Na natureza é encontrado em minérios, sendo os principais a galena, minim, cerussita e anglessita. O chumbo já teve diversas utilizações, desde a cunhagem de moedas na antiguidade, produção de tubulações e soldas, como antifúngico, antidetonante, e fixador de cores, até a produção de baterias acumuladoras de energia, atualmente (Needleman, 2000; Vahter et al., 2002).

Por ser de intensiva e longa utilização, os relatos de intoxicação por chumbo são inúmeros, sendo Hipócrates o primeiro a ligar os sintomas da sua intoxicação a seu fator causal. Durante a Idade Média, a intoxicação por chumbo foi totalmente esquecida e somente no século XVI, apareceu novamente na literatura, quando Paracelso descreveu a "doença dos mineiros", e a primeira descrição moderna de saturnismo foi realizada em 1839 por Tanqurel.

Pelo fato do chumbo ser virtualmente onipresente no meio ambiente, como resultado de sua ocorrência natural e sua utilização industrial, permanece associado a patologias em todo mundo, principalmente nos países em desenvolvimento.

1.1.2. Absorção e distribuição

O chumbo é absorvido primariamente pelos tratos gastrintestinal e respiratório. É transportado ligado aos eritrócitos e depositado na matriz óssea, deslocando o cálcio, o que pode causar efeitos tóxicos continuados mesmo depois de cessada a exposição. O chumbo tem uma meia-vida de 35 dias no sangue, 50 dias nos tecidos moles, e de 20 a 30 anos nos ossos (Landrigan, 1994).

Como esse metal afeta vários órgãos e sistemas do organismo, os mecanismos de toxicidade propostos envolvem processos bioquímicos fundamentais, que incluem a habilidade do chumbo de ligar-se a proteínas alvo que são, em geral, proteínas que naturalmente ligam cálcio ou zinco (Godwin, 2001). Mesmo tendo uma moderada afinidade por grupamentos tiólicos (Bondy and Guo, 1996), o chumbo é comumente encontrado nas

células e tecidos ligado à proteínas contendo tiol (-SH) e tióis de baixo peso molecular (Goering, 1993; Campagna et al., 1999). Porém, sob exposição moderada, é importante ressaltar que as mudanças bioquímicas e funcionais promovidas pelo chumbo podem ser revertidas.

As interações bioquímicas do chumbo com grupamentos tiólicos são consideradas de grande significado toxicológico, visto que, se tal interação ocorrer em uma enzima, sua atividade pode ser alterada e resultar em efeitos tóxicos. A aminolevulinato desidratase (δ-ALA-D) é uma enzima que contém zinco em seu sítio ativo, e é considerada uma molécula alvo clássica à metais pesados, sendo utilizada como indicativo de intoxicação (Gurba et al., 1972; Rocha et al., 1995 & 2001; Rodrigues et al., 1996), e sua inativação pode permitir o acúmulo do seu precursor o ácido aminolevulínico (ALA), levando a um aumento na produção de espécies reativas de oxigênio, o que pode ocasionar efeitos neurotóxicos ou hepáticos (Hermes-Lima et al., 1991; Bechara et al., 1993).

Os desvios hematológicos produzidos pelo chumbo, e que levam à anemia são o resultado de sua ação tóxica sobre as células vermelhas e eritropoiéticas na medula óssea. Esses efeitos incluem inibição da síntese da hemoglobina (Hb) e diminuição do tempo de vida dos eritrócitos circulantes, resultando na estimulação da eritropoese. Entretanto, a anemia não é uma manifestação precoce do envenenamento por chumbo, sendo rara sem outros efeitos detectáveis, e só é evidente quando o nível de Pb-S é significativamente elevado por períodos prolongados (Hu et al., 1994; WHO, 1995; ATSDR, 1999).

1.2. Sacarose

Estima-se que o consumo de sacarose na dieta ocidental corresponde a aproximadamente 35% do total de carboidratos ingeridos. Esta dieta rica em sacarose, além de um possível desenvolvimento de obesidade, está relacionada a um aumento na produção de espécies reativas de oxigênio, triglicerídios, hiperglicemia, e hipertensão (McDonald, 1995; Roberts et al., 2001; 2002; Busserolles et al., 2002a, 2002b, 2002c; Škottová et al., 2004), estando também associado a uma diminuição dos níveis de tiol e atividade de enzimas tiólicas (Škottová et al., 2004), sugerindo um efeito pró-oxidante das dietas ricas em sacarose.

Tanto humanos quanto roedores podem adotar um comportamento de hiperfagia mediante uma situação de estresse social. Rowland et al., (1976), Fullerton et al., (1985) e Kageyama et al., (2000) descreveram que a disponibilidade de uma dieta rica em sacarose pode facilitar a indução desta hiperfagia, e também atenuar o estresse.

1.3. Dano oxidativo

As espécies reativas de oxigênio (ERO) são produzidas normalmente durante o metabolismo celular e possuem grande reatividade, causando lipoperoxidação e oxidação de proteínas e do DNA. As membranas celulares, que contém grande quantidade de ácidos graxos poliinsaturados, podem sofrer danos mediados por radicais livres. A lipoperoxidação é iniciada por algum radical, que ao reagir com os lipídios insaturados das biomembranas resulta na formação de hidro ou lipoperóxidos, que são altamente reativos e podem

seguir uma cascata oxidativa, com severas conseqüências à integridade da membrana, liberando no meio produtos da degradação de ácidos graxos.

Os radicais livres podem induzir diretamente lipoperoxidação ou modificar os grupos sulfidrílicos de proteínas. Esta modificação pode ser reversível, enquanto a oxidação das membranas não o é (Pruijn et al., 1991). Sob condições normais, os sistemas antioxidantes celulares minimizam os danos causados pelas ERO, porém, quando a produção de radicais livres excede a capacidade protetora da célula, observa-se o estresse oxidativo.

As espécies reativas de oxigênio podem ser tóxicas, e determinantes do tempo de vida celular, e já está demonstrado que os metabólitos das espécies reativas de oxigênio estão associados com muitos processos degenerativos (Melov et al., 1998; Janssen et al., 1998). Alterações no *status* oxidativo do organismo estão envolvidas em danos celulares e disfunções teciduais, além de estarem envolvidas com diversas patologias, tais como catarata, isquemia, enfisema pulmonar, cirrose hepática, diabetes mellitus, envelhecimento, doenças neurodegenerativas, danos no DNA e câncer (Cohen, 1989; Halliwell & Gutteridge, 1990; Floyd, 1990).

1.4. Selênio

1.4.1. Histórico

O selênio foi descoberto em 1817 pelo sueco Jons. J. Berzelius enquanto investigava uma doença que acometia trabalhadores em uma fábrica de ácido sulfúrico. Anteriormente, ainda no século XIII, Marco Polo relatou que cavalos na região de Succuir, no oeste da China apresentavam perda de cascos e pêlos após a ingestão de certas plantas venenosas. Seis séculos após os relatos de Marco Polo, foram descritos os mesmos sintomas em animais

entre o Sul de Dakota e Nebraska. A descoberta do agente etiológico destes efeitos tóxicos data do ano de 1928, quando Dr. Kurt Franke estudou estas plantas e seus grãos, concluindo que se tratava do selênio.

O selênio é um elemento traço cuja essencialidade nutricional foi demonstrada em 1957, em ratos (Schwartz & Foltz, 1957). Até então o único interesse biológico prático para o selênio era a toxicidade causada por altos níveis deste elemento na dieta (Levander & Burk, 1994). Entretanto, a partir da década de 70, foram descritas a deficiência de selênio e uma patologia reportada como uma cardiomiopatia endêmica, que pode ser suprimida pela suplementação com selênio (Ge et al., 1983).

O selênio é portanto, um micronutriente cuja concentração pode ocasionar deficiência ou toxicidade, sendo importante determinar a influência da suplementação de diferentes formas de selênio e sua biodisponibilidade. Estudos demonstraram que a biodisponibilidade das formas orgânicas de selênio (Se-metionina) são maiores do que as obtidas nas formas inorgânicas (selenito e selenato) (Levander et al., 1983; Smith & Picciano, 1987; Litov & Combs, 1991; Favier, 1993).

O selênio apresenta um grande número de funções biológicas, sendo a mais importante como antioxidante, e sendo descrito como redutor da toxicidade de metais pesados, entre eles mercúrio, chumbo e prata (Frost, 1983; Cuvin-Aralar & Furness, 1991; Ellingsen et al., 1993; Levander & Burk, 1994).

Com a descoberta do papel essencial do selênio no centro ativo da enzima glutationa peroxidase (Rotruck et al., 1981) e com o aumento do entendimento do papel fisiológico do selênio na regulação do dano oxidativo (Cadenas & Sies, 1985; Ursini & Bindoli, 1987), aumentou o interesse na síntese de

compostos orgânicos contendo selênio que possuam propriedades biológicas e aplicações farmacológicas (Parnham & Graf, 1991, Nogueira et al., 2004)).

1.4.2. Disseleneto de Difenila $(PhSe)_2$ e Ebselen

Experimentos realizados in vitro revelaram que, o PZ 51(2-fenil-1,2-benzisoselenazol-3(2H)-ona) ou Ebselen, como este composto é chamado, exibe atividade glutationa peroxidase, *per se*, catalizando a redução da H_2O_2 e outros hidroperóxidos na presença de glutationa como substrato (Sies, 1993).

O Ebselen vem sendo descrito como antioxidante, neuroprotetor e antiinflamatório (Parnham & Graf, 1991; Tan et al., 1997, Saito et al., 1998, Yamaguchi et al., 1998; Kondoh et al., 1999).

Recentes estudos têm demonstrado que o disseleneto de difenila também apresenta atividade do tipo tiol peroxidase (Engman et al.,1992, Rossato 2002, Meotti, et al., 2003; Nogueira et al., 2003; 2004), o que poderá motivar o uso terapêutico deste composto. Parte destes efeitos protetores está ligado a capacidade dos mesmos em decompor peróxidos na presença de tióis e de reduzir a peroxidação lipídica em diversos modelos experimentais.

A toxicologia destes compostos é bastante similar. Em doses farmacológicas ambos os compostos apresentam baixa toxicidade tanto para ratos como para camundongos (Perottoni et al., 2005; Fachineto et al., 2006).

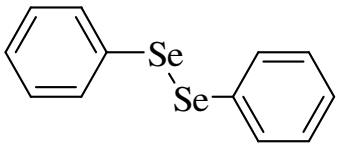

Figura 1. Disseleneto de Difenila $(PhSe)_2$

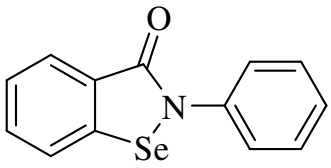

Figura 2. Ebselen

1.5. Avaliação comportamental

O aumento na produção de espécies reativas de oxigênio (ERO) ou a diminuição das defesas antioxidantes estão associadas com algumas manifestações comportamentais, entre elas a discinesia orofacial em animais (Naidu et al., 2003; Burger et al., 2003) e a discinesia tardia em humanos (Andreasen and Jorgensen, 2000; Lohr et al., 2003).

A discinesia tardia e a discinesia orofacial são consideradas síndromes neurológicas graves que podem ser observadas em humanos e animais experimentais. É uma desordem caracterizada normalmente por movimentos repetitivos involuntários, que envolvem a região da boca, face e língua; sendo algumas vezes também observada na musculatura do tronco e membros (Egan et al., 1997; Casey, 2000; Kulkarni and Naidu, 2001). Pouco se sabe acerca dos mecanismos moleculares promotores deste distúrbio, mas uma hipótese a ser considerada é a de que um aumento na produção de radicais livres possa ter efeitos sobre a discinesia orofacial. Este aumento na produção de radicais livres pode ter origem no metabolismo da dopamina, ou produzido durante a transmissão glutamatérgica (Lohr, 1991; Tsai et al., 1998; Naidu and Kulkarni, 2001) ou ainda, pela dieta (Fachinetto et al., 2005).

O glutamato é um neurotransmissor encontrado no cérebro de mamíferos, presente em mais da metade das sinapses no cérebro, envolvido em vários processos fisiológicos, tais como aprendizado, memória e formação de redes neuronais durante o desenvolvimento (Ozawa et al., 1998, Meldrum et al., 1999; Izquierdo and McGaugh, 2000). E é sabido que o chumbo pode bloquear o receptor NMDA (N-metil-D-aspartato), interferindo com a neurotransmissão do glutamato. Nesse contexto, tem sido relatado que o chumbo pode bloquear o receptor NMDA, um receptor glutamatérgico do tipo

ionotrópico, interferindo com a neurotransmissão glutamatérgica e desencadeando efeitos neurotóxicos (NourEddine et al., 2005).

Além de desencadear efeitos neurotóxicos, efeitos genotóxicos promovidos pelo chumbo vêm sendo estudados em vários sistemas biológicos, com resultados contraditórios. O acetato de chumbo já foi descrito como indutor de aberrações cromossômicas (Obe et al., 1975; Lin et al., 1994) e de danos no DNA (Sharma et al., 1985; Devi et al., 2000). Entretanto, Dunkel et al. (1984), e Zelikoff et al. (1988) demontraram resultados contraditórios, onde não se observou genotoxicidade induzida pelo metal.

2. Objetivos

Considerando que o chumbo e a sacarose administrados separadamente podem agir como pró-oxidantes, e também que o chumbo é considerado um inibidor clássico da δ-ALA-D, particularmente em modelos agudos de intoxicação, nós objetivamos avaliar o desenvolvimento de parâmetros oxidativos mediante a administração de acetato de chumbo, em diferentes modelos experimentais.

Em um modelo agudo, o objetivo foi estabelecer uma ligação entre a atividade da enzima δ-ALA-D, estresse oxidativo e transporte de glutamato no cérebro de camundongos intoxicados com acetato de chumbo. Além disso, avaliar os efeitos do tratamento com Ebselen ou disseleneto de difenila, uma vez que estes compostos apresentam atividade antioxidante.

Em um modelo crônico, considerando a possível atividade pró-oxidante do chumbo e da sacarose, o objetivo deste trabalho foi investigar se a exposição crônica e simultânea, durante 12 meses, a estes agentes, poderia apresentar um sinergismo no aparecimento de discinesia orofacial ou produzir distúrbios no comportamento locomotor de ratos.

Uma vez que o estresse oxidativo está relacionado à toxicidade induzida pelo chumbo em modelos agudos de intoxicação, o objetivo deste estudo foi investigar, os parâmetros oxidativos após a exposição ao chumbo, sacarose, ou ambos por 24 meses em ratas, ministrados na água de beber, e avaliar se este modelo crônico de longa exposição ao chumbo e/ou sacarose pode promover algum tipo de resposta adaptativa ao estresse oxidativo.

