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Papel de selenoproteínas na neurotoxicidade induzida por metilmercúrio, em camundongos, e potencial bioinseticida de uma alga da Antártica (*Prasiola crispa*) em modelo *de Drosophila melanogaster*

DISSERTAÇÃO DE MESTRADO

ANA PAULA PEGORARO ZEMOLIN

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Dissertação apresentada ao programa de Pós-graduação Stricto sensu em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

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São Gabriel

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PAPEL DE SELENOPROTEÍNAS NA NEUROTOXICIDADE INDUZIDA POR METILMERCÚRIO EM CAMUNDONGOS E POTENCIAL BIOINSETICIDA DE UMA ALGA DA ANTÁRTICA (*Prasiola crispa*) EM MODELO DE *Drosophila melanogaster*

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“Quando a gente acha que tem todas as respostas,

vem a vida e muda todas as perguntas ...”

Luis Fernando Verissimo

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RESUMO

O metilmercúrio (MeHg) é um agente tóxico que causa importantes prejuízos à saúde humana e ambiental. Parte desses efeitos está relacionado a sua capacidade de induzir estresse oxidativo. Os mecanismos precisos pelos quais o MeHg leva ao estresse oxidativo ainda não estão bem esclarecidos. Dados na literatura apontam para a participação de selenoproteínas como a glutationa peroxidase e a tioredoxina redutase neste processo. Neste estudo, buscou-se investigar o papel de isoformas de glutationa peroxidase (GPx1 e GPx4) e da tioredoxina redutase (TrxR1) na neurotoxicidade induzida por MeHg em camundongos, focando na atividade e expressão destas proteínas. Nossos resultados mostraram, que o tratamento de camundongos Swiss machos adultos com MeHg (40 mg/L na água de beber) durante 21 dias causa uma diminuição significativa na atividade das enzimasGPx e TrxR no córtex e cerebelo dos animais tratados, em comparação com os controles. Observou-se também uma significativa redução na expressão de GPx1, GPx4 e TrxR1 no cerebelo dos animais tratados, enquanto que no córtex apenas GPx4 e TrxR1 foram afetadas. Concomitantemente a estes resultados, observou-se um aumento significativo na atividade das enzimas antioxidantes SOD, CAT, GR e GST no cerebelo e da CAT no cortex. A expressão de HSP70 foi significativamente aumentada no cerebelo dos animais tratados. Estes dados denotam uma clara resposta celular antioxidante frente aos efeitos tóxicos do MeHg em nosso modelo, reforçando dados da literatura que indicam o estresse oxidativo como um importante mecanismos na neurotoxicidade induzida por este organometal. Além disso, nossos resultados apontam para isoformas de glutationa peroxidase e tioredoxina redutase como importantes alvos moleculares do MeHg e, ao menos, em nosso conhecimento, este é o primeiro estudo demosntrando o papel da GPx4 na neurotoxicidade induzida por este agente tóxico ambiental.

Outro objetivo deste trabalho foi investigar os efeitos biológicos do extrato da alga *Prasiola crispa*(PcE), oriunda do continente Antártico, nos modelos de *Drosophila melanogaster* e *Nauphoeta cinérea*. Organismos adaptados a ambientes extremos como a Antártica tendem a apresentarem uma constituição única em termos de metabólitos secundários. Desta forma, estudos que visem à elucidação de efeitos biológicos de organismos oriundos destas regiões tendem a apresentarem

relevância do ponto de vista biotecnológico. Nossos dados apontam para um potencial biocida de PcE nos modelos de mosca-da-fruta (*Drosophila melanogaster*) e barata cinerea (*Nauphoeta cinerea*), visto que a administração do extrato induziu toxicidade nos dois modelos. A toxicidade em *D. melanogaster* foi avaliada como percentagem de mortalidade, atividade locomotora (geotaxia negativa) e alterações bioquímicas incluindo atividade acetilcolinesterase (AChE) e marcadores de estresse oxidativo.Também foi investigada a ação cardiotóxica do extrato no modelo de coração semi-isolado de barata cinerea. A administração do extrato(2mg/ml) foi feita por 24 horas e, nas moscas, causou um aumento massivo na mortalidade (aumento de 7,6 vezes em relação ao controle). Também foi observado um aumento significativo na atividade locomotora, indicando uma ação neurotóxica do extrato. A atividade AChE, os níveis de glutationa e formação de hidroperóxido manteve-se inalterada. A atividade da glutationa S-transferase aumentou significativamente após a administração de PcE, já a atividade da catalase diminuiu significativamente em moscas que receberam o extrato. No modelo de coração semi-isolado de barata, foi observado uma diminuição da freqüência cardíaca. A incubação do extrato com DTNB, um forte agente oxidante, bloqueou significativamente o efeito cardiotóxico do extrato, sugerindo que compostos redutorespodem ser responsáveis pelo efeito observado. Desta forma, este estudo demonstrou os efeitos tóxicos dePcE, em dois modelos de inseto, sugerindo seu potencial como bioinseticida. Os mecanismos precisos relacionados a este efeito ainda necessitam de esclarecimentos, entretanto, alterações em sistemas antioxidantes vitais podem estar envolvidos.

Palavras-chave: metilmercúrio, neurotoxicidade, glutationa peroxidase, tioredoxina redutase, *Prasiola crispa*, Antártica, efeito bioinseticida.

ABSTRACT

Methylmercury (MeHg) is a toxic agent that causes severe damage to human health and the environment. These effects are related to its ability to induce oxidative stress. The precise mechanisms by which MeHg leads to oxidative stress are not well understood. Data from literature point to the involvement of selenoproteins such as glutathione peroxidase and thioredoxin reductase in this process. In this study, we sought to investigate the role of isoforms of glutathione peroxidase (GPx4 and GPx1) and thioredoxin reductase (TrxR1) in MeHg-induced neurotoxicity in mice, focusing on activity and expression of these proteins. Our results showed that treatment of adult male mice Swiss with MeHg (40 mg / L in drinking water) for 21 days causes a significant decrease in GPx and TrxR enzyme activity in the cerebral cortex and cerebellum of treated animals when compared to controls. We also observed a significant reduction in the expression of GPx1, GPx4 and TrxR1 in the cerebellum of the treated animals, whereas in the cortex only GPx4 and TrxR1 are affected..In parallel, we observed a significant increase in antioxidant enzymes SOD, CAT, GR and GST in the cerebellum and CAT in cortex. HSP70 expression was significantly increased in the cerebellum of the treated animals. These results show a clear antioxidant cell response against the toxic effects of MeHg in our model, reinforcing the literature data indicating oxidative stress as an important mechanism in the neurotoxicity induced by this organometal. Furthermore, our results point to isoforms of glutathione peroxidase and thioredoxin reductase as important molecular targets of MeHg and, at least, to our knowledge, this is the first study demonstrating the role of GPx4 in the neurotoxicity induced by this toxic environmental agent.

Another objective of this study was to investigate the biological effects of the extract of the alga *Prasiola crispa* (PcE), from the Antarctic continent, in the insect models *Drosophila melanogaster* and *Nauphoeta cinerea*. Organisms adapted to extreme environments such as Antarctica tend to present a unique composition in terms of secondary metabolites. Thus, studies aimed at elucidating the biological effects of organisms from these areas tend to be relevant in a biotechnological point of view. Our data demonstrates a potential biocide PcE effect in the fruit fly (*Drosophila melanogaster*) and lobster cockroach (*Nauphoeta cinerea*), since the administration of the extract induced toxicity in both models. Toxicity in *D. melanogaster* was

assessed as percentage of mortality, locomotor activity (negative geotaxis) and biochemical measurements including acetylcholinesterase (AChE) and markers of oxidative stress. We also investigated the cardiotoxic action of the extract in a cockroach semi-isolated heart model. The administration of the extract (2 mg / ml) for 24 hours toflies, caused a massive increase in mortality (7.6-fold increase compared to control). We also observed a significant increase in locomotor activity, indicating a neurotoxic action of the extract. AChE activity, glutathione levels and the formation of hydroperoxide remained unchanged. The activity of glutathione S-transferase significantly increased after administration of PcE while catalase activity was significantly decreased in flies that received the extract. A significant decrease in heart rate in the cockroach semi-isolated heart model was observed after PcE administration. The incubation of the extract with DTNB, a strong oxidizing agent, significantly blocked the cardiotoxic effect of the extract, suggesting that reducing compounds may be responsible for the observed effect. Thus, this study demonstrated, for the first time, the toxic effects of PcE, in two insect models, suggesting its potential as a bioinsecticide. The precise mechanisms related to this effect still need clarification, however, changes in vital antioxidant systems may be involved.

Keywords: methylmercury, neurotoxicity, glutathione peroxidase, thioredoxin reductase, *Prasiola crispa*, Antarctica, effect of insecticide.

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão da literatura sobre os temas trabalhados nesta dissertação.

A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, que se encontra no item **MANUSCRITO**. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

O item **CONCLUSÕES**, encontrado no final desta dissertação, apresenta interpretações e comentários gerais sobre os resultados dos manuscritos presentes neste trabalho.

As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **CONCLUSÕES** desta dissertação.

SUMÁRIO

1. INTRODUÇÃO

1.1 Espécies Reativas de Oxigênio (EROs) e Defesas Antioxidantes

A utilização do oxigênio para a produção mais eficiente de energia possibilitou o surgimento de organismos multicelulares complexos no ambiente terrestre. A maioria desses organismos utiliza o oxigênio como receptor final na cadeia transportadora de elétrons na mitocôndria, a fim de produzir trifosfato de adenosina (ATP) como fonte de energia. As células de mamíferos utilizam mais de 80% do ATP mitocondrial na manutenção de suas atividades metabólicas.

No entanto, ao mesmo tempo em que a utilização do oxigênio permitiu melhor aproveitamento de energia, trouxe desvantagens, visto que muitas espécies reativas originárias do seu metabolismo podem causar danos celulares (Halliwell et al., 2007; Omoni et al., 2005), além de estarem relacionadas a inúmeras condições patológicas, tais como artrite, câncer, inflamações, doenças cardíacas e degenerativas, além do envelhecimento. (Samant et al., 1998; Rojkind et al., 2002).

A toxidade do oxigênio decorre da interação das EROs com diversas biomoléculas podendo lesionar diferentes estruturas celulares. Na normalidade, é muito baixa a concentração de EROs dentro das células, devido a presença de sistemas antioxidantes celulares que catalisam a remoção ou impedem a formação de EROs (Oga S. et. al, 2008).

Uma substância antioxidante é tida com uma substância que inibe o processo de oxidação, ou, quando em baixa concentração em relação ao substrato oxidável, é capaz de inibir ou diminuir a capacidade de oxidação do substrato em questão. (Omoni et al., 2005) No decorrer da evolução, os seres vivos desenvolveram esses mecanismos adaptativos capazes de lhes permitirem co-existir com a exposição aos oxidantes. As defesas contra esses agentes envolvem diversos mecanismos, como os de prevenção e reparo, em que estão incluídas essas defesas antioxidantes. (Halliwell et al., 2007).

No decorrer da evolução, os seres vivos desenvolveram mecanismos adaptativos capazes de lhes permitirem co-existir com a exposição a EROs. Entre os

mecanismos de defesa antioxidante celular, estão os sistemas não enzimáticos como o tripeptídeo glutationa (GSH), vitamina E e C, albumina, polifenóis, flavonóides, entre outros. Já dentre os mecanismos de defesa enzimáticos, as enzimas antioxidantes como a superóxido dismutase (SOD), glutationa peroxidade (GPx), glutationa S-transferase (GST), catalase (CAT), Tiorredoxina Redutase (TrxR1) e HSP70 podem ser destacados (Halliwell et al., 2007, Oga S. et. al, 2008).

