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Tese de doutorado

ESTUDO DE MECANISMOS DE TOXICIDADE DO METILMERCÚRIO: EFEITO PROTETOR DE FLAVONÓIDES

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Santa Maria, RS, Brasil

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Por

Caroline Wagner

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, 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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ESTUDO DE MECANISMOS DE TOXICIDADE DO METILMERCÚRIO: EFEITO PROTETOR DE FLAVONÓIDES

Elaborada por

Caroline Wagner

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

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Á Annita da Rocha Gregory

(*in memoriam*)

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RESUMO

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

ESTUDO DE MECANISMOS DE TOXICIDADE DO METILMERCÚRIO: PAPEL PROTETOR DE FLAVONÓIDES

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CO-ORIENTADORA: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, agosto de 2010

O metilmercúrio (MeHg) é um importante agente tóxico ambiental que pode causar desordens neurocomportamentais e neurofisiológicas irreversíveis em humanos e animais experimentais. Os principais mecanismos pelos quais o MeHg induz toxicidade são: a ruptura na homeostase do cálcio intracelular, a indução de estresse oxidativo, a inibição da atividade da Na^+/K^+ ATPase neuronal e mudanças nos níveis das enzimas antioxidantes. Adicionalmente, dados recentes relatam o envolvimento do sistema da tiorredoxina como um dos alvos de toxicidade do MeHg. Por outro lado, os flavonóides possuem propriedades quelantes para metal divalente, atividade antioxidante e são permeáveis a barreira cérebro-sangue. Além disso, eles podem oferecer neuroproteção a uma variedade de modelos animais e celulares de doenças neurológicas, incluindo proteção contra a toxicidade do MeHg. Considerando que o exato mecanismo pelo qual o MeHg exerce toxicidade permanece desconhecido e que poucos e controversos dados sobre a interação do MeHg com flavonóides são encontrados na literatura, este estudo avaliou os mecanismos de toxicidade do MeHg em modelos *in vitro* e in *vivo* bem como, o desempenho de diferentes flavonóides: quercetina, quercitrina e rutina em diferentes modelos de toxicidade induzidos pelo MeHg. Nosso estudo mostrou que o MeHg (100µM) causou aumento na peroxidação lipídica e na produção de espécies reativas de oxigênio (EROs) em fatias de córtex de ratos. Os

flavonóides quercitrina (25 µg/mL) e quercetina (5, 10 e 25 µg/mL) protegeram contra esta toxicidade, e contra o aumento de ERO produzidas pelo MeHg (5µM) nas mitocôndrias. Diferentemente, o flavonóide rutina não obteve efeito protetor contra a indução da peroxidação lipídica e produção de ERO induzidas pelo MeHg em fatias corticais de cérebro. O aumento na produção de ERO, geradas pelo MeHg, foi dependente do aumento dos níveis intracelulares de cálcio (artigo 1). Já, estudos *in vivo* com camundongos tratados oralmente com MeHg (5mg/kg), durante 30 dias, mostraram um marcado aumento nos parâmetros de toxicidade (diminuição no ganho de peso, aumento na freqüência de micronúcleos e nefrotoxicidade), diminuição no desempenho do sistema motor (atividade locomotora e coordenação motora), e deficiência na memória espacial, bem como alterações em vários parâmetros bioquímicos (diminuição na atividade da glutationa peroxidase (GPx) e Na^+/K^+ ATPase e aumento na peroxidação lipídica). O co-tratamento com quercitrina (10mg/kg) pela via intraperitoneal, diminuiu as alterações comportamentais principalmente por diminuir os níveis de peroxidação lipídica e manter a atividade da GPx e da Na^+/K^+ ATPase iguais aos níveis do controle (manuscrito 1). Além disso, nosso estudo demonstrou, pela primeira vez, que o MeHg inibe a atividade da tiorredoxina redutase (TrxR). Uma única administração oral de MeHg (1, 5, 10 mg/kg), causou uma marcada inibição na atividade da TrxR renal, enquanto no fígad observou-se uma inibição significativa após exposição a 5 e 10 mg/kg (a atividade da TrxR foi determinada 24 horas após a administração de MeHg). No cérebro, o MeHg não inibiu a atividade da TrxR *in vivo* (artigo 2). Já os resultados *in vitro* revelaram que o MeHg causou uma inibição concentração dependente na atividade da enzima TrxR isolada de cérebro (0,05 – 1 µM) fígado (0,05 - 1 µM) e rim (0,025 – 1 µM). Assim, nós ampliamos a caracterização dos mecanismos associados com os efeitos neuroprotetores dos flavonóides quercetina e quercitrina na toxicidade induzida pelo MeHg. Adicionalmente, outros dados sobre a toxicidade do MeHg, foram obtidos, tais como: (1) o cálcio desempenha um papel central na toxicidade do MeHg, (2) em fatias de cérebro de ratos o MeHg induz estresse oxidativo mitocondrial via interação direta com as mitocôndrias, bem como via mecanismos mitocondriais indiretos. Além disso, (3) o MeHg (5mg/kg) pode levar a inúmeras alterações comportamentais que podem estar relacionadas à inibição da atividade das

enzimas GPx e Na^+/K^+ ATPase e (4) aumento na peroxidação lipídica. A alta afinidade do MeHg por grupos selenóis das moléculas endógenas pode levar (5) a inibição da TrxR o que pode contribuir para a toxicidade do MeHg. Podemos concluir que o MeHg leva a um aumento na produção de ERO pelas mitocôndrias, o que contribui para um aumento na peroxidação lipídica induzida pelo MeHg. Além disso, a inibição de importantes enzimas antioxidantes como a GPx e a TrxR podem contribuir para aumentar o dano oxidativo, que parece estar relacionado com o aparecimento dedanos comportamentais. Desta forma a atividade antioxidante dos flavonóides quercetina e quercitrina parece estar diretamente associada à capacidade destes compostos emproteger contra a toxicidade do MeHg.

Palavras-chaves: MeHg, flavonóides, estresse oxidativo, tiorredoxina redutase, antioxidantes, cálcio

ABSTRACT

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

Mechanisms of methilmercury toxicity: protective effect of flavonoids

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DATE AND PLACE OF THE DEFENSE: Santa Maria, August 2010

Methylmercury (MeHg) is an important environmental toxicant that may cause irreversible neurobehavioral and neuropsychological disorders in humans and experimental animals. The major mechanisms of MeHg-induced toxicity currently being explored are the disruption of intracellular calcium homeostasis, the induction of oxidative stress, inhibition of neuronal Na^+/K^+ -ATPase activity and change the status of antioxidant systems. In addition, recent data reported the involvement of MeHg toxicity with damage in thioredoxin system. On the other hand, flavonoids have been reported to possess divalent metal chelating properties, antioxidant activities and to readily permeate the blood–brain barrier. They can also provide neuroprotection in a wide array of cellular and animal models of neurological diseases, including protection against MeHg toxicity. However, the exact mechanism of MeHg toxicity remain unclear and limited data on the interaction of MeHg with flavonoids are avaliable in literature. In view of this, our study evaluated the mechanisms of MeHg toxicity *in vivo* and *in vitro* models and evaluated the performance of different flavonoids: quercetin, quercitrin and rutin in diferent models of MeHg toxicity. Our study showed that MeHg (100 µM) caused lipid peroxidation and reactive oxygen species (ROS) generation in brain cortical slices. Quercitrin and quercetin protected against this toxicity and mitochondria from MeHg (5 µM)-induced ROS generation. In contrast, rutin did not afford a significant protective effect against MeHg (100 µM)-induced lipid peroxidation and ROS production in cortical brain slices. MeHg-generated ROS in cortical slices was dependent upon an increase in

intracellular calcium levels. *In vivo* studies with mice treated during 30 days with MeHg (5mg/Kg) orally, presented a marked increase in toxicity parameters (loss in body weight gain, increased in micronucleis frequencies, nefrotoxicity), decrease in motor system performance (locomotor activity and motor coordination) and spatial memory deficiency as well as alteration in some biochemical parameters (decrease in glutathione peroxidase and Na^+/K^+ ATPase activity, increase in lipid peroxidation). The co-treatment with quercitrin (10mg/kg) intraperitoneally, decreased the behavior alterations manly by decreased lipid peroxidation levels, maintained the Na^+/K^+ ATPase and GPx activities. In addition, our study demonstrated, for the first time, that MeHg inhibited the activity of thioredoxin reductase. A single oral MeHg administration (1, 5 and 10 mg/Kg) caused a marked inhibition of kidney TrxR, while in liver a significant inhibition was observed after exposure to 5 and 10 mg/Kg of MeHg (TrxR was determined 24 hours after MeHg). In brain, MeHg did not inhibit TrxR. *In vitro* results demonstrated that MeHg inhibited brain (0.05 – 1 µM) , liver (0.05 – 1 µM) and kidney (0.025 – 1 µM) TrxR in a dose dependent manner Here, we have extended the characterization of mechanisms associated with the neuroprotective effects of flavonoids quercetin and quercitrin against MeHg-induced toxicity. In addition, we provided novel data establishing that (1) calcium plays a central role in MeHg toxicity, (2) in brain slices MeHg induces mitochondrial oxidative stress both via direct interaction with mitochondria as well as via mitochondria- indirect mechanisms. In addition (3) MeHg (5mg/kg) caused a number of behavioural alterations that are related with an inhibition of cerebelar and cerebral GPx and Na^+/K^+ ATPase activities and (4) increased in lipid peroxidation.The higly affinity of MeHg to selenol groups of endogenous molecules can lead to (5) inhibition of thioredoxin reductase that can contribute to MeHg toxicity. We conclude that MeHg lead to increase in mitochondria ROS generation that contributes to increase in lipid peroxidation. In addition, the inhibition of important antioxidant enzymes such as GPx ans TrxR can contribute to oxidative damage that can be related to development of behavioral damage. In this view the antioxidant activity of flavonoids quercetin and quercitrin seems to be direct associate with the capacity of flavonoids to confere protection against MeHg toxicity.

Key words: MeHg, flavonoids, oxidative stress, thioredoxin redutase, antioxidant, calcium

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Manuscrito 1

Artigo 2

LISTA DE ABREVIATURAS

Cys	cisteína
DNA	ácido desoxirribonucleíco
EO	estresse oxidativo
ERO	espécie reativa de oxigênio
GPx	glutationa peroxidase
GSH	glutationa
GSSG	glutationa oxidada
Hg^0	mercúrio metálico
Hg^{2+}	mercúrio inorgânico
$HOCl^-$	ácido hipocloroso
MeHg	metilmercúrio
NADPH	nicotinamida adenina dinucleotídeo fosfato
NMDA	N-metil D-aspartato
$NO_2^{\bullet}$	dióxido de nitrogênio
O_2^-	radical superóxido
$OH^{\cdot}$	radical hidroxil
$ONOO^-$	peroxinitrito
Sec	selenocisteína
SGLT1	transportadores de glicose dependentes de sódio
SOD	superóxido dismutase
TBARS	espécies reativas ao ácido tiobarbitúrico
Trx	tiorredoxina

TrxR	tiorredoxina redutase

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos e manuscrito, os quais se encontram no item **ARTIGOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam na íntegra o presente estudo.

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

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

1- INTRODUÇÃO

Os metais pesados são alguns dos principais contaminantes encontrados no meio ambiente. Estes metais têm ampla aplicação industrial, constituindo uma das principais formas de intoxicação ocupacional (Salgado, 1996). O mercúrio é considerado um dos principais metais tóxicos, devido ao seu elevado uso industrial, sendo de grande importância na exposição ocupacional e na poluição ambiental (Boischio e Henshel, 1996; Klaassen, 1996). Sabe-se que o mercúrio pode causar diversos prejuízos ao organismo, afetando os sistemas nervoso, renal e hepático (Larini e cols., 1997).

O metilmercúrio (MeHg) é um reconhecido poluente ambiental que, nas últimas décadas, causou contaminação e intoxicação humana em várias partes do mundo, como por exemplo, em Minamata, no Japão e também no Iraque (Robertson e Orrenius, 2000; Gochfeld, 2003). No Brasil, estudos têm evidenciado que várias espécies de peixes carnívoros da Amazônia apresentam altos níveis de MeHg (Malm, 1998; Pinheiro e cols., 2003). Consequentemente, comunidades ribeirinhas localizadas próximas as áreas de garimpo, podem sofrer exposição crônica a níveis relativamente elevados de MeHg em sua dieta, que é rica em peixes (Pinheiro e cols., 2003). Neste contexto, estudos epidemiológicos apontam para déficits neurológicos em comunidades pesqueiras que possuem uma dieta baseada no consumo de peixes (Granjean e cols., 1997; Clarkson e cols., 2003). Danos estes que envolvem disfunções sensoriais nas extremidade, ataxia cerebral, perturbações ao nível da visão e fala, dificuldades de audição, disfunção do equilíbrio (Clarkson e cols., 2003).

O MeHg afeta uma variedade de funções celulares, podendo causar danos em muitos órgãos e sistemas, particularmente, no sistema nervoso central (Chang, 1980; Clarkson, 1997). O mecanismo de ação envolvido na toxicidade do MeHg ainda não está completamente compreendido, entretanto, a alta afinidade do composto por grupos sulfidrílicos, o aumento na produção de espécies reativas de oxigênio (EROs) e distúrbios na homeostase do cálcio intracelular parece exercerem um papel central em seus efeitos tóxicos (Simpon, 1961; Bach e Weibel, 1976; Rooney, 2007).

Estudos *in vivo* associam à exposição ao MeHg a efeitos neurotóxicos severos tanto em animais como em humanos (Coluccia e cols., 2007; Díez, 2009). Vítimas da intoxicação por MeHg apresentam microencefalia, retardo no desenvolvimento, paralisia cerebral, fraqueza muscular, reflexos anormais além de danos intelectuais e no desenvolvimento (Choi e cols., 1981; Marsh e cols., 1987; Gilbert e Grant-Webster, 1995). Além disso, animais expostos ao MeHg desenvolveram deficiência na aprendizagem e memória, danos na locomoção, perda de tônus muscular, disfunção auditiva, motora e de aprendizagem (Chuu e cols., 2001; Goulet e cols., 2003; Farina e cols., 2005, Chuu e cols., 2007).

Estudos bioquímicos relacionam a toxicidade do mercúrio à inibição na atividade da enzima Na^+/K^+ ATPase (Huang e cols., 2008), avaria no sistema da glutationa (GSH), com mudanças na atividade das enzimas glutationa peroxidase (GPx) e decréscimo nos níveis de GSH (Farina e cols., 2003; Vicente e cols., 2004; Farina e cols., 2005; Franco e cols 2009) além de danos no sistema da tiorredoxina (Trx), diminuindo a atividade da enzima tiorredoxina redutase (TrxR) (Carvalho e cols., 2008; Wataha e cols., 2008). A alta afinidade do MeHg pelo grupo tiol da cisteína (Cys) bem como afinidade pela selenocisteína (Sec) presente no sítio catalítico de importantes enzimas como GPx a TrxR pode estar relacionado com estes efeitos tóxicos (Sugiura e cols., 1976; Hultberg e cols., 2001; Wataha e cols., 2008; da Conceição Nascimento e cols., 2009).

O estresse oxidativo também pode ser observado em intoxicações pelo mercúrio. A peroxidação lipídica pode ser verificada após exposição ao metal, sendo detectada através do aumento na concentração de espécies reativas ao ácido tiobarbitúrico (TBARS) (Huang e cols., 1996; Hoffman e Heinz, 1998; El-Demerdash, 2001). Além disso, a exposição ao MeHg pode levar ao aumento de EROs e diminuição das defesas antioxidantes (depleção de GSH, inibição de catalase, TrxR, GPx entre outras) (Hultberg e cols., 2001; Farina e cols., 2003, wataha e cols., 2008). Desta forma, acredita-se que a terapia por meio de agentes antioxidantes possa ser efetiva em proteger contra os danos causados pelo metilmercúrio (Farina e cols., 2005; Franco e cols., 2007; Lucena e cols., 2007 e 2010).

São diversos os antioxidantes utilizados na tentativa de reverter danos teciduais devido ao estresse oxidativo. Nas últimas décadas o uso de compostos naturais como possíveis agentes antioxidantes e neuroprotetores vem ganhado

atenção, entre eles os flavonóides. Os flavonóides possuem diversas atividades biológicas como efeitos contra doenças crônicas como câncer, doenças cardiovasculares e coronarianas, diabetes, doenças neurodegenerativas (como Parkinson e Alzheimer) entre outras (Middldeton e cols., 2000; Birt e cols., 2001; Havsteen, 2002; Zhao, 2009). As ações biológicas dos flavonóides sobre o organismo animal acontecem, principalmente, pela semelhança entre a estrutura química destes compostos e muitas moléculas do sistema biológico dos seres vivos, dentre elas: bases de ácidos nucléicos, coenzimas, hormônios esteróides, neurotransmissores. Além disso, a alta mobilidade dos elétrons no núcleo benzóico dos flavonóides contribui para sua ação antioxidante (Radouco –Thomas e cols., 1994; Casley-Smith, 1976; Abate e cols., 1990; Havsteen, 2002).

Trabalhos recentes (Franco e cols., 2007; Farina e cols., 2005; Lucena e cols., 2007 e 2010) sugerem que compostos naturais, principalmente a quercetina, podem exercer papel protetor contra a toxicidade do MeHg em modelos animais *in vitro* e *in vivo*. No entanto, outros trabalhos (Martins e cols., 2009) demonstram que a quercetina aumentou a toxicidade do MeHg, potencializando danos comportamentais e bioquímicos em camundongos tratados com este metal.

Considerando os aspectos acima mencionados, o presente estudo visou estudar os mecanismos tóxicos do MeHg em modelos *in vitro* relacionados com o aumento de EROs e peroxidação lipídica em mitocôndrias e em córtex cerebral bem como a inibição da enzima TrxR. Além disso, buscamos elucidar mecanismos de toxicidade do MeHg em modelos *in vivo* relacionados com danos comportamentais, inibição enzimática e estresse oxidativo (EO). Uma vez que não existem tratamentos efetivos contra os danos causados pelo MeHg, este estudo teve em vista também avaliar a atividade de flavonóides (quercetina, quercitrina e rutina) contra a toxicidade provocada pelo MeHg.

2 - REVISÃO BIBLIOGRÁFICA

2.1- Metais

Diversos metais, como o ferro, o zinco, o magnésio, e o manganês são considerados essenciais, pois são nutrientes requeridos por muitos organismos (Kabata-Pendias e Pendias, 1993). Entretanto, sabe-se que estes metais essenciais, dependendo de suas concentrações, podem apresentar efeitos tóxicos em determinados organismos (Kabata-Pendias e Pendias, 1993). Já outros metais, como mercúrio, alumínio, chumbo e cádmio, não são essenciais para os seres vivos, e podem apresentar toxicidade (Bruins e col., 2000).

A toxicidade decorrente da exposição a metais pesados pode ser devida ao deslocamento de metais essenciais de seus sítios de ligação ou devida a interações químicas com biomoléculas endógenas, como os grupos sulfidrílicos (SH). Os efeitos tóxicos geralmente resultam da alteração da estrutura de ácidos nucléicos e proteínas, interferência com o processo de fosforilação oxidativa e balanço osmótico, além de favorecerem o aparecimento do estresse oxidativo (Hughes e Poole, 1989).

O acúmulo de metais pesados no organismo humano representa um risco significativo para a saúde, levando a uma grande variedade de patologias, como a anemia, o câncer, a insuficiência renal crônica, a hipertensão, a gota, a infertilidade masculina, a gengivite e danos no sistema nervoso central (Choi e cols., 1981; Marsh e cols., 1987; Gilbert e Grant-Webster, 1995; Miller, 1998).

2.2 Mercúrio

O mercúrio é um metal obtido principalmente a partir do minério cinábrio. Podem ocorrer exposições ocupacionais durante a produção de cloro, soda cáustica, equipamentos elétricos e eletrônicos (baterias, retificadores e interruptores), aparelhos de controle (termômetros e barômetros), tinta látex, amálgamas dentárias, fungicidas, herbicidas, lâmpadas de mercúrio, entre outros (Salgado, 1996; Broussard e cols., 2002).

2.2.1 Formas de mercúrio

A população em geral está primariamente exposta ao mercúrio inorgânico, através das amálgamas dentárias, sendo os dentistas e seus auxiliares o grupo de maior risco de exposição (Brune e Evje, 1985). Dependendo do nível de contaminação, o ar e a água também podem se tornar importantes fontes de exposição ao metal. O uso de combustíveis de origem fóssil pode aumentar os níveis de mercúrio no ar (Vimy e Lorscheider, 1985). Industrialmente, a forma inorgânica do metal é utilizada em certos tipos de baterias e como componente de lâmpadas fluorescentes (Clarkson, 1997). Outras áreas de uso industrial do metal incluem a fabricação de plásticos, fungicidas e germicidas (Klaassen, 1996). Além disso, a alimentação também contribui para a contaminação pelo mercúrio, através da ingestão de peixes que acumulam o metilmercúrio, uma forma orgânica do metal (WHO, 1990).

As três principais formas químicas de mercúrio encontradas em nosso ambiente são o vapor de mercúrio (mercúrio elementar), mercúrio inorgânico e compostos orgânicos do metal (Klaassen, 1996).

2.2.2.1 Mercúrio metálico:

O mercúrio metálico ou mercúrio elementar (Hg^0) é utilizado em grande escala na indústria, sendo empregado na confecção de termômetros, amálgama dental, no processo de purificação do ouro explorado pelo garimpo, e em uma variedade de outros produtos de uso industrial e doméstico. Contudo, apesar do mercúrio metálico ser pouco absorvido por ingestão, ele tem uma alta capacidade de volatilização em temperatura ambiente, formando uma atmosfera de vapor de mercúrio a qual é facilmente absorvida pelos pulmões. Uma vez absorvida, essa forma lipossolúvel de mercúrio (Hg^0) pode atravessar a barreira hematoencefálica, a barreira placentária e as membranas biológicas onde pode ser oxidada à Hg^{2+} em uma reação que envolve a enzima catalase e o peróxido de hidrogênio. A forma inorgânica de mercúrio (Hg^{2+}) pode permanecer por vários anos no cérebro e outros tecidos, caracterizando assim o mercúrio como elemento bioacumulativo (Hargreaves e cols., 1988; Takeuchi e cols., 1989 ; Opitze cols., 1996; Braunwald e cols., 2001).

2.2.2.2 Mercúrio inorgânico:

O mercúrio inorgânico (Hg^{2+}) pode ser formado a partir do mercúrio metálico ou pela conversão de formas orgânicas de mercúrio a formas inorgânicas (Hg^{2+} e Hg^{+}) (Wood e cols., 1968). Entretanto, ele também é utilizado na forma iônica pela indústria, podendo ser encontrado em cosméticos, produtos de limpeza e outros produtos domésticos (Ozuah, 2000). Essa forma de mercúrio é facilmente absorvida por ingestão ou através da pele (Clarkson, 2002). Porém, relativamente pouco Hg^{2+} atravessa a barreira encefálica ou até mesmo as membranas biológicas, sendo assim excretado através da urina e/ou fezes (Takeuchi e cols., 1989). Devido a essa dificuldade de atravessar as membranas biológicas, a forma inorgânica de mercúrio tem como principal sítio de toxicidade o meio extracelular, bem como as membranas celulares (Friberg e Mottet, 1989).

2.2.2.3 Mercúrio orgânico:

A principal forma orgânica de mercúrio encontrada na natureza é o MeHg,proveniente principalmente da metilação do mercúrio metálico liberado pela indústria nos rios e córregos. O mercúrio metálico pode ser biometilado por bactérias metalogênicas em um processo relativamente simples: uma vez presente no meio ambiente, o mercúrio elementar pode ser facilmente incorporado por bactérias e organismos unicelulares, sendo então ionizado. No entanto, o mercúrio iônico é prejudicial a essas bactérias, que tentam eliminá-lo através da metilação, transformando-o em MeHg que por ser lipossolúvel é mais facilmente eliminado por organismos unicelulares (Wasserman e cols., 2001). A metilação do mercúrio ocorre pela transferência de um ou dois grupos metilcarboânions (CH_3^-) ao mercúrio inorgânico, e a vitamina B12 (metilcobalamina), a única coenzima reconhecida como possível doadora do grupo metil para o Hg^{2+} (Wood, 1974).