Artigo 1

EBSELEN AND DIPHENYL DISELENIDE DO NOT CHANGE THE INHIBITORY EFFECT OF LEAD ACETATE ON DELTA-AMINOLEVULINATE DEHYDRATASE

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Abstract

It is known that lead is toxic to several species of animals, and growing data support the participation of oxidation in lead toxicity. Selenium compounds, like diphenyl diselenide and Ebselen have a thiol-peroxidase like and other antioxidant properties. In this work, we determine whether these non-thiol-containing compounds with antioxidant properties could reverse the toxicity produced by Pb^{2+}. Lead acetate injection followed by injection with Ebselen or diphenyl diselenide did not change the levels of non- protein thiol groups (NPSH), whereas simultaneous treatment with lead plus Ebselen reduced NPSH levels in liver. Lead and Ebselen caused a marked reduction in thiobarbituric acid reactive species (TBARS) level in kidney, whereas lead or selenium compounds did not change TBARS levels in brain or liver. Lead acetate inhibited ALA-D activity in blood, liver, kidney and brain. Selenium compounds did not change enzyme activity nor the inhibitory effect of lead acetate in kidney and liver. Ebselen reversed brain ALA-D inhibition caused by Pb^{2+}. Reactivation index, indicated the extent of the reactivation of δ-ALA-D activity by a thiol donor, for ALA-D by DTT was higher in lead-treated groups than control groups in all tissues. Lead acetate or selenium compounds did not alter [^{3}H]-glutamate uptake by synaptosomes, whereas lead acetate plus Ebselen showed an increase on [^{3}H]-glutamate uptake. Lead induction of reactive oxygen species has been documented. The present study indicates that ALA-D inhibition precedes this event.

Keywords: lipoperoxidation, selenium, organocompounds, antioxidant, SH groups, TBARS

Introduction

Lead continues to persist throughout the environment and is one of the most common environmental and occupational contaminant (Tonner and Heiman, 1997). Routes of lead exposure include ingestion of contaminated drinking water, food or soil, and via inhalation of lead-contaminated dust. For children, intake of lead-containing paint is still an important source of intoxication (Godwin, 2001).

Several classes of molecular targets have been proposed to account for the symptoms associated with lead poisoning (Bouton et al., 2001). With few exceptions, these targets fall into two primary categories: proteins that naturally bind calcium and proteins that naturally bind zinc (Godwin, 2001). Despite lead having only a moderate affinity for sulfhydryl groups and not readily undergoing valence changes characteristic of transition metals (Bondy and Guo, 1996), it is commonly found in cells and tissues attached to thiol-containing proteins and small molecular weight thiols (Goering, 1993; Campagna et al., 1999). Zn^{2+}-containing enzymes, particularly those containing vicinal groups, such as δ-aminolevulinate dehydratase (ALA-D) are considered classical target molecules for this metal poisoning, (Gurba et al., 1972; Trevisan et al., 1980; Goering et al., 1986; Goering, 1993; Rocha et al., 1995 & 2001; Rodrigues et al., 1996; Jaffe et al., 2001). In line with this, the activity of the δ-ALA-D, commonly studied as a response test to lead,is considered an important index of subcritical effect (Secchi et al. 1974; Nordberg, 1976; Meredith et al. 1978; Simmonds et al. 1995; Polo et al., 1995).

ALA-D activity is highly sensitive to oxidants or situations associated with overproduction of free radicals (Flora et al., 2002; Folmer et al., 2002; Bolzan et al., 2002; Farina et al., 2003; Nogueira et al., 2003a; Soares et al.,

2003; Perottoni et al., 2004). Accumulation of δ-aminolevulinic acid (ALA), which results from ALA-D inhibition by Pb^{2+} or other agents, can have hepatic and neurotoxic effects (Bechara, 1996; Emanuelli et al., 2003) and part of these effects results from the overproduction of reactive oxygen species, (Monteiro et al., 1991; Hermes-Lima et al., 1991; Bechara et al., 1993). In line with this, data from different laboratories have indicated that oxidative stress can be an important factor for lead toxicity in different organs (Neal et al., 1997 & 1998; Gurer and Ercal, 2000). However, few studies that have evaluated simultaneously oxidative stress and ALA-D as indexes of lead toxicity in different organs.

Lead is described as a neurotoxic agent, and many of its molecular and cellular targets have been identified. However, the precise mechanisms of lead neurotoxicity are not all established (Bressler and Goldstein, 1991; Stohs and Bagchi, 1995; Bressler et al., 1999). This metal can cause alterations in neurotransmitter release (Bressler and Goldstein, 1991), activation of protein kinase C (Marcovac and Goldstein, 1988) and inhibition of glutamate uptake into astrocytes (Engle and Volpe, 1990). The amino acid glutamate is a common excitatory neurotransmitter, which in excessive amounts is toxic, causing the so-called excitotoxic reaction (Greenamyre, 1986). Agents that oxidize SH groups of the transporters can decrease glutamate uptake (Trotti et al., 1997; Nogueira et al., 2003a) and consequently can increase glutamate in the synaptic cleft. Although Pb^{2+} can change neurophysiologic responses to glutamate agonists (Braga et al., 1999), there are no data in the literature about the simultaneous effect of lead exposure and antioxidant treatment on glutamate uptake by brain synaptosomes.

The interest in organoselenide chemistry and biochemistry has increased in the last two decades mainly due to the fact that a variety of

organoselenium compounds possess antioxidant activity (Andersson et al., 1994). In fact, Ebselen (an organoselenium compound) is now considered as a pharmacological antioxidant agent for treatment of humans (Müller et al., 1984; Saito et al., 1998; Yamaguchi et al., 1998). Ebselen is a potent antioxidant agent and part of its antioxidative properties is linked to its glutathione-peroxidase like activity (Saito et al., 1998; Yamagushi et al., 1998, Rossato et al., 2002a). Other organoselenium compounds such as diphenyl diselenide also share with Ebselen both thiol-peroxidase like activity and other antioxidant properties (Wilson et al., 1989; Rossato et al., 2002b). Based on the fact that the pharmacological properties of Ebselen are related to its thiol peroxidase-like activity we have investigated the pharmacological properties of diphenyl diselenide. We observed that diselenide causes minimal toxicity when administrated acutely to mice and rats in doses that have anti-inflammatory and antinociceptive activity (Nogueira et al., 2003b).

Of particular importance, inorganic Se can protect rodents from the toxic effect of lead (Rastogi et al., 1976; Flora et al., 1983; Dhir et al., 1985; Othman and Missiry, 1998), possibly by affording additional antioxidant capacity via GPx (Flohé, 1973; Ursini et al., 1982; Behne and Kyriakopoulos, 1990; Bock et al., 1991). Since selenorgano compounds have antioxidant properties, we realized that they could be potential protective agents against metal intoxication.

As pointed out above, Pb2+ can yield toxicity by interfering with thiol-containing proteins, particularly with those containing Zn2+. Furthermore, oxidative stress can be an important outcome of Pb2+ exposure and the use of antioxidants and thiol protecting agents as possible therapeutic approaches against lead toxicity have been considered by basic investigators (McGowan, 1989; Flora et al., 1989 & 1991; Dhawan et al., 1988 & 1989; Gurer and

Ercal, 2000; Hsu and Guo, 2002). However, it must be emphasized that in the majority of these studies, at least one of these antioxidants used was a thiol-containing compound. Since Ebselen and other organochalcogenides, particularly diphenyl diselenide, have antioxidant properties we aimed to determine whether non-thiol-containing synthetic compounds with antioxidant properties could reverse the toxicity produced by Pb2+. Of particular importance for our working hypothesis, data from our group have indicated that Ebselen protect developing rat brain from methylmercury-induced neurotoxicity by acting as an antioxidant agent (Farina et al., 2003). A number of parameters indicative of lead poisoning in blood and soft tissues, like ALA-D activity, oxidative stress, non- protein thiol groups and glutamate uptake were determined in an attempt to establish a link between ALA-D, oxidative stress and brain glutamate transport in mice.

Material and Methods

Animals

Adult male Swiss albino mice (2-3 month old, 25-35 g) from our own breeding colony were maintained in an air-conditioned room (22-25ºC) under natural lighting conditions, with water and food (Guabi – RS, Brasil) *ad libitum*. Animals (varying of 5 to 19 animals/group) were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Medicine Veterinary and Animal Science of the University of São Paulo, Brazil.

Chemicals

Glacial acetic acid, *ortho*-phosphoric acid, hydrochloric acid, sodium selenite, sulfuric acid, perchloric acid, ethanol, $HgCl_2$, NaCl, K_2HPO_4, KH_2PO_4, 5,5'-dithio-bis-(2-nitrobenzoic acid), hydrogen peroxide, and ascorbic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil), butylated hydroxytoluene, sodium dodecyl sulfate, dimethyl sulfoxide, 2,4-dinitrophenylhydrazine, tris(hydroxymethyl)aminomethane, cysteine, thiobarbituric acid, δ-aminolevulinic acid, bovine serum albumin, reduced glutathione and comassie brilliant blue G were obtained from Sigma (St. Louis, MO). 1,1,3,3-Tetramethoxypropane was obtained from Aldrich (Milwaukee, WI). Tricholoroacetic acid was obtained from Reagen (Rio de Janeiro, RJ, Brazil). *p*-Dimethylaminobenzaldehyde was obtained from Riedel (Haën, Germany). Diphenyl diselenide $(PhSe)_2$ and Ebselen were synthesized by the method previously described (Paulmier 1986, Engmann, 1989). [^{3}H]-glutamic acid (49 Ci/mmol) was from Amersham International, UK. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Exposure

For the *in vivo* studies mice received a subcutaneous injection (10 mL/kg body weight) of vehicle (saline) or lead acetate (50 mg/kg), once a day for 15 days. Then, animals were injected (i.p) five days with vehicle (10 mL/kg body weight), Ebselen or diphenyl diselenide (25 μmol/kg) (Nogueira et al., 2003; Meotti et al., 2003). Diphenyl diselenide and Ebselen solutions were prepared in DMSO. Animals were anaesthetized with ether one day after the last injection for blood collection, and killed by decapitation. Tissues

(brain, liver and kidney) were removed for determination of ALA-D activity, TBARS, non-protein thiol groups (NPSH) and glutamate uptake.

ALA-D activity

Kidney, brain and liver of treated animals were quickly removed, placed on ice and homogenized in 150 mM NaCl 7, 7 and 10 volumes, respectively. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 minutes to yield a low-speed supernatant fraction (S_1) that was used for enzyme assay. Enzyme assay was carried out as described by Sassa (1982) by measuring the rate of product (porphobilinogen) formation, except that 200 mM potassium phosphate buffer, pH 6.4 and 2.5 mM aminolevulinic acid were used. Reaction was started 10 minutes after the addition of enzyme preparation by adding the substrate and carried out for 60, 120 and 180 minutes, for liver, kidney and brain, respectively, at 39ºC.

The blood ALA-D activity was determined as described above, except that 760 mM potassium phosphate buffer, pH 6.4 and 16 mM aminolevulinic acid were used, and the blood was preincubated with Triton 0.1% and incubation was carried out for 120 minutes.

The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 for the Ehrlich-porphobilinogen salt. Simultaneously, a set of tubes assayed using a similar incubation medium, except 1.4 mM dithiothreitol (DTT) was also added in order to obtain the reactivation index. This index indicates the extent of the reactivation of δ-ALA-D activity by DTT and is defined as:

$$\frac{\text{(ALA-D activity with DTT – ALA-D activity without DTT)}}{\text{ALA-D activity with DTT}} \times 100$$

TBARS determination

Kidney, brain and liver of treated animals were homogenized in 7, 7 and 10 volumes, respectively, of a medium containing 150 mM NaCl. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 min. and stored at -20ºC for a week, to further determination of thiobarbituric acid reactive substances (TBARS). TBARS were assayed as described by Ohkawa *et al*. (1979). In brief, samples were incubated at 100ºC for 120 min. in a medium containing 0.45 % sodium dodecyl sulfate, 100 mM hydrochloric acid, 1.4 M acetic acid, pH 3.4 and 0.3 % thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as standard.

Non- protein thiol groups (NPSH) determination

NPSH were determined as described by Ellman (1959) at 412 nm, as modified by Jacques-Silva et al. (2001). Non-protein thiol groups were determined in the fraction obtained after dilution of supernatants with 1 volume of 4 % trichloroacetic acid followed by centrifugation and neutralization with 0.5 M tris(hydroxymethyl)aminomethane/HCl, pH 7.5. A standard curve using cysteine was constructed in order to calculate the non-protein thiol groups in the tissue samples.

[3H]-Glutamate Uptake by crude synaptosomes

The brain crude synaptosomes were prepared as described by Rocha et al. (1990). The final pellet was incubated in HBSS (Hepes buffered salt solution, composition in mM: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0), pH 7.4 (adjusted with HCl), in the

presence of [^{3}H]-glutamate (final concentration 100 nM) for 1 min at 37^{o}C. The reaction was stopped by filtration through GF/B filters. The filters were washed 3 times with 3 ml of ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The radioactivity retained on the filters was measured in a Packard scintillation counter. Specific [^{3}H]-glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained in a similar incubation medium in which NaCl was substituted for choline chloride.

Protein quantification

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) (lead x selenium) followed by Duncan's Multiple Range Test when appropriate. Results with $P \leq .05$ were considered significant. Three-way ANOVA [tissue (4) x lead (2) x selenium (3)] were also carried out on data.

Results

NPSH levels

Treatment of mice with 50 mg/kg lead acetate or organochalcogenides (Ebselen or diphenyl diselenide) (25 μmol/kg) did not change the levels of non- protein thiol groups (NPSH) in brain (Figure 1A), kidney (Figure 1B) or

liver (Figure 1C). However, simultaneous treatment with lead plus Ebselen reduced NPSH levels in liver (P<.05).

TBARS levels

Intoxication with lead acetate and/or treatment with organochalcogenides did not change TBARS levels in brain (Figure 2A) and in liver homogenates (Figure 2B). In kidney homogenates, lead acetate intoxication caused a marked (about 40%) reduction in TBARS levels in control group (DMSO) and in diphenyl diselenide treated group (~36%). Lead acetate also tended to decrease the TBARS levels in kidney of Ebselen treated mice; however, since Ebselen caused a reduction in TBARS level in this organ, the effect of lead was not so evident as in other two groups (Figure 2C).

ALA-D activity

Treatment of mice with 50 mg/kg lead acetate inhibited ALA-D activity in all evaluated tissues, whereas Ebselen and diphenyl diselenide did not change enzyme activity nor the inhibitory effect of lead acetate in blood, liver and kidney (Figures 3A, 4A, 5A). In brain ALA-D activity, Ebselen reversed the lead-induced inhibition (Figure 6A).

In blood and liver, lead acetate inhibited ALA-D activity by 65% [$F(1,99)=141.93$ $P<.05$] and 55% [$F(1,83)=137.26$ $P<.05$], respectively (Figures 3A and 4A). In kidney and brain the reduction of ALA-D was 52% [$F(1,83)=87.95$ $P<.05$], and 23% [$F(1,49)=17.47$ $P<.05$], respectively (Figures 5A and 6A).

Reactivation index for ALA-D

The reactivation index for ALA-D by DTT was higher in the lead-treated animals than control group (Table 1). The hepatic reactivation index for ALA-D by DTT was 3- fold higher in the lead- treated group than in the control groups, whereas in kidney and brain differences between lead- treated and control groups were about 40 %. The blood ALA-D reactivation index was increased about 2 to 3 times in lead poisoned mice (Table 1). The cerebral ALA-D reactivation index revealed a significant main effect of lead exposure. However, a significant lead intoxication vs organochalcogenide interaction indicated that the reactivation index varied depending on lead and the class of chalcogenide considered. In fact, in lead intoxicated mice, Ebselen reduced significantly, whereas diphenyl diselenide increased the reactivation index in brain enzyme when compared with animals treated with DMSO (Table 1).

Mice treated with lead acetate (50 mg/kg), diphenyl diselenide or Ebselen (25 μmol/kg) did not modify [3H]-glutamate uptake by synaptosomes. Animals exposed to lead acetate and Ebselen showed an increase on [3H]-glutamate uptake when compared with control group (saline/DMSO) $p<.05$ (Figure 7).