A glutationa peroxidase (GPx) atua na eliminação do peróxido de hidrogênio a uma gama de peróxidos orgânicos (Nordberg e Arnér, 2001). Dos seis tipos conhecidos da GPx em mamíferos, cinco possuem selenocisteína na sua constituição: GPx 1, citosólica, Gpx 2, gastrointestinal, Gpx3, plasmática, Gpx 4 que encontra-se nas membranas celulares, com função, principalmente de proteger contra a peroxidação lipídica e reduzir os fosfolipídios de membranas, e GPx 6, encontrada no sistema olfatório humano. A Gpx5 se expressa no epidídimo de roedores e de alguns primatas e contém cisteína no lugar de selenocisteína. As diversas enzimas GPx em mamíferos adotaram diferentes funções. A GPx-1 tem atividade bem direcionada para equilibrar o estresse oxidativo celular (Brigelius-Flohé, 2006). A Gpx-4 bloqueia a o fator nuclear NF-kB e com isso inibe a biossíntese de leucotrienos e prostraglandinas, controlando também a inflamação (Brigelius-Flohé, 2006). Muitas das patologias que envolvem deficiências do selênio parecem estar associadas a danos oxidativos nos tecidos e à diminuição da atividade da GPx-1 (Arthur, 2000; Brigelius-Flohé, 2006).

O sistema tiorredoxina é composto pela tiorredoxina (Trx), pelas tiorredoxinas redutases e NADPH. Exerce um importante papel na manutenção do estado redox intracelular. Atuam na redução de pontes dissulfeto em proteínas. As TrxR catalisam a reação da redução da tiorredixina oxidada, consumindo NADPH(Arnér e Holmgren, 2000). Existem três isoformas descritas para mamíferos de TrxRs: TrxR1 (citosólico / nuclear) ; TrxR2 (mitocôndriais) e, TrxR3 é encontrados nos testículos.

Quando há limitação nestes sistemas antioxidantes, podem ocorrer diversas lesões cumulativas, levando a disfunção e morte celular (Atoui et al, 2005).

1.2 Compostos Naturais

Atualmente, vários estudos têm sido conduzidos objetivando a busca por novos compostos naturais para aplicação biotecnológica e na saúde. Parte destas pesquisas buscam descobrir suas atividades antioxidantes, com potencial para reverter o estresse oxidativo e assim atuar na cura e prevenção de uma série de doenças (Pereira et al., 2009; Sudatti et al. 2009; Bastianetto e Quirion, 2002; Ávila et al., 2008; Wagner et al., 2006; Williams et al., 2004; Patel et al., 2007), bom como encontrar compostos com atividades citotóxicas.

Organismos adaptados a ambientes extremos como a Antártica tendem a apresentarem uma constituição única em termos de metabólitos secundários. Neste contexto, plantas oriundas desses ambientes, são fontes singulares de compostos com potencial biotecnológico (Bravo et al., 2001).

1.2.1 *Prasiola crispa*

Os organismos que vivem em regiões polares são expostos às condições ambientais mais extremas do planeta. Este fato levou ao desenvolvimento de estratégias naturais que os permitem sobreviver nas condições mais adversas da Terra. Dentre estas estratégias de adaptação estão a produção de fotoprotetores, como micosporinas, aminoácidos, citoneminas secretada por cianobactérias e flavonóides secretados pelas plantas. (Pereira et al., 2009; Mostaert et al., 2006). Este fato demonstra a relevância de um estudo mais profundo sobre os efeitos biológicos desta alga, que pode apresentar em sua constituição uma combinação de compostos químicos que, normalmente, não são encontrados em nenhum outro organismo.

1.3 Mercúrio

O Mercúrio (Hg) está presente no ambiente de diferentes formas sendo: mercúrio elementar (Hg0), mercúrio inorgânico (HgII) e orgânico (MeHg). O Hg pode ser encontrado em pequenas concentrações nos diferentes compartimentos terrestres (biosfera, atmosfera, litosfera e hidrosfera) havendo fluxo contínuo deste metal, através de seu ciclo biogeoquímico (Johannesson, 2002). O mercúrio ocorre

também sob a forma de sais inorgânicos como o cloreto de mercúrio (HgCl2) e formas orgânicas como o metilmercúrio (MeHg) e dimetilmercúrio. O Hg precipitado junto com as chuvas atinge os ambientes aquáticos onde será metabolizado por microrganismos que produzirão as formas orgânicas de Hg (Clarkson et al., 2003;Nriagu e Pacyna 1988), desta forma, podendo atingir a cadeia trófica e ser incorporado aos organismos aquáticos através de um processo de bioacumulação (Lindstrom et al., 1991).As formas orgânicas de Hg também são produzidas pelo homem na indústria química.

Hg é considerado uma neurotoxina bem documentada que pode causar danos permanentes ao sistema nervoso central (Crespo-López et al., 2005). Os íons de Hg têm a capacidade de formar complexos muito fortes com os grupos sulfidrilas (–SH) das proteínas (presentes no aminoácido cisteína), afetando vários processos entre eles a formação do fuso mitótico. (Rodgers et al., 2001; Thier et al., 2003; Stoiber et al., 2004; Bonacker et al., 2005).

Além disso, o Hg apresenta-se como um elemento capaz de causar danos nefrotóxicos (Zalups, 2000), hepatotóxicos (Larini et al., 1997),aumento na concentração de espécies reativas de oxigênio (EROs) e diminuição das defesas antioxidantes (Hultberg et al., 2001; Farina et al., 2003; Wataha et al., 2008; Franco et al., 2008), bem como outros efeitos adversos em tecidos neurais (Mirzoian & Luetje, 2002) e no sistema imunológico onde auto-imunidade e imunosupressão têm sido relatados (Lalancette et al.,2003; Gagnaire et al., 2004; Mellegard et al., 2004).Neste sentido, a detecção e controle dos níveis de mercúrio no ambiente são de alta relevância para a saúde pública e no âmbito do desenvolvimento sustentável (Frasco et al. 2007; Malm et al., 1998).

1.3.1 Metil Mercúrio

O metilmercúrio (MeHg) é um reconhecido poluente ambiental que, nas últimas décadas, causou contaminação e intoxicação humana em várias partes do globo.(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 et al., 2003). Neste contexto, estudos epidemiológicos apontam para déficits neurológicos em comunidades pesqueiras

que possuem uma dieta baseada no consumo de peixes (Grandjean et al., 1997; Clarkson et al., 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). A exposição ao MeHg causa déficits severos na atividade locomotora tanto em humanos como em animais experimentais (Robertson e Orrenius, 2000; Gochfeld, 2003). Em roedores, esses distúrbios são mais pronunciados na fase de desenvolvimento (Franco et al., 2007).

Os mecanismos precisos pelos quais o MeHg causa citotoxicidade ainda não estão completamente esclarecidos. Porém, vários trabalhos sugerem que as diversas disfunções celulares causadas pelo MeHg estejam associadas a sua alta afinidade por grupos tiol (SH) e selenol (SeH) (Farina et al., 2011), dessa forma causando: (a) depleção de glutationa (GSH) (Rooney, 2007; Shanker et al., 2005); (b) inativação de enzimas (Zheng et al.,2003; Franco et al, 2009; Usuki et al., 2011); (c) dano ao DNA e morte celular (Nishioku et al., 2001).

Enfim, 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, como esclerose lateral, Alzheimer, esclerose múltipla e Parkinson (Clarkson,2002; Mutter et al., 2004).

1.4 Modelo experimental

Grande parte do entendimento dos mecanismos que levam ao desenvolvimento das doenças tem sido obtida graças aos modelos experimentais realizados com animais, sejam eles vertebrados ou invertebrados. O modelo experimental se faz valer pela capacidade deste em representar com fidelidade o fenômeno natural. Esse modelo animal, obrigatoriamente, deve permitir a avaliação de fenômenos biológicos naturais, induzidos ou comportamentais, que possam ser comparados aos fenômenos em questão. (Ferreira et al.,2005)

1.4.1 *Drosophila melanogaster*

O inseto, *Drosophia melanogaster,* conhecido popularmente como mosca da fruta, é um modelo biológico muito usado para realizar análises genéticas, mas

também é reconhecido por sua alta sensibilidade a substâncias tóxicas sendo considerado um bioindicador para detecção de poluentes e, também, para testar a ação biológica de substâncias naturais.

D. melanogaster e os humanos compartilham inúmeros genes, conservam vias metabólicas e sinalizadoras e, além disso, há uma crescente evidência de conservação a nível comportamental e de seus mecanismos moleculares, incluindo ritmos circadianos, aprendizagem, memória e sono, bem como, mais recentemente, estão sendo empregados como modelos animais de doenças que afetam o sistema nervoso como, por exemplo, a doença de Parkinson e Mal de Alzheimer (Nichols, 2006; Benton, 2008; Bagatini et al., 2011).

1.4.2 *Nauphoeta cinerea*

Nauphoeta cinerea, pertencente a ordem Blattodea, família Blaberidae, caracterizam-se por apresentarem pernas curtas, corpo oval e achatado dorso-ventralmente , em geral , coloração escura e por habitarem ambiente tropical e/ou temperado (Clopton, 2012). A maioria é solitária e apresentam hábito noturno sendo que neste período procuram por alimento e parceiros (as) para o acasalamento, e realizam oviposição e dispersão.

Recentemente, este inseto vem sendo usado como modelo biológico visto que apresentam sensibilidade a substâncias tóxicas. Desta forma, sendo considerado um bioindicador para detecção de poluentes e também para testar a ação biológica de substâncias naturais.

1.5 Seleproteínas

Selenoproteínas são proteínas que incorporam especificamente a selenocisteína na sua cadeia de aminoácidos. A maioria das selenoproteínas é enzima com o resíduo de selenocisteína responsável por suas funções catalíticas. As selenoproteinas são peças importantes em uma variedade de processos biológicos essenciais, atuando como oxirredutases que protegem e reparam componentes celulares, regulando o estado redox da proteína. (Rother et al., 2001; Finn et al., 2008).

As selenoproteínas compreendem pelo menos 20 proteínas eucarióticas, com expressão individual caracterizada pela alta especificidade com tecidos. A família da glutationa peroxidase (GPx) corresponde às selenoproteínas mais bem caracterizadas, e o grau de atividade dessa enzima no fígado ou no plasma é indicado pela presença de selênio no organismo (Lin & Shiau, 2005a, Kohrle et al., 2005).

Em linhas gerais, esta dissertação aborda em um primeiro capítulo a elucidação de mecanismos relacionados à neurotoxicidade induzida por MeHg *in vivo* em camundongos, focando na atividade e expressão de selenoproteínas, incluindo GPx1, GPx4 e TrxR1. Em um segundo capítulo, objetivou-se investigar os efeitos tóxicos do extrato de *Prasiola crispa*, uma alga da Antártica, em modelo de moscas-da-fruta (*Drosophila melanogaster*) e barata cinérea (*Nauphoeta cinerea*).

2. OBJETIVOS

2.1 Manuscrito 1:

- Avaliar a atividade e expressão de GPx1, GPx4 e TrxR1 em um modelo de camundongos expostos a MeHg*, in vivo.*

2.2 Manuscrito 2:

- Avaliar a toxidade e o potencial biocida do extrato da alga *Prasiola crispa* em *D. melanogaster* e *Nauphoeta cinerea*, bem como contribuir com a busca por inseticidas naturais que possam auxiliar a diminuir o uso de inseticidas sintético comerciais, minimizando os danos ambientais (Hernández et al., 1996), decorrentes de seu uso.

3. RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos manuscritos, **os quais estão dispostos na forma em que foram submetidos para publicação.**

4. MANUSCRITOS

4.1 Manuscrito 1

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Evidences for a role of glutathione peroxidase 4 (GPx4) in methylmercury induced neurotoxicity in vivo

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Running title: GPx4, GPx1, and TrxR1 inhibition by methylmercury in mouse brain.

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Abstract

We evaluated the activity and expression of antioxidant enzymes in the cerebellum and cortex of Swiss adult male mice exposed to methylmercury (MeHg) in drinking water (40mg/L) during 21 days. The activity of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD) and thioredoxin reductase (TrxR) were determined spectrophotometrically. The expression (protein levels) of GPx1 and GPx4 isoforms, TrxR1 as well as heat shock protein 70 (HSP70) were evaluated using specific antibodies and normalized by actin levels. The exposure of mice to MeHg caused a significant impairment in locomotor performance in the open field test (crossings and rearings). This result was followed by a significant reduction of GPx and TrxR activities in the cerebellum and cortex when compared to untreated animals. We also observed a substantial decrease in GPx1, GPx4 and TrxR1 protein levels in the cerebellum, while in the cerebral cortex, only GPx4 and TrxR1 were decreased after MeHg treatment. The activities of the antioxidant enzymes GR, GST, CAT and SOD were increased in the cerebellum after MeHg administration to mice. In contrast, only CAT was increased in the cerebral cortex of MeHg-treated animals. The expression of HSP70 was upregulated only in the cerebellum where MeHg-exposed mice showed a significant increase in the immunocontent of HSP70 when compared to controls. This is the first report showing a role for GPx4 in the neurotoxicity induced by MeHg in vivo. In addition, our data indicates that the selenoproteins GPx and TrxR as main targets during MeHg exposure, which may be considered in biomarker studies.

Keywords: methylmercury, selenoproteins, glutathione peroxidase, thioredoxin reductase, enzyme activity, protein expression.

1. INTRODUCTION

Reactive oxygen/nitrogen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical are known to induce damage of key biological components and cell membranes (Halliwell and Gutteridge, 2007). In order to counteract the deleterious effects of reactive species, cells developed a specialized machinery of antioxidant defense (Mugesh and Singh, 2000). Cellular defense against ROS requires the expression of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase which play central role in the detoxification of reactive species (Finkel and Holbrook, 2000; Arteel and Sies, 2001).

Methylmercury (MeHg) has been recognized as a ubiquitous environmental toxicant whose toxicity is associated to neurological and developmental deficits in animals and humans (Clarkson et al., 2003). Although environmental hazards such those occurred in the past in Japan and Iraq between the 50's and 70's, several anthropogenic sources of MeHg still pose high risk to human and environmental health (Hylander and Goodsite, 2006). Also important, it has been shown that mercury transport from more densely populated regions (lower latitudes) results in the accumulation of MeHg in the food chain of Arctic and Antarctic environments (Barkay and Poulain, 2007). Due to its potential bioaccumulation in fish, as well as its intensive applications in industry, coal fired power plants and mining, intoxication episodes are mainly related to diet and occupational exposures (Clarkson et al., 2003; Hylander and Goodsite 2006; Honda et al., 2006). The central nervous system (CNS) is highly susceptible to MeHg toxic effects and the developing brain has been shown to be largely sensitive to the neurotoxic actions of this organometal (Johansson et al., 2007; Grandjean and Herz, 2011).

The exact mechanisms underlying MeHg toxicity are not fully understood. However, it has been shown that oxidative stress plays a central role in this process (Aschner et al., 2007; Farina et al., 2011a). MeHg-induced oxidative stress seems to be related to direct oxidative properties of MeHg toward endogenous thiol and selenol groups in low molecular weight molecules as well as proteins (Shanker et al., 2005; Farina et al., 2011b). The inhibitory effects of MeHg toward selenoenzymes like glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) has been pointed out as a pivotal mechanism on MeHg induced ROS production and consequent oxidative stress (Franco et al., 2009; Wagner et al., 2010).

The selenoproteins GPx and TrxR have been described as important antioxidant enzymes in the cellular protection against damage caused by ROS (Reeves and Hoffmann 2009). The glutathione antioxidant system includes reduced glutathione (the most important low-molecular-weight sulfhydryl-containing antioxidant) and the GSH-related enzymes GPx and glutathione reductase (GR) (Dringen, 2000). Mammalian cells contain five isoforms of selenium-dependent GPx's: cytosolic GPx (GPx1), gastrointestinal GPx (GPx2), plasma GPx (GPx3), phospholipid hydroperoxide GPx (GPx4), and, in humans, GPx6, expressed only in the olfactory system (Brigelius-Flohe, 2006). GPx1, also called cytosolic or cellular GPx, is the most prominent GPx isoform and it is able to reduce hydrogen peroxide and a range of organic peroxides, including cholesterol and long-chain fatty acid peroxides, by expending GSH (Sunde, 1997; Arthur, 2000). GPx4 is expressed in a variety of tissues, however its subcellular localization is tissue dependent (Conrad et al., 2007). The main substrate for GPx4 is phospholipid hydroperoxides, a fact that may indicates the crucial role of GPx4 in the counteraction of lipid peroxidation (Brigelius-Flohe, 2006). Thioredoxin reductase (TrxR) enzymes are antioxidant

proteins that catalyze the reduction of oxidized thioredoxin by expenses of NADPH (Arner and Holmgren, 2000). There are three mammalian TrxRs described. TrxR1 (cytosolic/nuclear) and TrxR2 (mitochondria) are distributed in several tissues and TrxR3 is testes specific (Rundlof et al., 2004).

Although recent studies have demonstrated that MeHg causes decreases in the activity of GPx and TrxR, it is still unknown whether this process involves a protein expression alteration or a post-translational modification on the enzymes by this organometal. Thus, the aim of this study was to evaluate the activity and expression, in terms of protein levels, of GPx1, GPx4 and TrxR1 in a mouse model of MeHg exposure in vivo.

2. MATERIALS AND METHODS

2.1. Chemicals

Glutathione reductase (G3664), glutathione reduced (GSH), glutathione oxidized (GSSG), t-butyl-hydroperoxide (t-bOOH), 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate(NADPH)Methylmercury (II) chloride, protease inhibitor cocktail were purchased from Sigma-Aldrich (St Louis, MO, USA).All antibodies utilized in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other chemicals used in this work were from the highest analytical grade.

2.2. Animals and MeHg exposure protocol

Swiss mice were used from the Central Animal Facility of the Federal University of Santa Maria. The animals were kept in a vivarium in cages with free access to food and water at a controlled temperature (22 ± 3° C) and a light / dark cycle of 12:12 hours. Were maintained and used in accordance with the standards of the Animal Care Guidelines from the National Institutes of Health of the United States of America and the National Control of Animal Experimentation (CONCEA). Mice were treated with MeHg (40 mg/L) diluted in drinking water during 21 days. This protocol was previously published by our group and induces a significant increase in Hg levels in the mouse brain, followed by locomotor activity impairment (Farina et al., 2005; Dietrich et al., 2005). All experiments started 24 hours after MeHg exposure was finished. After treatment was finished the animals were acclimated to the experimental room for at least 2 hours prior to the beginning of the open field test. Open field tests were carried out in soundproof room without any human interference, as described elsewhere (Franco et al., 2007).

2.3. Western blot analysis

Western blotting was performed according to Franco et al., (2010) with minor modifications. The brain structures (cerebellum and cortex) were homogenized at 4 °C in 300 µL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na3VO4, 100 mM sodium fluoride and protease inhibitor cocktail (Sigma, MO). The homogenates were centrifuged at 1000 × g for 10 min at 4 °C and the supernatants (S1) collected. After total protein determination (Bradford, 1976) using bovine serum albumin as standard), β-

mercaptoethanol was added to samples to a final concentration of 8%. Then samples were frozen at −80 °C for further analysis. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Then, membranes were incubated with specific primary antibodies for the determination of GPx1, GPx4, TrxR1, HSP70, and β-actin protein expression. The blots were developed using secondary antibody linked to peroxidase and luminescence was captured in a Carestream Image Station 4000MM PRO molecular imaging system.

2.4. Assessment of antioxidant enzyme activity

Enzyme activity was determined in a Thermo Scientific Evolution 60S UV-Visible spectrophotometer. GR and GPx activity as described previously (Franco et., al 2007). Briefly, GR reduces GSSG to GSH, expending NADPH, the disappearance of which can be measured at 340 nm (Carlberg and Mannervik, 1985). The GPx1 and GPx4 activity was determined using the coupled assay described by Wendel, (1981) which indirectly monitors the consumption of NADPH at 340 nm using tert-butylhydroperoxide as GSSG generator in the assay conditions. Glutathione transferase (GST), activity was assayed by the procedure of Habig and Jakoby (1981) using 1-chloro-2,4-dinitrobenzene as substrate. Catalase (CAT) activity was measured according to Aebi (1984). Superoxide dismutase (SOD), activity was evaluated according to Kostyuk and Potapovich (1989). TrxR1 activity was measured based on the method of Holmgren and Bjornstedt (1995).

2.5. Statistical analysis

Statistical differences between groups were analyzed by Student's t-test. Differences were considered statistically significant when $P<0.05$.

3. RESULTS

3.1. MeHg induces locomotor deficits in mouse

The oral administration of MeHg (40mg/L in drinking water) caused a significant impairment in the mouse locomotor skills. As observed in Figure 1, the number of crossings (Figure 1A) and rearings (Figure 1B) were significantly ($p<0.05$) lower in MeHg-treated mice, when compared to untreated controls.

3.2. MeHg induces decreases in the activity and expression of selenoproteins in the mouse brain

There was a significant decrease in the activity of GPx in the cerebellum (Figure 2A; $p<0.001$) and cerebral cortex (Figure 2B; $p<0.05$) of MeHg-treated mice. TrxR activity was also decreased in both brain structures (cerebellum – $p<0.001$; cerebral cortex – $p<0.05$) of MeHg intoxicated animals (Figure 2C and 2D). We analyzed the expression (protein levels) of GPx1, GPx4 and TrxR1 by western blotting. As observed in Figure 3, there was a significant decrease in the levels of these selenoproteins in the cerebellum of treated mice, when compared to control. The Figure 3A shows representative blots of immunoreactive bands for GPx1, GPx4, TrxR1 and β-actin (loading control) in the cerebellum of controls and MeHg treated

animals. The Figures 3B to 3D represent the densitometric analysis of immunoreactive bands for GPx1 (Figure 3B), GPx4 (Figure 3C) and TrxR1 (Figure 3D) in the cerebellum. The results are expressed as ratio of target protein/ β-actin and controls were considered as 100%. The Figure 4A shows representative blots of immunoreactive bands for GPx1, GPx4, TrxR1 and β-actin in the cerebral cortex of controls and MeHg treated animals. The Figures 4B to 4D represent the densitometric analysis of immunoreactive bands for GPx1 (Figure 4B), GPx4 (Figure 4C) and TrxR1 (Figure 4D) in the cortex. In the cerebral cortex of MeHg-treated mice, we did not observe a significant change in GPx1 expression (Figure 4B), when compared to control.

3.3. MeHg induces changes in the activity of antioxidant enzymes in the mouse brain

The administration of MeHg to mice caused a significant increase ($p<0.05$) in the activity of GR (Figure 5A), GST (Figure 5B), CAT (Figure 5C) and SOD (Figure 5D) in the cerebellum, when compared to control. In contrast, in the cerebral cortex (Figure 6), only CAT activity was altered. It was observed a significant increase ($p<0.05$) in the activity of this enzyme in the MeHg-treated animals, when compared to untreated controls (Figure 6C). The expression of HSP70 was determined in the brain structures (Figure 7). As observed in Figure 7A, MeHg-treated mice showed an increased expression of this chaperone in the cerebellum. The levels of HSP70 were not changed in the cerebral cortex, when comparing MeHg versus control animals (Figure 7B).