Outra forma de mercúrio orgânico é o etilmercúrio ($C_2H_5Hg^+$), o qual está presente no timerosal (Mertiolate®), medicamento de uso tópico utilizado como anti-séptico (Braunwald e cols., 2001), cuja industrialização e dispensação foram recentemente proibidas. Porém a utilização do timerosal como conservante de vacinas ainda continua a ser utilizado, trazendo sérios riscos a população

(Clarkson, 2003). Também existe o dimetilmercúrio ($(CH_3)_2$ Hg), uma forma "supertóxica" de Hg encontrada quase que exclusivamente em laboratório, que é absorvido facilmente através da pele. Porém, de um modo geral as formas orgânicas de mercúrio não têm boa absorção cutânea, sendo melhores absorvidas por inalação e ingestão (Braunwald e cols., 2001).

2.2.2- Toxicocinética

A principal via de penetração do mercúrio no organismo humano é a via pulmonar, devido à exposição a vapores ou partículas de mercúrio (Larini e cols., 1997). Em menor proporção, a absorção ocorre pela via dérmica (Salgado e cols., 1996). O mercúrio elementar, após ser absorvido, é parcialmente oxidado a mercúrio iônico nos eritrócitos e nos tecidos. O mercúrio inorgânico distribui-se na corrente sangüínea, concentrando-se mais no plasma que nos eritrócitos. Já as formas orgânicas, lipossolúveis, concentram-se nos eritrócitos (Salgado e cols., 1996).

A excreção dos compostos de mercúrio se dá principalmente pelas vias fecal e urinária (Larini e cols., 1997). A eliminação pelos túbulos proximais é seguida por parcial reabsorção nos túbulos distais. A filtração glomerular é prejudicada em razão da formação de complexos Hg-proteínas. Já a eliminação fecal dos compostos mercuriais ocorre principalmente por via biliar (Salgado e cols., 1996). O mercúrio é também excretado na saliva, suor e leite (Larini e cols., 1997).

2.3 **Metilmercúrio**

2.3.1 *Exposição ao* metilmercúrio:

O mercúrio é naturalmente liberado no meio ambiente por erupções vulcânicas e pelo desgaste da crosta terrestre, no entanto esse mercúrio geralmente está ligado ao enxofre na forma de sulfeto de mercúrio (HgS). Esse composto é bastante estável e pouco reativo, e desta forma não é considerado tóxico (Chuu e cols., 2007). Devido a essas características químicas inertes o

sulfeto de mercúrio (cinabar) tem sido utilizado por mais de 2000 anos como sedativo pela medicina chinesa (Yeoh e cols., 1986).

A maioria do mercúrio liberado no meio ambiente é de origem antropogênica, por dejetos industriais e principalmente pelo garimpo de ouro (Lacerda, 1996). No Brasil, estima-se que aproximadamente 100 a 200 toneladas de mercúrio sejam despejadas no ambiente anualmente (Lacerda, 1996). A tabela abaixo mostra a emissão de mercúrio no Brasil por ano.

Tabela 1. Comparação entre as emissões antropogênicas de mercúrio para a atmosfera no Brasil, baseado em Lacerda (1996)[1] levando em consideração a situação em 1990.

Setor	Parâmetros de produção/consumo	Parâmetros de emissão para a atmosfera	Emissão ($t.ano^{-1}$)	% do total
Produção de cloro	25.7 $tHg.yr^{-1}$	45%	11.65	10.1
Produção de soda	125 $gHg.t^{-1}$ KOH	45%	0.37	0.3
Tintas & pigmentos	34.3 $tHg.yr^{-1}$	1%	0.34	0.3
Eletro-eletrônico	9.1 $tHg.yr^{-1}$	0.2%	0.02	<0.1
Combustão de carvão	27.2 x $10^9 MJ.yr^{-1}$	0.13 $\mu gHg.MJ^{-1}$	0.01	<0.1
Combustão de óleo	28.3 x $10^9 MJ.yr^{-1}$	0.33 $\mu gHg.MJ^{-1}$	0.01	<0.1
Combustão de biomassa	4.8 x $10^6 MJ.yr^{-1}$	0.03 $g.t^{-1}$	0.12	0.1
Pirometalurgia Pb	62.023 $t.yr^{-1}$	2- 4 $gHg.tPb^{-1}$	0.19	
Zn	163.000 $t.yr^{-1}$	8-45 $gHg.tZn^{-1}$	4.30	3.9
Cd	197 $t.yr^{-1}$	8-45 $gHg.tCd^{-1}$	0.05	
Produção de aço e ferro	15 x 10^7 $t.yr^{-1}$	0.08$gHg.t^{-1}$	12	10.4
Queimadas	11.100 $km^2.yr^{-1}$	7.8 $gHg.ha^{-1}$	8.7	7.5
Garimpos de ouro	87 $tAu.yr^{-1}$	0.92 $tHg.tAu^{-1}$	77.9	67.3
Total	-	-	115.7	100

Fonte: Lacerda 1996

Sabendo-se que a maioria do mercúrio liberado no ambiente é metilado e incorporado na base da cadeia alimentar (bactérias metalogênicas) pelo processo anteriormente mencionado, o mercúrio representa um sério risco ambiental (Lacerda, 1996). Uma vez que esse elemento se acumula na cadeia alimentar aquática através de um fenômeno chamado bioamplificação, ou seja, a concentração do metal aumenta à medida que ele avança os níveis tróficos (Boening, 2000). Portanto, por ter a capacidade de permanecer por longos períodos nos tecidos do organismo, o metilmercúrio pode ser encontrado em peixes predadores do topo da cadeia alimentar em concentrações elevadas (0,546 µgHg/g de peixe), e culminar finalmente na dieta humana (Malm, 1998; Boening, 2000; Pinheiro e cols., 2003). Na região do rio Tapajós, onde o consumo de peixe é a principal fonte de alimento diária, os níveis de exposição ao metilmercúrio, medidos em raiz de cabelo, variam de alguns µg/g até mais de 150 µg de Hg/g de cabelo (Malm, 1998; Pinheiro e cols., 2003). O patamar a partir do qual os primeiros sinais clínicos e sintomas de contaminação mercurial ocorrem é de 50 µg/g (IPCS., 1990).

Um dos casos mais famosos de contaminação por MeHg ocorreu na baía de Minamata, Japão na década de 50. A companhia Chisso Fertiliser descartava metilmercúrio, um subproduto do processo de produção de acetoaldeído, levando a contaminação de peixes, que eram posteriormente pescados e consumidos pela população local (Oyake e cols., 1966; Bakir e cols., 1973; Watanabe e Satoh, 1996). Na década de 70, no Iraque, Paquistão, Gana e Guatemala ocorreram vários casos de contaminação de agricultores e familiares que utilizavam grãos tratados com fungicidas a base de etil e metilmercúrio, os quais deveriam ter sido usados como sementes para o plantio, na preparação de pão caseiro. Particularmente no Iraque, 6.530 pessoas foram hospitalizadas e 459 mortes foram relacionadas diretamente com a exposição ao MeHg no país (Watanabe e Satoh, 1996; Oyake e cols., 1966; Bakir e cols., 1973).

2.3.2. Toxicidade do metilmercúrio:

Como relatado anteriormente, o MeHg é um conhecido poluente ambiental que nas ultimas décadas causou contaminação e intoxicação em diferentes regiões do mundo. O MeHg afeta uma variedade de funções celulares, podendo causar danos em muitos órgãos e tecidos, bem como em: células pulmonares de humanos e animais (Reichl e cols., 2001), monócitos e linfócitos de humanos (Insug e cols., 1997; Shenker e cols., 1997), células β pancreáticas (Chen e cols., 2006), tecido renal e hepático (de Freitas e cols., 2009), entre muitos outros. No entanto, o sistema nervoso central parece ser o alvo principal do MeHg, sendo o cerebelo a áreas mais afetada (Choi,1978; Atchison, 2005; de Freitas e cols., 2009).

Vários trabalhos sugerem que as diversas disfunções celulares causadas pelo MeHg estejam associadas a sua alta afinidade por grupos sulfidrílicos (Simpon, 1961; Bach e Weibel, 1976; Rooney, 2007), dessa forma causando diversas disfunções celulares, como por exemplo: (a) depleção de glutationa (GSH); (b) inativação de enzimas uma vez que várias enzimas possuem no seu sitio ativo grupos sulfidrílicos e ou selenocisteína onde o MeHg pode ligar-se inativando ou diminuindo a atividade das mesmas, assim deprimindo o metabolismo enzimático/celular (Zheng e cols., 2003; Carvalho e cols., 2008); (c) dano no DNA (Al-Sabti,1994), evidências indicam que o MeHg pode causar fragmentação no DNA e causar a ativação de fatores apoptóticos (caspases) (Nishioku e cols., 2001); (d) inativação de proteínas transportadoras: várias proteínas transportadoras que contém grupos (SH) ou selênio (Se) podem ser alvo do MeHg como o caso da g-glutamil transpeptidase (GGT) (Allen e cols., 2002). Outro importante exemplo de inativação de transportadores pelo MeHg é o caso dos transportadores de glutamato (GLT1 e GLAST), que contém respectivamente, nove e três resíduos de cisteína em seqüência (Pines e cols., 1992; Aschner e cols., 2000); (e) disfunção em organelas celulares: o MeHg causa disfunção em uma série de organelas celulares, tais como mitocôndrias (Seegal e cols., 2007) e retículo endoplasmático (Limke e cols., 2003).

Apesar de tais fenômenos de cito/neurotoxicidade representarem eventos distintos, parece haver uma relação entre eles. De fato, o MeHg leva a um aumento de glutamato na fenda sináptica, provavelmente poe diminuir a captação do glutamato pelos astrócitos, pricipalmente por inativar os transportadores de

glutamato (GLT1 e GLAST), que contém respectivamente, nove e três resíduos de cisteína em seqüência (Aschner e cols., 2000), . Tal fato leva a uma super ativação dos receptores glutamatérgicos do tipo N-metil D-aspartato (NMDA), que, por conseguinte, gera um aumento do influxo de Ca^{2+} e Na^{+} intracelular (Choi, 1992).

A citotoxicidade do Ca^{2+}, por sua vez, pode levar à disfunção mitocondrial, já que um aumento na entrada do Ca^{2+} celular leva à captação do excesso de Ca^{2+} pela mitocôndria, causando despolarização e inchaço mitocondrial. Estes fatores podem contribuir para uma elevação dos níveis de ERO, alteração da homeostase mitocondrial (Seegal e cols., 2007) e, conseqüentemente causar apoptose/morte celular. Por outro lado, recentes estudos com culturas neuronais indicam que o MeHg causa uma liberação primária dos estoques de Ca^{2+} do reticulo endoplasmático, seguido por um influxo de Ca^{2+} extracelular (Limke e cols*.,* 2003). Nesse caso, agentes quelantes de cálcio atenuaram a morte neuronal (Marty e Atchison, 1998). Além disso, os radicais livres, tais como algumas EROs, podem causar dano oxidativo direto na mitocôndria, levando à redução da atividade enzimática e comprometendo a funcionalidade mitocondrial (Radi e cols., 2002; Galindo e cols., 2003). Os radicais livres também podem ser produzidos pela mitocôndria através da fuga de elétrons da cadeia respiratória para o oxigênio, formando ânion superóxido O_2^{-} (Halliwell e Guitteridge, 1999). Finalmente, todos esses eventos isolados ou interligados podem culminar com perda neuronal e contribuir para o aparecimento de várias patologias relacionadas à contaminação com o MeHg (Clarkson, 2002; Mutter e cols., 2004).

2.3.2.1 Inibição enzimática

O metilmercúrio se mostrou capaz de iniber inúmeras enzimas com importante papéis fisiológicos como a GPx, a TrxR, $Na^{+}K^{+}$ATPase, entre outras (Zheng e cols., 2003; Carvalho e cols., 2008; Huang, 2008;Farina e cols., 2009; Franco e cols., 2009)

2.3.2.1.1 Tiorredoxina Redutase

O sistema da tiorredoxina é composto pela enzima tiorredoxina redutase, tiorredoxina (Trx) e NADPH. Este sistema desempenha importantes funções na regulação do metabolismo redox e de inúmeros processos celulares, tais como a síntese de DNA, proliferação celular e processos apoptóticos. Este sistema esta largamente distribuído em diferente tecidos e órgãos em mamíferos (Rozell e cols., 1985) e seu funcionamento é crítico para resposta celular a estresse, reparo de proteínas e proteção contra o dano oxidativo (Arner e Holmgren, 2000; Lillig e Holmgren, 2007). Além disso, trabalhos recentes (Carvalho e cols., 2008 ; Du e cols., 2009) indicam que a inibição da TrxR pode levar a efeitos deletérios nas células, levando a citotoxicidade e morte celular.

A atividade catalítica das selenoenzimas como a TrxR depende principalmente da bioquímica da selenocisteína (Sec) presente no seu sítio catalítico (Behne e cols., 2000). A singular capacidade de várias selenoenzimas ocorre principalmente porque a selenocisteína possui um alto potencial redox que permite a Sec fazer reações que a cisteína não realiza. Uma vez que o selenol pertencente à Sec está ionizado em pH fisiológico ele é bioquimicamente mais ativo que a cisteína (Ralston e cols., 2008). Infelizmente esta característica tão valiosa fisiologicamente pode tornar a TrxR muito vulnerável ao MeHg.

Alguns trabalhos mostram que o MeHg é capaz de inibir a atividade da TrxR em cultura celular e modelos *in vitro* utilizando TrxR recombinante (Carvalho e cols., 2008). Em cultura de monócitos expostos ao mercúrio foi observado uma inibição na atividade da TrxR após 24 horas de exposição (Wataha e cols., 2008). Apesar de evidências apontarem que a inibição da TrxR possa ser um dos mecanismos pelo qual o mercúrio exerça sua toxicidade, nenhum experimento *in vivo* está descrito na literatura, relacionando inibição da atividade da TrxR e exposição ao MeHg.

2.3.2.1.2 Na^+/K^+ ATPase

A enzima Na^+/K^+ ATPase exerce a importante função de manter o gradiente de Na^+ e K^+ entre o ambiente extra e intra-celular e desta forma mantêm o potencial de membrana neuronal. Esta enzima pode ser inibida pelo MeHg, levando a despolarização da membrana neuronal que por conseqüência leva a supressão da função neuronal e da transmissão excitatória (Rajanna e cols., 1990; Balestrino e cols., 1999).

Muitos estudos relatam que vários insultos, como por exemplo, os causados por altas doses de MeHg induzem significativas desordens neurológicas em modelos *in vivo e in vitro* e estes insultos são muitas vezes acompanhados pela inibição da atividade da Na^+/K^+ ATPase (Cheng e cols., 2005; Chuu e cols., 2001).

2.3.2.1.3 Glutationa Peroxidase

A glutationa peroxidase (GPx) é uma selenoenzima que apresenta propriedade antioxidante catalisando a redução de peróxido de hidrogênio e peróxidos orgânicos através do consumo do grupo tiol da glutationa (GSH), formando como produtos água e/ou glutationa oxidada (GSSG) (Engman e cols., 1989; Luo e cols.,1994; Ursini e cols.,1995). A enzima GPx possui quatro subunidades idênticas contendo, em cada uma, um sítio catalítico composto pelo resíduo de Sec, o qual está envolvido na formação do intermediário selenol (Enz-SeH), que reduz peróxidos à água ou álcool formando o ácido selênico (Enz-SeOH) (Wendel 1994; Ursini e cols.,1995). Assim como no caso da TrxR a presença de selenol no sítio ativo pode tornar esta enzima um alvo para toxicidade do MeHg. De fato, trabalhos mostram que o MeHg pode inibir a atividade da GPx em diferentes modelos experimentais (Sugiura e cols., 1976; Hultberg e cols., 2001; Zheng e cols., 2003; Farina e cols., a e b, 2009; Franco e cols., 2009; da Conceição Nascimento e cols., 2009; de Freitas e cols., 2009).

Além da GPx, também chamada de GPx clássica, citosólica ou celular (GPx1) existem mais cinco tipos de enzimas GPx (GPx2-GPx6) em mamíferos, as quais são divididas de acordo com a sequência de aminoácidos, especificidade por substrato e localização subcelular. Embora a GPx possa agir em uma ampla gama de substratos, dados experimentais *in vitro* sugerem que todos os tipos de GPx são

específicos para a GSH como agente redutor. A GPx1 é a mais abundante selenoenzima, pois é encontrada em quase todos os tecidos (CHU e cols., 1993). A GPx2 é tetramérica e ocorre no citosol. Em ratos, é mais proeminente no tecido gastrintestinal; porém, em humanos é presente no fígado e intestino grosso, mas não em outros órgãos. A GPx2 possui um importante papel nos mamíferos na proteção contra a toxicidade da ingestão de hidroperóxidos lipídicos, mas não para hidroperóxidos de fosfolipídios (CHENG e cols., 1997). A GPx3 foi primeiramente identificada no plasma humano e é uma glicoproteína compatível com sua função extracelular (TAKAHASHI e cols., 1987). A GPx4, também chamada de fosfolipídeo hidroperóxido, está associada à membrana celular e é descrita como uma proteína inibidora de peroxidação. Em contraste com as outras GPx, as quais possuem estrutura tetramérica, a GPx4 é um monômero e encontra-se principalmente no cérebro e coração de mamíferos (DUAN e cols., 1988; URSINI e cols.,1995). Todas as GPx1-GPx4 possuem um resíduo de selenocisteína no sítio ativo o qual é sucessivamente oxidado e então reduzido durante o ciclo catalítico (AUMANN e cols., 1997). Os outros dois tipos de GPx, GPx5 e GPx6, diferem das demais por serem selênio não-dependentes, ou seja, apresentam cisteína no sítio catalítico (KRYUKOV e cols., 2003). Suas funções biológicas ainda não estão bem estabelecidas.

2.3.2.2. Disfunção mitocondrial

Muitos estudos in vitro indicam que as mitocôndrias são importantes alvos para a toxicidade do MeHg (Verity e cols., 1975; Shenker e cols., 1999; Mori e cols., 2007). O MeHg diminui as funções mitocondriais e aumenta os níveis de EROs em sinaptossomas estriatais (Dreiem e Seegal, 2007). Este efeito pode ser um reflexo do acúmulo de MeHg intra mitocondrial, que pode levar a um colapso no potencial transmembrana mitocondrial (In sung e cols., 1997; Shenker e cols., 1999; Araragi e cols., 2003). O MeHg também pode induzir a geração de peróxido de hidrogênio e diminuir a viabilidade mitocondrial (Franco e cols., 2007).

EROs causam dano celular comprometendo a integridade das membranas celulares, proteínas e do DNA (Valko e cols., 2005). Além disso, EROs prejudica o metabolismo energético mitocondrial através da indução de mudanças oxidativas estruturais e subseqüente perda da atividade em inúmeras enzimas mitocondriais que possuem papel essencial na produção de ATP (Fiskum e cols., 2004). Além

disso, a direta ação de ERO nos lipídios e proteínas da membrana mitocondrial pode resultar na ativação da cascata apoptótica, através da abertura do poro de transição de permeabilidade mitocondrial (Lifshitz e cols., 2004).

2.3.2.3 Metilmercúrio e mudanças comportamentais:

A exposição ao MeHg durante a fase do desenvolvimento resulta em prejuízo na migração das células granulares e prejuízo na sinaptogênese, os quais causam uma desordem na arquitetura cerebelar (Choi, 1978). Já a exposição ao MeHg pós desenvolvimento, resulta em danos mais específicos que levam à perda dos grânulos cerebelares (Atchison, 2005). Essas evidências de um modo geral explicam o porquê das mudanças comportamentais em jovens serem mais acentuadas que em adultos (Atchison, 2005). A exposição a baixas doses de MeHg, particularmente em indivíduos jovens, pode provocar prejuízo motor súbito, deficiência na linguagem, problemas de aprendizagem, déficit de atenção e dificuldades na realização de tarefas (Grandjean, 1997). Todos esses sintomas estão relacionados à disfunção cerebelar, sendo também observados em jovens com neoplasia cerebelar ou com síndromes associadas à degeneração das células granulares do cerebelo (Levisohn, 2000; Riva e Giorgi, 2000). Tais dados, sugerem uma correlação entre baixas doses de exposição ao MeHg e alterações súbitas de comportamento, de origem cerebelar (Sarnati e Alcala, 1980). Distúrbios locomotores também são observados em animais de laboratório expostos a altas doses de MeHg (5 mg/kg/dia) e igualmente associados a patologias cerebelares (Sacamoto,1996). Assim os efeitos do MeHg em células cerebelares podem provocar um grande espectro de sintomas neurológicos que podem ser observados por mudanças comportamentais tais como: deficiência no aprendizado e memória, danos na locomoção, perda de tônus muscular, disfunção auditiva e disfunção motora (Chuu e cols., 2001; Goulet e cols., 2003; Farina e cols., 2005, Chuu e cols., 2007).

2.4.Flavonóides

Flavonóides são contituintes essenciais das células de vegetais superiores. Devido a sua coloração e odor, são responsáveis pela comunicação do vegetal com o ambiente, atraindo agentes polinizadores como pássaros, insetos e outros animais (Harbone, 1986 e 1988). Flavonóides também apresentam propriedades regulatórias semelhantes às vitaminas lipossolúveis, interferem no metabolismo celular e atuam juntamente com hormônios regulando o crescimento do vegetal (Fragner, 1964; Groteweld e cols., 1994; Jiang e cols., 1999). Estes compostos interferem ainda na transferência de elétrons durante a fosforilação oxidativa que ocorre no cloroplasto e possuem um importante papel na fixação do nitrogênio (Cantley e Hammes, 1976; Mukohata e cols., 1978; Mortenson e Thorneley, 1979).

Em mamíferos, os flavonóides ocorrem somente através da dieta. Estes compostos são largamente encontrados em frutas, vegetais, grãos, sementes, chás e vinho (Rice-Evans e cols., 1996 e 2004; Hollman e cols., 1999). Em indivíduos saudáveis, estima-se uma média de consumo de 1–2 g de flavonóides por dia. Apesar de o homem a várias décadas associar saúde com dieta de produtos de origem vegetal e usar ervas medicinais para o tratamento de algumas doenças, somente há poucos anos as propriedades bioquímicas e farmacológicas dos flavonóides começaram a ser desvendadas (Havsteen, 2002).

Os flavonóides são gamma-benzopironas e constituem uma família de compostos com pequenas variações em sua estrutura química. Alguns deles estão ligados a um monossacarídeo (maior parte encontrada na natureza), aumentando sua solubilidade em água. A porção aglicona (sem o monossacarídeo) constitui-se de difenilpropanos (C_6-C_3-C_6) (figura 1) (Harborn, 1989). O grupo compreende antocianidinas, flavonol, isoflavonol, flavona, isoflavona, flavana, isoflavana, flavanona, flavanol, isoflavanol, entre outros (Harborn, 1967; Croft, 1998). Assim, estes compostos compartilham a mesma estrutura primária e, conseqüentemente, compartilham algumas atividades biológicas; embora, devido a certas modificações estruturais, podem apresentar propriedades distintas (Havsteen, 2002).

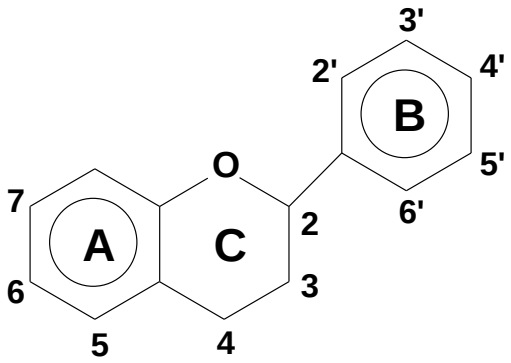

Figura 1: Estrutura química base (difenilpropano) dos flavonóides.