Discussion

The results of the present investigation demonstrated that lead acetate intoxication caused a marked inhibition of ALA-D from blood, liver and kidney which is in agreement with literature (Flora et al., 1982; Freeman et al., 1996; Ercal et al., 1996). Cerebral ALA-D was also inhibited by lead intoxication; however, the magnitude of brain ALA-D inhibition was less accentuated than that observed in the other tissues studied. The reactivation

index was significantly increased by lead acetate intoxication in all organs; however index values are higher in liver and blood, indicating that these tissues are the main target for lead in this short-term treatment protocol. Organochalcogenide treatment, as rule, did not change the inhibitory effect of lead acetate exposure. The only significant effect of organochalcogenides was observed in the brain. Lead acetate poisoning for 15 days followed by 5 days of treatment with Ebselen markedly decreased, whereas treatment with diphenyl diselenide increased the reactivation index of cerebral ALA-D. These results may indicate a protective effect of Ebselen on the neurotoxicity of lead. In line with this, Ebselen is an antioxidant compound effective against a variety of neuronal insults in humans and in animal models of neurotoxicity (Dawson et al., 1995; Takasago et al., 1997; Yamaguchi et al., 1998; Parnham and Sies, 2000; Porciúncula et al. 2001; Rossato et al. 2002b).

Oxidative stress is associated with lead intoxication and data from different laboratories have indicated that lead increases lipid peroxidation in different tissues (Hermes-Lima et al., 1991; Stohs and Bagchi, 1995; Gurer et al., 1998; Adanaylo and Oteiza, 1999; Tandon et al., 2002). However, the effect of lead poisoning on prooxidant/ antioxidant balance seems not to be homogeneous in all tissues, and depends on the developmental period of exposure (Moreira et al., 2001). In the present study, lead acetate did not change the manifestation of lipid peroxidation (quantified as thiobarbituric reactive species) in liver and brain. Unexpectedly, TBARS levels were markedly reduced in the kidney of lead intoxicated mice. The reasons for this decrease is still unclear to us but may be related to the properties of renal accumulated Pb2+ to compete with iron ions as initiator of lipoperoxidation. Data concerning the pro-oxidant effect of Pb2+ in vitro are scarce and controversial in the literature. In fact, the literature indicates that Pb2+ did not

increase lipoperoxidation (Willis, 1965) or at high non-toxicological significant concentrations causes an increase in lipid peroxidation (Shafiq-ur-Rehman, 1984; Yiin and Lin, 1995; Shafiq-ur-Rehman, et al., 1995; Sandhir and Gill, 1995).

Further support that our schedule of intoxication had not yet generated signs of oxidative stress in mice was evidenced from the results of non-protein –SH (NPSH) group quantification. NPSH was not modified by lead treatment in all the tissues analyzed. A reduction of NPSH was observed only in liver of lead- and Ebselen-treated mice. We speculate that this effect is a consequence of the fact that liver is the primary target organ for lead intoxication and this possibly is promoting an oxidative state that still has not modified TBARS hepatic levels. Ebselen is an organic compound with thiol-peroxidase like activity and can decompose peroxide using GSH or other reduced thiols as electron donor (Müller et al., 1984; Maiorino et al., 1988; Sies, 1993; Parnham and Sies, 2000; Daiber et al., 2000; Wendel et al., 1984). Thus, if lead acetate is starting to produce peroxide in liver, Ebselen can consume NPSH in order to decompose the excess of peroxide.

Lead is a neurotoxic agent and changes in the glutamatergic neurotransmission are a potential target for lead neurotoxicity. In line of this, literature data clearly indicate that lead can interfere with glutamate receptors (Mantovani et al., 1999; Braga et al., 1999), In the present investigation, lead alone was not able to change glutamate uptake by brain synaptosomes indicating that for our schedule of intoxication the glutamate transporters are not the primary target for lead. However, treatment of mice with organochalcogenides after the end of lead intoxication caused a significant increase in the glutamate uptake by brain synaptosomes. The implications of this increase for the neurotoxicity of lead are difficult to predict. In fact,

excess of glutamate in the synaptic cleft can produce a neurotoxic effect that can be followed by neurodegeneration (Lipton & Rosemberg, 1994; Price, 1999). However, more recent studies have clearly indicated that reduction of glutamate neurotransmission can also cause neurotoxic effects (Obrenovitch, 1999; Obrenovitch et al., 2000).

In conclusion, the results of the present investigation indicate that ALA-D inhibition antecedes the overproduction of ROS in different tissues, which is well documented in the literature (Gurer and Ercal, 2000; Hsu and Guo, 2002). Although lead poisoning can result in the installation of a state of oxidative stress in mammals it is still not clear whether oxidative stress is the cause or the consequence of the toxic effect of lead. Our data suggest that ALA-D inhibition can have a primary role in the development of lead toxicity. We speculate that ALA-D inhibition causes an increase in ALA, which is a pro-oxidant under physiological conditions (Bechara et al., 1993 & 1996), and subsequently contributes to the development of oxidative stress in lead exposed organisms.

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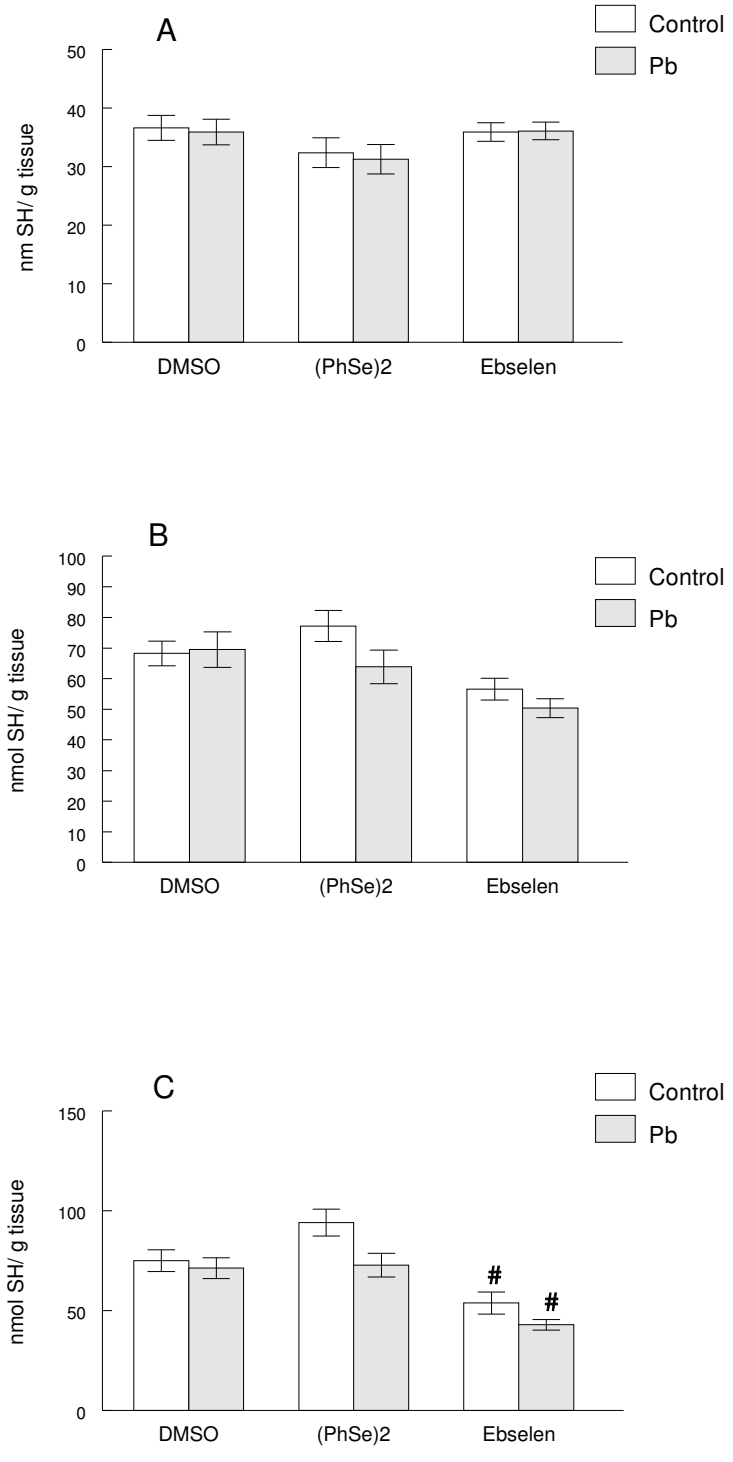
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Figure 1.

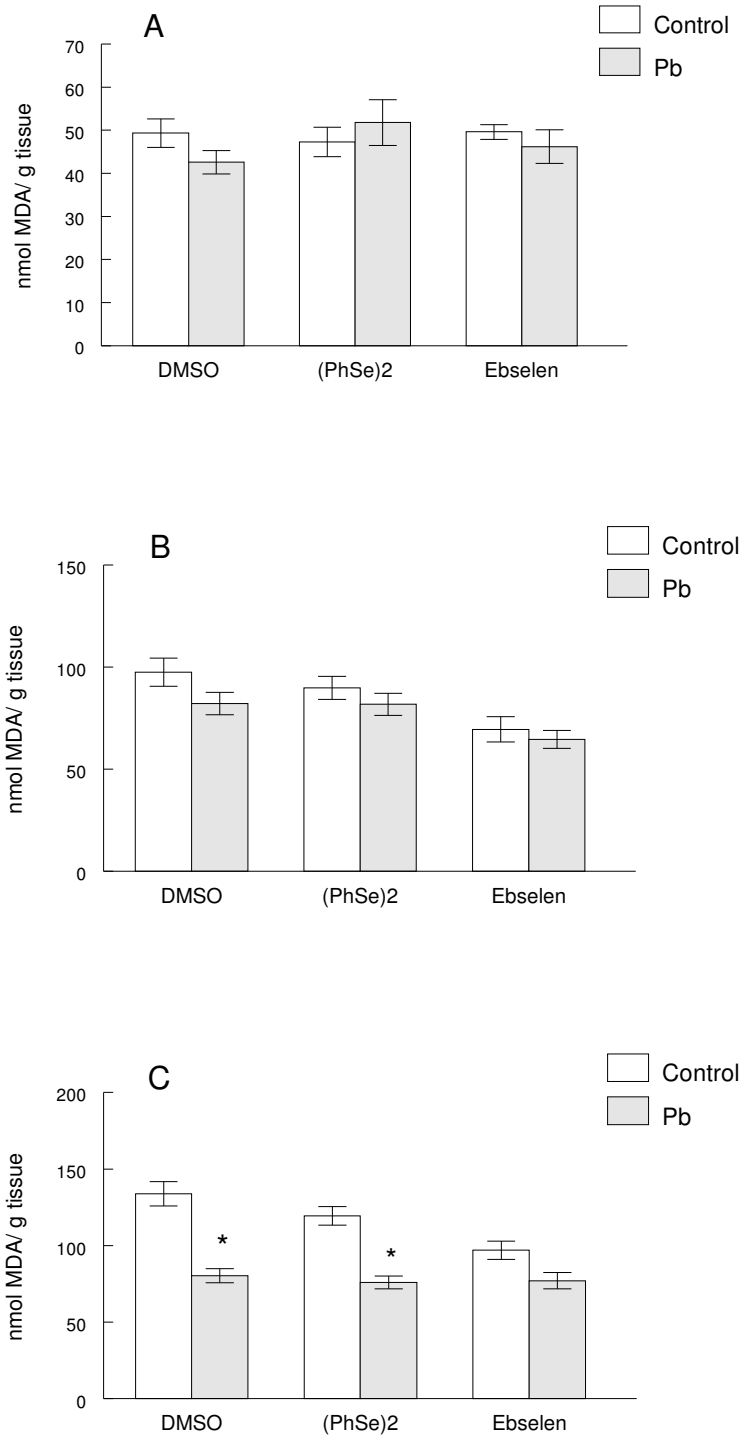
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Figure 2.

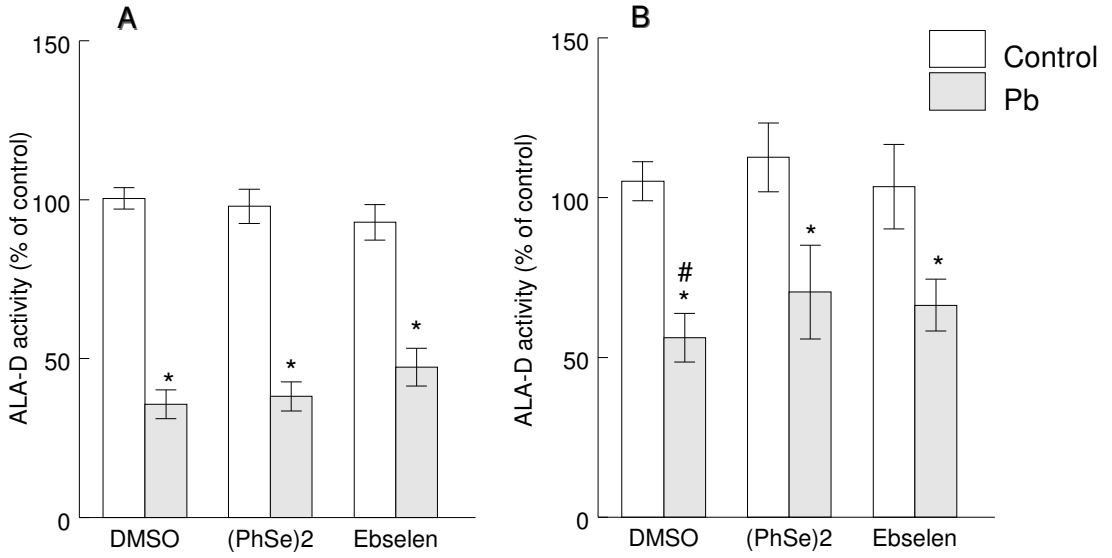
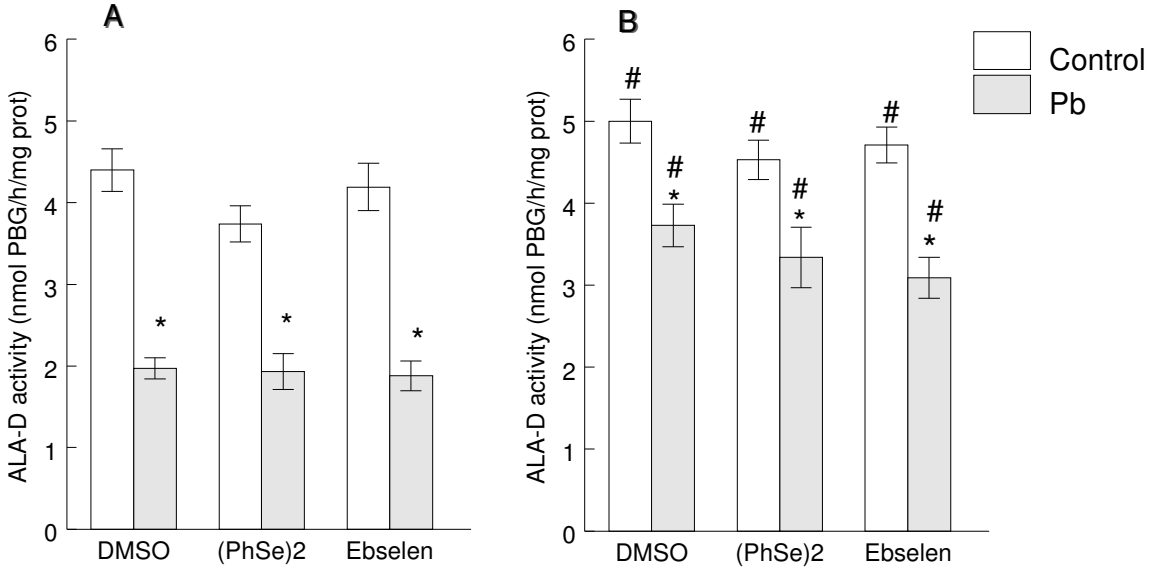