4. DISCUSSION

In the last years, reports in literature have pointed oxidative stress as a main mechanism by which MeHg exerts it deleterious effects to the CNS (reviewed by Farina et al., 2011a; 2011b). It was previously demonstrated that inhibition of important antioxidant enzymes activity could, at least in part, be responsible for the oxidative damage caused by this organometal (Carvalho et al., 2008; Carvalho et al., 2011; Farina et al., 2009; Franco et al., 2009; Glaser et al., 2010; Wagner et al.,2010; Branco et al., 2011). Because several of these primary targets, such as the antioxidant selenoenzymes, GPx and TrxR, are involved in the maintenance of the redox cell balance, their inhibition by MeHg can predispose cells to oxidative stress. Our goal was to demonstrate, for the first time in vivo, the participation GPx-4 as a main target during MeHg poisoning events.

In previous publications our group have shown the central role of glutathione peroxidase in the toxicity of MeHg in vivo and in vitro (Franco et al., 2009; Farina et al., 2009). Considering the high affinity of Hg by thiols and selenols (Hughes, 1978; Onyido et al., 2004), the inhibitory action of Hg towards selenoprotein such as GPx and TrxR may be related to a direct interaction of this metal with the selenol portion of these enzymes. In a physiological point of view, selenols retain an increased affinity for strong electrophile groups such as mercury, when comparing to thiol groups (Sugiura et a., 1976), thus, selenoproteins may be considered as primary targets during poisoning events with this organometal. Parallel to a decrease in the activity of GPx and TrxR in the brain of MeHg-treated mice, we also found a marked reduction in the expression levels of these proteins. Our data shows that, in addition to a putative post-translational modification of selenol moieties in the molecular

structures of GPx and TrxR proteins, the inhibitory effect of mercury compounds towards these selenoproteins in brain is related to a decrease in protein levels of different GPx and TrxR isoforms, a fact that can be seen as a novel mechanistic elucidation of MeHg neurotoxic outcomes, corroborating previous studies in literature (Usuki et al., 2011).

The inhibitory action of mercury compounds towards the thioredoxin system has been previously shown. In a series of elegant studies using a fish model, it was demonstrated that MeHg inhibits TrxR in several organs, including fish brain (Branco et al., 2011; Branco et al., 2012). Our study expands those contributions to literature and demonstrates that the inhibitory effects of MeHg on the thioredoxin system occur in vivo, and reports for the first time inhibition of TrxR in the brain of mammals. This seems to be a relevant phenomenon, since the thioredoxin system is reported to modulate a vast network of cell signalling pathways, and its inhibition, is likely to compromise the overall cell function and viability (Branco et al., 2011; Farina et al., 2011).

One main finding of our study was the decreased GPx4 protein expression in the brains of MeHg treated mice. Glutathione peroxidase 4 (GPx4) is ubiquitously expressed in mammals and appears to be the only known GSH-dependent enzyme that is essential for life (Yant et al., 2003). It is a versatile enzyme which is the only one out of seven isoforms in mammals able to reduce phospholipid hydroperoxides and repair oxidative damage to biomembranes (Roveri et al., 1994; Liang et al., 2009). In the absence of GSH as a reducing cofactor, GPx4 also utilizes protein-thiols in its catalytic cycle (Conrad, 2005).

The neurotoxicity induced by MeHg is in part attributed to its ability to promote lipid peroxidation (Farina et al., 2011). In addition, mitochondrial dysfunction also play central role in the toxic events elicited by this organometal (Mori et al., 2007; Franco et al., 2007). The apoptotic cell death induced by MeHg is in part attributed to release of apoptotic factors from mitochondria (Cecatelli et al., 2010) and lipid peroxidation of mitochondrial membranes may play a central role in this process (Franco et al., 2009, 2010). It has been shown that a GPx4 variant is localized to mitochondrial membranes (Pfeifer et al., 2001) and lipid peroxidation is shown to be a major trigger of cell death downstream of GPx4 deletion in animal models, a fact that is corroborated by results showing the protective effects of lipophilic antioxidants such as α-tocopherol (Conrad, 2009). Taking this into account, we suggest GPx4 to be a central modulator of cell death during pro-oxidative events and the inhibitory effects (direct inhibition and lowering protein expression) of MeHg towards this protein may be indicated as a prominent molecular mechanism of toxicity.

The inhibitory effects of MeHg towards the selenoproteins GPx1, GPx4 and TrxR1 correlates with the triggering of a cellular response cascade in order to counteract the pro-oxidative outcomes induced by exposure to the organometal. We have shown here that in addition to an increase on HSP70 levels, several antioxidant enzymes including SOD, CAT, GST and GR were up-regulated cerebellum, with a less pronounced response in the cerebral cortex of MeHg poisoned mice. This phenomenon appears to be a common response in several animal models, including rodents and fish (Franco et al., 2009; Branco et al., 2011, 2012; Yang et al., 2012), as well as in invertebrates (de Paula et al., 2012). The NF-E2-related factor 2 (Nrf2) is thought to be a pivotal regulator of the ARE-driven cellular defense against oxidative stress and its regulation appears to cell specific (Lee et al., 2005). This

transcription factor binds to the “antioxidant responsive element” – ARE (Nrf2-ARE pathway) and has been shown to regulate the expression of several antioxidant proteins such as glutathione-S-transferase (GST), GPX, GR, SOD, CAT and the thioredoxin system (Tanito et al., 2007; Schülke et al., 2012). The antioxidant responses after MeHg exposure may be related to an activation of Nrf2-ARE pathway. Reports in literature have demonstrated in cultured cells that MeHg activates Nrf2, which appears to be a limiting factor in the reduction of MeHg toxicity (Wang and Zang, 2009; Ni et al., 2011). Notwithstanding, further studies are necessary to clarify the role of Nrf2 in the protection against MeHg-induced deleterious effects under in vivo conditions.

It was observed a differential effect of MeHg exposure on the activities of antioxidant enzymes in the cerebral and cerebellar cortex. The cerebellum is shown to be selectively affected by mercury compounds (Leyshon and Morgan, 1991; Manto, 2012). In this regard, it has been shown that MeHg exposure causes specific degeneration of cerebral and cerebellar granule cells which are more densely distributed in the cerebellum as compared with the cerebrum (Leyshon-Sorland et al. 1994; Nagashima 1997). Also comparing cerebellum to other brain areas, Mori et al., (2007) have elucidated that rodent cerebellum mitochondria presents higher oxygen consumption and lower levels of antioxidants, such as glutathione, a fact that is likely to exacerbate the susceptibility of this brain structure to oxidative damage. Increased levels of GSH may act as a buffer allowing less “free” mercury to attack additional cellular targets, however, further studies are necessary to clarify the observed differential tissue specific effect of MeHg on the mouse brain antioxidant system.

Summarizing, our results, together with literature data indicates the selenoproteins GPx1, GPx4 and TrxR1 as central targets during MeHg poisoning events. Our data also points to a primary role for GPx4 during MeHg poisoning in vivo. The inhibition of enzyme activity and protein expression of these molecular targets may be toxicologically relevant and should be taken into account in biomarker studies.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

Authors declare no conflict of interest

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Figure legends

Fig. 1.Effects of MeHg exposure on mouse locomotor performance. Animals were orally treated with MeHg (40 mg/L in drinking water) for 21 days as described under Materials and methods and assayed for locomotor activity in the open field apparatus where the (A) number of crossing and (B) rearings were counted during 5 min. Data are expressed as the means±SD (n=8). * $p<0.05$ compared to controls.

Fig. 2.Effects of MeHg on GPx and TrxR activity. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished GPx and TrxR activity was determined in the cerebellum and cerebral cortex. (A) and (B) GPx activity in the cerebellum and cortex, respectively. (C) and (D) TrxR activity in the cerebellum and cortex, respectively. Results are expressed as enzyme activity (mU/mg total protein). Data are means±SD (n=8). *$p<0.05$; ***$p<0.001$ compared to controls.

Fig. 3.Effects of MeHg on GPx1, GPx4 and TrxR1 expression in mouse cerebellum. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished the immunocontent of GPx1, GPx4 and TrxR1 was determined in the cerebellum. (A) representative immunoblots showing the expression levels of GPx1, GPx4 and TrxR1. (B) densitometric analysis of GPx1 immunoreactive bands. (C) densitometric analysis of GPx4 immunoreactive bands. (D) densitometric analysis of TrxR1 immunoreactive bands. Specific protein levels were normalized by b-Actin immunocontent and expressed as a percentage of control. Data are means±SD (n=8). *$p<0.05$; ***$p<0.001$ compared to controls.

Fig. 4.Effects of MeHg on GPx1, GPx4 and TrxR1 expression in mouse cerebral cortex. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21

days, and 24 h after treatment was finished the immunocontent of GPx1, GPx4 and TrxR1 was determined in the cerebral cortex. (A) representative immunoblots showing the expression levels of GPx1, GPx4 and TrxR1. (B) densitometric analysis of GPx1 immunoreactive bands. (C) densitometric analysis of GPx4 immunoreactive bands. (D) densitometric analysis of TrxR1 immunoreactive bands. Specific protein levels were normalized by b-Actin immunocontent and expressed as a percentage of control. Data are means±SD (n=8). *p<0.05 compared to controls.

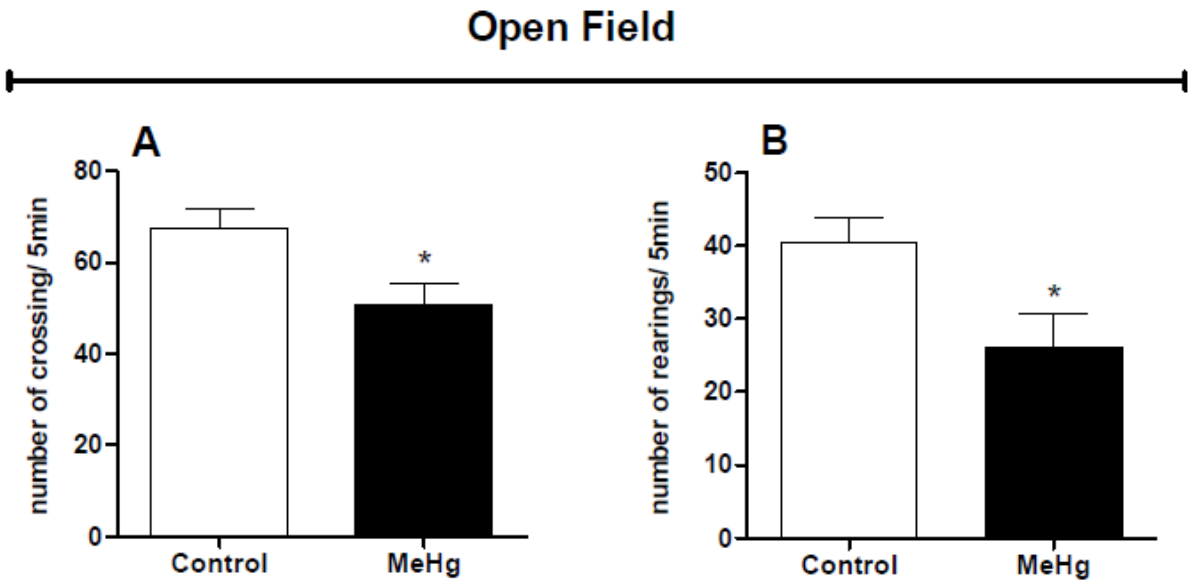
Fig. 5.Effects of MeHg on GR, GST, CAT and SOD activity in mouse cerebellum. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished (A) GR, (B) GST, (C) CAT and (D) SOD activity was determined in the cerebellum. Results are expressed as enzyme activity (mU/mg total protein). Data are means±SD (n=8). *p<0.05; **p<0.01; ***p<0.001 compared to controls.