2.4.1. Biodisponibilidade de flavonóides oriundos da dieta

A absorção intestinal e metabolismo de flavonóides da dieta ainda não estão bem estabelecidos, pois uma série de divergências a respeito deste processo é encontrada na literatura. A grande maioria dos estudos encontrados na literatura trata da biodisponibilidade da quercetina, provavelmente, pelo grande número de atividades biológicas deste flavonóide (Podhajcer e cols., 1980; Manach e cols., 1998; Hollman e cols., 1999; Graefe e cols., 1999; Yamamoto e cols., 1999; Aziz e cols., 1998; Walle e cols., 2001).

A maior parte dos flavonóides encontrada na natureza está sob a forma glicosilada, poucas quantidades são encontradas como aglicona, uma vez que esta é mais instável; sendo assim, a forma glicosilada a mais comumente ingerida na dieta (Walle, 2004; Yokomizo e Moriwaki, 2005). Quando ingeridos, os flavonóides chegam ao lúmen intestinal e, parte sofre ação de β-glicosidases de bactérias intestinais, liberando a molécula de monossacarídeo. A porção aglicona pode ser absorvida com as micelas biliares nas células epitelias e então, passar para a linfa (Dai e cols., 1997; Spencer e cols. 1999); ou podem atravessar a membrana do enterócito através de difusão passiva (Murota e Terao, 2003). Os flavonóides que

não sofrerem hidrólise serão absorvidos para o interior do enterócito através dos transportadores de glicose dependentes de sódio (SGLT1) (Murota e Terao, 2003) e, ao atingirem o enterócito, sofrem ação das β-glicosidases contidas nesta célula (Walle, 2004).

O pico de concentração plasmática para flavonóides como a quercetina ocorre 1,5 a 2 horas após a ingestão (Aziz e cols., 1998) e a meia-vida de eliminação plasmática é cerca de 22 horas (Hollman e cols., 1999); embora alguns autores relatem que a meia-vida de eliminação plasmática possa chegar a 72 horas (Walle, 2004). Os flavonóides são degradados á ácidos orgânicos (ácido cafeico e cinâmico, entre outros) e então, excretado com a urina (Graefe e cols., 1999; Bourne e Rice-Evans, 1999). Estudos com a quercetina [^{14}C] marcada radiotivamente, mostram que o CO_2 expirado através dos pulmões é o maior metabólito deste flavonóide (Walle e cols., 2001).

2.4.2 Atividade antioxidante dos flavonóides

Lembrando que as EROs incluem radicais livres, $OH^{\bullet}$, $O_2^{\bullet -}$, dióxido de nitrogênio ($NO_2^{\bullet}$); bem como moléculas não radicalares como o H_2O_2, peroxinitrito ($ONOO^-$); ácido hipocloroso ($HOCl^-$), entre outros. Estas moléculas possuem uma grande reatividade e podem levar à lipoperoxidação, oxidação de carboidratos, proteínas e DNA (Pratico e Delanty, 2000).

A sobrevivência da célula frente à toxicidade dos radicais livres no curso normal do metabolismo celular se dá graças à ação dos antioxidantes endógenos. Os antioxidantes são substâncias que direta ou indiretamente protegem os sistemas celulares dos efeitos tóxicos produzidos por espécies reatrivas (Halliwell, 1995). Compostos com ação biológica e função antioxidante podem ser moléculas protéicas com grupos tiólicos como as metalotioneínas, enzimas glutationa peroxidase, superóxido dismutase e catalase, moléculas não-protéicas como a glutationa e substâncias exógenas como a vitamina C e os flavonóides (Krishna e cols., 1996; Evans e cols., 1997; Mckenzie e cols., 1998; Halliwell, 1999; Edenharder e Grunhage, 2003; Hawse e cols., 2006). Assim, sob condições normais, os sistemas antioxidantes celulares minimizam os danos causados pelas

EROs, porém, quando a produção excede a capacidade protetora da célula tem-se o estresse oxidativo (Quig, 1998).

Trabalhos sugerem que os compostos fitoquímicos fenólicos exercem um efeito positivo no tratamento do câncer e doenças neurodegenetativas, e atribuem esses efeitos benéficos à atividade antioxidante e a capacidade de detoxificar radicais livres apresentada pelos flavonóides. (Rice-Evans e cols., 1996).

Esta atividade antioxidante ocorre principalmente porque os grupos hidroxilas dos anéis fenólicos dos flavonóides agem como doadores de elétrons, sendo responsáveis pela capacidade de seqüestrar radicais livres como $OH^{\cdot}$ (Husain e cols., 1987) e $O_2^{\cdot -}$ apresentada por estes compostos (Bors e cols., a and b, 1990). A estrutura catecol (estrutura o-dihydroxyl), a qual possui dois grupos hidroxil em posições vizinhas, é marcadamente mais eficiente que as demais configurações em doar elétrons, assim a quercetina e os demais flavonóides que apresentam a estrutura catecol podem exercer uma poderosa atividade em seqüestrar radicais livres (Bors e cols., 1990; Rice-Evans e cols., 1996). A atividade antioxidante dos flavonóides pode também ser associada ás suas propriedades quelantes, uma vez que os metais de transição, como o ferro, pode exercer um papel crucial na geração de espécies reativas de oxigênio através de reações como a de Fenton. Somando-se a isso, os flavonóides podem levar também a um aumento nas defesas antioxidantes do organismo (Halliwel e Gutteridge, 1999). Além disso, os flavonóides também possuem alta capacidade de remover íons de metais pesados com potencial de oxi-redução. Então, esta propriedade também confere proteção à toxicidade das EROs, uma vez que a formação de radicais livres e oxidação de biomoléculas pode ser catalisada por íons de metais pesados (Afanas'ev e cols., 1989; Halliwell e Gutteridge, 1990; Brown e cols., 1998)

2.4.3 Quercetina

A quercetina (figura 2) (3,3',4',5,7-pentahydroxyflavone), um dos mais abundantes flavonóides encontrados nas frutas e vegetais (Hertog e cols.,1992; Richter e cols., 1999), é um flavonol. Estima-se que em torno de 25-50 mg de

quercetina são consumidos diariamente em uma dieta equilibrada (Formica e Regelson, 1995).

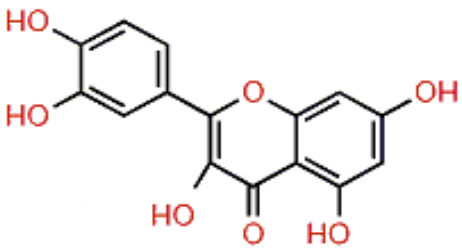

Figura 2. Estrutura da quercetina

Inúmeros estudos *in vitro* têm revelado diversos efeitos biológicos da quercetina, incluindo efeitos antimutagênicos, inibição da proteína quinase C (Lindahl e Tagesson, 1997), inibição de lipoxigenases, atividade mimética da superoxido dismutase (SOD), tratamento de inflamações, arteriosclerose, hemorragias, processos alérgicos e inchaço (Formica e Regelson, 1995; Havsten. 1983). A quecertina pode seqüestrar radicais livres (Morel e cols. 1993), prevenir o desenvolvimento de tumores e inibir a ativação carcinogênica (Richter e cols., 1999).

Uma forma de a quercetina exercer seus efeitos na célula está associada com as mudanças nas propriedades da membrana celular (Pawlikowska-Pawlega e cols., 2003) que parecem estar relacionadas com o influxo de cálcio e/ou com seu metabolismo (Morales e Lozoya, 1994; Pawlikowska-Pawlega e cols. 2000).

Estudos recentes também mostraram que a quercetina pode proteger as células neuronais imaturas e células corticais do dano oxidativo induzido por glutamato (Ishige e cols., 2001; Kim e cols., 2002). Além de apresentar papel protetor contra neurotoxicidade induzida por H_2O_2, MeHg e danos mitocondriais (Franco e cols., 2007, Heo e Lee, 2004; Arredono e cols., 2010)

2.4.4 Quercitrina

A quercetina é encontrada na dieta humana principalmente em sua forma glicosídica, como por exemplo, a quercitrina, a qual um ou mais grupos açúcares estão ligados ao grupo fenólico através de uma ligação glicosídica (Herrmann, 1976; Hermann e Lebensm, 1988). Estudos têm mostrado que a absorção intestinal da forma glicosídica da quercetina é superior a da forma aglicona (Hollman e cols., 1995). Além disso, a porção açúcar ligada à porção aglicona aumenta a solubilidade em solventes polares e conseqüentemente aumenta a absorção, através da utilização de transportadores de glicose que estão presentes na mucosa intestinal (Gee e cols., 1998).

A quercitrina (3-O-alpha-L-rhamnopyranoside) se mostrou capaz de atenuar a peroxidação lipídica induzida por diferentes agentes pró-oxidantes (Wagner e cols., 2006), melhorar a homeostase da glicose em animais (Babujanarthanam e cols., 2009), inibi a produção de peroxinitrito e superóxido (Kim e cols., 2007) e aumenta a atividade da glutationa-S-transferase (Apáti e cols., 2006).

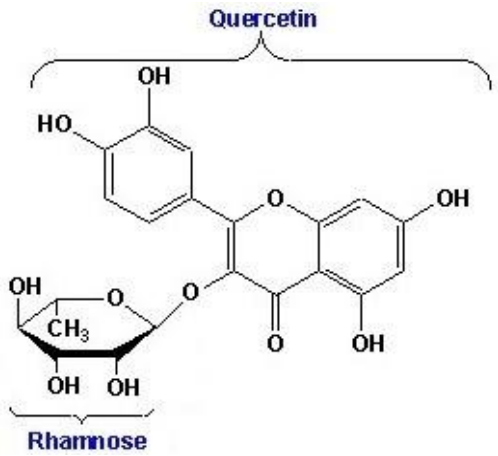

Figura 3 Estrutura química da Quercitrina

2.4.5 Rutina

Pesquisas avaliando a atividade antioxidante da rutina (Figura 4) concluíram que este flavonóide tem uma ação terapêutica em patologias que envolvem a produção de radicais livres. A rutina pode inibir a formação de radicais livres em diversos estágios, por reagir com íons superóxido e radicais lipídicos, além de poder formar complexo com ferro, que é um catalisador da formação de radicais de oxigênio ativo (Pathak, 1991; Yokozawa e cols., 1997).

Entre as diferentes atividades farmacológicas apresentadas pela rutina estão: conferir proteção *in vivo* e *in vitro* a mitocôndrias cardíacas contra dano causado por isoproterenol (Punithavathi e cols., 2010), a rutina se mostrou eficaz em proteger da morte celular induzida por isquemia/reperfusão, aumentou a expressão de proteínas anti-apoptóticas, bem como protegeu contra o aumento nos níveis de marcadores de estresse no retículo endoplasmático (Kim e cols., 2010).

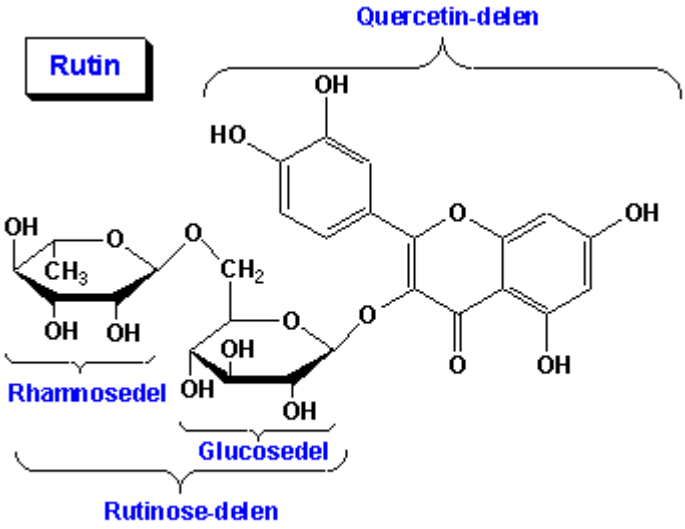

Figura 4: Estrutura química da Rutina

2.4.6 Outras atividades farmacológicas apresentada pelos flavonóides

Os flavonóides podem quelar metais divalentes, possuem atividade antioxidante e antiinflamatória, são permeáveis a barreira cérebro-sangue e oferecem neuroproteção a uma variedade de modelos de desordens neurológicas em modelos animais e celulares (Mande e cols., 2006; Youdim e cols., 2004). Administrações *in vitro* de quercetina exibem citoproteção em diferentes modelos de morte apoptótica (Dajas e cols., 2005). No entanto, resultados contraditórios são observados na literatura, alguns trabalhos mostram que a quercetina apresentou efeito protetor em modelos de doença de Parkinson, enquanto que em outros trabalhos, a quercetina foi ineficaz (Kaariaien e cols., 2008; Zbarsky e cols., 2005). Outros trabalhos mostram que a quercetina pode apresentar toxicidade em inúmeros modelos animais. Além disso, é sugerido que a presença de ligações glicosídicas pode levar a quecetina a apresentar diferentes efeitos terapêuticos e tóxicos (Ossola e cols., 2009).

Corroborando com estes dados estão as diferentes atividades farmacológicas apresentadas pela quercetina em associação com MeHg. Trabalhos mostram que extratos de plantas, que apresentam altas concentrações do flavonóide quercetina, protegem os animais expostos ao MeHg contra o dano oxidativo, alterações no sistema da glutationa, além de proteger contra danos comportamentais, tais como: danos locomotores e na coordenação motora (Farina e cols., 2005; Lucena e cols., 2007). Franco e colaboradores (2007) mostraram que a quercetina protege mitocôndrias isoladas de cérebro de camundongos contra os danos causados pelo mercúrio. De fato, a quercetina protegeu asmitocôndrias contra uma diminuição na viabilidade mitocondrial induzida pelo MeHg, protegeu contra a oxidação da glutationa e o aumento de ERO causada pelo MeHg, efeito relacionado com a capacidade deste flavonóide em remover espécies oxidantes produzidas por compostos de mercúrio (Franco e cols., 2007). Apesar de os flavonóides se mostrarem como compostos promissores no uso contra a toxicidade do MeHg, Martins e cols. (2009) demonstraram que a quercetina apresenta efeito neurotóxico sinérgico ao MeHg em experimentos *in vivo*. Assim é de fundamental importância novos estudos que avaliam os mecanismos de a interação entre os flavonóides e o MeHg.

3 OBJETIVOS

3.1 Objetivo geral:

Os objetivos deste trabalho foram avaliar diferentes mecanismos de toxicidade do metilmercúrio, bem como, avaliar um possível papel protetor de flavonóides neste efeitos tóxicos.

3.2 Objetivos específicos:

(1) Avaliar os mecanismos pelos quais o MeHg exerce seus efeitos tóxicos: em modelos *in vitro* de fatias de córtex cerebral e em mitocôndrias, bem como avaliar os efeitos tóxicos na atividade da enzima TrxR. Além disso, avaliar a toxicidade do MeHg em modelos experimentais *in vivo* com ênfase nas alterações comportamentais, inibição da atividade enzimática e na produção de ERO.

(2) Uma vez que não existe tratamentos efetivos contra os danos causados pelo MeHg este estudo também teve por objetivo propor uma terapia alternativa utilizando para isso três diferentes flavonóides: quercetina, quercitrina e rutina, em diferentes modelos de toxicidade induzidos pelo MeHg.

4- ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos e manuscritos, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos.

Os **Artigos 1 e 2** estão dispostos da mesma forma que foram publicados na edição das revistas científicas. O **manuscrito 1** está em fase de redação .

4.1 Artigo 1

Estudo comparativo da quercetina e duas derivações glicosídicas a quercitrina e a rutina, na produção de ERO induzida pelo metilmercúrio (MeHg)

Comparative study of quercetin and its two glycoside derivatives quercitrin and rutin against methylmercury (MeHg)-induced
ROS production in rat brain slices

Caroline Wagner, Alessandra P. Vargas, Daniel H. Roos, Ademir F. Morel, Marcelo Farina, Cristina W. Nogueira, Michael Aschner e João B. Rocha

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INORGANIC COMPOUNDS

Comparative study of quercetin and its two glycoside derivatives quercitrin and rutin against methylmercury (MeHg)-induced ROS production in rat brain slices

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Abstract The hypothesis that methylmercury (MeHg) potently induces formation of reactive oxygen species (ROS) in the brain is supported by observations on the neuroprotective effects of various classes of antioxidants. Flavonoids have been reported to possess divalent metal chelating properties, antioxidant activities and to readily permeate the blood–brain barrier. They can also provide neuroprotection in a wide array of cellular and animal models of neurological diseases. Paradoxically, in vivo administration of quercetin displays unexpected synergistic neurotoxic effect with MeHg. Considering this controversy and the limited data on the interaction of MeHg with other flavonoids, the potential protective effect of quercetin and two of its glycoside analogs (i.e., rutin and quercitrin) against MeHg toxicity were evaluated in rat cortical brain slices. MeHg (100 μM) caused lipid peroxidation and ROS generation. Quercitrin (10 μg/mL) and quercetin (10 μg/mL) protected mitochondria from MeHg (5 μM)-induced changes. In contrast, rutin did not afford a significant protective effect against MeHg (100 μM)-induced lipid peroxidation and ROS production in cortical brain slices. MeHg-generated ROS in cortical slices was dependent upon an increase in intracellular Ca^{2+} levels, because the over-production of MeHg-induced H_2O_2 in mitochondria occurred with a concomitant increase in Ca^{2+} transient. Here, we have extended the characterization of mechanisms associated with the neuroprotective effects of quercetin against MeHg-induced toxicity in isolated mitochondria, by performing an array of parallel studies in brain slices. We provide novel data establishing that (1) Ca^{2+} plays a central role in MeHg toxicity and (2) in brain slices MeHg induces mitochondrial oxidative stress both via direct interaction with mitochondria (as previously reported in in vitro studies) as well as via mitochondria-independent (or indirect) mechanisms.

Keywords MeHg toxicity · Quercetin · Quercitrin · Rutin · ROS · Mitochondria

Electronic supplementary material The online version of this article (doi:10.1007/s00204-009-0482-3) contains supplementary material, which is available to authorized users.

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Introduction

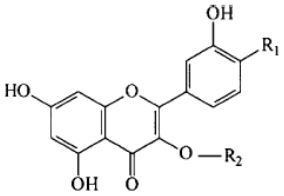
Methylmercury (MeHg) is a highly toxic environmental pollutant that causes neurological and developmental deficits in animals and humans (Clarkson et al. 2003). MeHg-induced neurotoxicity is an extensively reported phenomenon, but the precise molecular mechanisms underlying its cytotoxicity have yet to be fully clarified. The central nervous system (CNS) is considered the primary target for MeHg toxicity (Clarkson et al. 2003) and currently the

major mechanisms involved in its neurotoxicity are believed to be related to impairment of intracellular calcium homeostasis (Sirois and Atchison 2000), alterations of glutamate homeostasis (Allen et al. 2001; Aschner et al. 2000; Fonfria et al. 2005) and oxidative stress (Aschner et al. 2007; Franco et al. 2007).

Mercury is known to induce the formation of reactive oxygen species (ROS), cell death and DNA damage (Aschner et al. 2007; Grotto et al. 2009; Reichl et al. 2006a, b; Schmid et al. 2007). There are a number of reports concerning oxidative stress and the protective role of antioxidant enzymes against MeHg neurotoxicity (Allen et al. 2001, 2002; Shanker and Aschner 2003). In vitro studies have consistently demonstrated that MeHg can disrupt neuronal and astrocytic functions (Allen et al. 2001, 2002; Aschner et al. 2000; Yee and Choi 1994). Exposure of cortical rat brain slices and cortical astrocytes to MeHg have been shown to increase the ROS formation (Dreiem and Seegal 2007; Roos et al. 2009; Yee and Choi 1994).

Several in vitro studies have indicated that mitochondria are critical subcellular target for MeHg toxicity (Mori et al. 2007; Shenker et al. 1999; Verity et al. 1975). For example, MeHg decreases mitochondrial function and increases ROS levels in striatal synaptosomes (Dreiem and Seegal 2007). These effects may reflect the intra-mitochondrial accumulation of MeHg, leading to the collapse of the mitochondrial transmembrane potential (Araragi et al. 2003; InSug et al. 1997; Shenker et al. 1999) or can be related to an indirect effect of MeHg on glutamate homeostasis at the synaptic level (Allen et al. 2002; Aschner et al. 2000, 2007). MeHg can also induce hydrogen peroxide generation and decrease mitochondrial viability in isolated mouse brain mitochondria (Franco et al. 2007).

Flavonoids are widely found in vegetables, fruits, juices and tea and represent important components of the human diet (Hollman and Katan 1999; Rice-Evans et al. 1996). It has been proposed that phenolic phytochemicals exert a positive health effects in chronic diseases, such as cancer and neurodegenerative disorders. Many physiological benefits of flavonoids have been attributed to their antioxidant and free radical scavenging properties (Rice-Evans et al. 1996). Flavonoids possess divalent metal chelation properties, antioxidant and anti-inflammatory activities, readily permeate the blood–brain barrier (BBB) and afford neuroprotection in a wide array of cellular and animal models of neurological diseases (Mande et al. 2006; Youdim et al. 2004). In vitro quercetin administration has been reported to exhibit cytoprotection in different pro-oxidant models of apoptotic death (Dajas et al. 2003). However, contradictory results can also be found in the literature (Kaariaien et al. 2008; Zbarsky et al. 2005), where quercetin fails to provide protection against model of Parkinson's disease. Quercitrin (a glycoside rhamnose of quercetin) and rutin (a glycoside rutinose from quercetin) can also exhibit in vitro antioxidant properties in different pro-oxidant models (Pereira et al. 2009; Spanos and Wrolstad 1992; Wagner et al. 2006). Notably, recent reports have indicated that the pro-oxidant effect of MeHg can be counteracted by plant extracts rich in flavonoids and by purified quercetin (Farina et al. 2005; Franco et al. 2007). Paradoxically, simultaneous in vivo exposure to MeHg and quercetin caused synergistic neurotoxic effects in mice (Martins et al. 2009).



Flavonol	R_1	R_2
Rutin	OH	Rutinose
Quercitrin	OH	Rhamnose
Quercetin	OH	H

Fig. 1 Flavonoids structures

Given the lack of efficacious treatments that fully abolish MeHg-induced toxicity and that natural and synthetic antioxidants compounds afford protection in a variety of in vitro and in vivo models associated with oxidative stress (Gugliucci and Stahl 1995; Gupta et al. 2003; Sudati et al. 2009), the present study was designed to test the potential protective effects of antioxidant compounds against MeHg toxicity. Furthermore, the discrepant effects of quercetin against MeHg neurotoxicity after in vitro and in vivo studies indicate that the effect of structurally related quercetin flavonoids should be investigated in order to determine their potential toxic or protective effects. In this study, the potential protective effect of quercetin and its two glycoside structurally related flavonoids (rutin and quercitrin) (Fig. 1) against MeHg toxicity were evaluated in cerebral cortical slices, brain isolated mitochondria directly exposed to MeHg.

Materials and methods

Chemicals

Thiobarbituric acid, malonaldehyde bis-(dimethyl acetal) (MDA), dichlorofluorescin diacetate (DCFHDA), dichlorofluorescein (DCF), rutin, ethylene glycol tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), succinic acid and ionophore A23187 were obtained from Sigma (St. Louis, MO, USA). Trichloroacetic acid, sodium phosphate, sucrose, mannitol and L-glutamate were obtained from Vetec (Rio de Janeiro, RJ,

Brazil). MeHg was obtained from Merck (Rio de Janeiro, RJ, Brazil). Quercitrin and quercetin were isolated from *Solidago microglossa* D.C., and the purity of the isolated compounds was >99.3% (Morel et al. 2006).

Animals

Male Wistar rats (2–4 months) maintained under standard conditions (12-h light/dark, 22 ± 2°C) with food and water ad libitum were used. The Animal Care Committee approved all handling and experimental conditions.

Preparation of brain cortical slices

Rats were decapitated and the two cerebral hemispheres were used for preparation of coronal slices (0.4 mm) from the parietal cortical area using a McIlwain tissue chopper.