Figure 3



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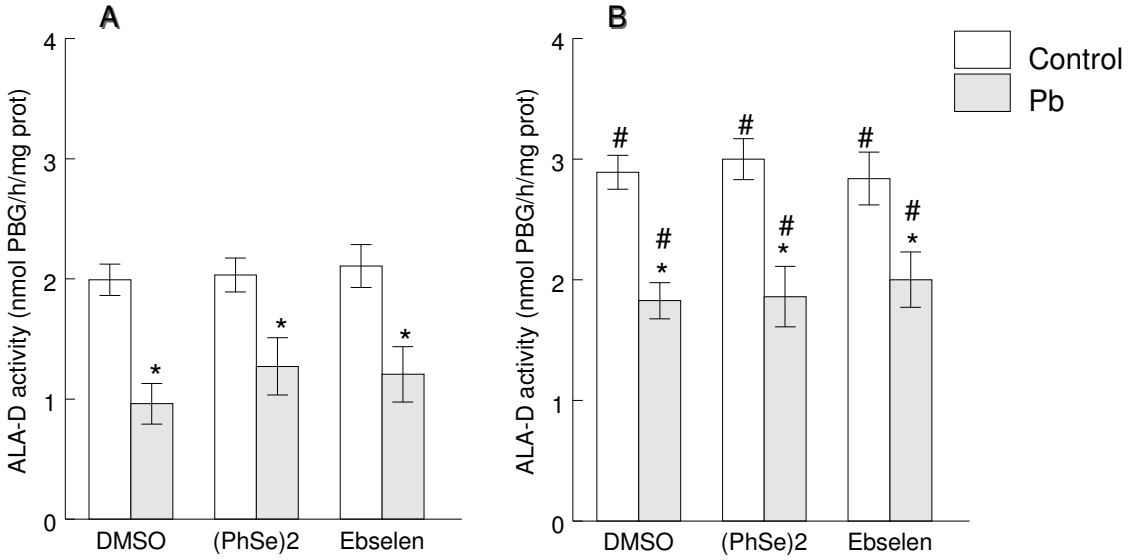

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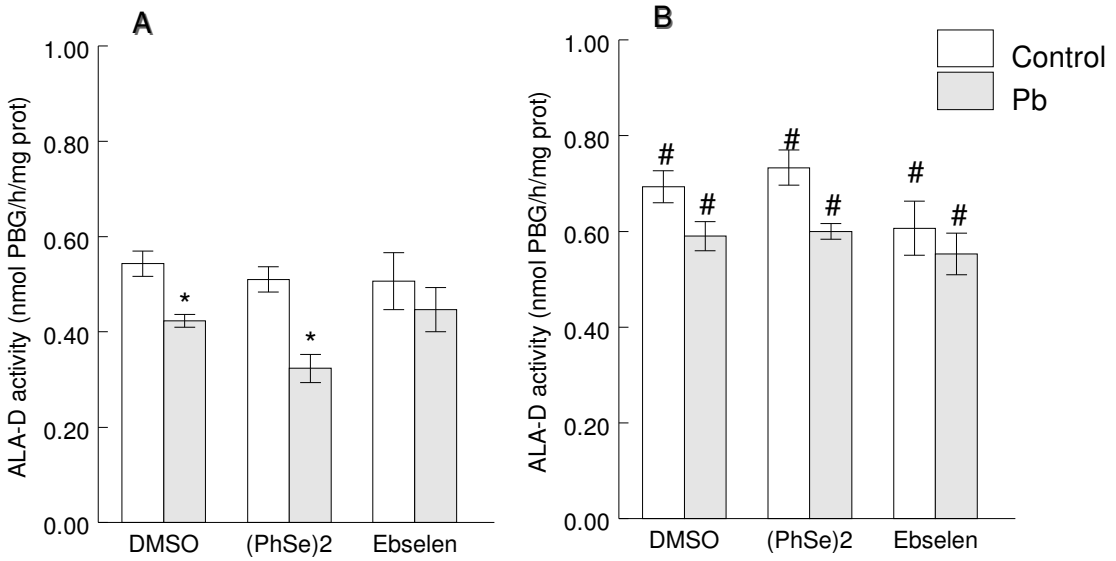

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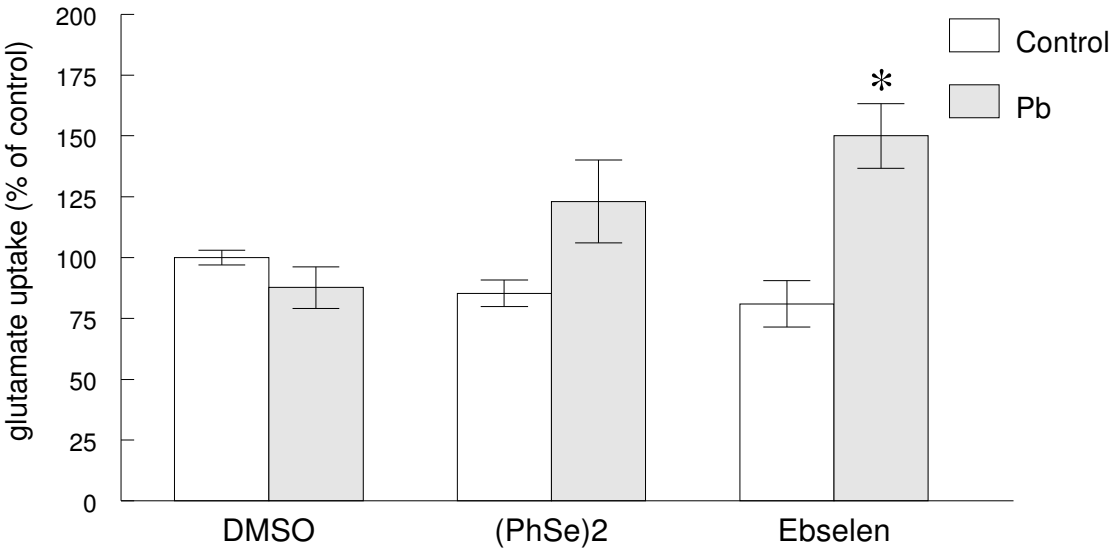
glutamate uptake (% of control)
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75
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Figure 7.

Table 1. ALA-D activity reactivation index

		Liver			Kidney			Brain			Blood		
		Mean	n	SEM	Mean	n	SEM	Mean	n	SEM	Mean	n	SEM
Control	$(PhSe)_2$	17.65	17	3.88	31.63	17	3.68	29.17	8	3.63	11.73	15	3.34
	Ebselen	11.65	8	2.79	25.18	8	3.18	16.74	6	2.74	8.02	10	2.84
	DMSO	12.33	18	2.77	30.21	18	3.76	20.16	12	3.93	9.23	15	3.28
Pb	$(PhSe)_2$	42.10	11	2.87	34.63	11	4.36	45.74	4	3.4	34.68	8	4.90
	Ebselen	37.89	10	3.65	39.85	10	4.23	19.96	6	3.41	20.16	10	4.68
	DMSO	45.68	21	3.47	47.77	21	4.46	27.45	15	2.91	27.32	19	4.61

Legends of figures

Figure 1. Effects of treatment with lead (50 mg/kg) and/or selenium compound (Ebselen or diphenyl diselenide) (25 μmol/kg) on brain (A), kidney (B) and liver (C) NPSH determination. NPSH were determined in the fraction obtained after dilution of homogenates supernatants with 1 volume of 4 % trichloroacetic acid followed by centrifugation and neutralization with 0.5 M tris(hydroxymethyl)aminomethane/HCl, pH 7.5. Values are expressed at means ± SEM (n = 5-18). #Significantly different from groups without selenium (P<.05).

Figure 2. Effects of treatment with lead (50 mg/kg) and/or selenium compound (Ebselen or diphenyl diselenide) (25 μmol/kg) on brain (A), liver (B) and kidney (C) TBARS production. Samples were incubated at 100ºC for 120 min. in a medium containing 0.45% sodium dodecyl sulfate, 100mM hydrochloric acid, 1.4M acetic acid, pH 3.4 and 0.3% thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm. (Values are expressed at means ± SEM (n = 5-19). *Significantly different from control group (P<.05).

Figure 3. Effects of treatment with lead acetate (50 mg/kg) and/or selenium compound (Ebselen or diphenyl diselenide) (25 μmol/kg) in blood ALA-D activity, in the absence (A) or presence (B) that DTT. Blood was pre- incubated with Triton 0.1%, and ALA-D activity was determined in a medium containing 760 mM potassium phosphate buffer pH 6.4. Reaction was started at addiction that 16 mM aminolevulinic acid, and the incubation was carried out for 120 minutes, in the absence or DTT presence (1.4 mM). Values expressed at percent of control. Values for control was 43.75 μmol PBG/gHb/h. (Data are expressed as means ± SEM for n= 10-17). *Significantly different from control group (P<.05). #Significantly different from group without DTT (P<.05).

Figure 4. Effects of treatment with lead acetate (50 mg/kg) and/or selenium compound (Ebselen or diphenyl diselenide) (25 μmol/kg) on liver ALA-D activity, in the absence (A) or presence (B) that DTT. Tissue was homogenized in 150 mM NaCl 10 volumes. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 minutes to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in presence that 200 mM potassium phosphate buffer, pH 6.4. Reaction was started 10 minutes after the addition of enzyme preparation by adding the substrate (2.5 mM aminolevulinic acid) and carried out for 120 minutes, at 39ºC, in the absence (A) or DTT presence (B) (1.4 mM). (Data are expressed as means ± SEM for n= 10-19). *Significantly different from control group (P<.05). #Significantly different from group without DTT (P<.05).

Figure 5. Effects of treatment with lead acetate (50 mg/kg) and/or selenium compound (Ebselen or diphenyl diselenide) (25 μmol/kg) on kidney ALA-D activity, in the absence (A) or presence (B) that DTT. Tissue was homogenized in 150 mM NaCl 7 volumes. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 minutes to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in presence that 200 mM potassium phosphate buffer, pH 6.4. Reaction was

started 10 minutes after the addition of enzyme preparation by adding the substrate (2.5 mM aminolevulinic acid) and carried out for 120 minutes, at 39ºC, in the absence (A) or DTT presence (B) (1.4 mM). (Data are expressed as means ± SEM for n= 10-19). *Significantly different from control group (P<.05). #Significantly different from group without DTT (P<.05).

Figure 6. Effects of treatment with lead acetate (50 mg/kg) and/or selenium compound (Ebselen or diphenyl diselenide) (25 µmol/kg) on brain ALA-D activity, in the absence (A) or presence (B) that DTT. Tissue was homogenized in 150 mM NaCl 7 volumes. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 minutes to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in presence that 200 mM potassium phosphate buffer, pH 6.4. Reaction was started 10 minutes after the addition of enzyme preparation by adding the substrate (2.5 mM aminolevulinic acid) and carried out for 120 minutes, at 39ºC, in the absence (A) or DTT presence (B) (1.4 mM). (Data are expressed as means ± SEM for n= 6-13). *Significantly different from control group (P<.05). #Significantly different from group without DTT (P<.05).

Figure 7. Effects of pre-treatment with diphenyl diselenide and Ebselen on [^{3}H]-glutamate uptake by mice brain synaptosomes treated with lead acetate. Adult mice were treated by s.c route whit lead acetate or saline by fifteen days. After, received diphenyl diselenide or Ebselen 25 µmol/kg or DMSO (1mL/kg) by i.p route by five days. The synaptosomes were prepared second Rocha et al. (1990). Data expressed at percent of control. Data for control was 9.63 pmol glutamate uptake/min/mg of protein. Data are mean ± SEM of six animals. *Denoted P<.05 as compared to control group (ANOVA/Duncan).

Manuscrito 2

Ingestion of Lead and/or Sucrose Do Not Change Oral diskynesia In Rats

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Abstract

It is known that lead is toxic to several species of animals, and growing data support the participation of oxidative stress in lead toxicity. In the same vein, ingestion of diet rich in free-sugars or in fat can be associated with increased oxidative stress and can also facilitate orofacial dyskinesia in rats. Considering that Pb and sucrose could have a pro-oxidant activity, the aim of this study was to investigate whether the simultaneous exposure to these agents could enhance the appearance of orofacial dyskinesia (OD) in rats. The incidence of OD increased significantly as a function of age (4 to 12 month) in rats. However, sucrose ingestion was not associated with an increase in OD incidence. Lead exposure (400 ppm), when associated with sucrose, contrary that one would expect, caused a decrease in OD incidence. The increase in OD as a function of age is in agreement with literature data and the absence of effect of sucrose and/or lead is possible related to an adaptation after long-term exposure to pro-oxidants or, alternatively, to antagonistic effect of lead at the level of NMDA receptor.

Keywords: lead acetate, sucrose, orofacial dyskinesia, locomotor behavior, rat.

Introduction

Lead is a common occupational and persistent environmental contaminant (Kakkar and Jaffery, 2005). Routes of lead exposure may include ingestion or inhalation of lead-contaminated dust (Godwin, 2001) and its effects in humans vary depending on the dose and the age of the exposed person (Agency for Toxic Substances and Diseases Registry, 1988). It is known that oxidative stress can be an important factor in lead toxicity (Neal et al., 1998; Gurer and Ercal, 2000), and neurotoxicity (Monteiro et al., 1991; Bechara et al., 1996; Emanuelli et al., 2003). In line with this, lead is a well-know neurotoxic agent and many of its molecular and cellular targets have been identified. Lead exposure can cause alterations in neurotransmitter release (Bressler and Goldstein, 1991 protein kinase C activation (Marcovac and Goldstein, 1988) and inhibition of glutamate uptake into astrocytes (Engle and Volpe, 1990). However, the precise mechanisms involved in lead neurotoxicity are still not definitively established (Bressler et al., 1999).

Hypercaloric diet intake has been shown to be important in the development of obesity (Warwick and Schiffman, 1992) and studies from our group have shown that ingestion of diets rich in fat or in free-sugar can be associated with increased oxidative stress in rodents (Folmer et al., 2002, 2003). Recently, literature data have reported that high fat diet ingestion decrease the levels of striatal dopamine (Choi et al., 2005) and facilitate the appearance of orofacial dyskinesia in rats (Fachinetto et al., 2005). In the same vein, data from our laboratory have also demonstrated that a simultaneous exposure to Cd2+ and sucrose causes an increase in Cd2+ toxicity (Folmer et al., 2004). In line with this, over production of reactive oxygen species (ROS)

and depletion in antioxidant defenses has been associated with diabetes and cadmium intoxication (Hunt et al., 1988; Wolff and Dean, 1987).