Fig. 6.Effects of MeHg on GR, GST, CAT and SOD activity in mouse cerebral cortex. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished (A) GR, (B) GST, (C) CAT and (D) SOD activity was determined in the cerebral cortex. Results are expressed as enzyme activity (mU/mg total protein). Data are means±SD (n=8). *p<0.05 compared to controls.

Fig. 7.Effects of MeHg on HSP70 expression in mouse brain. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished the immunocontent of HSP70 was determined in the cerebellum and cerebral cortex. (A) representative immunoblots showing the expression levels of HSP70 and its respective densitometric analysis in mouse cerebellum. (B)

representative immunoblots showing the expression levels of HSP70 and its respective densitometric analysis in mouse cerebral cortex. Specific protein levels were normalized by b-Actin immunocontent and expressed as a percentage of control. Data are means±SD (n=6). *$p<0.05$ compared to controls.

Figure 1. Zemolin et al



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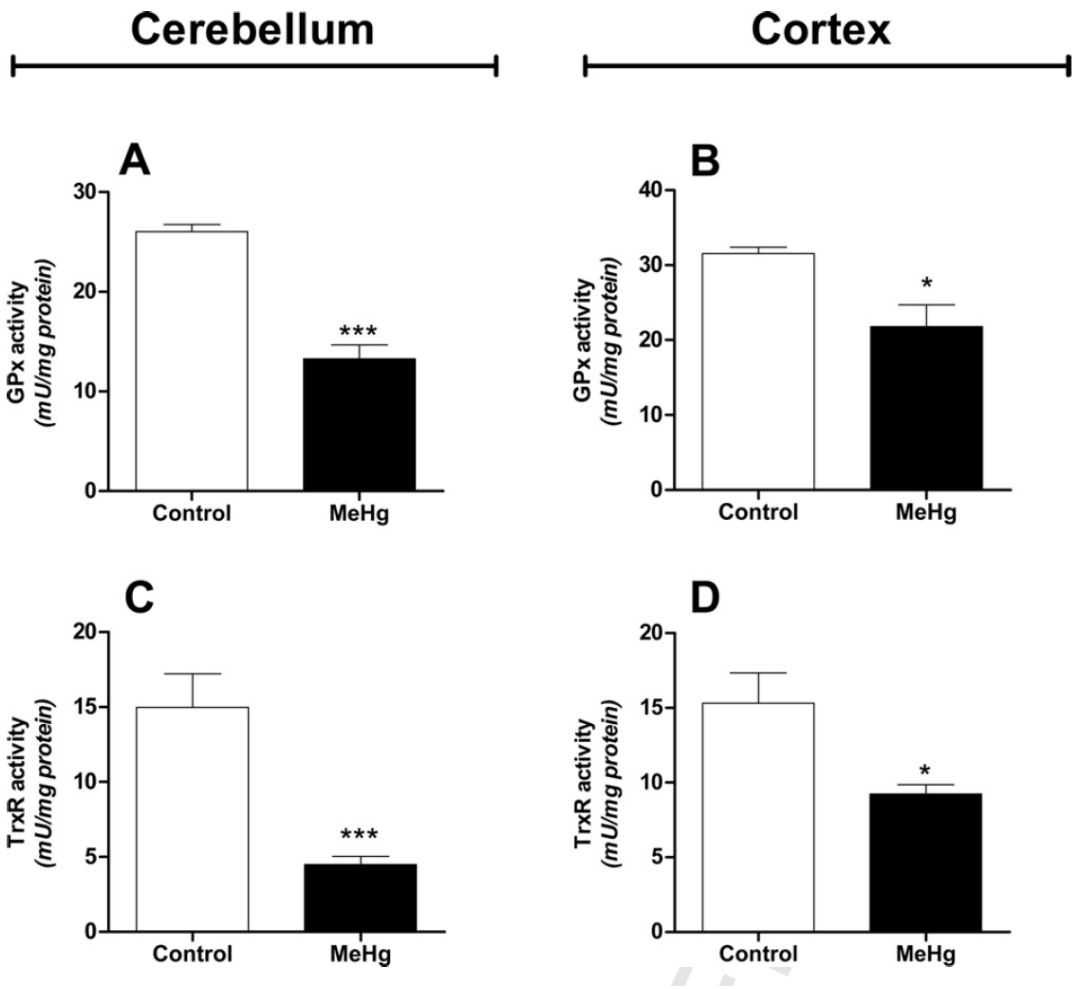

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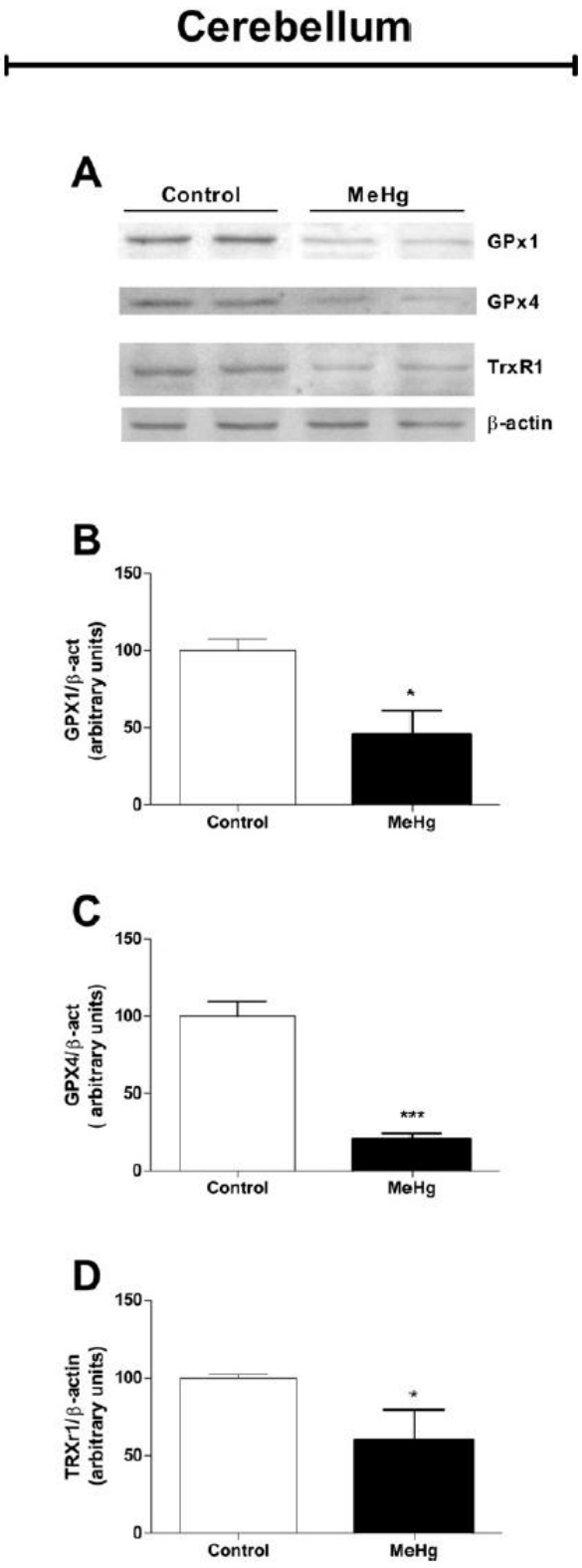

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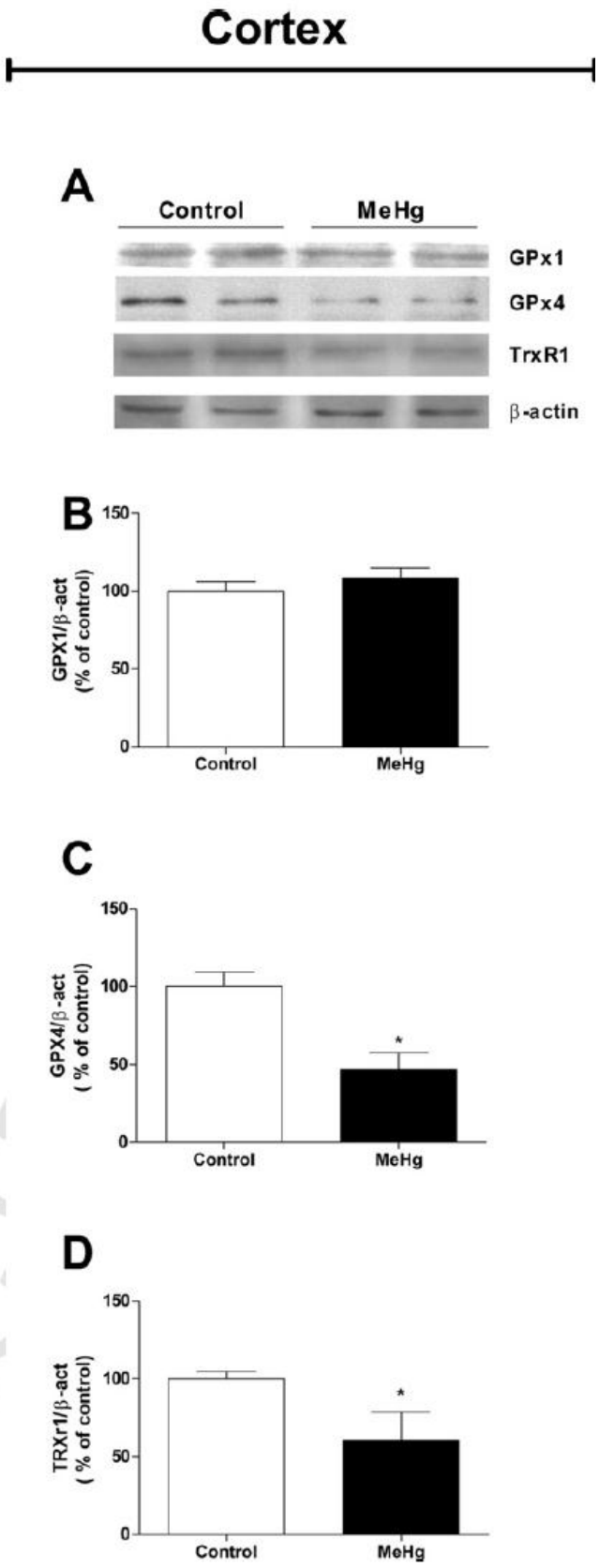