Determination of lipid peroxidation

Lipid peroxidation was measured in the cortical slices by detection of TBA-reactive substances, according to previous reports (Santamaría et al. 1997).

The slices (5 slices per tube) were pre-incubated in a PBS buffer in the presence or absence of MeHgCl (100 μM) and three concentrations (5, 10 or 25 μg/mL) of each flavonoids (quercetin, quercitrin and rutin) were tested. The time of pre-incubation was 2 h. After exposure to the pre-incubation conditions, the slices were removed from the media and homogenized in PBS buffer. The homogenates were used for measurement of peroxidation by addition of 2 mL of the color reagent (0.375 g of thiobarbituric acid (TBA) + 7.5 g of trichloroacetic acid + 2.5 mL of HCl in 100 mL of water). The final solution was heated in a boiling water bath for 30 min. After cooling the samples on ice, they were centrifuged at 3,000*g* for 15 min, and the absorbance of the respective supernatants was measured spectrophotometerically at 532 nm. The results were calculated as nmol of thiobarbituric reactive substances (TBARS) per mg of protein and expressed as percentage of control (unstimulated).

Determination of ROS formation in slices

The levels of ROS in cortical slices were measured by the oxidation of 2,7-dichlorofluorescein diacetate (DCFHDA) (Wang and Joseph 1999). Slices were maintained in ice in a PBS medium containing (in mM) 10 glucose, 124 NaCl, 10 $NaHPO_4$, 5 NaH_2PO_4, 5 KH_2PO_4, pH 7.4. The slices were pre-incubated in the PBS buffer for 10 min containing DCFHDA (5 μM) and then exposed to flavonoids and 100 μM MeHg for 2 h. To quantify the extracellular ROS level, an aliquot (400 μL) of the incubation medium was mixed with 2.1 mL of buffer and the formation of the oxidized fluorescent derivative 2′,7′-dichlorofluorescein (DCF) was monitored using excitation and emission wavelengths of 488 and 525 nm, respectively (fluorescence spectrophotometer, Hitachi F-2000). For quantification of intracellular ROS, the slices were washed three times in ice-cold buffer and homogenized in a PBS buffer. Aliquots of 400 μL were mixed with 2.1 mL of PBS buffer for fluorescence quantification. The results were expressed as percentage of control.

Isolation of fresh rat brain mitochondria

Brain mitochondria were isolated as previously described by Brustovetsky and Dubinsky (2000) with minor modifications. Wistar rats were killed by decapitation. The brains were rapidly removed and placed on ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA; free fatty acid) and 10 mM HEPES pH 7.2. The tissue was then homogenized and the resulting suspension centrifuged for 7 min at 2,000×*g*. Next, the supernatant was centrifuged for 10 min at 12,000×*g*. The pellet was resuspended in isolation buffer II containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 10 mM HEPES pH 7.2 and centrifuged at 12,000×*g* for 10 min. The supernatant was discard and the final pellet gently washed and resuspended in buffer III containing 50 μM of EGTA, 10 mM sucrose, 65 mM KCl and 10 mM HEPES, pH 7.2, to a protein concentration of 0.5 mg/mL.

Determination of ROS production in brain mitochondria

Experiments were carried out in a standard reaction medium containing 10 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 50 μM EGTA and 100 μg of mitochondrial protein. The DCFHDA (2 μM) was added to monitor ROS formation in the presence or absence of $CaCl_2$ (60 μM), which can induce ROS generation (Hansson et al. 2008). The formation of the oxidized fluorescent derivative (DCF) was monitored using excitation and emission wavelengths of 488 and 525 nm, respectively.

Protein estimation

Protein concentration was measured second method described by Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Statistical analysis

The results are expressed as means ± standard deviations and were analyzed by one-way analysis of variance (ANOVA). The Duncan's multiple range test was applied

post hoc to determine the significance of the difference between the various groups. Differences were considered statistically significant at $P < 0.05$. Data from DCFHDA oxidation were obtained directly from the spectrofluorimeter software and for sake of clarity part of the continuous reading data were not included in the statistical analysis.

Results

Lipid peroxidation in cortical brain slices

MeHg (100 μM) caused a statistically significant increase in lipid peroxidation when compared to the control (Fig. 2a–c). Quercitrin did not change basal levels of lipid peroxidation. However, it caused a statistically significant decrease in MeHg-induced lipid peroxidation at 25 μg/mL (Fig. 2a).

Under basal conditions, a concentration-dependent increase in TBARS production was observed after rutin treatment; however, the changes were statistically indistinguishable from the control group (Fig. 2b). Rutin also failed to protect against MeHg-induced oxidative stress (Fig. 2b).

Quercetin caused a significant reduction both in the basal and in the MeHg-induced TBARS levels in a concentration-dependent manner (Fig. 2c).

ROS production in cortical brain slices

MeHg (100 μM) had no significant effect on DCFHDA oxidation (ROS production; $P = 0.063$) in the supernatant of the incubation medium (data not shown). Quercitrin, rutin or quercetin did not modify DCFHDA oxidation in the slices' incubation medium in the presence or absence of MeHg (data not shown).

In contrast to the results obtained with the incubation medium, MeHg caused a significant increase (~60%) in DCFHDA oxidation (ROS production) in brain cortical slices (Fig. 3a–c). Quercitrin and quercetin counteracted the pro-oxidant effect of MeHg (Fig. 3a and c, respectively). Quercitrin caused a significant protective effect against MeHg-induced ROS generation at the highest concentrations (25 μg/mL) and quercetin caused a decrease in ROS production at all tested concentrations (Fig. 3a and c, respectively). Rutin did not attenuate the pro-oxidant effects of MeHg, levels remaining statistically indistinguishable from cortical slices treated with MeHg alone (Fig. 3b).

ROS production in brain mitochondria

Mitochondrial oxidation of DCFHDA was markedly stimulated by calcium (Fig. 4a–c). MeHg (5 μM) alone (Fig. 4a–c) did not cause an increase in ROS production when compared to the control. However, when MeHg was added in the presence of calcium (60 μM) (Fig. 4a–c), it caused a rapid increase in ROS production that was followed by a reduction in the rate of DCFHDA oxidation.

Quercetin (10 μg/mL) by itself caused a decrease in the rate of DCFHDA oxidation and significantly counteracted the pro-oxidant effect of calcium and that of calcium plus MeHg (Fig. 4c).

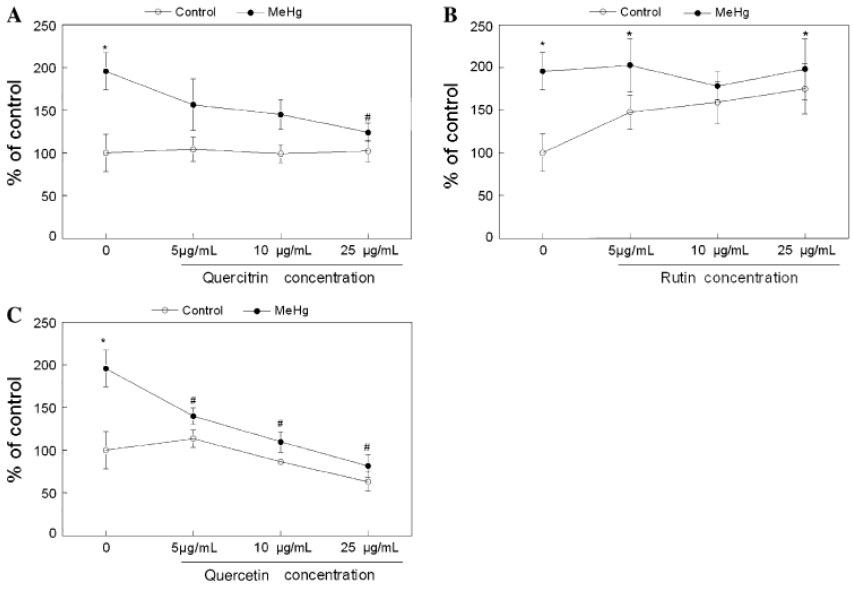


Fig. 2 Effect of different concentrations of flavonoids on MeHg (100 μM)-induced TBARS production in cortical slices. The slices were incubated for 2 h with MeHg in the presence or absence of quercitrin (**a**), rutin (**b**) and quercetin (**c**). Data are expressed as means ± SEM ($n = 4$ independent experiments performed in duplicates).*Represent a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

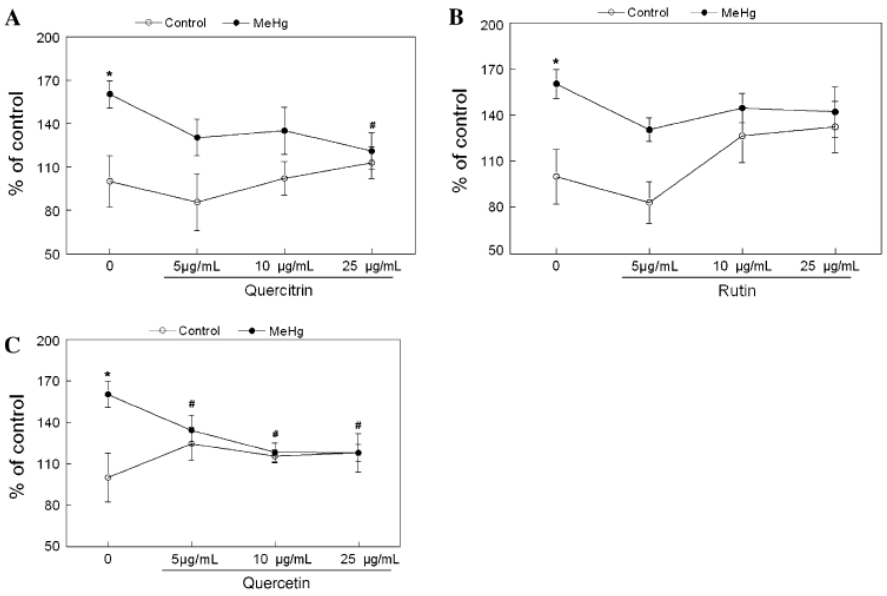


Fig. 3 Effects of the quercitrin (**a**), rutin (**b**), and quercetin (**c**) on MeHg-induced ROS generation in cortical slices. The brain cortical slices were incubated with DCFHDA in the presence or absence of MeHg (100 µM). Data are expressed as mean ± SEM and are calculated as percent control for five independent assays. *Statistically different from control; #statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

Quercitrin (10 µg/mL) had a partial protective effect against calcium-induced ROS formation and also protected against calcium plus MeHg-induced DCFHDA oxidation. In the absence of Ca^{2+}, quercitrin reduced the oxidation of DCFHDA determined in the presence of MeHg (Fig. 4a).

Rutin (10 µg/mL) did not afford a significant protective effect against MeHg-generated ROS formation either under basal condition or in the presence of calcium ion. (Fig. 4b).

In order to establish whether the rapid burst of DCFHDA oxidation was a consequence of fast Ca^{2+} entry into mitochondria and to exclude a possible inhibition of esterases by MeHg in the presence of Ca^{2+} (i.e., the intense increase in fluorescence that was followed by a significant slowdown in the rate of DCFHDA oxidation), we tested the effect of the calcium ionophore (A23187) on DCFHDA oxidation (Fig. 5). Addition of the calcium ionophore generated a trace that is similarly shaped to that observed after addition of MeHg to a medium containing Ca^{2+}(Fig. 4 and supplementary material), i.e., it produced an intense stimulation in the rate of DCFHDA oxidation that was followed by a significant slowdown in the DCFHDA oxidation. In the absence of Ca^{2+}, A23187 caused no change in the rate of DCFDA oxidation (data not shown), which indicated that DCFDA oxidation is Ca^{2+}-dependent.

Discussion

In agreement with earlier reports (Allen et al. 2001; Aschner et al. 2007; Shanker and Aschner 2003), we have established that MeHg causes increased lipid peroxidation and DCFHDA oxidation in brain rat cortical slices (Figs. 2, 3). MeHg-induced lipid peroxidation has been invoked to occur secondarily to elevations in intracellular Ca^{2+} concentrations (Denny et al. 1993; Komulainen and Bondy 1987), which can trigger the generation of ROS (Hansson et al. 2008). Here, we have obtained novel evidence in support of the critical role for Ca^{2+} in MeHg-induced ROS production in mitochondria. Our data established that MeHg increase the rate of DCFHDA oxidation only in the presence of Ca^{2+} (Fig. 4) suggesting that fast entry of Ca^{2+} into mitochondria is a primary event and a prerequisite for MeHg-induced ROS formation. In addition, MeHg has been shown to inhibit astrocyte glutamate transporter function resulting in increases glutamate concentrations in the extracellular fluid. The ensuing activation of NMDA (*N*-methyl D-aspartate) glutamate receptors leads to increased Na^+ and Ca^{2+} influx into neurons (Choi 1992). Accordingly, MeHg-induced lipid peroxidation likely reflects over-stimulation of the glutamatergic system with sustained elevation in intracellular free Ca^{2+} levels (Limke et al. 2004; Marty and Atchison 1997).

The exposure of cortical slices to the flavonoids, quercetin and quercitrin (Fig. 1), was associated with a protective effect against lipid peroxidation induced by MeHg (Fig. 2). These results are analogous to those obtained for quercetin in isolated brain mitochondria (Fig. 4), and this protective effect is correlated with the capacity of this flavonoid to detoxify the H_2O_2 generated in the presence of mercurials (Franco et al. 2007). In agreement with this data, flavonoids have been widely reported as effective scavengers of H_2O_2 (Cai et al. 1997). Furthermore, myrecitryn, a flavonoid structurally related to quercetin and quercitrin, has been shown to block Ca^{2+} influx into brain slices (Meotti et al.

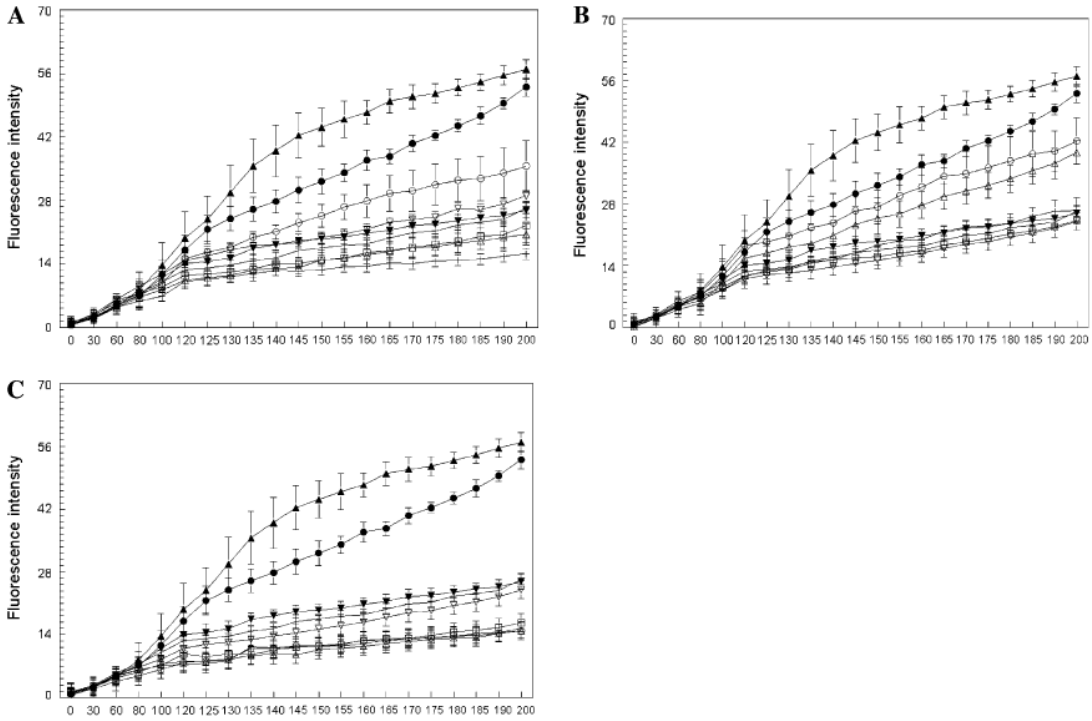


Fig. 4 **a** Effects of quercitrin on brain mitochondrial DCFHDA oxidation: MeHg and Ca^{2+} interactions. Mitochondria were incubated in a medium containing 50 µM EGTA 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2. The symbols represent the following: (*solid line*) control, (*filled circle*) $CaCl_2$ (60 µM), (*filled inverted triangle*) MeHg (5 µM), (*filled triangle*)$CaCl_2$ (60 µM) plus MeHg (5 µM), (*open square*) quercitrin (10 µg/mL), (*open inverted triangle*) ethanol (0.2%), (*dagger*) quercitrin (10 µg/mL) plus MeHg (5 µM), (*open circle*) quercitrin (10 µg/mL) plus MeHg (5 µM) plus $CaCl_2$ (60 µM) and (*open triangle*) quercitrin (10 µg/mL) plus $CaCl_2$ (60 µM). $CaCl_2$, MeHg and quercitrin were added at 50, 70 and 120 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation. **b** Effects of rutin on brain mitochondrial DCFHDA oxidation: MeHg and Ca^{2+} interactions. Mitochondria were incubated in a medium containing 50 µM EGTA 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2 The symbols represent the following: (*solid line*) control, (*filled circle*) $CaCl_2$ (60 µM), (*filled inverted triangle*) MeHg (5 µM), (*filled triangle*) $CaCl_2$ (60 µM) plus MeHg (5 µM), (*open square*) rutin (10 µg/mL), (*open inverted triangle*) ethanol (0.2%), (*dagger*) rutin (10 µg/mL) plus MeHg (5 µM), (*open circle*) rutin (10 µg/mL) plus MeHg (5 µM) plus $CaCl_2$ (60 µM) and (*open triangle*) rutin (10 µg/mL) plus $CaCl_2$ (60 µM). $CaCl_2$, MeHg and rutin were added at 50, 70 and 120 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation. **c** Effects of quercetin on brain mitochondrial DCFHDA oxidation: MeHg and Ca^{2+} interactions. Mitochondria were incubated in a medium containing 50 µM EGTA, 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2. Data were obtained in a Simatuzu Spectrofluorimeter (readings were made at every 0.1, i.e., continuous traces were obtained, but for sake of clarity an interval of 30 s was used for statistical data analysis. The symbols represent the following: (*solid line*) control, (*filled circle*) $CaCl_2$ (60 µM), (*filled inverted triangle*) MeHg (5 µM), (*filled triangle*) $CaCl_2$ (60 µM) plus MeHg (5 µM), (*open square*) quercetin (10 µg/mL), (*open inverted triangle*) ethanol (0.2%), (*dagger*) quercetin (10 µg/mL) plus MeHg (5 µM), (*open circle*) quercetin (10 µg/mL) plus MeHg (5 µM) plus $CaCl_2$ (60 µM) and (*open triangle*) quercetin (10 µg/mL) plus $CaCl_2$ (60 µM). $CaCl_2$, MeHg and quercetin were added at 50, 70 and 120 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation

2007). Thus, in addition to a direct interaction with H_2O_2, flavonoids could reduce H_2O_2 production via inhibition of Ca^{2+} influx into brain slices or mitochondria. Flavonoids may also form redox inactive complexes with iron (Fe), rendering this pro-oxidant unavailable for Fenton reaction. Corroborating this hypothesis, both quercitrin and quercetin effectively block Fe-induced TBARS production in brain homogenates (Pereira et al. 2009; Wagner et al. 2006).

In contrast to quercetin and quercitrin, the third flavonoid tested, rutin, failed to protect against MeHg-induced lipid peroxidation. The antioxidant activities of flavonoids are influenced by their chemical structure (Rice-Evans et al. 1996). A comparison of quercetin with rutin (Fig. 1) indicates the possible influence of the 3-OH in combination with the adjacent double bond in the C ring. If one is dispensed with, the other apparently loses its impact on the antioxidant activity (Rice-Evans et al. 1996). Thus, the absence or low antioxidant effect of rutin is possibly related to its structure. Moreover, the glycoside group present in rutin increases its hydrophilicity (Saija et al. 1995), thus

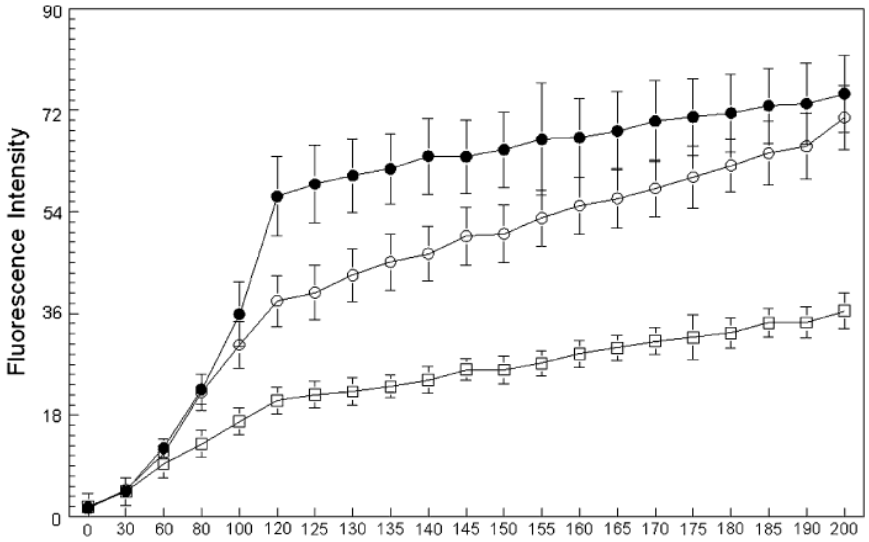


Fig. 5 Effects of the calcium ionophore, A23187, on DCFADA oxidation. Mitochondria were incubated in a medium containing 50 µM EGTA 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2. The symbols represent the following: (*open square*) control, (*open circle*) $CaCl_2$ (60 µM), (*filled circle*) $CaCl_2$ (60 µM) plus ionophore. $CaCl_2$ and ionophore was added at 60 and 80 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation

decreasing its permeability across membranes. As shown, rutin failed to protect both the cortical slices and the brain mitochondria from MeHg-induced ROS generation (Figs. 3, 4). Rutin also tended to exhibit a pro-oxidant effect by itself (Fig. 2b), which is consistent with earlier data from literature (Cotelle 2001).

In contrast, quercetin and quercitrin were protective against MeHg-induced ROS production (Figs. 2, 3, 4). This effect likely reflects the presence of the *o*-dihydroxy group in the B ring of their structures (Fig. 1). This confers higher stability to the radical form and participates in electron delocalization; the 2,3 double bond in conjugation with a 4-oxo bond in the C ring are responsible for electron delocalization from the B ring. Thus, the antioxidant potency reflects electron delocalization of the aromatic nucleus. When these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus and the 3- and 5-OH groups with 4-oxo function in the A and C rings are required for maximal radical scavenging potential (Rice-Evans et al. 1996).

ROS cause cell injury by compromising the integrity of cell membrane, proteins and by cleaving the DNA (Valko et al. 2005). Moreover, ROS impair mitochondrial energy metabolism by inducing oxidative structural changes and the ensuing loss of activity in a number of mitochondrial enzymes that play critical roles in ATP production (Fiskum et al. 2004). Additionally, the direct action of ROS on mitochondrial membrane lipids and proteins results in the activation of apoptotic cascades, through both opening of the mPTP (mitochondrial permeability transition pore) and mPTP-independent mechanisms (Lifshitz et al. 2004). Accordingly, an important objective of the present study was to better understand the pro-oxidant effect of MeHg. As a first step to achieve this goal, we investigated the effect of MeHg on ROS generation in freshly isolated brain mitochondria and mitochondria isolated from brain cortical slices. MeHg alone did not cause an increase in ROS production in brain mitochondria, but in the presence of Ca^{2+} it led to an over-stimulation in ROS production (Fig. 4). Notably, we found the same effects with the Ca^{2+} ionophore, A23187. In fact, the responses to MeHg and to the ionophore in the presence of Ca^{2+} were qualitatively similar (compare Figs. 4, 5 and supplementary material). Dubinsky and Levi (1998) have demonstrated that in the presence of an ionophore, large Ca^{2+} loads lead to immediate mitochondrial depolarization and Ca^{2+} sequestration. These observations are consistent with our hypotheses that both the Ca^{2+} ionophore, A23187 and MeHg lead to rapid and massive increase in mitochondrial Ca^{2+} influx, which secondarily triggers the over-stimulation of ROS production. The latter, in turn, facilitates mPT induction, loss of the electron transport chain and the ensuing mitochondrial death.