Similarly, orofacial dyskinesia in animal models (Naidu et al., 2003; Burger et al., 2003) and tardive dyskinesia in humans (Andreasen and Jorgensen, 2000; Lohr et al., 2003) have been connected to oxidative stress. These extrapyramidal disorder are characterized by repetitive involuntary movements, involving mouth, face, and tongue, and sometimes limb and trunk musculature (Egan et al., 1997; Casey, 2000; Kulkarni and Naidu, 2001). The molecular mechanisms that underlie the neuropathophysiology of orofacial dyskinesia are still not completely understood. One hypothesis that has been reinforced by experimental data is that free radical derived from the metabolism of dopamine and/or from an enhancement of glutamatergic neurotransmission caused by blocking presynaptic dopamine receptor participates in the genesis of orofacial dyskinesia (Lohr, 1991; Tsai et al., 1998; Naidu and Kulkarni, 2001; Burger et al 2005).

Considering that Pb and sucrose may have pro-oxidant activity, the aim of this study was to investigate whether the simultaneous exposure to these agents could enhance the appearance of orofacial dyskinesia in rats.

Material and methods

Animals

Female Wistar rats (60 days old) were maintained on a natural cycle in a controlled temperature room (22-26^{o}C). They received food *ad libidum* (Guabi, RS, Brazil) and Pb and/or sucrose) via drink water. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the University of Santa Maria, Brazil.

Exposure to Lead and Sucrose

Rats were distributed in six treatments: (1) control, (2) 20% sucrose, (3) 100 ppm lead acetate, (4) 20% sucrose + 100 ppm lead acetate, (5) 400 ppm lead acetate, (6) 20% sucrose + 400 ppm lead acetate. This experimental exposure was carried out for 12 months. To prevent lead acetate precipitation, 0.5ml/L of glacial acetic acid was added to the water of all groups. The animals body weight were measured monthly.

Behavioral analysis

Orofacial Dyskinesia

Orofacial Dyskinesia was analyzed as described in Burguer et al (2003). In short. rats were observed in a glass cage (20 x 20 x 19cm) equipped with mirror under the floor and behind the back wall of the cage to allow behavioral quantification when the animal was faced away from the observer. The animal behavior was observed by 6 min after 2 min of adaptation to the cage. The trained observer scored and analysed the following behavioral categories: vacuous chewing movements (VCM - purposeless mouth openings) and tongue protrusion. These parameters were not scored during grooming or rearing.

Open field

The ethological test open field is used to analyze behavior with base on natural conflict-situations between exploration of and the aversion against open or bright areas (Whimbey and Denenberg, 1967, Asano, 1986). It is used to analyze activity and anxiety profiles in rodents (Archer, 1973; Whimbey and Denenberg, 1967).

The open field test arena used consisted of an white wood cage measured 50 x 50 x 40 cm^3 and was divided into 9 squares by black lines. In trials (2 min of duration), locomotion was assessed by the numbers of lines crossed with the four paws and rearing when the rat reared on its hind paws. The arena was cleaned with alcohol and rinsed with water after every test.

Biochemical analysis

After 12 months of treatment, the blood collection was performed by eye vein puncture, and the blood samples were centrifuged for plasma glucose, insulin, aspartate aminotransferase (AST) and alanino aminotransferase (ALT) quantification.

Statistical analysis

Body weight and the behavioral parameters were analyzed by a three-way analysis of variance (ANOVA) (2 Pb x 2 sucrose x 4 age) with the age factor treated as a repeated measure. Statistical analysis was followed by Duncan's Multiple Range test when appropriate. Results with $P<.05$ were considered significant.

Results

Body weight gain

The statistical analysis of body weight gain demonstrated a significant statistical interaction between treatments group and time of exposure ($F(6,165)=2.75$, $P<.01$). The sucrose group had an increase in body weight gain ($F(1,55)=11.61$, $P<.01$) in comparison to other groups. The lead acetate

treatment did not promote any difference in the body weight gain ($F(6,165)=1.17$, $P>.05$), when compared to the control group (TABLE. 1).

Biochemical parameters

ANOVA for hematocrit revealed a significant main effect of sucrose ingestion ($F(1,18)=11.06$, $P<.01$). Sucrose consumption, regardless of lead treatment, was associated with a reduction in hematocrit (TABLE 1). ANOVA for plasma glucose indicated a main effect of lead ($F(2,54)=3.43$, $P<.05$). In fact, exposure to lead tended to increase the blood glucose. The increase was significant for rats treated with Pb 100ppm, but not with Pb 400 ppm.

ANOVA of insulin levels, revealed a significant main effect of sucrose, Insulin levels increased after sucrose consumption and the increase was significant in the sucrose and sucrose+100 ppm lead groups. ANOVA of AST and ALT revealed no significant effects (TABLE. 1).

Open Field

There is no effect on open field in all groups (TABLE. 2).

Rearing

ANOVA for rearing revealed a main effect of age ($F(4,220)=31.6$, $P<.01$) which was a consequence of an increase in rearing incidence as a function of age (TABLE. 2) independent of lead or sucrose treatment.

Orofacial Dyskinesia

Vacuous Chewing Movements

ANOVA of VCM yielded a main effect of age ($F(4,220)=12.64$, $P<.01$), because VCM incidence increased as a function of age in all groups, excepting

in the group exposed simultaneously to lead (400 ppm) and sucrose (20%). In line with this, the statistical analysis revealed a significant sucrose x lead x age interaction ($F(8,220)=2.10$, $P<.05$) (TABLE. 3).

Tongue Protrusion

ANOVA for tongue protrusion showed a significant between sucrose vs lead interaction ($F(2,55)=6.65$, $P<.01$). Post hoc comparisons revealed that rats exposed to lead (400 ppm) and sucrose had a decrease in the incidence of tongue protrusion (TABLE. 3).

Discussion

Exposure to lead is sometimes associated with an increase in locomotor activity (Silbergeld et al., 1974; Ma et al., 1999). Here, we observed no significant differences in female rat activity after exposure to lead. In fact, the only consistent effect was obtained in rearing, where aging was associated with an increase in the incidence of this behavior. These results indicate that the rearing behavior, differing from general activity that was not modified significantly by age, increase as a function of age in female rats.

The OD incidence was not modified by sucrose consumption, whereas the effect of lead on OD varied dependent on the dose of exposure. In fact, the OD incidence increased with aging and the exposure to the highest dose of lead (400 ppm) abolished the increase in the incidence of OD. The increase in OD with age in close agreement with literature data showing that the OD increased as a function of age (Bergamo et al., 1997; Abílio et al., 2004). Although no previous study had investigated the effect of lead or sucrose consumption on OD, the results of the present investigation, indicated that neither sucrose consumption nor lead exposure facilitated the appearance of

OD. These results are in sharp contrast to our expectation, because the exposition to lead and sucrose were supposed to increase the oxidative stress and the OD incidence. In the present study, we did not determine the oxidative stress status of the groups and to the best of our knowledge there are only limited evidence in the literature that exposure to sucrose can cause oxidative stress (Busserolles et al., 2002), but there are some studies showing that ingestion of diets rich in free-sugar is associated with oxidative stress (Folmer et al 2002). However, there are some points of evidence in the literature that lead exposure is associated with an increase in oxidative stress (Flora et al., 1991; Gurer & Ercal, 2000; Hsu & Guo, 2002). Thus, we were expecting that lead was associated with an increase in OD; however, contrary to our expectation, simultaneous exposure to the highest dose of lead (400 ppm) and sucrose decreased the OD incidence. One plausible explanation to this results is that lead can interfere with glutamatergic transmission, particularly with NMDA receptor activation (Albuquerque, 1995; Braga et al., 1999).

OD is thought to be associated with an increase in the glutamatergic transmission in different brain structures (Burger et al., 2004, 2005), particularly with an overactivation of NMDA. Since literature data clearly indicate that lead can inhibit NMDA activation (Cory-Slechta, 1995; NourEddine, et al., 2005), it is plausible to speculate that the absence of an increase in OD in lead treated rats is a result of a complex interaction between its pro-oxidant and NMDA antagonist effects. Alternatively, the ingestion of sucrose and/or lead could cause a compensatory response in the antioxidant system that precluded the increase in OD. Support for this speculation can be found in the literature, in fact, Frussa-Filho and co-workers have elegantly demonstrated that spontaneously hypertensive rats did not display an increase in OD (Queiroz et al., 1998) and recently they have demonstrated that the

cerebral catalase activity of these hypertensive rats is higher than controls, which could explain the absence of an increase in OD in these animals (Abílio et al., 2004).

In conclusion, our results suggest that lead and sucrose exposure for about one year did not induced an increase in OD incidence. Although we have no explanation to this, we can speculate that lead and sucrose are activating some compensatory mechanism(s) that protect the rats from the toxicity and the secondary effects of oxidative stress, such as the increase in OD.

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Table 1. Effect of age, sucrose and/or lead administration on body weight and blood parameters in rats.

Treatment	Body Weight(gr)	Hematocrit (%)	Glucose (mg/dL)	Insulin (U/dL)	AST (U/L)	ALT (U/L)
Control	283.78 + 9.46	49.34 + 3.68	71.63 + 6.36	1.31 + 0.27	178.4 + 30.88	52.14 + 2.70
Sucrose	342.8 + 16.25*	45.00 + 0.99*	87.36 + 11.96	3.45 + 1.16*	170.55 + 23.00	40.33 + 2.42
Pb 100ppm	293.5 + 6.30	48.50 + 0.28	97.85 + 6.54*	1.88 + 0.51	230.42 + 81.85	83.85 + 33.0
Suc + Pb 100ppm	323.34 + 9.69*	43.25 + 2.05*	100.45 + 11.61*	3.98 + 0.89*	130.42 + 16.91	49.67 + 7.47
Pb 400ppm	295.78 + 6.10	47.25 + 1.60	92.3 + 4.51	1.44 + 0.28	165.6 + 29.70	56.50 + 7.08
Suc + Pb 400ppm	302.8 + 8.25	45.34 + 1.45*	68.72 + 7.41	2.03 + 0.54	161.37 + 35.28	42.37 + 2.25

Effect of sucrose and/or lead acetate treatments in body weight, hematocrit, glucose, insulin, aspartate aminotrasferase (AST) and alanino aminotransferase (ALT). The blood collection was performed by eye vein puncture at 12 months of treatment and was used for hematocrit determination, the blood samples were also centrifuged for plasma determinations. The results are means $\pm$ S.E. for 9-11 animals per group. *Significantly different from the control group ($P < .05$).

Table 2. Effect of age, sucrose and/or lead administration on exploratory behavior in rats.

Treatments	Open Field		Rearing	
	Months of treatment			
	4	12	4	12
Control	24.5 ± 2.07	19.1 ± 3.03*	9.0 ± 1.30	13.1 ± 2.28 *
Pb 100ppm	24.5 ± 2.19	23.8 ± 1.93	9.8 ± 0.99	14.2 ± 1.46 *
Pb 400ppm	23.4 ± 3.11	22.2 ± 3.05	9.8 ± 1.65	17.8 ± 2.67 *
Sucrose	25.0 ± 3.27	18.2 ± 1.37	11.5 ± 1.75	15.2 ± 1.23 *
Sucrose + Pb 100ppm	21.7 ± 3.45	19.3 ± 2.35	9.2 ± 2.23	13.2 ± 2.18 *
Sucrose + Pb 400ppm	18.7 ± 2.95	15.7 ± 1.96	10.2 ± 1.51	13.3 ± 1.83 *

Effect of age or treatment with sucrose and/or lead acetate on locomotor activity of rats. The rats received during 12 months in drink water lead acetate 100ppm or 400ppm, and 20% sucrose, sucrose plus lead acetate 100 or 400ppm. The rats were observed during 2 minutes in an open field apparatus (a white wood cage measured 50 x 50 x 40 cm^3, divided into 9 squares) and the numbers of squares crossed with the four paws were recorded. The behavioral test was performed 4 times, starting 4 months after beginning the exposure. The total time of the behavioral experiments was 12 months. The results show the measures of 4 and 12 months of exposure, and were presented how means + S.E. for 9-11 animals per group. *Significantly different from first measure (4months old) ($P < .05$).

Table 3. Effect of age, sucrose and/or lead administration on orofacial dyskinesia in rats.

Treatment	Vacuous Chewing Movements		Tongue Protrusion	
	Months of treatment			
	4	12	4	12
Control	23.66 ± 2.73	30.22 ± 6.50*	2.33 ± 0.42	2.55 ± 0.49
Pb 100ppm	25.75 ± 3.06	29.88 ± 4.56*	1.62 ± 0.86	1.37 ± 0.68
Pb 400ppm	18.44 ± 3.01	41.33 ± 7.32*	2.89 ± 0.81	5.78 ± 2.81
Sucrose	23.50 ± 3.35	31.90 ± 4.51*	3.10 ± 0.84	2.20 ± 0.62
Sucrose + Pb 100ppm	19.11 ± 5.34	39.89 ± 10.6*	2.33 ± 0.73	2.55 ± 1.01
Sucrose + Pb 400ppm	16.70 ± 1.96	16.60± 1.36#	1.30 ± 0.4	1.20 ± 0.34 *

Effect of age or treatment with sucrose and/or lead acetate on orofacial dyskinesia behavior of rats. The rats received during 12 months in drink water lead acetate 100ppm or 400ppm, and 20% sucrose, sucrose plus lead acetate 100 or 400ppm. The rat was maintained in a glass cage (20 x 20 x 19cm) equipped with mirror under the floor and behind the back wall. The animal behavior was observed by 6 min after 2 min of adaptation to the cage. It was counted the frequency of vacuous chewing movements and the number of tongue protrusion movements. The behavioral test was performed 4 times, starting 4 months after beginning the exposure. The total time of the behavioral experiments was 12 months. The results were presented as means + S.E. for 9-11 animals per group. *Significantly different from the first measure (4 months old) ($P < .05$). # Significantly different from control the group ($P < .05$).

Manuscrito 3

Lead and sucrose long-term administration did not increase oxidative parameters nor cause inhibition of d-ALA-D.

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Abstract

Pb is an important occupational and environmental toxicant, and attacks a variety of cellular targets. The molecular events involved in lead toxicity are not completely understood, but oxidative stress seems to play an important role in its toxicity. High sucrose- consumption has been associated with obesity and with increased oxidative stress. Recent data from our laboratory have indicated that simultaneous exposure to cadmium and sucrose potentiates the metal toxicity. Considering the possible pro-oxidant activity of lead and sucrose, the aim of this work was to investigate whether lead and sucrose long- term exposure could have synergistic toxic effects to female rats. Female rats were exposed to lead acetate (100 or 400ppm via drink water) and/or were provided with 20% sucrose solution or tap water for 2 years. Sucrose ingestion caused an increase in body weight gain and in plasma triglycerides. Hepatic Non Protein Thiol Groups (NPSH) were decreased in sucrose group, but were increased in brain 400ppm lead and high- lead plus sucrose groups. Simultaneous exposure with sucrose and lead (400ppm) caused a significant increase in splenic TBARS levels and ALA-D activity. Hepatic, renal and blood ALA-D activities and TBARS were not modified by sucrose and/or lead exposures. The results of the present study suggest that long-term exposure to lead and sucrose can cause biochemical alterations compatible with oxidative stress. However, these changes were tissue-specific. Furthermore, ALA-D activity, which is a classical marker of lead intoxication, was not inhibited by lead or sucrose in brain, kidney, liver and blood. And in contrast to what one would expect, the enzyme was increased in spleen. These results may indicate that ALA-D is an indicator of acute or sub-chronic lead exposure and that

some adaptations to lead and/or sucrose toxicity occur after long-term exposure to these substances.