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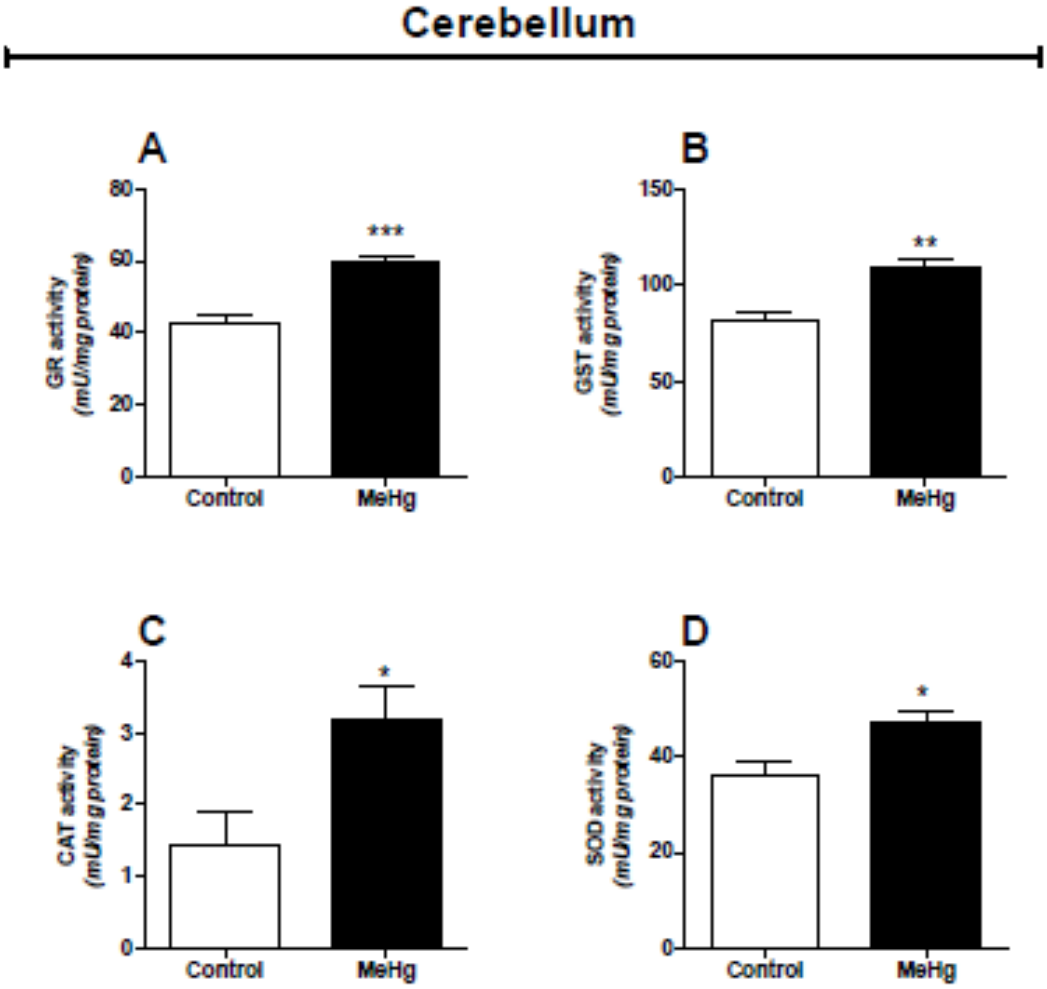

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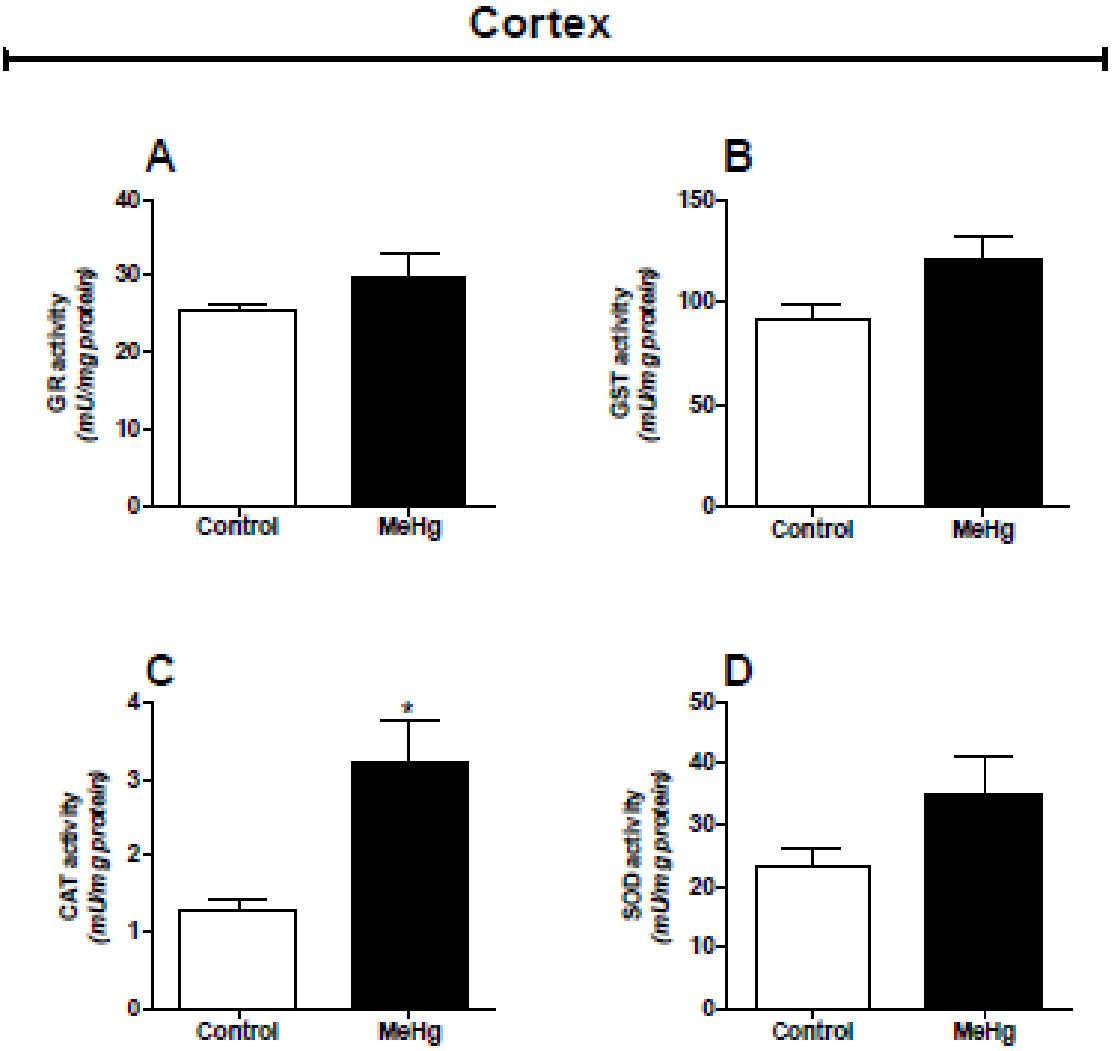

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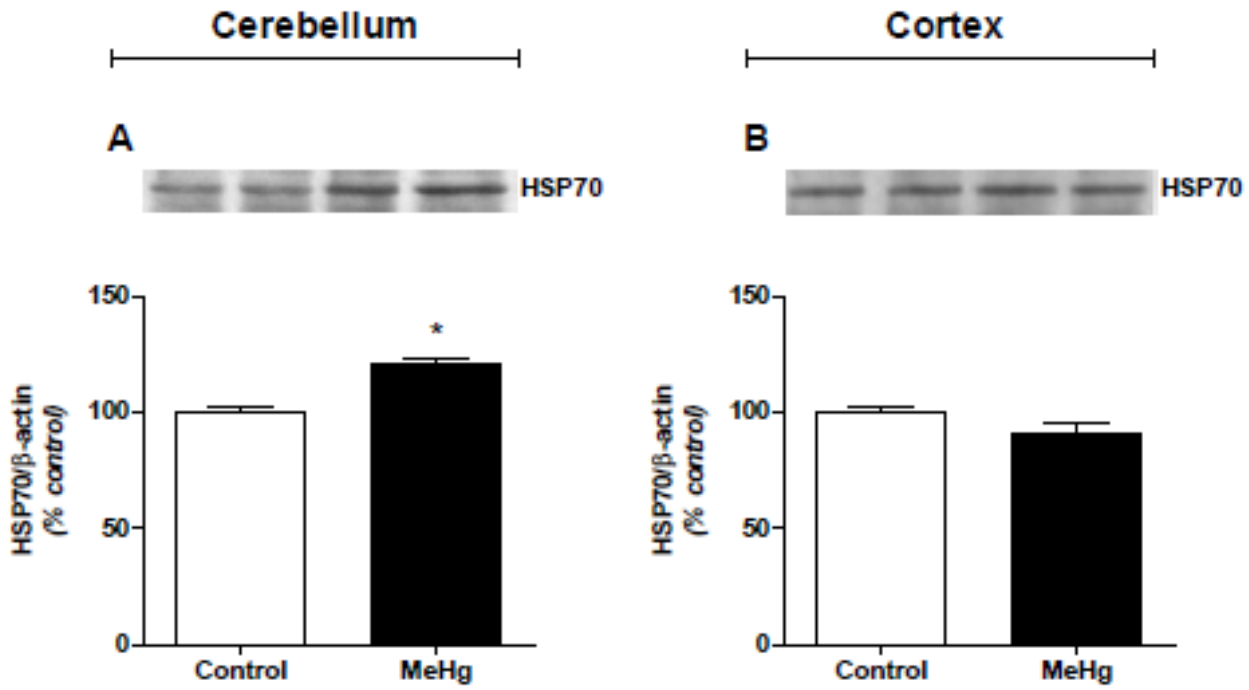

4.2 Manuscrito 2

Submetido à **Journal of Biosciences (ISSN 0973-7138)**

Toxicity-Induced by an Antarctic Alga (*Prasiola Crispa*) on Fruit Fly *Drosophila melanogaster* and Cockroach *Nauphoeta cinerea*: Evidences for Bioinsecticide Action

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Running title: Bioinsecticide effect of Antarctic alga Prasiola crispa

Abstract

We investigated the toxic effects of *Prasiola crispa* extract (PcE), an Antarctic alga on a fruit fly (*Drosophila melanogaster*) and cockroach (*Nauphoeta cinerea*) model. Toxicity was assessed in flies as mortality, negative geotaxis and biochemical alterations including acetylcholinesterase (AchE) as well as oxidative stress markers. We also examined the cardiotoxic action of PcE in a model of semi-isolated cockroach heart. The administration of PcE (2 mg/ml) to flies for 24 hours resulted in a massive increase in mortality (7.6 fold increase, compared to control). It was also observed a significant alteration in negative geotaxis behavior. The AchE activity, glutathione levels and hydroperoxide formation remained unchanged. Flies glutathione S-transferase and catalase activity was significantly altered after PcE treatment for 24 hours. It was observed a substantial decrease in cockroach semi-isolated heart function. The addition of DTNB, an oxidizing agent, concomitant with the extract significantly blocked this effect, suggesting that reduced compounds may be involved in the cardiotoxic action delivered by the PcE. Our results show for the first time the toxic effects of an Antarctic alga in two insect models, *Drosophila melanogaster* and *Nauphoeta cinerea*. The insecticide properties of PcE may be related to changes on important antioxidant/detoxifying systems as well as to changes on cardiac function of insects.

Keywords: *Prasiola crispa*, bioinsecticide, toxicity, *Drosophila melanogaster*, cockroach, Antarctic alga.

1. Introduction

The insecticidal properties of a number of plants have been investigated for thousands of years, and some plants can substitute many synthetic means of control (Sujatha, 2010). In this respect, it is important to emphasize that natural agents are environmentally less harmful than synthetic pesticides. Moreover, natural agents can act in many insects in different ways (Sujatha, 2010).

Prasiola crispa, a terrestrial eukaryotic green alga can be found at the Antarctic continent (Kovácik and Pereira, 2001). Although there are no studies targeting the biological effects of this alga, interesting characteristics, like adhesive properties has been described for plant of the genus Prasiola sp. (Mostaert et al., 2006), highlighting the biotechnological importance of this organism. A study carried out with three Antarctic plant species (Deschampsia antarctica Desv., Colobanthus quitensis (Kunth) Bartl., and Polytrichum juniperinum Hedw), have demonstrated low toxic effects for mammalian and non-mammalian cells, associated with protective effects against UV-induced damage (Pereira et al., 2009).