In summary, our results establish that MeHg caused lipid peroxidation and ROS generation in mitochondria and brain slices. The flavonoids quercetin and quercitrin afforded protective effects with the following rank order: quercetin > quercitrin. Rutin failed to attenuate MeHg-induced ROS formation. Our results establish that quercetin and quercitrin offer possible therapeutic potential in MeHg toxicity; however, more in vivo experiments are needed to validate the possible use of these flavonoids against MeHg-induced injuries, particularly in view of the recently published synergistic toxic effect of quercetin and MeHg in adult mice.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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4.1.2 Manuscrito 1

Quercitrina atenua os efeitos neurotóxicos causados pela exposição *in vivo* ao MeHg

Quercitrin attenuates the neurotoxic effect caused by *in vivo* exposure to MeHg.

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Quercitrin attenuate the neurotoxic effect caused by *in vivo* exposure to MeHg.

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ABSTRACT

Exposure to methymercury (MeHg) can cause irreversible neurobehavioral and neuropsychological disorders in humans and animals. The major mechanisms of MeHg-induced neurotoxicity currently being explored are the disruption of intracellular calcium homeostasis, induction of oxidative stress, depletion of endogenous thiols pool, inhibition of neuronal Na^+/K^+ -ATPase and negative modulation of antioxidant enzymes. Flavonoids can counteract the in vitro lipid peroxidation and ROS production induced by MeHg. However, data about the potential in vivo neuroprotective effects of flavonoids against MeHg-induced toxicity are scarce and, recently, it was demonstrated that quercitrin caused an exacerbation of MeHg neurotoxicity (Martins et al. 2009, Food Chem. Toxicol. 47, 645). In view of the apparent discrepancy between in vitro and in vivo data, here we evaluated the potential neuroprotective effect of quercitrin (10mg/kg) against MeHg. Mice were treated during 30 days with MeHg (5mg/kg, p.o.) and co-treated with quercitrin (10mg/kg, i.p.). MeHg reduced body weight gain, increased micronucleos frequencies, caused nefrotoxicity, impaired motor performance (locomotor activity and motor coordination), disrupt spatial memory deficiency and altered biochemical endpoints of toxicity (i.e., it caused a decrease in glutathione peroxidase and Na^+/K^+ ATPase activity and an increase in lipid peroxidation). The co-treatment with quercitrin mitigated some of the toxic consequences of MeHg exposure. This study suggest that quercitrin can protect against MeHg neurotoxicity possibly by its antioxidant activity. The *in vivo* neuroprotective effects of quercitrin described here are at variance with the recent study of Matins et al (2009) which indicated a synergic neurotoxic interaction between MeHg and quercitin.

INTRODUCTION

Methylmercury (MeHg, methylated form of mercury) is a highly lipophilic and deleterious environmental contaminant (Clarkson, 1993) that can be bioaccumulated in piscivorous fish and aquatic mammals (Clarkson et al., 2003). Consequently, consumption of contaminated fish can represent an important source of human exposure to MeHg.

MeHg can cause irreversible neurobehavioral and neuropsychological disorders in humans and animals (Gilbert and Grant-Webster, 1995; Rice and Barone, 2000). Consistent with these observations, various studies have reported that brain is particularly vulnerable to MeHg toxicity after high-dose exposure (0.5–11 ppm or total dose of 0.4–50 mg), which can cause abnormal walking ability, motor deficits (impaired rota-rod performance), hearing loss and pathological changes in the cerebellum (Choi et al., 1981; Franco et al., 2006, Goulet et al., 2003; Rice and Gilbert 1992; Roegge et al., 2004; Sakamoto et al., 2002).

The major mechanisms of MeHg-induced neurotoxicity currently being explored are the disruption of intracellular calcium homeostasis, induction of oxidative stress which can be related, at least in part, to mercury high affinity for endogenous thiols, inhibition of neuronal Na^+/K^+-ATPase (Anner and Moosmayer, 1992; Sirois and Atchison, 2000; Yee and Choi, 1996) and negative modulation on antioxidant enzymes (Aschner et al. 2007; Farina et al., 2003; Farina 2005; Vicente et al., 2004; Wataha e cols., 2008). Oxidative stress, associated with MeHg exposure can contribute for its neurotoxicity and can trigger cell death or tissue injury within the central and peripheral nervous system (Inoue et al., 2004).

Flavonoids possess metal chelating properties, antioxidant and anti-inflammatory activities, readily permeate the blood–brain barrier (BBB) and can afford neuroprotection in a wide array of cellular and animal models of neurological diseases (Mande et al. 2006; Youdim et al. 2004; Wagner et al., 2006 and 2010). Consistent with these observations, several studies have shown the antioxidant effects of quercetin and its derivative compounds against pro-oxidative damage

(Wagner et al., 2006; Meotti et al., 2007), including metal-induced toxicity (Mishra and Flora, 2008; Wagner et al. 2010).

In relation to MeHg toxicity, literature data have indicated that quercetin counteracted the lipid peroxidation and ROS production induced in vitro by MeHg (Franco et al., 2007; Wagner et al. 2010). In addition, similar protection was observed with its glycoside derivative, quercitrin (Wagner et al, 2010). In sharp contrast with *in vitro* data, Martins et al. (2009) have demonstrated that quercetin had synergistic neurotoxic effects with MeHg after *in vivo* exposure in adult mice. In addition a number of *in vitro* controversial results of protective or toxic effect of quercetin (and its metabolites and glycoside forms) can be found in the literature (Ossola et al., 2009;Vafeiadou et al. 2008). Therefore, in view of the limited number of *in vivo* studies about the interaction of flavonoids with mercurial, the main objective of this study was to evaluate the potencial protective effect of quercitrin (a glycoside form of quercetin) against MeHg-induced toxicity.

MATERIALS AND METHODS

Animals and Treatment

Adult (2-months-old) male mice (Swiss albino) obtained from the Central Biotery (UFSM,Santa Maria, Brazil) were maintained at 25ºC on a 12:12 h light/dark cycle, with free access to food. All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology. Mice were divided into fourth experimental groups (n=6 per group): control (DMSO 1%), quercitrin (10 mg/kg), MeHg (5mg/Kg), MeHg + quercitrin. The flavonoid diluted in DMSO (1%) was administrated daily by intraperitonial (i.p.) injections and MeHg was diluted in water and administrated daily by oral gavages. Animals were exposed to MeHg and treated with quercitrin during 30 consecutive days.

MeHg doses were based in previous studies (Martins et al.2009; De Freitas et al. 2009). Dimethylsulfoxide (DMSO) was used as vehicle because quercitrin is hydrophobic and has no toxicity at 1 mL/kg body weight (Farina et al., 2003).

Behavioral test

Along the treatment animals were subjected to behavioral/ functional tests for evaluating locomotor activity (open field), coordination (rota road) and spatial memory (plus maze tasks).

Open-field tests were performed on days 4, 16 and 30 in an isolated facility with no interference noise or human activity. An open-field test was carried out as described by Kim et al. (2000) in a box [56 (long) _ 42 (wide) _ 40 cm (high)] with the floor divided into 12 squares. Each mouse was transferred from its home cage directly to the open field and observed for 2 min.Locomotor activity was determined by the number of crossing (squares crossed with the 4 paws) and rearing responses. The time spent in the central area was also quantified. After the open-field test, mice were subjected to the rotarod task (Dunham and Miya (1957)). The bar rotated at a constant speed of 21 rpm and each mouse was subjected to three

different trials with 3 min of interval between them. Mean of falling latency and the number of falls values were recorded.

The elevated plus maze was used to evaluate spatial memory, following the procedure described earlier by Reddy and Kulkarni (1998). On the day before testing (days 4, 14 and 29), each animal was submited to training. The animal was placed at the end of open arm of the elevated plus maze and the transfer latency (TL), the time taken by the rat to move into one of the closed arms, was recorded on the training day. If the animal did not enter a closed arm within 90 s, it was gently pushed into one of the closed arm and the TL latency assigned as 90 s. The rat was allowed to explore the maze for 20 s and then returned to the home cage. Rats were placed again on the maze 24 h after the first exposure (days 5, 15 and 30) and TL was recorded.

Tissue preparation for biochemical analyses

After the last behavioral test, animals were killed by decapitation and cerebellum, brain and kidney were removed and homogenized in tris – HCl 10mM, pH 7.4. Tissue homogenates were centrifuged at 2500g for 10 min and the supernatants were used for biochemical analyses. The total blood was used for micronucleus test analysis.

Protein and nonprotein thiols levels

Protein and nonprotein thiol was measured based on Ellman (1959) with minor modifications. For protein thiol quantification, cerebellum, brain and kidney supernatants were incubated with DTNB in a 0.5M phosphate buffer, pH 8.0 and the absorbances were measured at 412 nm. For nonprotein thiol quantificantion, the supernatants were first precipitated with cold 10 % trichloroacetic acid and then centrifuged at 15,000g for 2 min. The clear supernant fraction was incubated with DTNB using the same conditions described above for protein thiol quantification.

Lipid peroxidation

Lipid peroxidation (LPO) was measured as thiobarbituric acid reactive substances (TBARS) based on Ohkawa et al. (1979). Briefly, samples were incubated in a reaction media containing 0.28% 2-thiobarbituric acid (TBA), 1.2% SDS, and 0.45 M/0.12 M acetic acid/HCl buffer (pH 3.4). After that, samples were incubated at 95 ºC for 60 min for color development. TBARS were measured at 532 nm and compared to a standard curve of malondialdehyde (MDA).

Na^+/K^+ATPase activity

The reaction mixture for Na^+/K^+-ATPase assay contained 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, and 50 mM Tris–HCl, pH 7.4, in the presence or absence of 0.1 mM of ouabain, was pre-incubated at 37ºC for 20 min with an aliquot of 50 µL of brain or cerebellum supernatant. The reaction was started by adding ATP to a final concentration of 3mM. Na^+/K^+ATPase activity was calculated by the difference between the activity determined in the absence to that determined in the presence of oubain. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow (1922).

Estimation of reactive species production

Formation of reactive species was estimated according to a previous report (Ali et al., 1992) with minor modifications. Aliquots of 20µL were incubated in tris-HCl buffer (pH 7.4) in the presence of 5 µM 2,7-dichlorofluorescein diacetate at 37 ºC for 60 min in the dark. Fluorescent signals were recorded at the end of the incubation at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Results were expressed as fluorescence units.

Superoxide dismutase activity.

Superoxide dismutase activity was performed according to the method of Misra and Fridovich (1972). Briefly, epinephrine rapidly autooxidizes at pH 10.5 producing adrenochrome, a pinkcoloured product that can be detected at 480 nm. The addition of samples (10, 20 and 30µL) containing superoxide dismutase inhibits the autooxidation of epinephrine. The rate of inhibition was monitored during 180 seconds at intervals of 30 sec. The amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity (UI).

Glutathione Peroxidase activity

Renal, cerebral and cerebelar GPx activity was assayed spectrophotometrically by the method of Wendel (1981), using the glutathione (GSH) – NADPH–glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. Supernatant was added to the GSH–NADPH–glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity is indirectly measured by means of NADPH oxidation. H_2O_2 is decomposed, generating GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase present in the assay media at the expenses of NADPH.

Mutagenic tests

Micronucleus test

Two whole blood smears from each animal were made on pre-cleaned microscope slides, air dried at room temperature and stained in 5% May-Grunwald-Giemsa in buffered water for 5 min.

The criteria used for the identification of MN were their size smaller than one-third of the main nucleus, no attachment with the main nucleus, same color and intensity as the main nucleus, and so forth. The MN frequency was calculated as: %

MN= Number of cells containing micronucleus X100 / Total number of cells counted. This percentage was based on 2000 cells counted.

Micronuclei presence was determined by three investigators that were blind to the treatment of the animals using an optical microscopy at a magnification of 1000×.

Statistical analyses

The results are expressed as means ± standard error of means and were analyzed by one-way or two-way analysis of variance (ANOVA). The Duncan's multiple range tests were applied *post hoc* to determine the significance of the difference between the various groups. Differences were considered statistically significant at $p<0.05$.

RESULTS

Body weight gain

MeHg treatment (5mg/Kg) caused a significant decreased in body weight gain in comparison to control animals that received DMSO 1%. The differences to control became significant after 20 days of MeHg treatment and persisted thereafter (Table 1). The co–treatment with quercitrin (10mg/Kg) abolished the toxic effect of MeHg on body weight gain (Table 1). The body weight gain in animals trated with quercitrin alone was similar to control animals.

Locomotor activity

In the open-field test (table 2), MeHg caused significant decrease in locomotor activity (crossing was decreased after 16 and 30 days, whereas rearing was decreased after 30 days of MeHg treatment). For crossing, quercitrin co-treatment prevented MeHg-induced behavioral impairments and the total number of crossings of MeHg+quercitrin group was similar to that of control group.The same results was observed for quercitrin alone. In relation to number of rearing, quercitrin did not protect against MeHg neurobehavioral toxicity.

Motor coordination

MeHg decreased the time latency of animals into apparatus (this was statistically significant at 16 days and 30 days of treatment. Quercitrin co-treatment protected against this behavioral impairment and maintained the latency to fall similar to that of control mice both in day 16 and 30 of treatment. In relation to number of falls, MeHg treatment significantly increased the number of fall after 30 days of treatment and co-treatment with quercitrin protected against this effect (table 3).

Spatial memory

MeHg caused a significant impairment in spatial memory of mice determined 30 days of MeHg treatment. In fact, MeHg increased latency to escape from the open arm in the plus maze (table 4). The co-treatment with quercitrin caused a partial protection and the latency of MeHg+quercitrin group was smaller than that of MeHg group.

Biochemical analyses

Na^+/K^+ ATPase activity

MeHg treatment decreased significantly brain Na^+/K^+ ATPase activity in comparison to DMSO control (figure 1A). Quercitrin co-treatment protected against MeHg - induced enzyme inhibition. Quercitrin treatment alone, did not caused a significantly alteration in enzyme activity. In cerebellum (figure 1B), MeHg caused a non-significant decreased in Na^+/K^+ ATPase activity. Similarly, quercitrin or MeHg+quercitrin treatment did not altered enzyme activity.

Lipid peroxidation and ROS levels

Exposition of animals to MeHg caused an increase in brain LPO of mice (figure 2A). Co-treatment with quercitrin did not protect against MeHg-induced brain LPO. In constrast to brain, cerebellum LPO was not modified by MeHg, quecritrin or MeHg+quercitrin (figure 2B). In addition, when DCFA oxidation was employed as a marker of ROS production (figure 3), there was no significant alteration in its oxidation in brain or cerebellum of treated mice in relation to control group (figure 3 A and B).

Any alteration was observed in kidney LPO and DCFA oxidation (data not show).

Thiol content

Quercitrin produced a statistically significant decreased in brain protein thiol levels (figure 4 A). Brain non protein thiol levels was not modified by MeHg and/or quercitrin treatment (figure 4B). In kidney, non protein thiol was not modified by MeHg and/or quercitrin treatments, whereas MeHg produced a statistically significant decrease in protein thiol content and co-treatment with quercitrin protected against this decrease (data not show).

Antioxidant enzymes

SOD and GPx

Cerebral SOD activities were not altered by MeHg, quercitrin or combined treatment (figure 5).

Brain GPx activity tended to decrease after MeHg treatment, but the difference was not statistically significant (figure 6A). In cerebellum, GPx activity markedly inhibited by MeHg and co-treatment with quercitrin did not protect against MeHg-induced inhibition of GPx activity (figure 6B). In Kidney, MeHg caused a marked decreasein GPx activity and co-treatment with quercitrin effectively protected against MeHg-induced GPx inhibition (data not show).

Mutagenic tests

Micronucleus frequencies

MeHg treatment increased the frequency of micronucleus and quercitrin co-treatment caused a modest protective effect, decreasing the micronucleus frequencies in comparison to MeHg treatment (figure 7).

DISCUSSION

MeHg (5mg/kg) treatment for 30 days provoked neurotoxicity as determined by biochemical and behavioral alterations in mice and quercitrin (10mg/kg) treatment conferred neuroprotection against different endpoints of toxicity. In fact, MeHg promoted a decrease in body weight gain (table 1), a classical marker of toxicity. In addition MeHg treatment increased micronucleos frequency (figure 7) and changed some renal biochemistry parameters (data not show). These results indicate a general toxicity promoted by MeHg intoxication. In addition, quercitrin co-treatment could afford some protection against MeHg- induced toxicity in various of these parameters.

Numerous studies have documented the efficacy of MeHg as a neurotoxic agent in experimental animals. Indeed, MeHg exposure can cause severe movement or postural disorders, learning and memory dysfunction and rota-rod impairment (Goulet et al., 2003; O'Kusky et al., 1988; Sakamoto et al., 2002, 1993). In this study, animals treated with MeHg presented a decrease in locomotor activity and impairments of motor coordination (table 2 and 3). There is some evidence that cerebellar cells are selectively targeted by mercury compounds in vivo (Sanfeliu et al., 2003) and that MeHg neurotoxicity affects the motor system (Grandjean et al., 1997). Indeed, the relationship between MeHg-induced motor deficit and MeHg-induced cerebellar damage is a well-described process (Sakamoto et al., 1993). In this study, in addition to locomotors and coordinations impairments, MeHg caused marked decrease in cerebellar GPx activity (figure 6B), suggesting a possible involment of cerebellar injury in motor alteration caused by MeHg. Quercitrin co-treatment protected against motor coordination damage (table 3) and maintained locomotor activity of MeHg-treated mice similar to that of control group (table 2), but failed to protect against changes caused by MeHg exposure in rearing movements. Similarly, quercitrin failed to protect against MeHg – induced decreased in cerebellar GPx activity (figure 6B), suggesting that the absence of neurobehavioral protection by quercitrin unprotection is related with inability of quercitrin in maintained the cerebellar GPx levels.

Neuronal Na^+/K^+ATPase activity is crucial for the maintenance of Na^+ and K^+ gradients between the intra- and extra-cellular milieus and for maintaining neuronal membrane potential and excitability. Consequently, inhbition of Na^+/K^+ ATPase by MeHg, can disrupt membrane depolarization and can suppress neuronal function and excitatory transmission (Balestrino et al., 1999; Rajanna et al., 1990). Consistent with these findings, exposure to MeHg, can significantly induce *in vivo* and *in vitro* neurological disorders and which can be associated with the inhibition of Na^+/K^+ATPase activity in the brain (Cheng et al., 2005; Chuu et al., 2001). Here we have observed that MeHg treatment caused an inhibition in brain Na^+/K^+ATPase (figure1A), which can be associated with locomotor impairments and spatial memory deficits (table 4) caused by MeHg treatment. Additionally, quercitrin co-treatment protected Na^+/K^+ ATPase against MeHg inhibition, suggesting a mechanism via which quercitrin could afford behavioral protection against MeHg neurotoxicity.

In mammals, brain cells appeared to be more susceptible to oxidative injury triggered by MeHg which causes disruption of mitochondrial permeability, hampers intracellular antioxidant enzyme function and stimulates the formation of free radicals and LPO (Aschner et al., 2007; Cheng et al., 2005; de Freitas et al., 2009 Yee and Choi, 1996). From these findings, it can be infered that oxidative stress play an important role in the neurodegenerative processes associated with MeHg intoxication. Corroborating this, MeHg exposure can increase LPO (Huang et al., 2008), which can change brain biochemistry and function.In adittion the protective effect exerted by quercitrin against brain LPO can be one mechanism via which quercitrin promotes neuroprotection against pro-oxidant neurotoxicants.

In contrast to quercetin (Martins et al., 2009), which had synergic neurotoxic effects with MeHg, its glycoside form of quercitrin had no over toxic effects in adult mice and could protect against MeHg neurotoxicity. We realize that the molecular structure of quercitrin (i.e., presence of glucose residue in the C ring of quercitin) confered less toxicity to quercetin.

Despite massive efforts in search for new drugs that could counteract mercurial toxicity, there are no effective treatments available that completely abolish its toxic effects. In MeHg poisoning, supportive care is given when necessary to maintain

vital functions. In addition, the use of chelating agents assists the body's ability to eliminate mercury from the tissues (Pingree et al., 2001; Carvalho et al., 2007). However, these drugs appear to be of limited use, because of their adverse side effects (Tchounwou et al., 2003) and limited ability to cross the blood-brain barrier (Aposhian et al., 1995). Therefore new compounds that can protect against mercury toxicity, and have, that present reduced side effects, such as quercitrin can be considered promissig neuroprotective agents and can impart new insights in the development of new drugs for treatment of mercury toxicity.

In conclusion MeHg caused motor impairment and spatial memory deficits that can be related with cerebral decrease in GPx and Na^+/K^+ ATPase activities and LPO production. Quercitrin (10mg/Kg) co-treatment confered neuroprotection mainly by conteracting the oxidative stress caused by MeHg, and by protecting Na^+/K^+ ATPase from inactivation by MeHg.

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RESULTS

Table 1: Effects of MeHg and quercitrin treatment on the body weight gain of mice.

	Body weight (g)			
Treatment	Day 4	Day 12	Day 20	Day 30
Control	29.7 ± 1.9	33.3 ± 1.4	35.2 ± 1.4	36.3 ± 1.4
MeHg	29.2 ± 1.6	26.7 ± 5.5	29.6 ± 1.7 *	27.8 ± 2.8*
Quercetrin	29.2 ± 1.7	33.3 ± 2.0	35.5 ± 1.7	37.0 ± 2.3
MeHg + Quercitrin	30.5 ± 1.6	32.7 ± 2.0	35.4 ± 1.9 #	38.0 ± 1.7#

Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by two-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

Table 2: Effects of MeHg and quercitrin treatment on locomotor activity evaluated in the open field test.

	Rearing (nº)			**Crossing (nº)**		
Treatment	Day 4	Day 16	Day 30	Day 4	Day 16	Day 30
Control	11.7 ± 2.5	6.5 ± 3.3	5.5 ± 1.1	33.3 ± 5.2	16.3 ± 3.2	15.5 ± 1.9
MeHg	8.0 ± 1.9	2.0 ± 0.8	0.6 ± 0.4 *	24.8 ± 5.1	4.4 ± 1.3 *	3.5 ± 2.2 *
Quercetrin	8.3 ± 2.5	5.8 ± 1.7	4.3 ± 1.5	23.0 ± 5.8	14.7± 3.9	13.8 ± 2.1
MeHg + Quercitrin	8.5 ± 0.8	0.5 ± 0.3 *	1.4 ± 0.7*	30.2 ± 1.8	5.8 ± 1.5 *	12.6 ± 4.0 #

Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

Table 3 Effect of MeHg and quercitrin treatment on motor coordination test performed in rota ro.d apparatus in different days of treatment (4, 16 and 30).