Introduction

Pb is still an important occupational and environmental hazard throughout the world. Pb can adversely affect the nervous, hematopoietic, endocrine, renal and reproductive systems and its toxic effects can vary depending on several factors including the developmental stage, time, route and level of exposure. (ATSDR, 1993).

Different molecular targets have been implicated in lead poisoning (Bouton et al., 2001), of particular toxicological significance, lead is commonly found in cells and tissues associated with thiol-containing proteins and small molecular weight thiols (Goering, 1993; Campagna et al., 1999). Zn2+-containing enzymes, particularly those containing vicinal groups, such as d-aminolevulinate dehydratase (ALA-D) are considered classical targets for lead (Gurba et al., 1972; Goering et al., 1986; Goering, 1993; Rocha et al., 1995 & 2001; Rodrigues et al., 1996; Jaffe et al., 2001, Nogueira et al., 2004). In line with this, the blood d-ALA-D activity is commonly used as a marker of lead intoxication and considered an important index of its subcritical effect (Secchi et al. 1974; Nordberg, 1976; Meredith et al. 1978; Simmonds et al. 1995; Polo et al., 1995).

It is known that oxidative stress can be an important factor in lead toxicity in mammals (Monteiro et al., 1985; Hermes-Lima et al., 1991; Bechara et al., 1993; Neal et al., 1998; Gurer and Ercal, 2000).

It is not worthy that lead intoxication can be associated with increased levels of blood aminolevulinic acid (ALA) and with an increase in antioxidant

enzymes activity in human erytrocytes (Bechara, 1996). Importantly, Bechara and co-workers have clearly demonstrated that ALA can have pro-oxidant activity under physiological conditions (Pereira et al., 1992; Bechara et al., 1993)

Hypercaloric diet intake has been shown to be important in the development of obesity (Warwick and Schiffman, 1992) and experimental studies suggest that high-sugar or fat diets can be associated with increased oxidative stress (Faure et al., 1997; Shinozaki et al., 1999; Roberts et al, 2000, 2002; Folmer et al., 2002, 2003). In the same vein, we have recently demonstrated that simultaneous exposure to Cd2+ and sucrose can cause a significant potentiation of metal toxicity (Folmer et al., 2004).

The over production of reactive oxygen species (ROS) or the antioxidant depletion can cause cellular damage and tissue dysfunction by a variety of mechanisms and have been found to be associated with numerous pathological conditions, such as the diabetes, cancer, atherosclerosis and neurodegenerative diseases (Hunt et al., 1988; Wolff and Dean, 1987; Dean et al., 1997; Gonçalves et al., 2005).

Considering that Pb and sucrose given separately could have pro-oxidant activities, and that lead is a classical inhibitor of ALA-D (Perottoni et al., 2005), particularly in short- term animal model of exposure, the aim of this study was to investigate whether simultaneous long-term exposure to these agents could produce synergistic toxic effects in female rats. Although some animal studies have indicated the participation of oxidative stress in short-term exposure to lead (Neal et al., 1998 & 1999; Ni et al., 2004), limited data are available for long-term exposure (Ito et al., 1985; Sugawara et al., 1991.). Most importantly, long- term human exposure to lead can be associated with an increase in antioxidant enzymatic defenses (Bechara, 1996) which can be

an adaptative response to chronic exposure to oxidative stress. Thus, the present study was also undertaken to determine whether long- term exposure to lead and/or sucrose could be associated with adaptative response.

Material and Methods

Animals

Female rats (60 days old, 155-160g) from our own breeding colony were maintained for 24 months in an air-conditioned room (22-25ºC) under natural lighting conditions, with water and food (Guabi – RS, Brasil) ad libitum. Animals (11 to 12 animals/group) were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the University of Santa Maria, Brazil.

Chemicals

Lead acetate, glacial acetic acid, *ortho*-phosphoric acid, hydrochloric acid, sulfuric acid, perchloric acid, $HgCl_2$, NaCl, K_2HPO_4, KH_2PO_4, 5,5'-dithio-bis-(2-nitrobenzoic acid), hydrogen peroxide, and ascorbic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil), buthylated hydroxytoluene, sodium dodecyl sulfate, dimethyl sulfoxide, 2,4-dinitrophenylhydrazine, tris(hydroxymethyl)aminomethane, cysteine, thiobarbituric acid, δ-aminolevulinic acid, bovine serum albumin and comassie brilliant blue G were obtained from Sigma (St. Louis, MO). 1,1,3,3-Tetramethoxypropane was obtained from Aldrich (Milwaukee, WI). Trichloroacetic acid was obtained from Reagen (Rio de Janeiro, RJ, Brazil). *p*-Dimethylaminobenzaldehyde was obtained from Riedel (Haën, Germany). All

other chemicals were of analytical grade and obtained from standard commercial suppliers.

Exposure

The rats were divided in the groups: control, 20% sucrose, 100ppm lead acetate, 20% sucrose + 100ppm lead acetate, 400ppm lead acetate, 20% sucrose + 400ppm lead acetate. Lead and/or sucrose exposures were made via drink water for a period of 2 years. Was added to water 0.5ml/L of glacial acetic acid to prevent lead acetate precipitation. A group of one- year old females was used as additional control group. This group did not receive sucrose or lead.

Animals were killed by decapitation and tissues (brain, liver and kidney) were removed for determination of ALA-D activity, lipid peroxidation (TBARS), and non-protein thiol groups (NPSH).

ALA-D activity

The kidney, spleen, brain and liver were quickly removed, placed on ice and homogenized in 150 mM NaCl 7, 7, 5 and 10 volumes, respectively. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 minutes to yield a low-speed supernatant fraction (S_1) that was used for enzyme assay. Enzyme assay was carried out as described by Sassa (1982) by measuring the rate of product (porphobilinogen) formation, except that 200 mM potassium phosphate buffer, pH 6.4 and 2.5 mM aminolevulinic acid were used. Reaction was started 10 minutes after the addition of enzyme preparation by adding the substrate and carried out for 60, 120 and 180 minutes, for liver and spleen, kidney and brain, respectively, at 39ºC.

Blood ALA-D activity was determined as described above, except that 760 mM potassium phosphate buffer, pH 6.4 and 16 mM aminolevulinic acid were used. Blood was pre-incubated with triton 0.1% for 10 minutes and incubation was carried out for 60 minutes.

The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 for the Ehrlich-porphobilinogen salt. Simultaneously, a set of tubes were assayed in the presence of 1.4 mM dithiothreitol (DTT) to obtain the reactivation index. This index indicates the extent of the reactivation of δ-ALA-D activity by DTT and is defined as:

$$\frac{\text{(ALA-D activity with DTT – ALA-D activity without DTT)}}{\text{ALA-D activity with DTT}} \times 100$$

TBARS determination

Kidney, spleen, brain and liver of were homogenized in 7, 7, 5 and 10 volumes, respectively, of a medium containing 150 mM NaCl. The homogenate was centrifuged at 4,000 x g at 4ºC for 10 min. and stored at -20ºC for one week to posterior determination of thiobarbituric acid reactive substances (TBARS). TBARS were assayed as described by Ohkawa *et al*. (1979). In brief, samples were incubated at 100ºC for 120 min in a medium containing 0.45 % sodium dodecyl sulfate, 100 mM hydrochloric acid, 1.4 M acetic acid, pH 3.4 and 0.8 % thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as standard.

Blood TBARS were assayed as described by Eserbauer & Cheeseman (1990). In brief, after precipitation of tissue with trichloroacetic acid, samples

were incubated at 100ºC for 30 min in an acid medium containing 0.3 % thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as standard.

Non- protein thiol groups (NPSH) determination

NPSH were determined as described by Ellman (1959) at 412 nm, as modified by Jacques-Silva et al. (2001). Non-protein thiol groups were determined in the fraction obtained after dilution of supernatants with 1 volume of 4 % trichloroacetic acid followed by centrifugation and neutralization with 0.5 M tris(hydroxymethyl)aminomethane/HCl, pH 7.5. A standard curve using cysteine was constructed in order to calculate the non-protein thiol groups in the tissue samples.

Protein quantification

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by Duncan's Multiple Range Test when appropriate. Results with $P \leq .05$ were considered significant.

RESULTS

At the end of two years of treatment, there was a tendency to increase the incidence of death in the group treated with 400ppm plus 20% sucrose. However, the difference was not statistically significant (TABLE 01).

ANOVA demonstrated that ingestion of a 20% of sucrose for 24 months was associated with significant increase (approximately 28%) in the body weight. ($F(7,45)=13.41$, $P<.01$). Liver-to-body weight ratio was not modified by treatments with lead or sucrose, whereas kidney ($F(7,45)=20.43$, $P<.01$) and spleen ($F(7,45)=3.49$, $P<.01$) simultaneously exposed to lead (100 or 400ppm) and sucrose exhibited a significant organ/body ratio (TABLE 1).

Exposure to lead acetate or sucrose did not change TBARS levels in liver, kidney, brain (FIGURE 01) and blood (FIGURE 02). In spleen homogenates, the association between sucrose and 400ppm lead acetate caused an increase ($F(7,45)=2.41$, $P<.05$) in TBARS levels (about 42%) (FIGURE 02).

Exposure to lead acetate did not change non-protein thiol groups (NPSH) in liver or kidney (FIGURE 03), whereas in brain homogenate the dose of 400ppm of lead acetate, and its association with sucrose consumption induced a significant ($F(7,43)=7.59$, $P<.01$) increase in cerebral NPSH (FIGURE 03). Sucrose consumption caused a marked reduction in the quantity of NPSH in liver ($F(7,44)=2.84$, $P<0.5$) (FIGURE 03). In kidney and brain (FIGURE 03), a significant effect of ageing was observed. In fact, the renal and cerebral NPSH contents from the 12 month old group were significant higher than that of 24 month old rats.

Basal and DTT reactivated hepatic, renal, cerebral and blood ALA-D activities were not modified by sucrose or lead treatments (FIGURES 4, 5, 7 and 8, respectively). Renal ALA-D tended to be inhibited in the group treated with 400ppm of lead plus sucrose (activity 33.6% lower than control group) (FIGURE 05).

In contrast to the other organs, splenic basal ($F(8,46)= 12.02$, $P<.01$) and DTT reactivated ($F(8,46)=9.56$, $P<.01$) ALA-D activities were significantly

increased in the group exposed to lead (100 or 400ppm) and sucrose (FIGURE 06).

Reactivation index of hepatic ALA-D was not modified by treatments (TABLE 2). In kidney, the reactivation index of one year old group was significantly higher than that of two year old rats (TABLE 2). The index of reactivation of ALA-D, in spleen was decreased in groups exposed simultaneously to lead (100 and 400ppm) and sucrose. In contrast, isolated exposure to lead or sucrose did not change the reactivation index in spleen (TABLE 02).

DISCUSSION

Literature data have indicated that lead exposure can be associated with increased oxidative stress in animal models of intoxication (Adonaylo & Oteiza, 1999a,b; Gurer and Ercal, 2000; Silbergeld, 2003). However, there are also negative reports in the literature (Perottoni, et al., 2004; 2005). For the case of humans, there are only scarce data available and the results are not coincident. In fact, the classical and elegant study of Bechara and co-workers demonstrated that antioxidant enzymes were increased in occupationally lead-exposed subjects (Monteiro et al., 1985; Bechara et al., 1996).

Furthermore, they demonstrated that ALA levels, the substrate of ALA-D, were increased in these workers and correlated with oxidative stress (Costa et al., 1997). In contrast with this, studies have indicated that antioxidant defenses were reduced in lead exposed (Meredith et al., 1978; Moreira et al., 2001).

The results of the present investigation suggest that the pro-oxidant effects of lead after a very long- period of treatment was observed only in spleen and when animals were feed with sucrose. These results indicate that

the toxicity of lead and sucrose are tissue- specific and may be related differences in the antioxidant defenses of each tissue. Furthermore, exposure to lead and other pro-oxidants can produce adaptative responses that are specific for each tissue (Sandstead et al. 2000; Roney & Colman, 2004).

Although some animal studies have demonstrated that relative short term exposure to lead caused oxidative stress in brain, kidney and liver, there are no details on time-course studies in the literature about the development of oxidative stress after exposure to lead. Consequently, the absence of a clear pro-oxidant effect of lead after two years of exposure can be related to adaptative mechanisms. Alternatively, as the long term exposure to lead and sucrose was associated with a high incidence of death (Van Esch & Kroes, 1969; Eyden et al. 1978; Vogiatzis et al. 1999; Heike et al. 2002; Lustberg et al., 2002), we can speculate that the animals more susceptible to oxidative stress were excluded from the study.

Ingestion of diets containing high levels of free sugars can be associated with increased oxidative stress in rodent models (Faure et al., 1997; Shinozaki et al., 1999; Roberts et al, 2000, 2002; Folmer et al., 2002, 2003). In line with this, recent data from our laboratory have indicated that short- term exposure to cadmium and sucrose was associated with deleterious changes in enzymes and increased oxidative stress in specific tissues (Folmer et al., 2004). In the present study, sucrose caused only a clear decrease in hepatic NPSH levels and, in association with lead, increased splenic TBARS levels. Thus, it seems that the toxicity of sucrose intake is dependent on the tissue considered and on the end- point of toxicity used to determine it. Additional, time- course studies will be necessary to establish whether some adaptation to sucrose toxicity can occur after long-term sucrose consumption.

ALA-D is a classical marker of lead and other metals toxicity (Gurba et al., 1972; Goering et al., 1986; Goering, 1993; Rocha et al., 1995, 2001). Unexpectedly, long-term exposure to lead was not associated with ALA-D inhibition in liver, brain, kidney and blood. We found an increase in ALA-D activity in spleen of rat exposed to lead and sucrose. The reasons for these results may be linked to the increase in defenses against lead and oxidative stress, including the synthesis of metallothioneins or lead- binding proteins (Goering et al., 1986; Sandstead et al. 2000; Roney & Colman, 2004).

Regardless of the mechanism, the results of the present report are the first demonstration that ALA-D was not inhibited in several tissues and was even increased in spleen of rats exposed to a very high dose of lead (400ppm) when combined with sucrose ingestion. Of particular importance, and in sharp contrast to our own hypothesis that ALA-D inhibition could be a sensor of oxidative stress (Folmer et al., 2003; Gonçalves et al., 2005), the results of the present investigation for spllenic ALA-D indicate that the enzyme can be activated by oxidative stress. To respond whether this increase in spleenic ALA-D and the absence of hepatic, cerebral, renal and blood ALA-D inhibition were a consequence of adaptative responses to long- term exposure to pro-oxidant will be necessary detailed time- course studies with these and other pro-oxidants.

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TABLE 1. Survival index, body weight and organ/body weight ratio of 2 year old rats

Treatment	Survival	Body Weight (gr)	n	Tissue	Organ/ Body (%)
Old control	7/11	345.30 ± 34.69	7	Liver	2.82 ± 0.60
				Kidney	0.69 ± 0.09
				Spleen	0.22 ± 0.08
20% sucrose	9/11	439.50 ± 82.02 *	9	Liver	3.15 ± 1.03
				Kidney	0.67 ± 0.15
				Spleen	0.20 ± 0.06
Pb 100ppm	7/11	350.00 ± 23.43	7	Liver	2.65 ± 0.29
				Kidney	0.63 ± 0.04
				Spleen	0.23 ± 0.05
20%+100ppm	7/11	348.00 ± 52.87	7	Liver	3.08 ± 0.22
				Kidney	0.96 ± 0.22 *
				Spleen	0.32 ± 0.09 *
Pb 400ppm	7/11	340.00 ± 32.90	7	Liver	2.69 ± 0.24
				Kidney	0.68 ± 0.41
				Spleen	0.24 ± 0.04
20%+400ppm	5/12	346.00 ± 37.71	5	Liver	2.83 ± 0.28
				Kidney	1.47 ± 0.28 *
				Spleen	0.37 ± 0.13 *
Adult control	6/6	281.20 ± 12.30 *	6	Liver	2.58 ± 0.09
				Kidney	0.63 ± 0.07
				Spleen	0.22 ± 0.04

Data show as mean ± S.D. * Statistical difference of control at P<.05.