Plants have evolved and adapted to different environmental conditions and their secondary metabolites appear to play essential role in this process (Kennedy, 1993). It has been recognized that organisms living in Polar Regions, are subject to extreme environmental conditions. This fact has lead to the developing of natural strategies enabling these organisms to survive under the most extreme environmental conditions on Earth (Pereira et al., 2009). Among these strategies of adaptations is the production of photoprotective compounds, such as mycosporine-like amino acids, scytonemim secreted by cyanobacteria and flavonoids secreted by

plants (Pereira et al., 2009). This fact emphasizes the importance of studies concerning the biological effects of these organisms, which may present in its cellular constitution a combination of chemical compounds normally not found in other organisms.

Considering the scarcity of studies exploring the biotechnological potential of Antarctic organisms, the main aim of this study was to evaluate the effects of the methanolic extract of the terrestrial eukaryotic green alga from Antarctica, *Prasiola crispa* (PcE) on survival of adult *D.melanogaster*, and in parallel, to verify a possible modulation of antioxidant enzymes activity and locomotor performance in response to the exposure of this organism to the *Prasiola crispa* extract. The fruit fly *Drosophila melanogaster* belongs to the order Diptera and family Drosophilidae. This model is recognized for its high sensitivity to toxic substances, thus being considered a bioindicator for detection of pollutants and also to test the biological action of natural substances. We also investigated the cardiotoxic actions of PcE in *Nauphoeta cinerea*, a cockroach species which we are developing as a new model for toxicological studies.

2. Material and Methods

2.1 Plant material and chemicals

Prasiola crispa was collected in the ice free-areas near Arctowski Polish Station Region, Admiralty Bay, King George Island (61°50' - 62°15' S and 57°30' - 59°00' W), Antarctica. The plants were dried in dark chamber with circulating air at 40 °C and stored in dark bags in a freezer. The dried and powdered plant material (about 100 g) was submitted to extraction using methanol (powder/solvent ratio = 1:10 w/v) by maceration at room temperature. After 24 h of extraction the sample was filtered through Whatman number 1 filter paper and the same plant material was extracted again with another 1000 ml of methanol. This procedure was repeated for 3 days, after which the methanolic solutions were combined and evaporated to dryness under reduced pressure at rotary evaporator at 40–50 °C to obtain the methanolic extracts.

MAPK antibodies and secondary anti-rabbit antibody was purchased from Cell Signaling Technology (Uniscience do Brasil, São Paulo, SP, Brazil). PARP antibody was from Santa Cruz Biotechnology (Santa Cruz, CA 95060 USA). All other chemicals were from Sigma (São Paulo, SP, Brazil) and were of analytical grade.

2.2 Drosophila culture and *Prasiola crispa* extract treatment

Flies were maintained at 25° C on a standard diet (Golombieski et al., 2008). For P. *crispa extract* exposure experiments, 60 male adult flies were placed in a vial containing a paper filter soaked in 1% sucrose with or without dissolved *Prasiola crispa* extract (2 mg/ml). The flies were maintained under these conditions

up to 24 hours. Finished the period of treatments, 15 individual flies were submitted to behavioral test and a total of 45 flies were homogenized for biochemical analysis. All experiments using flies were repeated thrice using different fly cultures.

2.3 Cockroach *Nauphoeta cinerea* maintenance and treatment

Adult cockroach *Nauphoeta cinerea* were purchased from specialized breeding company (Nutrinsecta, Betim – MG, Brazil). After ether anesthesia, semi-isolated heart preparations were obtained in accordance with pre-established methods (Baumann and Gersch, 1982) and heart rate was monitored for 40 min with the aid of a stereomicroscope. Plant extract concentrations (0.5-2 mg/ml) were dissolved in saline and added to the heart preparation at a final volume of 200 µl.

2.4 Flies Mortality and Negative Geotaxis

In each experiment, 45 flies were separated in culture vials (control and PcE). Finished the treatments, the number of dead flies was counted and expressed as fold increase comparing to controls (control was considered 1±SD). Locomotor activity was determined by the negative geotaxis assay as described by Bland et al., (2009), with some modifications. For the assays, 15 adult flies were anesthetized and placed separately in vertical glass column (length, 25 cm; diameter, 1.5 cm) (Jimenez-Del-Rio et al., 2010). The assays were repeated three times at 1 min intervals. After 30 min recovery, individual flies were gently tapped to the bottom of the column and the time required to reach 8 cm in the columns was registered.

2.5 Biochemical measurements

Flies were homogenized in 0.02 M HEPES buffer pH 7.0 and centrifuged at 1000 g for 5 minutes (4°C). The supernatant was isolated and an aliquot separated for determination of acetylcholinesterase activity, glutathione and hydroperoxide content based on protocols previously described (Franco et al., 2009). The remained supernatant was then centrifuged at 20,000 g for 30 minutes. The resulted supernatant was used for determination of glutathione S-transferase (GST) and catalase (CAT) activity according to methods described earlier (Franco et al., 2009). Superoxide dismutase (SOD) activity was evaluated according with Kostyuk and Potapovich (1989). PARP cleavage, MAPK kinase (ERK1/2, JNK and p38) phosphorylation and total content was determined according to protocols described previously (Posser et al., 2009) using specific antibodies.

2.6 Statistical analysis

Statistical differences between groups for analysis of behavior and mortality were analyzed by Student's t-test. Differences were considered statistically significant when *P<0.05; ***p< 0.001. For analysis to the heart semi-isolated cockroach statistical significance was assessed by One-Way ANOVA followed by Duncan's post hoc test. *p<0.05.

3. Results

Treatment of flies with PcE (2 mg/ml in 1% sucrose) resulted in a substantial increase (7.6 fold increase, p<0.001) in mortality after 24 hours (Figure 1A). It was also observed an increase in locomotor activity, assessed by negative

geotaxis behavior. In this task, flies that fed on alga extract diluted on sucrose showed and improved ($p<0.05$) climbing performance (Figure 1B).

Acetylcholinesterase activity (AchE), glutathione levels (GSH) and hydroperoxide formation was not changed after PcE administration to *Drosophila melanogaster* for 24 hours (Table 1). However, it was possible to observe a significant increase ($p<0.05$) in glutathione S-transferase (GST) activity while catalase (CAT) was significant inhibited ($p<0.05$) in flies treated with 2 mg/ml of alga extract (Table 1). The activity of SOD was also unchanged (Table 1). PARP cleavage, an indicator of apoptotic cell death, MAPK kinase (ERK1/2, JNK and p38) phosphorylation as well as total content was unchanged (data not shown).

The administration of *Prasiola crispa* extract to cockroach (*Nauphoeta cinerea*) semi-isolated heart caused a significant decrease in heart rate, which was dependent on concentration of alga extract tested (Figure 3A). The co-administration of alga extract with DTNB (2mM), an oxidizing agent blocked the cardiotoxic action of the extract (Figure 3B).

4. Discussion

Chemical insecticides used for insect control are especially dangerous in urban areas since these products can affect man and others animals, pollute the air, water and even enter the food chain. So along with the appearance of insect resistance and other negative side effects, the search for alternative forms of control has become important. (Mendonça et al., 2011).

Commercial insecticides and repellents with lower mammalian toxicity and less aggressive to the environment are desirable and studies focusing the search for novel biopesticides are ongoing. Approximately 2000 species of plants representing more than 170 families are said to have insecticidal properties (Kamaraj et al., 2008). In the present study, we investigated the potential insecticidal action of an Antarctic alga extract. It has been reported that plant derived compounds may pose toxicity to a wide range of insects including flies and cockroaches (Ravi et al., 2007). According to Miyazawa et al., (1994), substances extracted from plants may interfere directly with all development stages of fruit fly *Drosophila melanogaster* and cockroaches. Flavonoids, saponins, terpenes, alkaloids and steroids are important phytochemical classes when considering the insecticide activity of plant extracts (Bélo et al., 2009). Plant derived chemicals such as terpenoids and flavonoids have been studied for their insect repellent activity as well as antimicrobial action (Bell et al., 1990; Ndemah et al., 2002). In this context, prior knowledge on the effects of chemical agents present in the alga *Prasiola crispa* to ecosystems and other living organisms would help improve the assessment of environmental impacts (Berry et al., 2008).

Our results demonstrated that exposure of fruit flies to a 2 mg/ml solution of PcE, for 24 hours caused signs of toxicity related to disruption of neurobehavioral function as well as important cell defense antioxidant systems. Reports have used mortality/lethal concentration as reliable tools for the investigation of insecticide properties of plant/fungi derived compounds (Pohleven et al., 2011). Notwithstanding, in addition to mortality, secondary effects such as repellence, ovicidal, oviposition deterrent activity, changes on feeding behavior among others are considered valuable properties for natural compounds pest control (Upsani et al.,

2003; Kweka, 2008; Pohleven et al., 2011). The dose employed in the present report caused a massive mortality. Taking these facts into account, it remains to be explored whether lower dosages do PcE would induce such alterations.

The mortality effect was accompanied by changes in negative geotaxis, a commonly used behavior addressed to assess neurolocomotor function in *Drosophila melanogaster*. (Hosamani and Muralidhara, 2009). This result suggests that PcE toxicity may be related to a potential interaction of phytochemicals derived from this plant with neurotransmission systems involved in the regulation of such behavioral task. In this regard, we checked whether acetylcholinesterase would be involved in the observed phenomenon. The administration of PcE for 24 h did not cause any interference with cholinesterase activity, indicating that at least for the period tested this enzyme is not related to the neurolocomotor action of the extract. There is evidence for the involvement of serotonine and dopamine pathways in the regulation of negative geotaxis behavior in *D. melanogaster* (Pendleton et al., 2002; Pendleton et al., 2005). It was also shown that atropine, a cholinergic antagonist, was less effective than dopaminergic modulators in the recovering of negative geotaxis in a fruit fly model of Parkinson's disease (Pendleton et al., 2002). This is in agreement with our data, showing that cholinergic system may not be primarily involved in the neurolocomotor action of PcE extract.

Glutathione S- trasferase is an important antioxidant enzyme and is involved in phase II detoxification systems (Sau et al., 2010). GSTs belong to a family of multifunctional enzymes that catalyze the conjugation of glutathione to various other molecules and have a role in mechanisms intracellular detoxification of endo and xenobiotic compounds. (Chelvanayagam et al., 2001; Walters et al., 2009). The

observed increased GST activity in *Drosophila melanogaster* exposed to *Prasiola crispa* extract may be related to an adaptive response related to an increased elimination of toxic plant derivatives (Agiani et al., 2003; Wei et al., 2001). It has been demonstrated that natural compounds are able to increase the expression of GSTs that together with endogenous glutathione acts favoring the elimination of plant metabolites from the organisms (Singh et al., 2000). The observed increase in GST activity by PcE in fruit flies may be explained by a potential activation of the Nrf2-ARE pathway. The up-regulation of detoxifying enzymes by natural compounds is being strongly related to activation of Nrf2-ARE pathway (Nguyen et al., 2003; Liu et al., 2011). The Nrf2 nuclear translocation and subsequent DNA binding may be triggered by dissociation from the inhibitory protein Keap1 as well as by phosphorylation by upstream kinases such as PKC and MAPK (Liu et al., 2011). At least in part, our results showed that MAPK (Figure 2) may not be involved with GST modulation induced by PcE.