	Nº of fall			**Latency (seconds)**		
Treatment	Day 4	Day 16	Day 30	Day 4	Day 16	Day 30
Control	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	57.7 ± 5.2	57.6 ± 1.5	57.5 ± 1.6
MeHg	3.3 ± 0.8*	0.9 ± 0.4	6.7 ± 2.1*	18.3 ± 4.7*	35.2 ± 8.3*	17.7 ± 10.8*
Quercetrin	3.4 ± 0.8*	0.9 ± 0.2	1.0 ± 0.4	28.6 ± 7.0*	44.8± 2.4	46.9 ± 6.7
MeHg + Quercitrin	4.2 ± 1.0*	0.5 ± 0.4	1.1 ± 0.5 #	19.3 ± 9.3 *	52.7 ± 5.7#	42.5 ± 8.1#

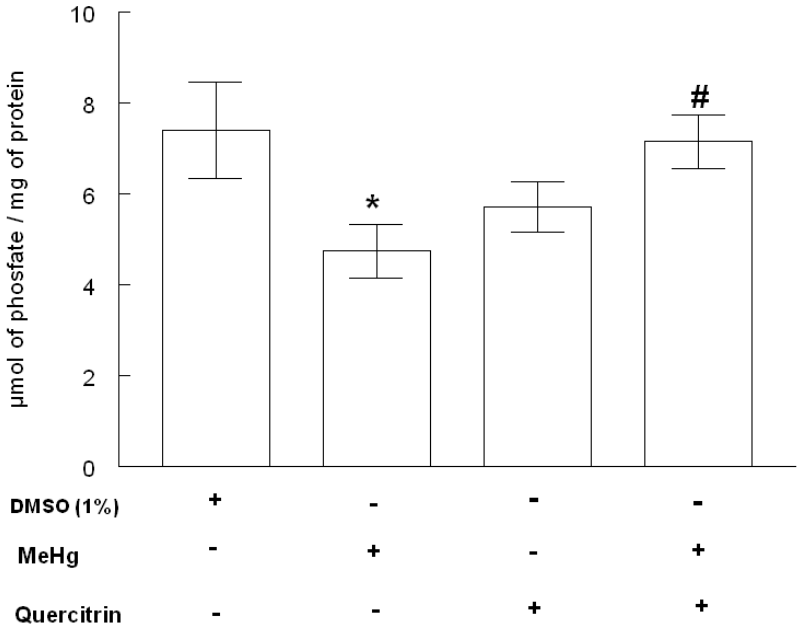
Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by two-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

Table 4: Effect of MeHg and quercitrin treatment on spatial memory test performed in elevated plus maze apparatus in different days of treatment (5, 15 and 30).

	Time latency (seconds)		
Treatment	5	15	30
Control	42.2 ± 15.7	37.2 ± 9.7	20.8 ± 3.8
MeHg	44.3 ± 9.8	56.0 ± 17.2	64.0 ± 16.2 *
Quercetrin	31.7 ± 9.1	29.3 ± 6.7	21.5 ± 4.8
MeHg + Quercitrin	35.2 ± 1.8	49.7 ± 3.2	44.0 ± 2.8 #

Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by two-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

A


µmol of phosfate / mg of protein
10
8
6
4
2
0
*
#
DMSO (1%) + - - -
MeHg - + - +
Quercitrin - - + +

B

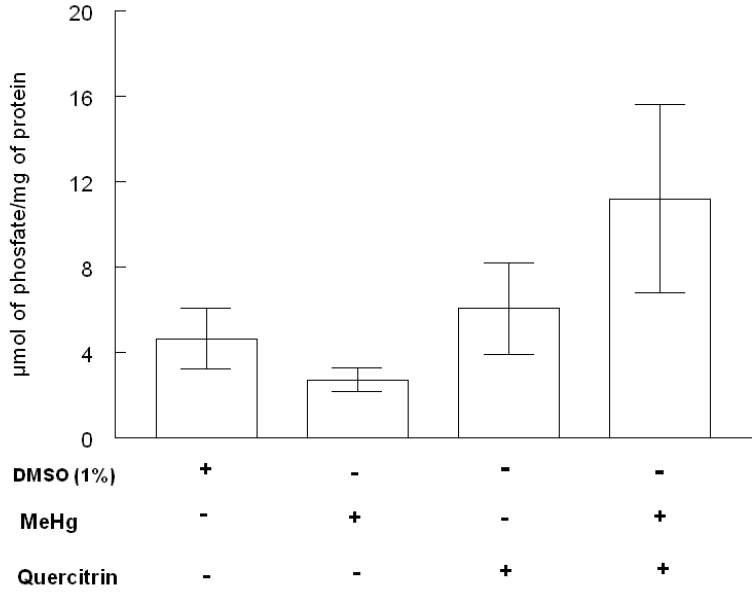


Figure 1: Na^+/K^+ATPase activity in cerebrum (a) cerebellum (b) of mice treated with MeHg (5mg/Kg) and quercitrin (10mg/Kg) for 30 days. Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p< 0.05$ were considered statistically significant).

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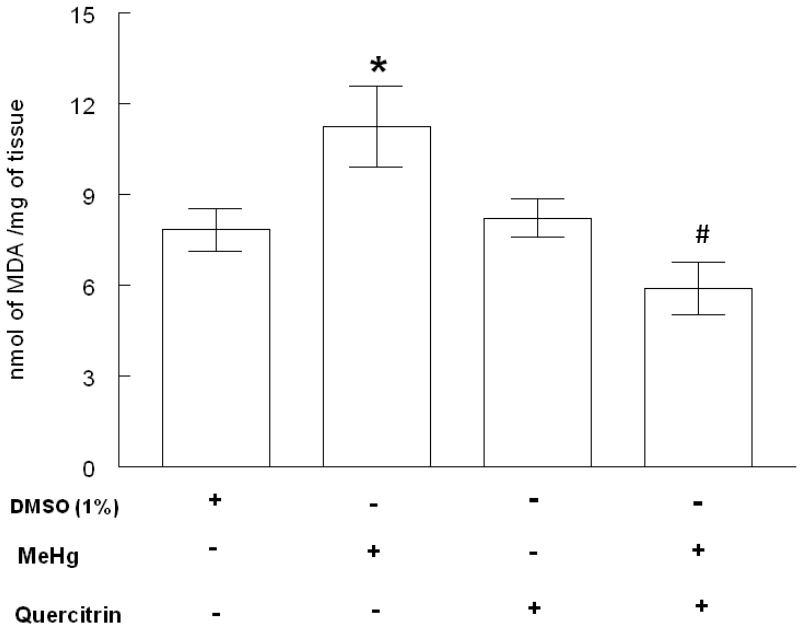

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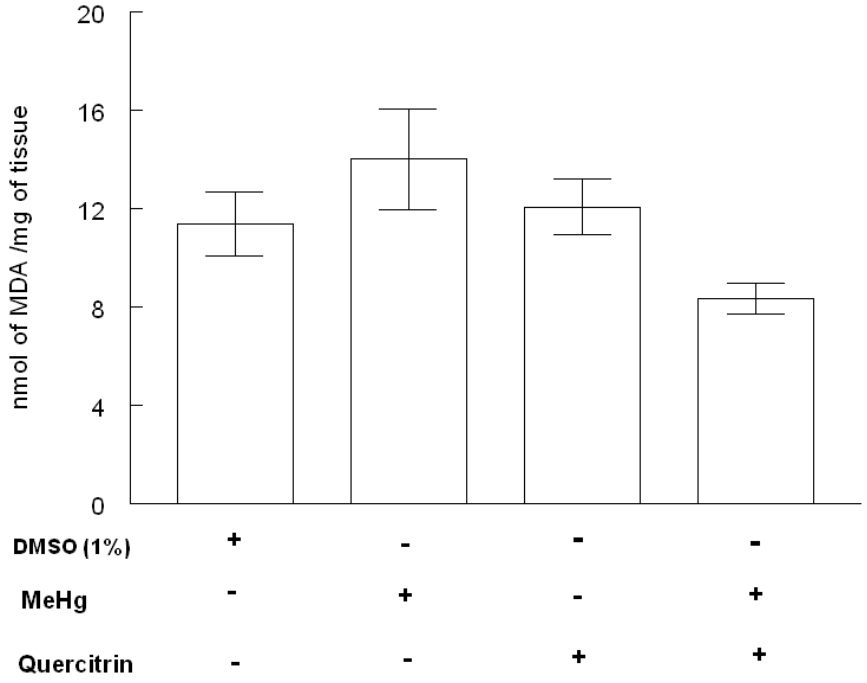

Figure 2: Lipid peroxidation in cerebrum (a) and cerebelum (b) of mice treated with MeHg (5mg/Kg) and quercitrin (10mg/Kg) for 30 days. Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

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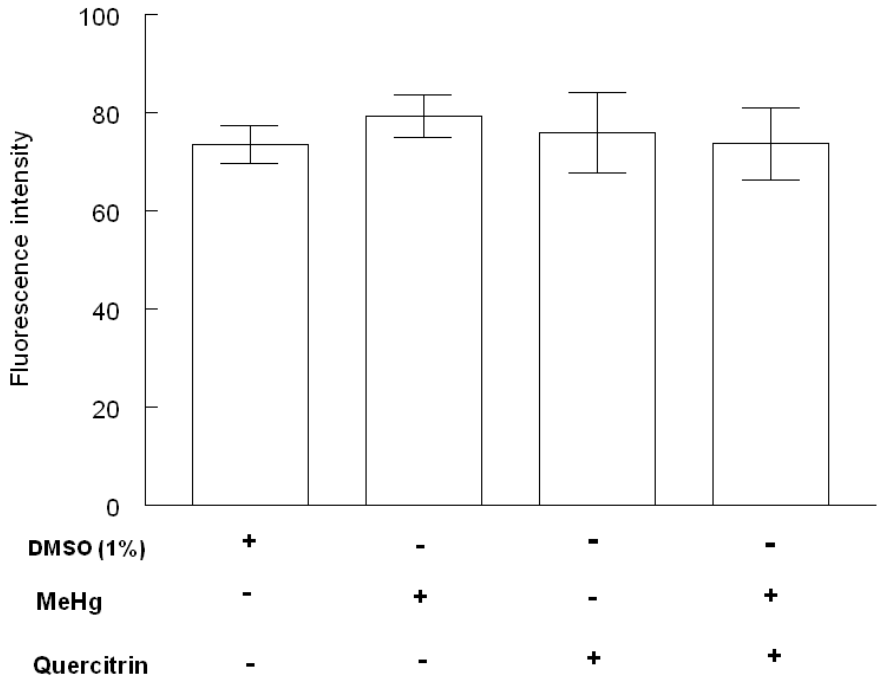
100
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60
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20
0
Fluorescence intensity
DMSO (1%)
MeHg
Quercitrin
+ - - -
- + - +
- - + +

B

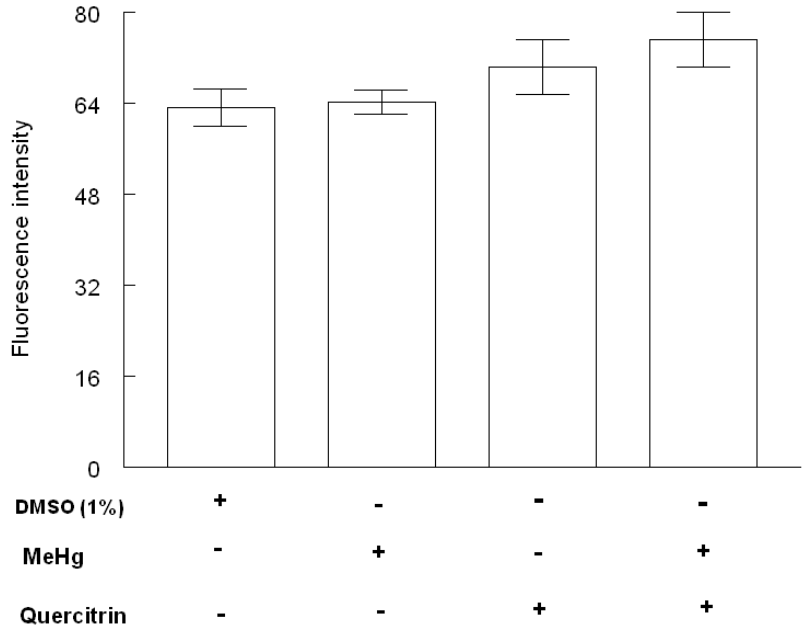

Figure 3: ROS production in cerebrum (a) and cerebelum (b) of mice treated with MeHg (5mg/Kg) and quercitrin (10mg/Kg) for 30 days. Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

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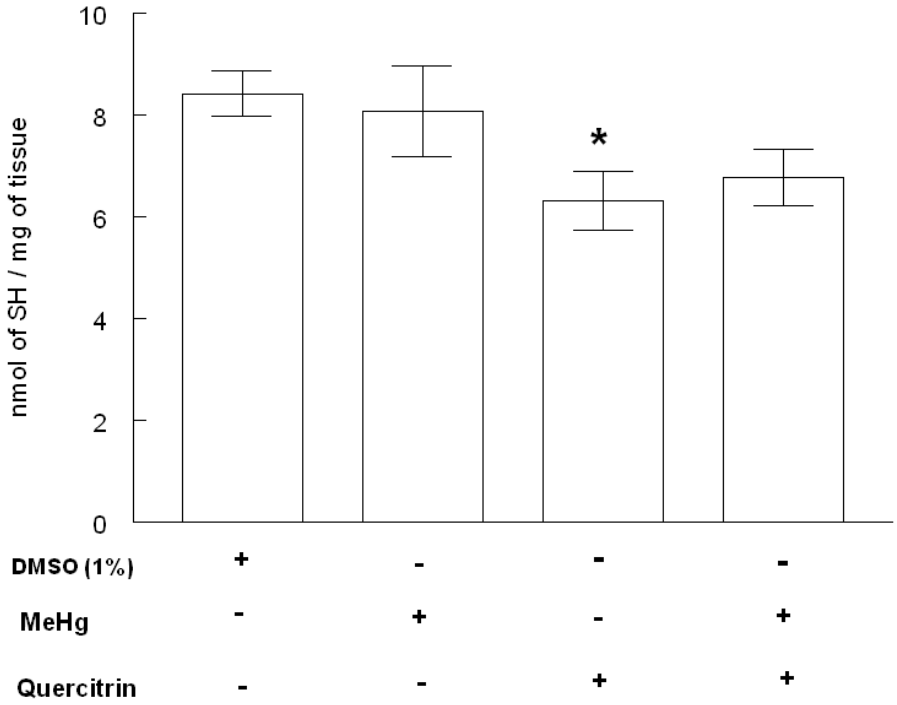

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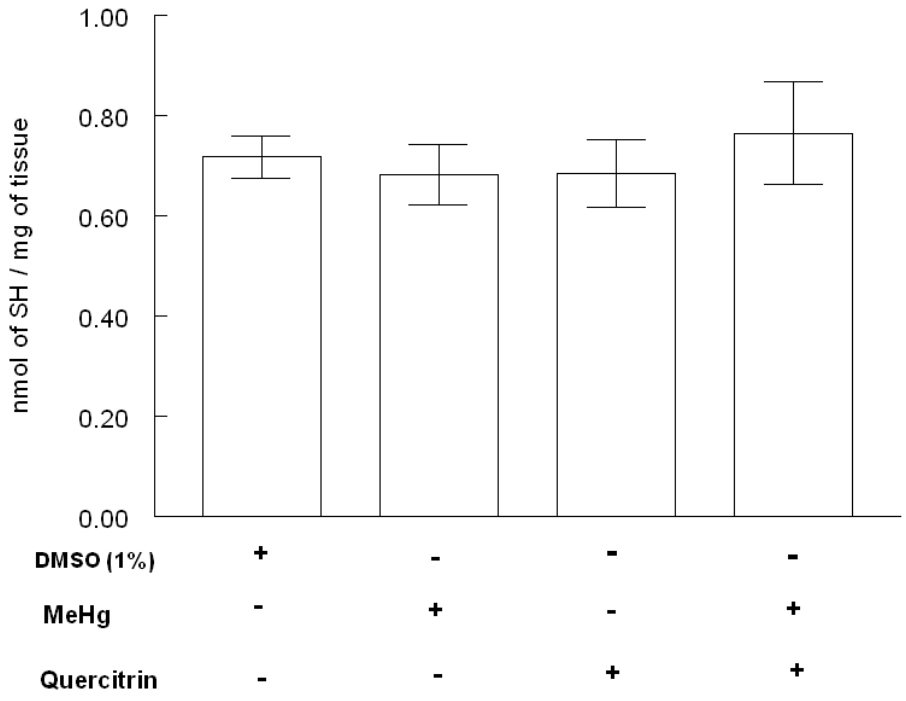


Figure 4: Protein (A) and nonprotein (B) thiol levels in cerebrum of mice treated with MeHg (5mg/Kg) and quercitrin (10mg/Kg) for 30 days. Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p< 0.05$ were considered statistically significant).

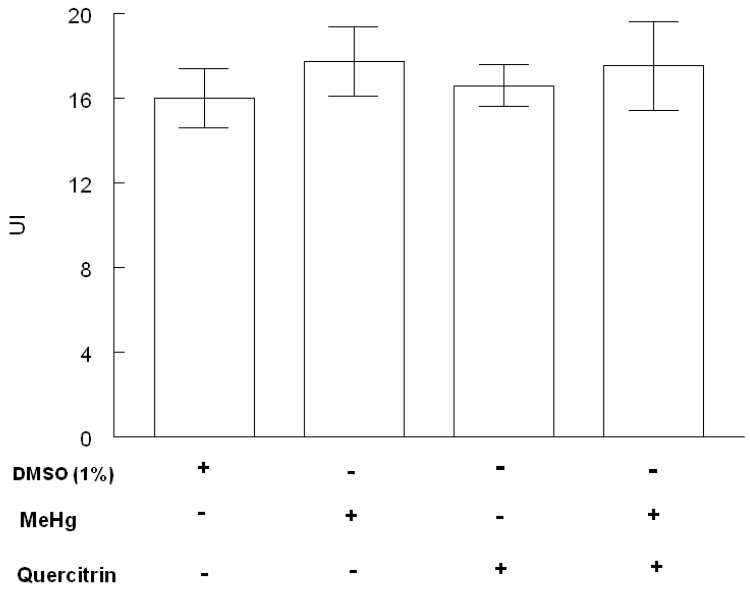
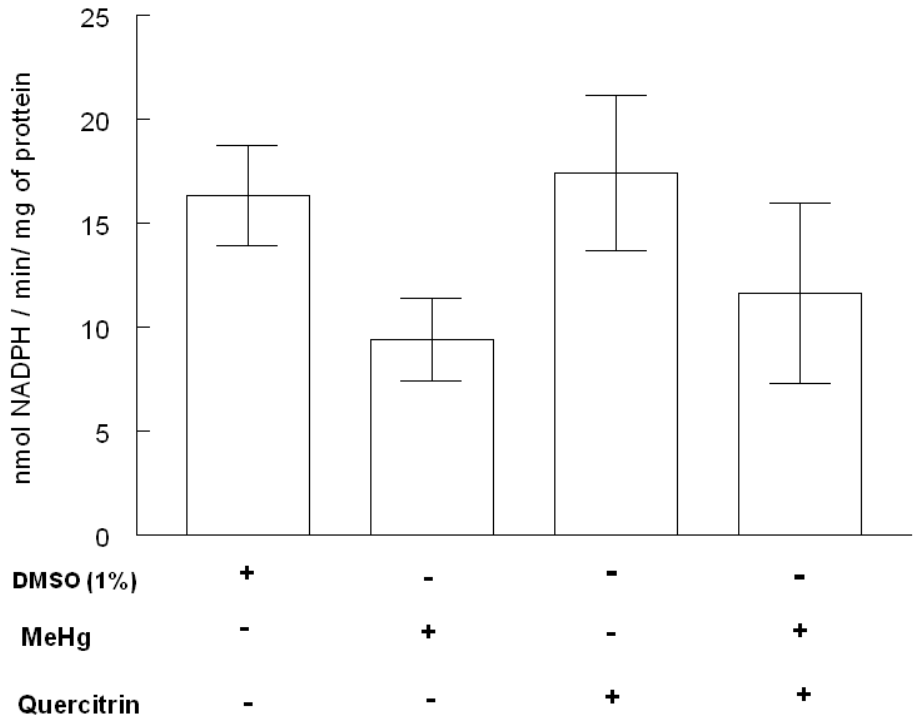


Figure 5: SOD activity in cerebrum of adult mice treated with MeHg (5mg/Kg) and quercitrin (10mg/Kg). Data are expressed as mean ± S.E.M of six different animals. * Represents a significant difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

A


nmol NADPH / min/ mg of prottein
25
20
15
10
5
0
DMSO (1%)
MeHg
Quercitrin
+ - - -
- + - +
- - + +

B

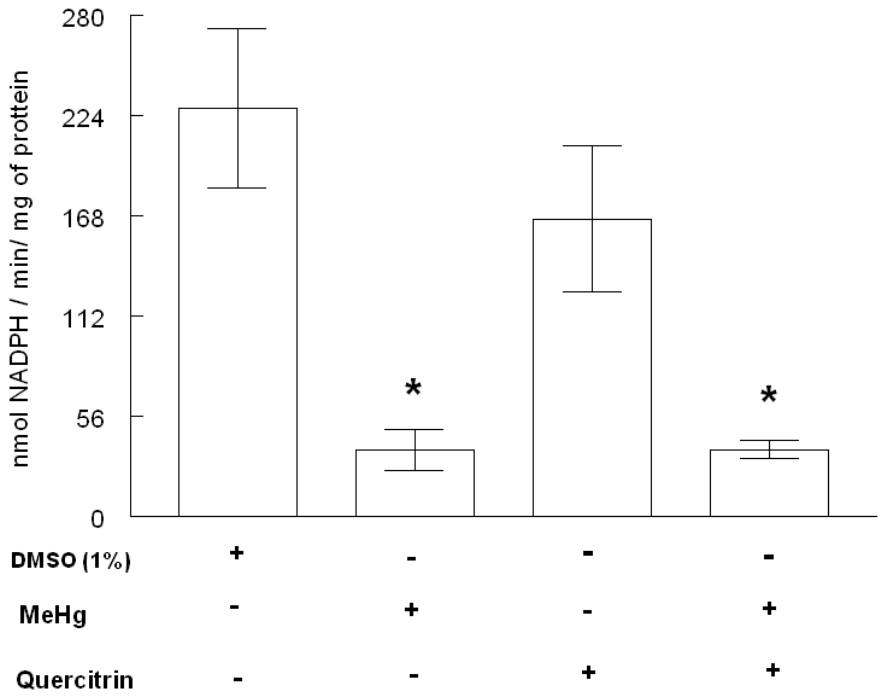


Figure 6: Glutathione peroxidase activity in cerebrum (A) and (B) cerebellum of adult mice treated with MeHg (5mg/Kg) and quercitrin (10mg/Kg). Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

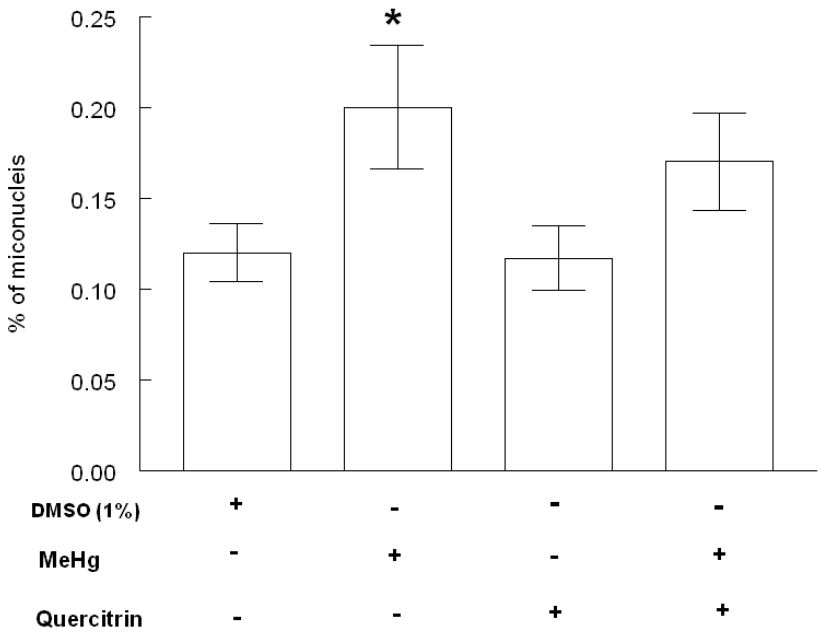


Figure 7: Micronucleos frequencies of mice treated with MeHg and quercitrin. Animals receive 30 days treatment with 10 mg/Kg of quercitrin and 5mg/Kg of MeHg. Data are expressed as mean ± S.E.M of six different animals. * Represents a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

4.3 Artigo 2

Inibição da thiorredoxina redutase de camundongos pelo MeHg em modelos *in vivo* e *in vitro*

In vivo and in vitro **Inhibition of mice thioredoxin reductase by Methylmercury**

Caroline Wagner *, Jéssie H Sudati, Cristina W Nogueira and João B T Rocha*

Accepted article
Biometals (xx)

***In vivo and in vitro* Inhibition of mice thioredoxin reductase by methylmercury**

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ABSTRACT

The thioredoxin (Trx) system, involving redox active Trxs and thioredoxin reductases (TrxRs), sustain a number of important Trx-dependent pathways. These

redox active proteins support several processes crucial for cell function, cell proliferation, antioxidant defense, and redox-regulated signaling cascades. Methylmercury (MeHg) is an important environmental toxicant that has a high affinity for thiol groups and can cause oxidative stress. The Trx system is the major system responsible for maintaining the redox state of cells and this function involves thiol reduction mediated by selenol groups in TrxRs. MeHg has a great affinity to thiols and selenols, thus the potential toxic effects of MeHg on TrxR inhibition were determined in the current study. A single administration of MeHg (1, 5, and 10 mg/Kg) caused a marked inhibition of kidney TrxR activity, while significant inhibition was observed in the liver after exposure to 5 and 10 mg/Kg of MeHg. TrxR activity was determined 24 hours after MeHg. In the brain, MeHg did not inhibit TrxR activity. *In vitro* exposure to MeHg indicated that MeHg inhibits cerebral (IC_{50}, 0.158µM), hepatic (IC_{50}, 0.071µM), and renal TrxR activity (IC_{50}, 0.078µM). The results presented herein demonstrated for the first time that renal and hepatic TrxRs can serve as an *in vivo* target for MeHg. This study suggests that MeHg can bind to selenocysteine residues present in the catalytic site of TrxR, in turn causing enzyme inhibition that can compromise the redox state of cells.