FIGURE 01.

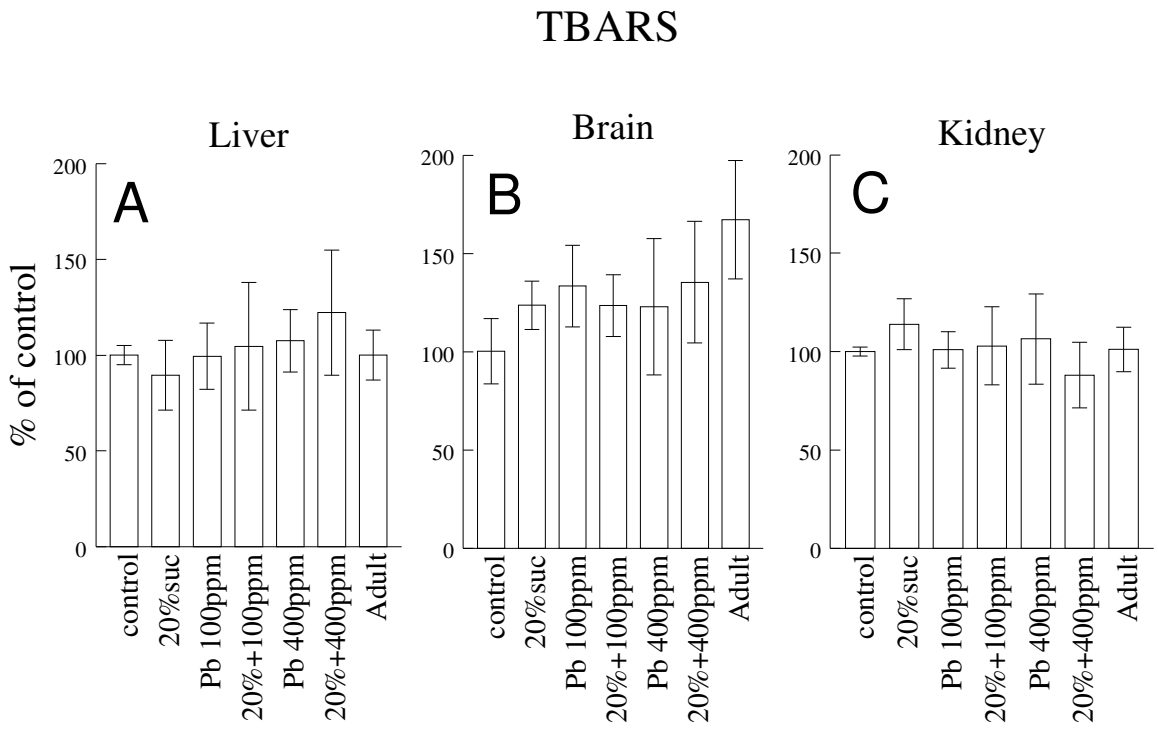

Figure 1. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on liver (A), brain (B) and kidney (C) TBARS production. Samples were incubated at 100ºC for 120 min. in a medium containing 0.45 % SDS, 100 mM hydrochloric acid, 1.4 M acetic acid, pH 3.4 and 0.8% thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm. Values are expressed as mean ± S.E.M. (n=5-9). Control values are 130, 76 and 180 nmol MDA/g tissue to liver, brain and kidney, respectively.

Figure 02.

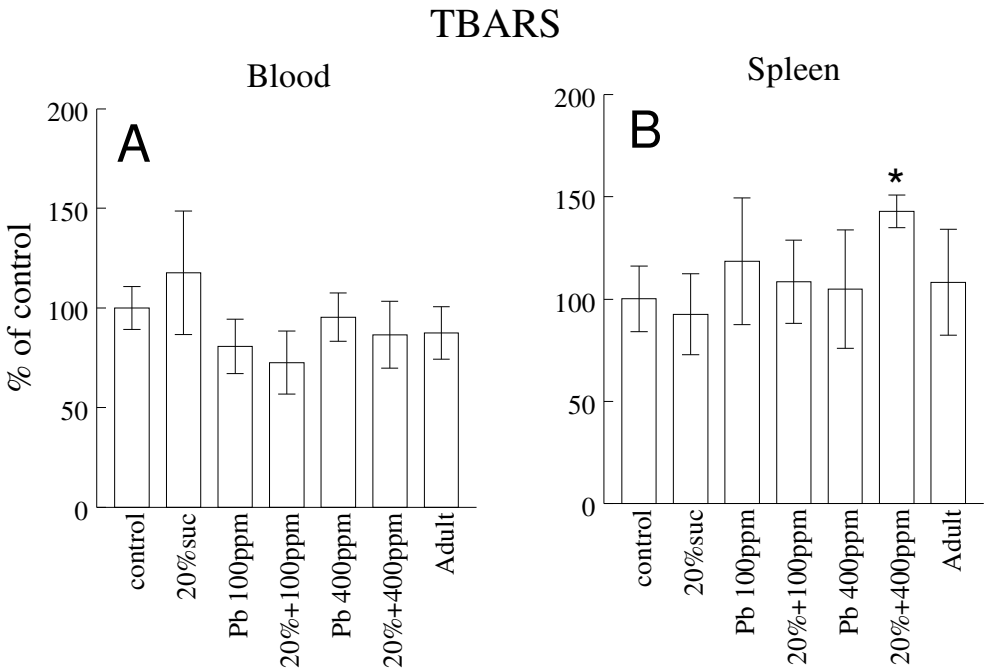

Figure 2. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on blood (A) and spleen (B) TBARS production. Tissue samples were incubated at 100ºC for 120 min. in a medium containing 0.45 % SDS, 100 mM hydrochloric acid, 1.4 M acetic acid, pH 3.4 and 0.8 % thiobarbituric acid. The blood was precipitated with trichloroacetic acid, and incubated at 100ºC for 30 min. in an acid medium containing 0.8 % thiobarbituric acid. After centrifugation the reaction product was determined at 532 nm. Values are expressed as mean ± S.E.M. (n=5-9) * significantly different from the control group.

FIGURE 03

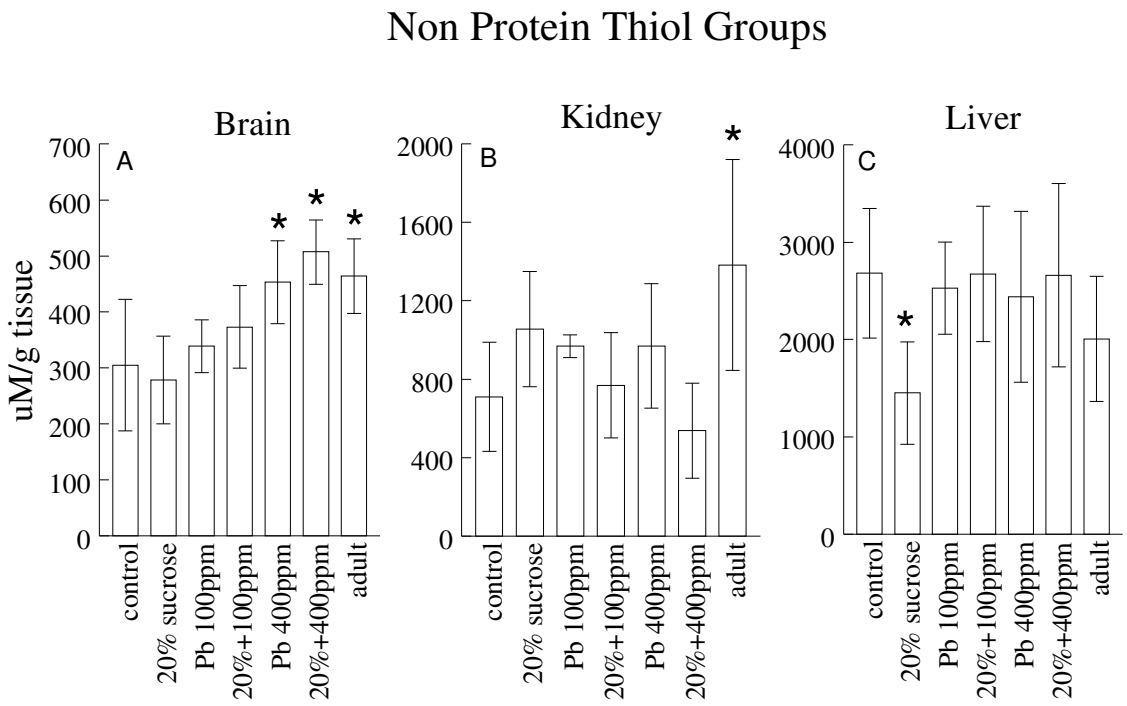

Figure 3. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on brain (A), kidney (B) and liver (C) NPSH production. Non-protein thiol groups were determined in the fraction obtained after dilution of supernatants with 4% trichloroacetic acid followed by centrifugation and neutralization with 0.5 M tris(hydroxymethyl)aminomethane/HCl, pH 7.5. Values are expressed as mean ± S.E.M. (n=4-9). * significantly different from the control group.

TABLE 02. ALA-D activity reactivation index

	Liver			Kidney			Spleen			Brain			Blood		
	Mean	n	S.E.M.	Mean	n	S.E.M.	Mean	n	S.E.M.	Mean	n	S.E.M.	Mean	n	S.E.M.
Contro	7.23	7	2.35	20.77	7	5.25	54.17	7	6.84	25.09	7	4.23	11.82	6	2.65
20% sucrose	5.16	9	1.88	14.66	8	5.66	47.10	9	4.38	10.82*	9	2.21	19.02	9	3.26
Pb 100ppm	10.38	7	1.70	19.36	7	2.42	49.24	7	4.02	8.11*	7	2.01	12.93	7	3.07
20% + Pb 100ppm	5.38	7	0.94	14.49	7	3.37	37.12*	7	4.98	18.74	7	5.04	31.70*	7	5.11
Pb 400ppm	9.71	7	2.24	22.06	7	3.44	51.86	7	3.27	10.95*	7	1.21	24.04	7	7.52
20% + Pb 400ppm	7.29	4	2.15	14.21	4	1.93	22.67*	4	4.29	17.64	4	3.43	38.97*	4	3.35
Adult control	13.58	6	3.55	36.51*	6	8.51	57.03	6	7.45	16.05	6	5.18	28.09	6	9.85

*Significantly different from the control group (P<.05).

FIGURE 04.

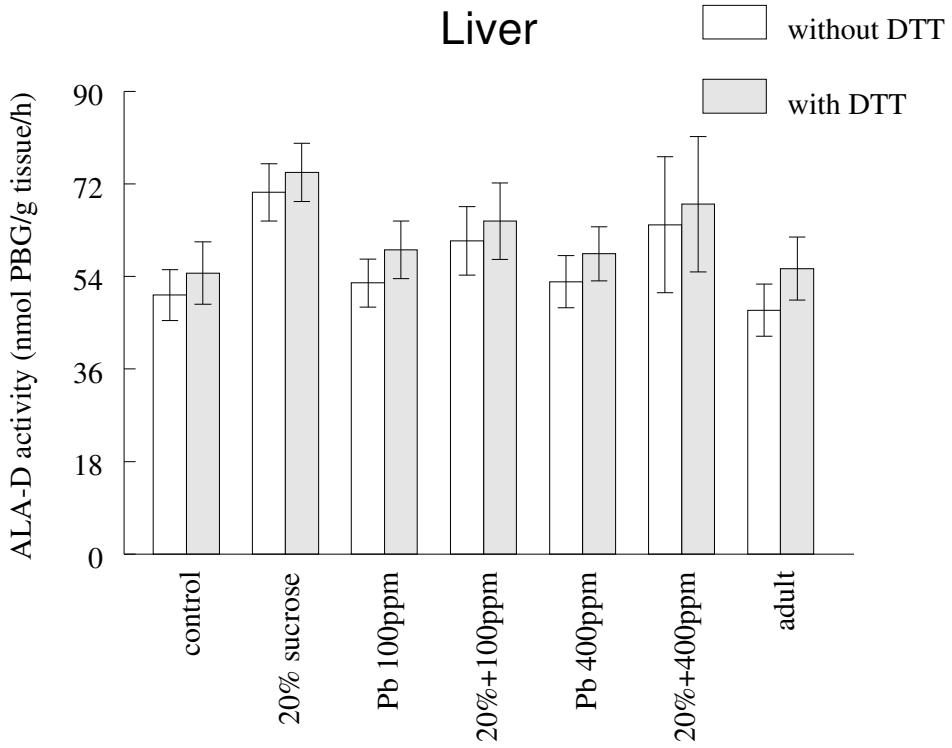

Figure 4. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on liver ALA-D activity. Tissue was homogenized in 150mM NaCl 10 volumes. The homogenate was centrifuged at 4000 × g at 4°C for 10 min to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in the presence of 200mM potassium phosphate buffer, pH 6.4. Reaction was started 10 min after the addition of enzyme preparation by adding the substrate (2.5mM aminolevulinic acid) and carried out for 60 min, at 39°C, in the absence or DTT presence (1.4 mM), (data are expressed as means ± S.E.M. for n = 4-9).

FIGURE 05.

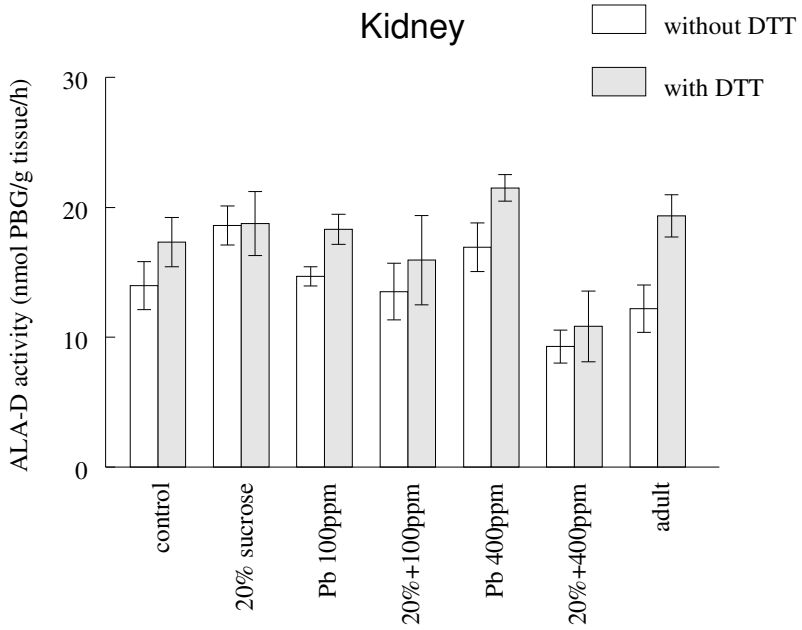

Figure 5. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on kidney ALA-D activity. Tissue was homogenized in 150mM NaCl 7 volumes. The homogenate was centrifuged at 4000 × g at 4^{o}C for 10 min to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in the presence of 200mM potassium phosphate buffer, pH 6.4. Reaction was started 10 min after the addition of enzyme preparation by adding the substrate (2.5mM aminolevulinic acid) and carried out for 90 min, at 39^{O}C, in the absence or DTT presence (1.4 mM), (data are expressed as means ± S.E.M. for n = 4-9).