The inhibition of CAT activity may also be an important mechanism of toxicity of the extract, since this enzyme has crucial role in the clearance of hydrogen peroxide from cells as well as to the oxidative stress defense (Aebi, 1984). The disruption of cell defense antioxidant systems is been pointed as central mechanisms of action in a variety of models of investigation of drug/compound toxicity (Franco et al., 2009). Despite the well known antioxidant properties of natural compounds, it has been demonstrated that some plant derivatives may act as pro-oxidants, inducing reactive oxygen species (Liu et al., 2011) and also by inhibiting catalase activity (Yang et al., 2011). In addition, plant secondary metabolites including polyphenols are shown to inhibit iron-dependent enzymes such as isoforms of CYP450 (Cermak and Wolffram, 2006). Thus, one could suppose that the inhibitory effect of PcE on

fruit fly catalase activity may be related to a direct interaction of PcE compounds and the enzyme.

Prasiola crispa extract also showed cardiotoxicity signs in a semi-isolated cockroach heart. The addition anticholinesterase agents in the preparation of semi-isolated heart of cockroaches can cause an increase or decrease in heart, depending on the concentration tested (Polsinelli et al., 2010). Considering that in our study PcE had no effect in the acetylcholinesterase activity, this mechanism appears to be not involved in the observed cardiotoxic effect. Since the impaired cardiac function after PcE was blocked by co-administration with an oxidizing agent (DTNB), one could suppose that plant metabolites with reducing capability may be involved in the cardiotoxic action of the Antarctic alga extract.

In conclusion, our results show preliminary data on the insecticidal effects of *Prasiola crispa* extract in a *Drosophila melanogaster* model and *Nauphoeta cinerea*. Our results suggest that *Prasiola crispa* may be used as an alternative tool for control of pests with potentially less harm effects to the environment. At least in our knowledge, this is the first report showing the bioinsecticide effect of an Antarctic plant. The exact mechanisms of toxicity still remain to be elucidated, however, interaction with antioxidant systems may be pointed out as a clue in further studies. In addition, studies are ongoing in order to characterize the phytochemical profile of PcE aiming to identify potential metabolites responsible for its insecticidal action.

This study comprehends part of the work of Brazilian researchers from the Antarctic National Institute for Science and Technology (“Instituto Nacional de Ciência e Tecnologia Antártico de Pesquisas Ambientais - INCT-APA”) related to Antarctic plant chemistry and its biotechnological applications. It is believed that

knowledge on the biotechnological potential of Antarctic plants, in addition to research on plant biology and evolving processes are essential to the management and preservation of these unique natural resources.

5. Acknowledgements

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6. Note

Authors declare that partial results from this study were presented at the Activity Report Workshop of the “Instituto Nacional de Ciência e Tecnologia Antártico de Pesquisas Ambientais - INCT-APA” held in Natal – RN, Brazil from October 25 and 26, 2010 in the form of an expanded abstract.

7. Conflict of interest

Authors declare no conflict of interest

8. References

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Figure legend

Figure 1.*Drosophila melanogaster* mortality and behavior after treatment with PcE. Flies were treated for 24 hours with PcE 2 mg/ml. After treatment, (A) mortality was observed for a period of 24 hours. Survived flies were used for determination of (B) negative geotaxis behavior. Statistical significance was assessed by Student's t-test. * $p<0.05$; *** $p<0.001$.

Figure 2. PcE induced cardiotoxicity in semi-isolated cockroach (Nauphoeta cinerea) heart. (A) PcE caused a significant decrease in cockroach heart rate at concentration of 2mg/ml. (B) DTNB, an oxidizing agent, significantly blocked the PcE induced decrease in heart rate. Statistical significance was assessed by One-way ANOVA followed by Duncan's post hoc test. * $p<0.05$.

Table

Table 1. Enzyme activities, glutathione and hydroperoxide levels.

	AchE (mU/mg protein)	**GST** (mU/mg protein)	**CAT** (mU/mg protein)	**SOD** (mU/mg protein)	**GSH** (μmol/mg protein)	**Hydroperoxides** (nmol/mg protein)
Control	30.2±4.3	90.7±12.3	156.1±7.2	84.2±5.2	0.20±0.01	0.20±0.02
PcE	27.3±0.9	155.9±19.5*	123.8±13.4*	91.7±14.1	0.22±0.01	0.23±0.05

Figure 1. Zemolin et al

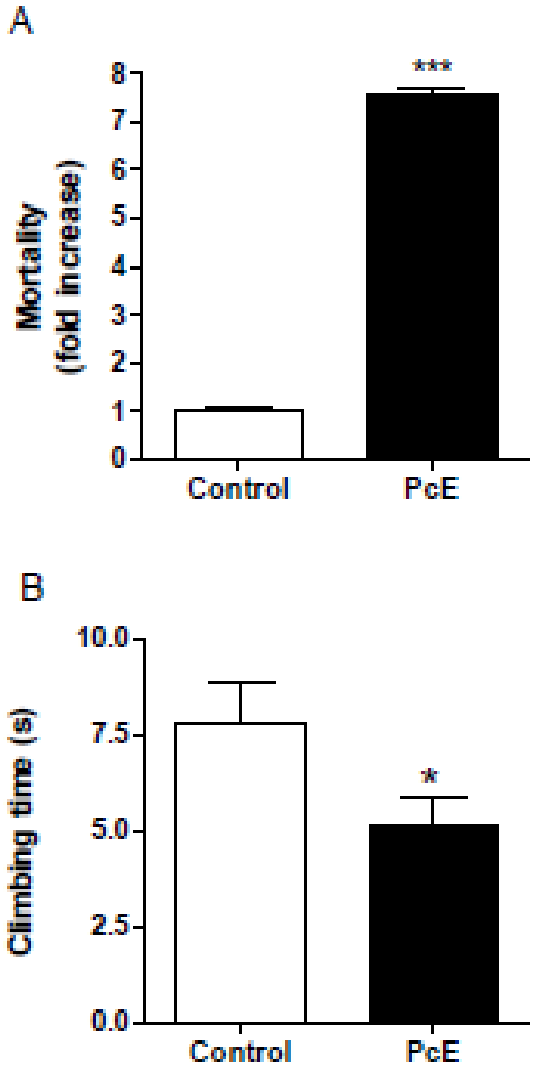
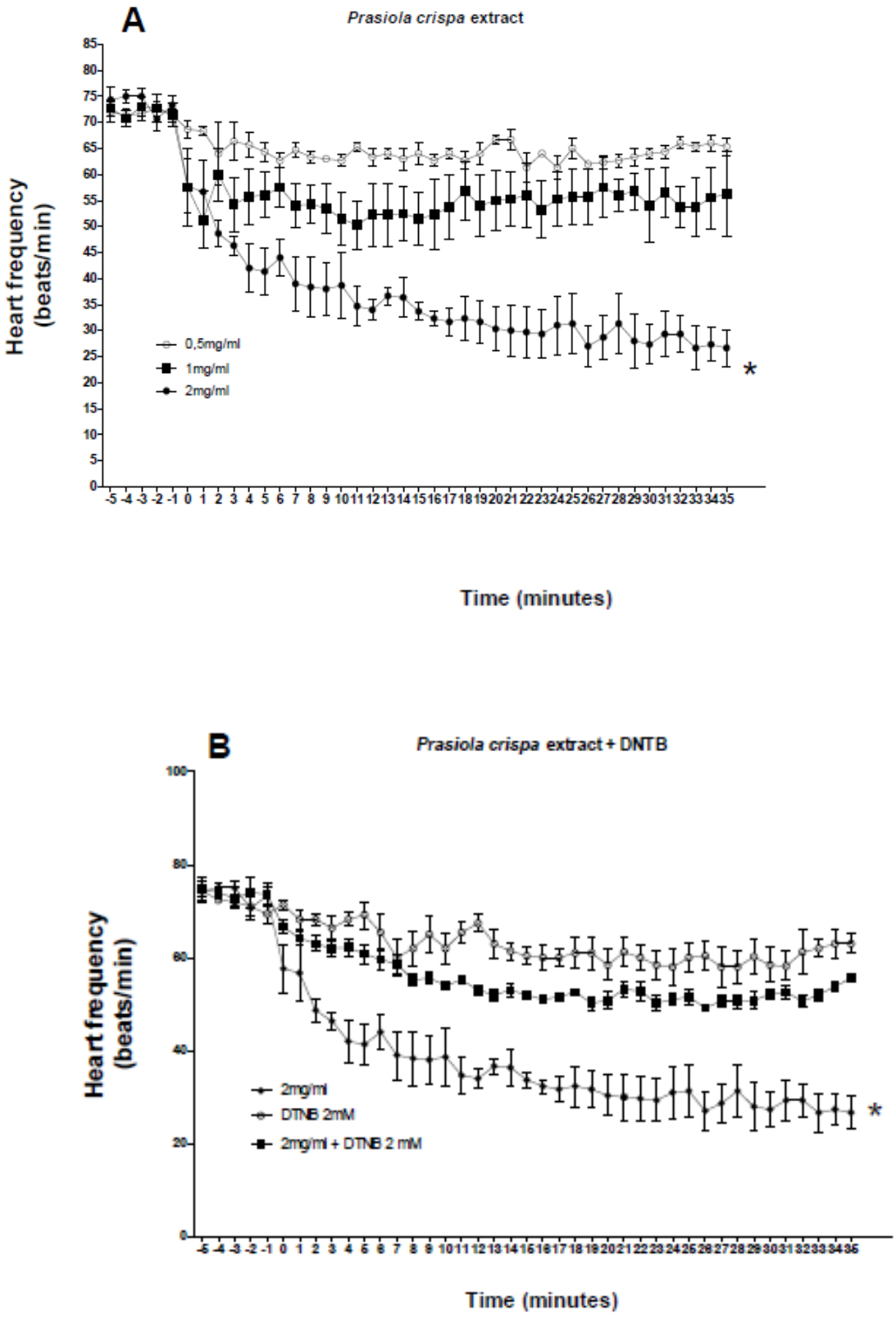


Figure 2. Zemolin et al



5. CONCLUSÕES

5.1 Manuscrito 1

O tratamento com MeHg, em camundongos (40 mg / L na água de beber por 21 dias) demonstrou:

- Significativo prejuízo no desempenho locomotor e indução de alterações na atividade e expressão de importantes enzimas antioxidantes;

- Inibição da atividade e expressão das selenoproteínas GPx1, GPx4 e TrxR1 no cerebelo e GPx4 e TrxR1 no córtex cerebral, demonstrando que estas selenoproteínas são alvos centrais na neurotoxicidade induzida por este organometal;

- O aumento na atividade das enzimas GR, GST, CAT e SOD no cerebelo e CAT no córtex cerebral, além do aumento na expressão de HSP70 no cerebelo, pode estar relacionada a uma resposta compensatória à inibição da atividade e expressão das selenoproteínas GPx1, GPx4 e TrxR1 causada pelo MeHg;

5.2 Manuscrito 2

A exposição de *Drosophila melanogaster* e *Nauphoeta cinerea* ao extrato da alga *Prasiola crispa* demonstrou:

- Sinais de toxicidade através do aumento na mortalidade e alteração na atividade locomotora, indicando seu potencial uso como bioinseticida;

- Significativo aumento na atividade da enzima Glutationa-S-transferase (GST), indicando uma resposta adaptativa relacionada com a eliminação de metabólitos tóxicos presentes no extrato;

- Inibição da atividade da enzima catalase (CAT) apontando para este fenômeno como um importante mecanismo de toxicidade do extrato, uma vez que esta enzima tem um papel crucial na defesa contra o estresse oxidativo;

- Uma diminuição significativa na frequência cardíaca, apontando para um efeito cardiotóxico como um importante mecanismo de ação inseticida do extrato.

- O agente oxidante DTNB bloqueou a ação cardiotóxica do extrato, desta maneira indicando que compostos com potencial redutor podem estar envolvidos na ação cardiotóxica do extrato da alga *Prasiola crispa*.

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