Keywords: Thioredoxin reductase, methylmercury toxicity, thiol, enzyme inhibition, kidney damage

INTRODUCTION

The thioredoxin (Trx) system, composed of thioredoxin reductase (TrxR), Trx, and NADPH, plays an important role in regulating redox metabolism and other

cellular processes, such as DNA synthesis, cell proliferation, and apoptosis. This system is widely distributed in different mammalian organs and tissues (Rozell et al. 1985) and is critical for the cellular stress response, protein repair, and protection against oxidative damage (Arner and Holmgren 2000; Lillig and Holmgren 2007).

Human Trx has a conserved dithiol active site, Cys32-Gly-Pro-Cys35, and contains three structural cysteine residues (Cys62, Cys69, and Cys73). These additional residues make Trx susceptible to oxidation via generation of a second disulfide (Cys62–Cys69), which leads to loss of catalytic activity (Holmgren 1985; Lillig and Holmgren 2007; Watson et al. 2003). Trx is the major disulfide reductase responsible for maintaining cytosolic proteins in their reduced state (Fang and Holmgren, 2006).

Mammalian TrxR is a selenoenzyme containing a unique, catalytically-active selenolthiol/selenenylsulfide in the conserved C-terminal sequence (-Gly-Cys-Sec-Gly; Zhong et al., 2000; Sandalova et al., 2001). TrxR has a broad range of functions; specifically, TrxR exhibits broad substrate specificity, reducing many low molecular compounds, including hydrogen peroxide, lipid hydroperoxides, ascorbate, lipoic acid, ubiquinone and Trx (Li et al. 2008). Of particular importance, it has been reported that inhibition of mammalian TrxR can cause deleterious cellular effects, leading to cytotoxicity and cell death (Carvalho et al. 2008 ; Du et al. 2009).

Mercury contamination is a critical public health problem. Mercury is considered one of the most toxic metals in the environment. Inorganic mercury exists in different oxidation states, and inorganic mercury can be converted to organic mercury by aquatic microorganisms (Clarkson and Magos 2006).

Differences in the reactivity and transport of the different forms of mercury are responsible for variations in tissue and organ distribution, patterns of biological effects, and toxic potencies (Clarkson and Magos 2006; de Freitas et al. 2009). The kidneys are the primary target organ for inorganic Hg (II); whereas organic mercuric compounds, such as methylmercury (MeHg), are strong neurotoxicants, primarily damaging the central nervous system (Clarkson et al. 2003; Fonfria et al. 2005; Roos et al. 2009; Aschner et al. 2007; Wagner et al. 2010).

Mercury toxicity has been related to the formation of stable complexes with sulfhydryl-containing molecules, such as the cysteine residues of proteins and non-protein molecules (Ballatori 2002; Rooney 2007). Methylmercury can disrupt cellular redox balance in human monocytes, thus triggering a decrease in Trx1 levels (Wataha et al., 2008). Recently, it has been demonstrated that MeHg can directly inhibit TrxR and Trx activity in HeLa and HEK 293 cells (Carvalho et al. 2008). Horai et al. (2008) have suggested that a change in TrxR levels may be an important mechanism by which Hg can cause hepatic toxicity.

Despite the apparent involvement of disruption of the redox system in MeHg toxicity and the capacity of mercury compounds to interact with thiols and senols, reports on MeHg interactions with the Trx system are limited. Of particular importance, there are no reports on *in vivo* MeHg poisoning exploiting TrxR as the molecular end point of MeHg toxicity. Therefore, the objective of this study was to investigate the effects of *in vivo* exposure to MeHg in cerebral, renal, and hepatic TrxR activity in adult mice. For comparative purposes, we also determined the *in vitro* effect of MeHg on TrxR from liver, brain, and kidney.

MATERIALS AND METHODS

Chemicals

Methylmercury (II) chloride was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of the highest available commercial grade.

Animals

Male mice (2 months) were maintained under standard conditions (12 h light/dark, 22 ± 2 °C) with food and water *ad libitum*. The Animal Care Committee approved all animal handling and experimental conditions

In vivo treatment

Sixteen mice were equally divided into 4 experimental groups: control; MeHg 1 mg/kg; MeHg 5 mg/kg; and MeHg 10 mg/kg. The mice were treated by oral gavage. Control mice received a daily oral dose of water. After 24 hours of administration, the animals were sacrificed. The livers, brains, and kidneys were quickly removed from the mice, placed on ice, and homogenized.

Tissue preparation

Thioredoxin reductase was partially purified by a modification of the method described by Holmgren and Bjornstedt (1995). Tissues were homogenized in buffered saline (137 mM NaCl, 2.7mM KCl, 4.3 mM Na_2HPO_4; and 1.4 mM KH_2PO_4 [pH=7.3]). Livers (0.5 g) were homogenized in 10 volumes of buffered saline, brains (0.5 g) were homogeneized in 3 volumes of buffered saline, and kidneys (0.5g) were homogeneized in 5 volumes of buffered saline. Homogenates were

centrifuged at 13,000 g for 30 min. The protein in the supernatant was measured and adjusted to 10 mg/ml. The supernatant was dialyzed against buffered saline for 16 hours to remove endogenous glutathione and Trx. The dialyzate was heated at 55º C for 10 min, cooled, and centrifuged at 13,000g for 30 min to remove denatured protein.

TrxR activity

Thioredoxin reductase activity was measured by the method described by Holmgren and Bjornstedt (1995). The reaction mixture consisted of the following (in mM): 0.24 NADPH, 10 EDTA, 100 potassium phosphate buffer (pH 7.0), 2 mg/ml DTNB, and 0.2 mg/ml of BSA. The partially purified TrxR was added to a cuvette containing the reaction mixture and the absorbance were followed at 412 nm for a maximum of 4 min.

In vitro assays

Mice were decapitated, and the liver, brain, and kidneys were rapidly removed and homogenized in buffered saline according to the tissue preparation procedure (*vide supra*). The TrxR activity was determined as described above, with the exception that MeHg was added to the reaction medium (final MeHg concentrations were 0.025, 0.05, 0.075, 0.1, 0.5, or 1 µM).

Protein estimation

The protein concentration was measured by the Lowry method (1951) using bovine serum albumin (BSA) as a standard.

Statistical analysis

The results are expressed as the mean ± standard deviation, and were analyzed by one-way analysis of variance (ANOVA). The Duncan's multiple range test was applied *post hoc* to determine the significance of the difference between the various groups. Differences were considered statistically significant at a $p<0.05$.

RESULTS

In vitro Inhibitory effects of MeHg

To evaluate the inhibitory potency of MeHg on TrxR activity, the partially purified enzyme from liver, kidney, and brain was incubated in the presence and absence of MeHg. In liver (Figure 1), MeHg (0.05, 0.1, 0.5, and 1 µM) caused a significant inhibition of TrxR in a concentration-dependent manner. The IC_{50} values (0.071 µM ± 0.005) for TrxR inhibition by MeHg in liver tissue was the lowest of the three tissues tested (Table 1).

In brain (Figure 2), MeHg (0.05, 0.1, 0.5, and 1 µM) caused a statistically significant inhibition in TrxR, but with significantly lower potency than determined for liver and kidney. The IC_{50} value for inhibition of brain TrxR was 0.158 µM ± 0.029 (Table 1).

In kidney (Figure 3), MeHg significantly inhibited TrxR (as low as 0.025 µM) in a concentration-dependent manner. The IC_{50} value was 0.078 µM ± 0.011 (Table 1).

In vivo Inhibitory effects of MeHg

To analyze the behavior of MeHg as a potential *in vivo* inhibitor of TrxR, we exposed mice to three different doses of MeHg (1, 5, and 10 mg/kg), and the activity of partially purified TrxR from liver, kidney, and brain was evaluated 24 hours after MeHg administration. MeHg caused a significant inhibitory effect on liver TrxR activity in doses of 5 and 10 mg/kg (Figure 4). Methylmercury, at a dose of 1 mg/kg, decreased TrxR activity, but not statistically different from the control group values (Figure 4). In contrast to liver and kidney, MeHg treatment did not cause alteration

in cerebral TrxR (Figure 5). In kidney, MeHg caused a marked inhibition of TrxR activity at all doses. Furthermore, MeHg at a dose of 10 mg/kg caused a pronounced inhibitory effect of approximately 80% (Figure 6).

DISCUSSION

This study showed that MeHg can effectively inhibit TrxR after *in vitro* and *in vivo* exposure. However, the inhibitory potency varied depending on the type of exposure and on the tissue. Indeed, *in vivo* treatment with MeHg (1-10 mg/kg) inhibited renal TrxR and *in vitro* experiments confirmed that MeHg inhibits this important redox regulatory enzyme. Taking into account that mercuric ions have a greater affinity to bind to reduced sulfur atoms, especially those on endogenous thiol-containing molecules (glutathione, metallothionein, homocysteine, and *N*-acetylcysteine; Hultberg et al. 2001), the biological effects of MeHg can be related to interactions with sulfhydryl-containing residues in the active site of TrxR. In addition, mercurial compounds can also inhibit TrxR by binding to selenol groups. In fact, as a consequence of its softness, mercury has a higher affinity for selenols than thiols (Sugiura et al. 1976; da Conceição Nascimento et al. 2009).

Several *in vivo* and *in vitro* studies have suggested that exposure of experimental animals to organic forms of mercury can be accompanied by the induction of oxidative stress (Aschner et al. 2007). The role of TrxR and Trx, at the core of cellular thiol redox control and antioxidant defense (including here the ability to reduce peroxiredoxins; Seo et al. 2000), implicate that inhibition of the Trx system can increase ROS levels. Thus, one of the mechanisms by which MeHg can induce oxidative stress could be via TrxR inhibition. Mahboob et al. (2001) have shown that Hg(II) treatment enhances lipid peroxidation in several tissues, but kidney is one of the most sensitive organs to Hg toxicity. In agreement with this finding, our *in vivo* results demonstrated that renal TrxR was more inhibited by MeHg than the brain and liver enzymes. Therefore, the inhibition of kidney TrxR may be a new

mechanism via which MeHg exerts nephrotoxicity and increases lipid peroxidation (de Freitas et al. 2009).

Herein we have demonstrated a significant inhibition of renal and hepatic TrxR which may be due to a higher deposition of mercury in these organs after MeHg exposure in mice (de Freitas et al. 2009). In addition, the *in vitro* results indicated that brain TrxR had higher IC_{50} values for the inhibition by MeHg, and consistent with *in vivo* MeHg exposure, did not cause inhibition of cerebral TrxR, suggesting that brain TrxR is less sensitive to MeHg inhibition after *in vivo* and *in vitro* exposure. The absence of cerebral TrxR inhibition after *in vivo* exposure to MeHg can also be related to a low level of mercury deposition in the brain when compared to liver and kidney. de Freitas et al. (2009) have shown that exposure to MeHg caused accumulation of Hg in the liver, kidney, cerebrum, and cerebellum, but the level of hepatic and renal Hg deposition was approximately 10 times higher than in brain.

The catalytic activity of selenoenzymes, such as TrxR, depends upon the biochemistry of the selenocysteine present at the active sites (Behne et al., 2000). The unique capabilities of the various selenoenzymes, like reduction of hydroperoxides, occur because of the selenocysteine residues. Because the selenol of selenocysteine is ionized at a physiologic pH, it is often more reactive than a cysteine thiol. Unfortunately, these features that make TrxR so valuable physiologically, also make it more vulnerable to MeHg toxicity (Ralston et al., 2008).

An irreversible inhibitor is one that forms covalent bonds with components of the active site of an enzyme. Because selenocysteine is the principal active site catalytic component of TrxR, MeHg is by definition a highly specific irreversible

selenoenzyme inhibitor; MeHg forms covalent bonds between its mercury moiety and the selenium of the selenocysteine of the enzyme (Farina et al. 2009; Ralston et al. 2008). In this case, however, the inhibitor–enzyme complex not only abolished the activity of the inhibited selenoenzyme, it also restricteds selenium release from the MeHg–SeCys complex, severely limiting the bioavailability of selenium for participation in future intracellular cycles of SeCys synthesis (Ralston et al. 2008).

In summary, this study demonstrated, for the first time, that *in vivo* exposure to MeHg can cause inhibition of liver and kidney TrxR, but not brain TrxR. *In vitro* exposure caused a dose-dependent inhibition of TrxR, and kidney and liver TrxR were more sensitive to inhibition by MeHg than brain TrxR. Methylmercury can bind to the selenium moiety of selenocysteine, thus directly inhibiting the activity of selenoenzymes (Farina et al. 2009b) and, consequently, disrupting the antioxidant functions and redox state maintained by these enzymes. The results presented here reinforce the central role of selenoproteins in the toxicity of mercurials compounds and strongly indicate that TrxR is an important *in vivo* molecular target for MeHg.

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TABLE

Table 1

IC $_{50}$ values of *in vitro* inhibition of TrxR activity by MeHg in different tissues.

Tissue	Ic_{50} values (μM)
Liver	0.071 ± 0.005
Brain	0.158 ± 0.029 *
Kidney	0.078 ± 0.011

Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. * represent statistical differences between the tissues. (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p< 0.05$ were considered statistically significant).

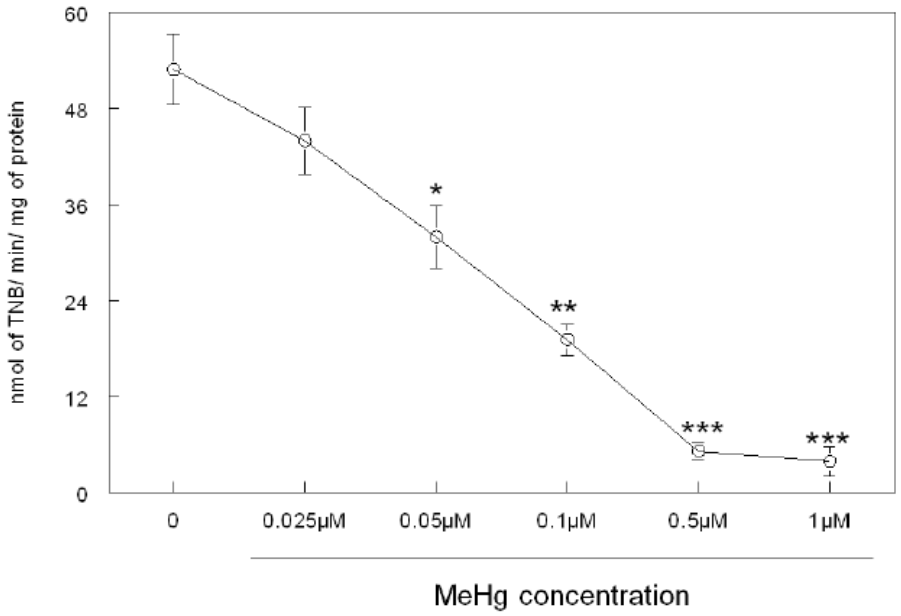

Figure 1: *In vitro* inhibition of liver TrxR by different MeHg concentration. MeHg was incubated in reaction medium in different concentrations:0.025, 0.05, 0.1, 0.5 and 1 µM and the reaction were started with the addition of purified enzyme. Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

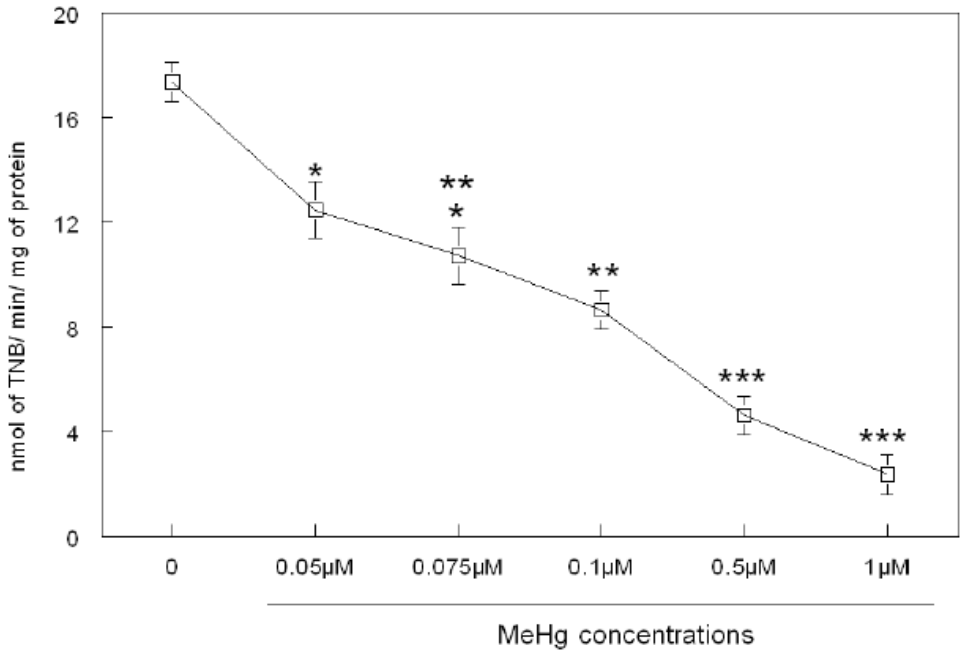

Figure 2: *In vitro* inhibition of brain TrxR by different MeHg concentration. MeHg was incubated in reaction medium in different concentrations: 0.05, 0.075, 0.1, 0.5 and 1 μM and the reaction were started with the addition of purified enzyme. Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

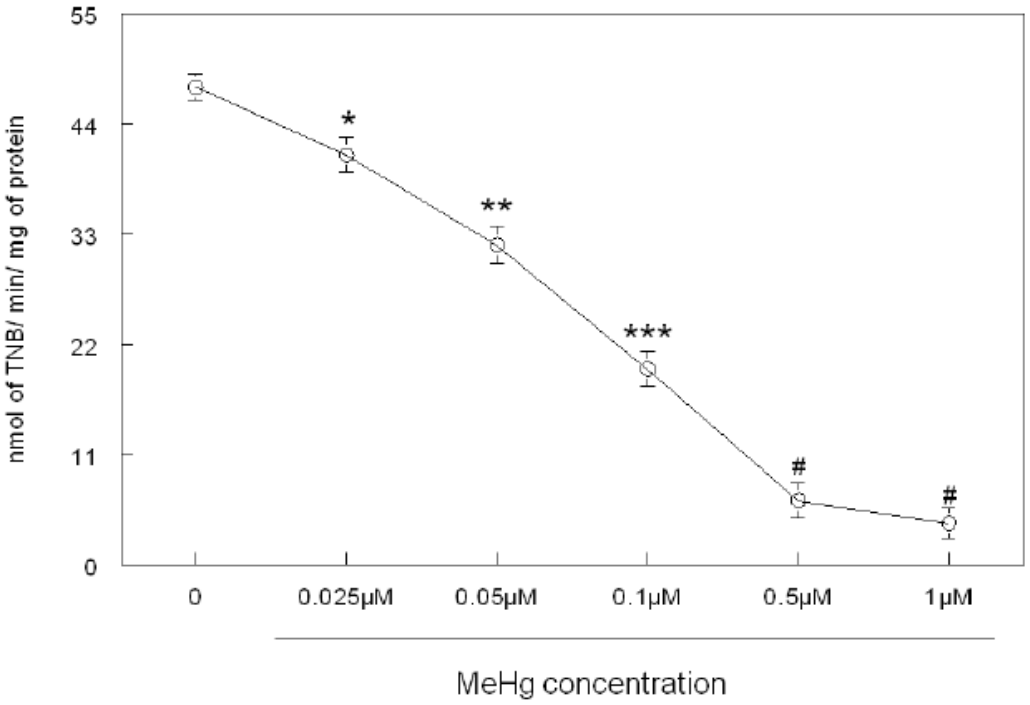

Figure 3: *In vitro* inhibition of kidney TrxR by different MeHg concentration. MeHg was incubated into reaction medium in different concentrations:.0.025, 0.05, 0.1, 0.5 and 1 μM and the reaction were started with the addition of purified enzyme. Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p< 0.05$ were considered statistically significant).