FIGURE 06

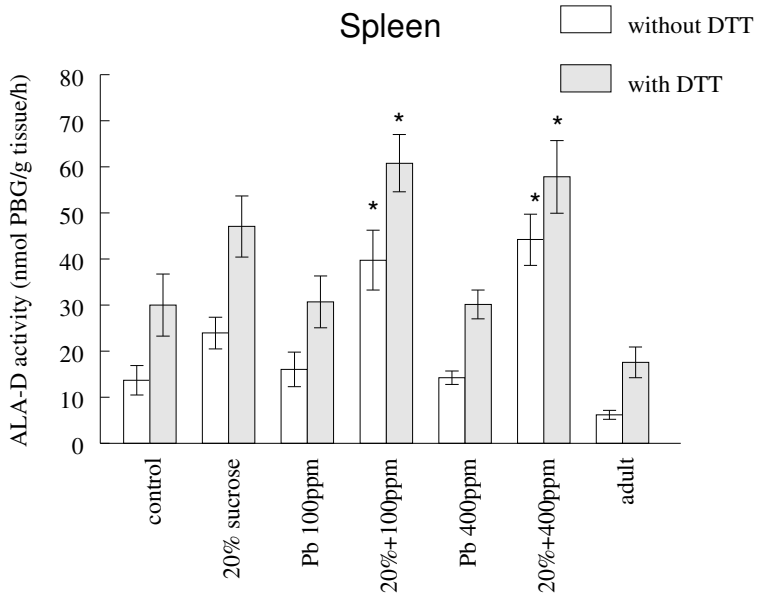

Figure 6. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on spleen ALA-D activity. Tissue was homogenized in 150mM NaCl 7 volumes. The homogenate was centrifuged at 4000 × g at 4^{o}C for 10 min to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in the presence of 200mM potassium phosphate buffer, pH 6.4. Reaction was started 10 min after the addition of enzyme preparation by adding the substrate (2.5mM aminolevulinic acid) and carried out for 90 min, at 39^{O}C, in the absence or DTT presence (1.4 mM), (data are expressed as means ± S.E.M. for n = 4-9). *Significantly different from the control group ($P < .05$).

FIGURE 07

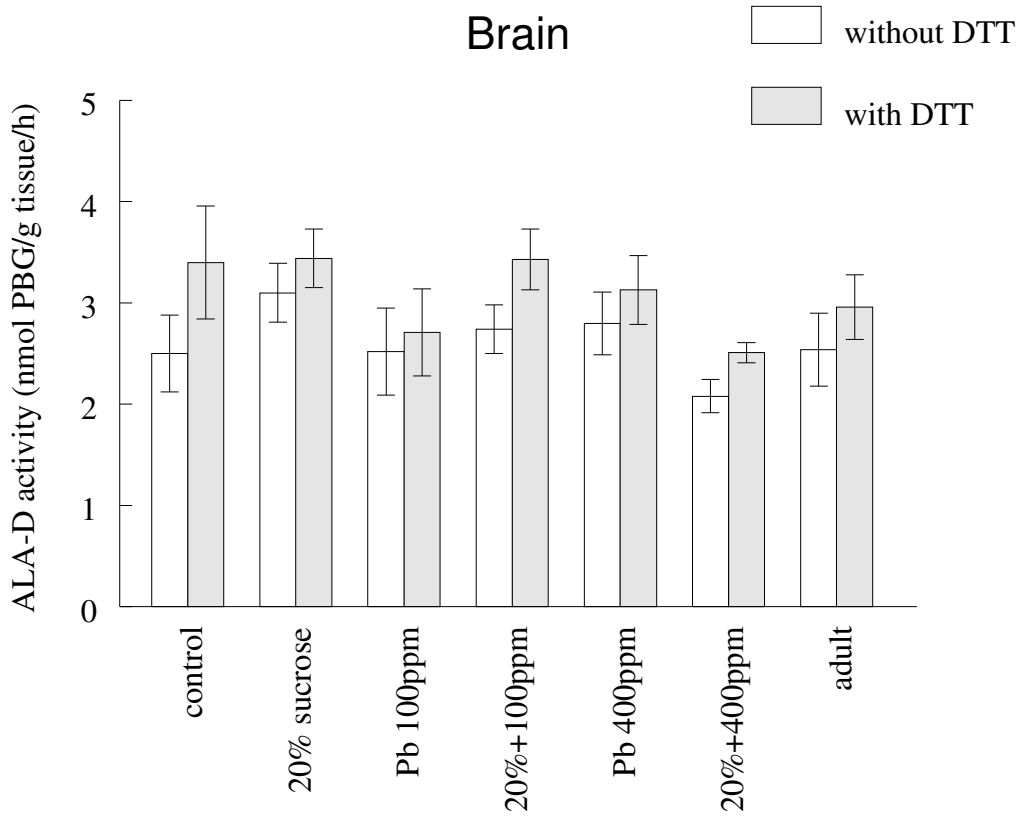

Figure. 07. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on brain ALA-D activity. Tissue was homogenized in 150mM NaCl 7 volumes. The homogenate was centrifuged at 4000 × g at 4°C for 10 min to yield a low-speed supernatant fraction that was used for enzyme assay. Enzyme assay was carried in the presence of 200mM potassium phosphate buffer, pH 6.4. Reaction was started 10 min after the addition of enzyme preparation by adding the substrate (2.5mM aminolevulinic acid) and carried out for 120 min, at 39°C, in the absence or DTT presence (1.4 mM), (data are expressed as means ± S.E.M. for n = 4-9).

FIGURE 08

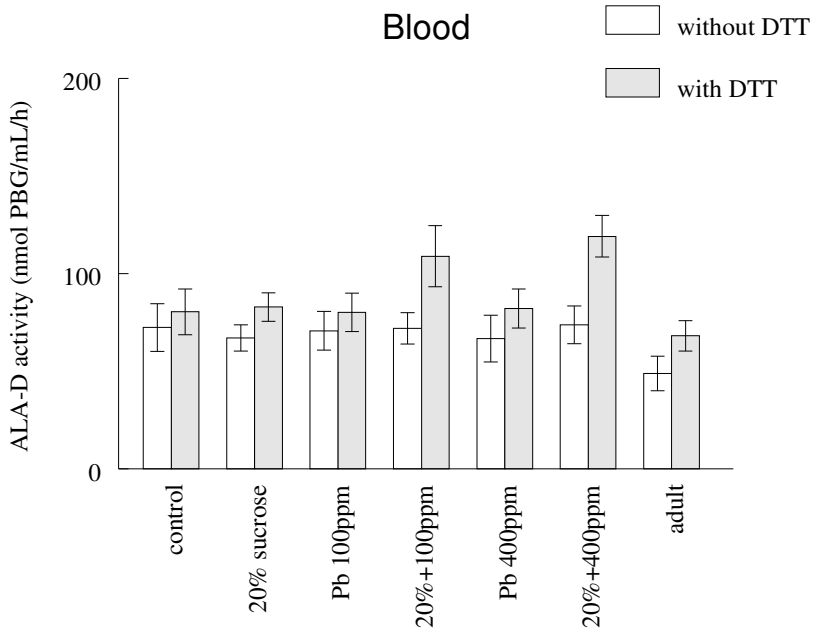

Figure 08. Effects of treatment with lead acetate (100 or 400ppm) and/or sucrose (20%) on blood ALA-D activity. Blood was pre-incubated with Triton 0.1%, and ALA-D activity was determined in a medium containing 760mM potassium phosphate buffer pH 6.4. Reaction was started at addition of 16mM aminolevulinic acid, and the incubation was carried out for 120 min, in the absence or DTT presence (1.4 mM). Values were expressed as percent of control. Values for control was 15.75 nmol PBG/gHb/h. (Data are expressed as means ± S.E.M. for n = 4-9).

Discussão

Como relatado anteriormente, o Pb^{2+} pode causar toxicidade devido a interferência com proteínas tiólicas, particularmente as que contêm zinco. Desta forma, o estresse oxidativo pode ser um importante efeito à exposição ao chumbo, e o uso de antioxidantes contra os efeitos tóxicos do chumbo tem sido uma constante na literatura (McGowan, 1989; Flora et al., 1989 & 1991; Dhawan et al., 1988 & 1989; Gurer and Ercal, 2000; Hsu and Guo, 2002). Uma vez descrito que alguns organocalcogênios (Andersson et al., 1994), tais como o Ebselen (Müller et al., 1984; Saito et al., 1998; Yamaguchi et al., 1998) e o disseleneto de difenila (Wilson et al., 1989; Rossato et al., 2002b) apresentam propriedades antioxidantes, nós avaliamos se estes compostos poderiam reverter a toxicidade produzida pelo chumbo (**ARTIGO 1**). Mediante a admistração de 50mg/kg acetato de chumbo via subcutânea, durante 15 dias, foi observado uma significativa inibição da atividade da enzima δ-aminolevulinato desidratase em todos os tecidos avaliados (fígado, rins, cérebro e sangue) de camundongos. Pelo fato de ter havido uma maior reativação da atividade da δ-ALA-D no fígado e no sangue, na presença de DTT, um agente redutor, podemos inferir serem os grupos tiólicos da enzima os alvos da intoxicação por chumbo. A subseqüente administração dos compostos orgânicos de selênio, (25umol/kg) intraperitonealmente, durante 5 dias não foi eficiente para modificar o quadro de inibição enzimática induzido pelo chumbo. Após a administração do chumbo e do Ebselen, foi observada a diminuição do Índice de reativação no homogeneizado de cérebro, enquanto o tratamento com disseleneto de difenila aumentou este índice. Estes resultados podem indicar um efeito protetor do Ebselen sobre a neurotoxicidade promovida pelo chumbo, o que não foi observado para o composto disseleneto

de difenila. Apesar de ser reportada a associação entre o estresse oxidativo e a intoxicação por chumbo, nós não encontramos peroxidação lipídica no fígado e cérebro. No tecido renal, observamos uma diminuição dos níveis de TBARS. Especula-se que o motivo desta diminuição seja pelo acúmulo renal do chumbo, o qual estaria competindo com os íons Fe, que são iniciadores de peroxidação lipídica. Outra evidência de que os possíveis danos oxidativos promovidos pelo chumbo não são generalizados é a ausência de alteração nos níveis de grupos tiólicos não protéicos. Porém, observamos a diminuição deste parâmetro no fígado de camundongos tratados com chumbo e Ebselen. Isto poderia ser motivado pelo fato de que o fígado é descrito como um dos alvos primários dos metais pesados. Considerando-se que o ebselen se utiliza de compostos tiólicos como doador de elétrons para exercer sua atividade antioxidante, podemos inferir que ele esteja utilizando-se destes grupos –SH não protéicos para reduzir os danos oxidativos promovidos pelo chumbo. Apesar de a literatura citar efeitos neurotóxicos promovidos pelo chumbo, neste modelo de intoxicação em camundongos nós não observamos efeitos na captação de glutamato. Isto pode indicar que o transporte glutamatérgico não é um alvo primário da intoxicação pelo chumbo.

Um aumento na produção de espécies reativas de oxigênio, ou uma diminuição nas defesas antioxidantes do organismo podem estar relacionadas com a manifestação de discinesia orofacial em modelos animais (Naidu et al., 2003; Burger et al., 2003) e até com discinesia tardia, em humanos (Andreasen and Jorgensen, 2000; Lohr et al., 2003). Os mecanismos moleculares causadores da discinesia orofacial não são conhecidos, mas uma hipótese é a de que o aumento na produção de radicais livres durante o metabolismo da dopamina participe da iniciação da discinesia orofacial (Lorh, 2003). Considerando que o chumbo e uma dieta rica em sacarose podem ter

uma atividade pró oxidante, no MANUSCRITO 2 investigou-se se a simultânea exposição a estes agentes promoveria o aparecimento de discinesia orofacial ou distúrbios locomotores. Os resultados apresentados no MANUSCRITO 2 indicam que os animais não apresentaram distúrbios locomotores durante o período experimental. Observou-se um aumento significativo na atividade exploratória em decorrência do envelhecimento. Os animais também desenvolveram um incremento de discinesia orofacial em função da idade. Não foram observados efeitos promovidos pelo chumbo. Apenas na maior concentração de acetato de chumbo na presença da solução de sacarose, encontrou-se uma diminuição na discinesia orofacial. Apesar de não ter sido avaliado parâmetros oxidativos neste trabalho, era de se esperar que um evento pró- oxidante viesse a aumentar os índices destes comportamentos estereotipados (Flora et al., 1991; Folmer et al., 2002, Fachinetto et al., 2005). Uma possível explicação para a diminuição na discinesia orofacial encontrada neste estudo, seria a interferência do chumbo na transmissão glutamatérgica (Albuquerque, 1995; Braga et al., 1999), o que poderia estar ativando algum mecanismo protetor compensatório.

Em um período mais longo de tratamento, observamos que a associação do chumbo e sacarose afeta os órgãos de forma diferenciada, o que pode indicar diferenças na capacidade antioxidante de cada tecido. Os níveis de grupos tiólicos não protéicos apresentaram-se diminuidos no fígado de animais que receberam apenas sacarose, a qual associada com chumbo promoveu um aumento no parâmetro de avaliação de estresse oxidativo no baço (MANUSCRITO 3). Apesar da ALA-D ser um marcador clássico da intoxicação por chumbo, neste modelo longo de intoxicação apenas observou-se um aumento na atividade desta enzima no baço. Este resultado não era esperado, mas pode estar relacionado a um aumento nas defesas antioxidantes.

Conclusões

Os presentes resultados de um tratamento sub-crônico indicam que a inibição da δ-ALA-D antecede o aumento da produção de ERO em diferentes tecidos. Apesar da exposição ao chumbo poder resultar na instalação de um estado oxidativo em mamíferos, não está claro se o estresse oxidativo é a causa ou a conseqüência dos efeitos tóxicos do metal. Nossos resultados sugerem que a inibição da δ-ALA-D tem uma função primária no desenvolvimento da toxicidade por chumbo. Nós especulamos que a inibição da δ-ALA-D causa um aumento no seu precursor ALA, e que isto é pró-oxidante sob condições fisiológicas, com conseqüente contribuição para o desenvolvimento do estresse oxidativo em organismos expostos ao chumbo.

Durante 12 meses de tratamento, nossos resultados sugerem que chumbo e sacarose não induzem a um aumento de comportamento estereotipado. Apesar de não termos uma explicação para este fato, especulamos que estes agentes ativam algum mecanismo compensatório que protege os ratos da toxicidade e dos efeitos secundários do estresse oxidativo, como a discinesia orofacial.

Esta ausência de efeitos pró-oxidativos neste protocolo de tratamento longo, pode estar relacionada com algum tipo de resposta adaptativa. Podemos considerar também, que pelo fato de um longo período sob exposição ao chumbo promover uma elevada mortalidade, os animais mais sensíveis ao estresse oxidativo foram naturalmente excluídos deste estudo.

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ANEXO