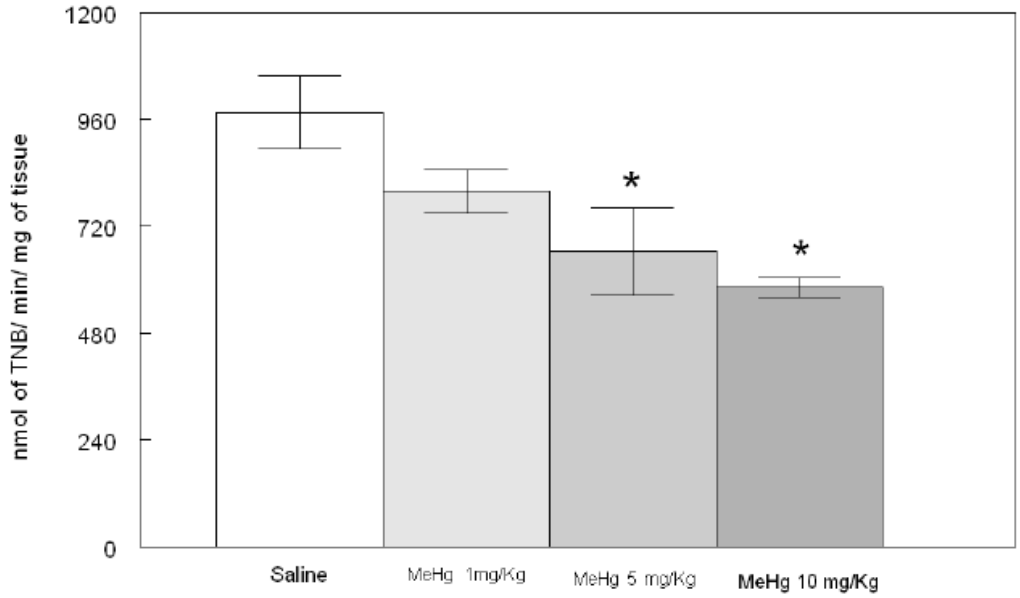

Figure 4: Inhibition of liver thioredoxin reductase (TrxR) activity by MeHg exposure. Animals were exposed to tree different MeHg concentration: 1, 5, and 10 mg/Kg, during 24 hours. TrxR activity was assessed by DTNB reduction method. Data are expressed as mean ± S.E.M. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

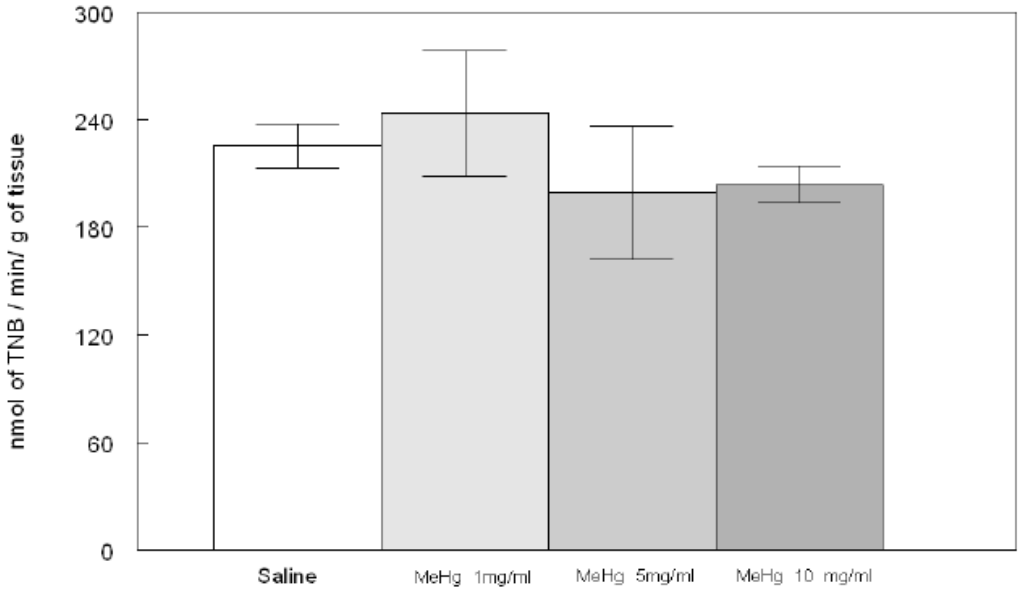

Figure 5: Inhibition of brain thioredoxin reductase (TrxR) activity by MeHg exposure. Animals were exposed to tree different MeHg concentration: 1, 5, and 10 mg/Kg, during 24 hours. TrxR activity was assessed by DTNB reduction method. Data are expressed as mean ± S.E.M. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

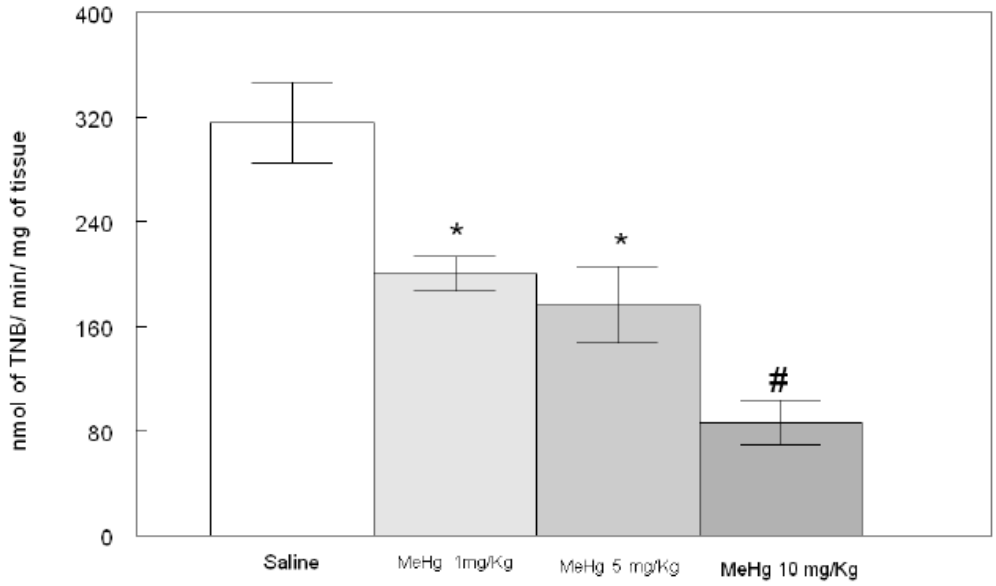

Figure 6: Inhibition of kidney thioredoxin reductase (TrxR) activity by MeHg exposure. Animals were exposed to tree different MeHg concentration: 1, 5, and 10 mg/Kg, during 24 hours. TrxR activity was assessed by DTNB reduction method. Data are expressed as mean ± S.E.M. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $p < 0.05$ were considered statistically significant).

5. DISCUSSÃO

O MeHg é um reconhecido poluente ambiental que, nas últimas décadas, causou contaminação e intoxicação humana em várias partes do mundo, como por exemplo em Minamata, no Japão, e também no Iraque (Robertson e Orrenius, 2000; Gochfeld, 2003). No Brasil, estudos têm evidenciado que várias espécies de peixes carnívoros da Amazônia apresentam altos níveis de MeHg (Malm, 1998; Pinheiro e cols., 2003). Consequentemente, comunidades ribeirinhas localizadas próximas a áreas de garimpo, sofrem exposição crônica a níveis relativamente elevados de MeHg em sua dieta, que é rica em peixes (Pinheiro e cols., 2003). Neste contexto, estudos epidemiológicos apontam para déficits neurológicos em comunidades pesqueiras que possuem uma dieta baseada no consumo de peixes (Granjean e cols., 1997; Clarkson e cols., 2003). Assim a exposição humana a este metal é bastante grande no mundo todo, tornando de grande valia estudos que procuram elucidar os mecanismos pelos quais o MeHg exerce seus efeitos tóxicos bem como, possíveis compostos que venham a ser usados no tratamento da intoxicação com MeHg.

Este estudo demonstrou que os principais mecanismos pelos quais o MeHg exerce seus efeitos tóxicos ocorrem via aumento na produção de espécies reativas de oxigênio nas mitocôndrias (figura 4, artigo 1), que esta intimamente relacionado com a desregulação do metabolismo do cálcio. Este aumento na produção de espécies reativas leva então a um aumento na peroxidação lipídica, observado tanto na exposição *in vitro* (figura 4, artigo1) quanto na *in vivo (*figura 2, manuscrito 1) ao MeHg. Além disso, este trabalho mostrou, pela primeira vez, que o MeHg é capaz de inibir a atividade da enzima TrxR em camundongos tratados com diferentes concentrações de MeHg. A alta afinidade do MeHg a grupos tióis (como os presentes na cisteína), bem como ao selenol (como o presente na selenocisteína), presentes nos sítios ativos de inúmeras enzimas, provavelmente é o responsável pela inibição observada na atividade da TrxR e da glutationa peroxidase. Todos esses danos a inúmeras moléculas com papel fundamental no metabolismo, acaba desencadeando danos comportamentais, como distúrbio na locomoção e na coordenação motora (tabela 2 e 3, manuscrito 1) em animais expostos ao MeHg.

Outros resultados importantes obtidos por este trabalho foram o papel dos flavonóides quercetina e quercitrina na prevenção da toxicidade causada pelo MeHg. Estes compostos mostraram-se capazes de diminuir a produção de ERO causadas pelo MeHg nas mitocôndrias , e assim diminuir a peroxidação lipídica tanto em experimentos *in vitro* quanto *in vivo* (neste caso só a quercitrina foi testada). Além disso, a quercitrina se mostrou capaz de proteger da inibição causada pelo MeHg à enzima Na^+/K^+ ATPase (figura 1, manuscrito 1), bem como conferiu proteção contra alterações comportamentais causadas pela exposição ao MeHg.

De fato, este trabalho mostrou que a produção de ERO mitocondriais induzidas pelo MeHg é dependente de cálcio (figura 4, manuscrito 1), uma vez que o MeHg sozinho não aumentou a produção de ERO mitocondrial, e quando em presença de cálcio ocorreu uma super estimulação na produção de ERO mitocondrial, acompanhado por danos mitocôndrias. Além disso, foi observado os mesmos efeitos causados pelo MeHg quando as mitocôndrias foram expostas ao ionóforo de cálcio A23187 (figuras 4 e 5, artigo 1). Já é descrito na literatura (Dubinsky e Levi, 1998) que na presença de um ionóforo, grandes quantidades de cálcio são carregadas para dentro da mitocôndria levando a uma imediata despolarização da mitocôndria. Assim, da mesma maneira que o ionóforo, o MeHg leva a um rápido e massivo aumento no influxo de cálcio pela mitocôndria, o qual leva a uma super estimulação na produção de ERO, levando a uma transição na permeabilidade mitocondrial e colapso na cadeia de transporte de elétrons, podendo levar a morte mitocondrial.

Todo este processo de aumento na produção de ERO via cálcio causada pelo MeHg, está relacionada também, a um aumento na produção de ERO nas fatias corticais de cérebro de ratos (figura 3, artigo 1). Este aumento nas ERO vai levar a um aumento no dano as membranas biológicas, levando a um aumento na peroxidação lipídica, observados na figura 2 do primeiro artigo.

Os flavonóides quercetina e quercitrina foram capazes de proteger contra o aumento na produção de ERO causado pelo MeHg. Estes dois flavonóides diminuiram a produção de ERO estimuladas pelo cálcio e MeHg em mitocôndrias (figura 4 A e C, artigo 1), bem como protegeram contra a indução de ERO nas fatias corticais de cérebro de ratos (figura 3A e C, artigo 1) e também contra a peroxidação lipídica (figura 2 A, artigo 1).

Estes efeitos protetores apresentados pela quercetina e quercitrina foram atribuídos a capacidade destes flavonóides de detoxificar a espécie reativa H_2O_2 (peróxido de hidrogênio) geradas durante a exposição ao mercúrio (Franco e cols., 2007; Cai e cols., 1997). Além disso, parte da proteção exercida pelos dois flavonóides pode ser atribuída a efeitos relacionados ao influxo de cálcio, uma vez que a miricitrina, um flavonóide com estrutura similar a quercetina e a quercitrina, se mostrou capaz de bloquear o inflluxo de cálcio em fatias de cérebro (Meotti e cols., 2007). Assim, a quercetina e a quercitrina podem proteger contra o influxo de cálcio para o interior da mitocôndria provocado pelo MeHg, e assim, proteger contra o aumento de ERO.

Por outro lado, o flavonóide rutina, não foi capaz de proteger contra a peroxidação lipídica induzida pelo MeHg (figuras 2B, artigo1), além de apresentar um modesto efeito protetor contra a produção de ERO (3B e 4B, artigo 1). Este modesto ou ausente efeito protetor da rutina pode ser atribuído a sua estrutura química, uma vez que as atividades antioxidantes dos flavonóides são influenciadas por sua estrutura (Rice-Evans e cols., 1996). Uma comparação entre a quercetina e a rutina (figura 1, artigo 1) indica uma possível influência do grupo 3-OH em combinação com a dupla ligação adjacente presente no anel C. Na ausência de um destes grupamentos pode ocorrer uma perda na atividade antioxidante (Rice-Evans e cols., 1996). Desta forma a perda ou diminuição da atividade antioxidante da rutina esta diretamente relacionada à sua estrutura. Além disso, o grupo glicosídico presente na estrutura da rutina torna este composto mais hidrofílico (Saija e cols., 1995) e, desta forma, diminui sua permeabilidade através das membranas. Além desta falta ou diminuição da atividade antioxidante, a rutina também mostrou efeitos pro-oxidantes (figura 2B, artigo1), estes resultados também estão de acordo com outros dados da literatura que sugerem uma atividade pró-oxidante da rutina (Cottele, 2001).

Visto que a quercitrina se mostrou capaz de proteger contra a toxicidade do MeHg *in vitro*, que há dados controversos na literatura quanto à capacidade protetora da quercetina contra a toxicidade do MeHg e que modificações na estrutura da quercetina pode diminuir sua toxicidade (Vafeiadou e cols., 2008; Ossola e cols., 2009) o flavonóide quercitrina foi utilizado para os testes *in vivo* de proteção contra a toxicidade causada pelo MeHg.

Em camundongos expostos ao MeHg (5 mg/kg) foi possível observar que este metal causou inúmeros efeitos tóxicos, tais como: perda no ganho de peso (tabela 1, manuscrito 1), formação de micronúcleo (figura 7, manuscrito 1) e danos renais (diminuição na atividade da enzima GPx e aumento na peroxidação lipídica).

A exposição ao MeHg também levou a mudanças comportamentais, tais como, perda na atividade locomotora (tabela 2, manuscrito 1), perda na coordenação motora (tabela 3, manuscrito 1) e alteração na memória espacial (tabela 4, manuscrito 1). Além dos danos comportamentais o MeHg causou uma marcada diminuição na atividade da enzima GPx em cérebro e cerebelo (figura 6A e C, artigo 2). A relação entre deficiência motora e o MeHg induzir dano cerebelar é um processo já descrito na literatura. Assim nós atribuímos parte dos danos comportamentais causados pelo MeHg a inibição da atividade da GPx cerebelar e também cerebral.

Não só a GPx sofreu alteração na atividade, mas também a enzima Na^+/K^+ATPase (figura 1, manuscrito 1). A inibição da atividade desta enzima pode levar a despolarização da membrana, supressão da função neuronal e da transmissão excitatória (Balestrino e cols., 1999; Rajanna e cols., 1990). Além disso, outros trabalhos mostraram que o MeHg em altas doses pode induzir desordens neurológicas acompanhadas pela inibição da atividade da enzima Na^+/K^+ATPase no cérebro (Cheng e cols., 2005; Chuu e cols., 2001). Desta forma a inibição da atividade da Na^+/K^+ATPase pelo MeHg apresentada neste trabalho pode corroborar como os danos motores e na memória espacial dos animais tratados com MeHg.

Além de inibição enzimática, a exposição ao MeHg levou a um aumento na peroxidação lipídica. Em relação a isto, as células cerebrais de mamíferos parecem ser mais suscetíveis as injúrias oxidativas causadas pelo MeHg. O MeHg causa alteração na permeabilidade da mitocôndria e nas funções das enzimas antioxidantes que leva a formação de radicais livres e peroxidação lipídica (Aschner e cols., 2007; Cheng e cols., 2005; de Freitas e cols., 2009; Yee and Choi, 1996). Além disso, há trabalhos demonstrando que o estresse oxidativo pode contribuir para a indução de alterações neurológicas causadas pela intoxicação com MeHg, além de causar a inibição da enzima da Na^+/K^+ATPase (Huang e cols., 2008). Assim, as inúmeras alterações nas funções neuronais (alteração na atividade

locomotora, na coordenação motora e distúrbios na memória espacial) causadas pela exposição ao MeHg podem estar relacionadas com o estresse oxidativo.

Neste trabalho o co-tratamento com o flavonóide quercitrina, mostrou efeitos benéficos contra a toxicidade do MeHg. A quercitrina (10mg/kg) protegeu contra parâmetros tóxicos tais como: diminuição no ganho de peso (tabela 1, manuscrito 1), aumento na formação de micronúcleos(figura 7, manuscrito 1) e danos renais.

Além disso, a quercitrina protegeu contra a maior parte dos danos comportamentais, como alteração na coordenação motora, distúrbio na memória espacial e parte dos danos locomotores. Nós atribuímos estes efeitos protetores da quercitrina a sua capacidade de prevenir contra peroxidação lipídica causada pelo MeHg, bem como manter os níveis de atividade da GPx cerebral e da Na^+/K^+ATPase iguais ao controle, protegendo contra a inibição provocada pela exposição ao MeHg (figuras 1, 2 e 6, manuscrito 1). Assim como observado no artigo 1, a quercitrina diminui a peroxidação lipídica causada pelo MeHg, provavelmente por proteger as mitocôndrias e desta forma diminuir a produção de espécies reativas que iram causar o dano aos lipídios das membranas. Desta forma o alto potencial antioxidante da quercitrina (em outros trabalhos do grupo, este flavonóide se mostrou um potente neuroprotetor, Wagner e cols., 2006), esta diretamente ligado aos efeitos protetores apresentados nesse trabalho. Além disso, os flavonóides podem remover íons de metais pesados (Afanas've e cols., 1989; Halliwell e Gutteridge, 1990; Brown e cols., 1998), e em relação a isso a quercitrina poderia estar complexando o MeHg diminuindo a quantidade de MeHg livre para interagir com as biomoléculas como, por exemplo, as enzimas GPx e Na^+/K^+ATPase. Porém, não há dados na literatura em relação à capacidade dos flavonóides em complexar íons mercúrio, estas observações são incertas.

A um massivo esforço em pesquisar novas drogas que possam proteger contra a toxicidade do MeHg uma vez que não há um tratamento efetivo que elimine completamente os efeitos tóxicos deste metal. O uso de agentes quelantes pode auxiliar na manutenção das funções vitais e ajudar o organismo a eliminar o mercúrio dos tecidos (Pingree e cols., 2001; Carvalho e cols., 2007). Porém, estas drogas possuem uso limitado devido aos seus efeitos colaterais (Tchounwou e cols., 2003), além da limitação em atravessar a barreira cérebro-sangue (Aposhian e cols., 1995). Desta forma, novos compostos que possam proteger contra a toxicidade do MeHg, como por exemplo, os observados pela quercitrina, que

apresentam poucos efeitos colaterais, que são permeáveis a barreira cérebro-sangue e que apresentam marcada atividade neuroprotetora são de grande valia no desenvolvimento de novas drogas para o tratamento de intoxicação por MeHg.

Inúmeros trabalhos mostram que o mercúrio pode formar complexos estáveis com moléculas que contenham grupamentos sulfidrílicos, como por exemplo, com os resíduos de cisteína presentes em moléculas protéicas e não protéicas (Ballatori, 2002; Rooney, 2007), bem como com selenol em resíduos de selenocisteína, presente no sítio ativo de inúmeras selenoezimas. E que a afinidade do MeHg pode levar a ruptura do balanço redox da célula (Wataha e cols., 2008; Carvalho e cols., 2008; Fang and Holmgren, 2006), nos chamou a atenção a escassez de trabalhos relacionando a toxicidade do MeHg com uma importante enzima responsável por grande parte do balanço redox da célula, da defesa antioxidante e da síntese de DNA, a enzima tiorredoxina redutase (TrxR) (Rozell e cols., 1985; Arner e Holmgren, 2000; Lillig e Holmgren, 2000). Desta forma o segundo artigo desta tese trata da toxicidade do MeHg na atividade da selenoenzima TrxR.

O MeHg inibiu efetivamente a atividade da tiorredoxina redutase tanto na exposição *in vivo* quanto *in vitro*. Esta capacidade inibitória variou de acordo com o modelo de exposição e do tecido utilizado.

Nós encontramos uma significativa inibição na atividade da TrxR renal e hepática (figuras 4 e 6, artigo 2), esses dois tecidos apresentaram altos níveis de deposição de mercúrio após os camundongos serem tratados com MeHg (de Freitas e cols., 2009). Além disso, o MeHg não inibiu a atividade da TrxR cerebral na exposição *in vivo* (figura *5*, artigo 2) , concordando com os resultados *in vitro* que mostraram que a atividade da TrxR cerebral apresenta maiores valores de IC_{50}, sugerindo que a TrxR cerebral é menos sensível a inibição provocada pelo MeHg do que os tecidos renais e hepáticos. Essa falta de inibição da TrxR cerebral também pode ser atribuída aos menores níveis de deposição de mercúrio no cérebro quando comparados com o rim e o fígado (de Freitas e cols., 2009)

Os íons de mercúrio possuem grande afinidade para ligar-se a átomos de enxofre, como por exemplo, aos grupos tióis presentes nas moléculas endógenas (Hultberg e cols., 2001), desta forma os efeitos tóxicos do MeHg podem estar relacionados com sua interação com os resíduos sulfidrílicos presentes no sítio

ativo da TrxR. Contribuindo, os compostos de mercúrio também podem inibir a TrxR através da ligação com o grupo selenol também presente no sítio ativo dessa enzima. Além disso, o mercúrio apresenta maior afinidade por selenol do que tiol (Sugiura e cols., 1976; da Conceição Nascimento e cols., 2009).

A atividade catalítica das selenoenzimas como a TrxR depende da bioquímica da selenocisteína presente em seu sítio catalítico (Behne e cols., 2000). O grupo selenol presente na selenocisteína está ionizado em pH fisiológico, o que o deixa mais reativo do que o tiol presente na cisteína. Infelizmente, esta característica que torna a molécula de selenol tão valiosa fisiologicamente também a torna mais vulnerável a toxicidade do MeHg (Ralston e cols., 2008).

Assim este trabalho mostrou pela primeira vez, que a exposição *in vivo* ao MeHg inibiu a atividade da TrxR renal e hepática, mas não a TrxR cerebral. Os resultados *in vitro* confirmam que a TrxR renal e hepática foram mais sensíveis do que a cerebral. O MeHg pode se ligar ao átomo de selênio presente na selenocisteína inibindo a atividade desta selenoenzima, levando a perda na atividade antioxidante e desequilíbrio do estatus redox mantido por esta enzima. Assim sendo, a inibição da TrxR adiciona um novo mecanismo pelo qual o MeHg exerce seus efeitos tóxicos.

De forma geral, este estudo demonstrou que o MeHg causa alteração na homeostase do cálcio levando a um aumento na produção de ERO pelas mitocôndrias e o que contribui para o aumento na peroxidação lipídica causada pelo MeHg, além de aumentar a produção de ERO, o MeHg leva a inibição de importantes enzimas antioxidantes, como a glutationa peroxidase e thiorredoxina redutase, principalmente, por interagir com grupos tióis e selenóis presentes no sítio catalítico destas enzimas. A inibição destas enzimas antioxidantes pode contribuir para aumentar o dano oxidativo tal como a peroxidação lipídica. A associação do aumento na peroxidação lipídica e inibição da atividade das enzimas GPx e Na^+/K^+ATPase parece estar diretamente relacionado com o aparecimento de danos comportamentais. A atividade “scavenger” e antioxidante dos flavonóides quercetina e quercitrina parecem estar diretamente associadas à capacidade destes compostos em proteger contra a toxicidade do MeHg. A quercitrina se mostrou eficaz tanto *in vivo* quanto *in vitro*, e desta forma pode ser uma possível candidata para ser usada como uma droga promissora no tratamento de intoxicação pelo MeHg.

6. CONCLUSÃO

De acordo com os resultados apresentados nesta tese podemos concluir que:

- O MeHg causou aumento na produção de ERO mitocondrial e este processo esta diretamente ligado a presença de cálcio. Este aumento na produção de ERO levou a peroxidação lipídica.
- O MeHg inibiu a atividade de enzimas antioxidantes como a TrxR e GPx o que pode contribuir para o aumento na peroxidação lipídica e demais danos oxidativos causados pela intoxicação com este metal.
- O MeHg reduziu a atividade locomotora, a coordenação motora e a memória espacial de camundongos tratados com MeHg(5mg/kg, durante 30 dias), que parecem estar relacionados com parâmetros bioquímicos tais como: inibição da atividade das enzimas Na^+/K^+ ATPase e GPx e aumento na peroxidação lipídica.
- O MeHg exerceu toxicidade via inibição da enzima TrxR tanto *in vivo* quanto *in vitro*. E que a TrxR cerebral foi mais resistente ao dano causado pelo MeHg do que a TrxR de fígado e rim.
- Os flavonóides quercetina e quercitrina podem protegeram contra a toxicidade in vitro do MeHg. E esta proteção parece estar diretamente relacionada à capacidade antioxidante destes flavonóides.
- A rutina não se mostrou eficiente em proteger contra o aumento na peroxidação lipídica causada pela exposição in vitro ao MeHg, além de apresentar atividade pró-oxidante. Esta falta de proteção parece estar relacionada à sua estrutura química.
- A quercitrina protegeu contra os danos causados pelo MeHg *in vitro* e *in vivo*, revertendo os danos comportamentais e bioquímicos causados pelo MeHg, mostrando que este flavonóide pode vir a ser utilizado no tratamento de intoxicação por MeHg.

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