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MARCIO GONÇALVES CORRÊA

INVESTIGAÇÃO DOS EFEITOS BIOQUÍMICOS, PROTEÔMICOS E ESTRUTURAIS DA EXPOSIÇÃO PROLONGADA AO CLORETO DE MERCÚRIO SOBRE A MEDULA ESPINHAL DE RATOS

BELÉM

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Dissertação apresentada ao Programa de Pós-Graduação em Neurociências e Biologia Celular da Universidade Federal do Pará, como requisito parcial para obtenção do grau de Mestre em Neurociências e Biologia Celular.

Área de concentração: Neurociências.

Orientador: Prof. Dr. Rafael Rodrigues Lima.

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Nós poderíamos ser muito melhores se não quiséssemos ser tão bons.

Sigmund Freud

RESUMO

O Cloreto de mercúrio (HgCl2) é um poluente amplamente encontrado no meio ambiente. Esta espécie de mercúrio é capaz de promover diversos prejuízos ao Sistema Nervoso Central (SNC), incluindo danos no córtex motor, área relacionada ao planejamento e execução da atividade motora, no entanto, permanecem desconhecidos os efeitos do HgCl2 na medula espinhal, uma importante via de comunicação entre o SNC e a periferia. Administramos HgCl2 para ratos adultos, por 45 dias, via oral, a fim de investigarmos os efeitos na bioquímica oxidativa, no perfil proteômico e em estruturas da medula espinhal. Nossos resultados mostraram que a exposição a este metal promoveu aumento dos níveis de Hg no parênquima medular, prejuízo na bioquímica oxidativa, alteração em proteínas do sistema antioxidante, do metabolismo energético e na mielina; bem como causou desorganização na bainha de mielina e redução na densidade neuronal. Apesar da baixa dose, concluímos que a exposição prolongada ao HgCl2 provoca alterações bioquímicas e na expressão de diversas proteínas, culminando em danos na bainha de mielina e redução de neurônios na medula espinhal.

Palavras-chave: Mercúrio, cloreto de mercúrio, medula espinhal, neurotoxicologia, proteômica, SNC.

ABSTRACT

Mercury chloride (HgCl2) is a pollutant widely found in the environment. This form of mercury is able to promote several damages to the Central Nervous System (CNS), including changes to the motor cortex, an area related to the planning and execution of motor activity. However, the effects of HgCl2 on the spinal cord, an important pathway for the communication between the CNS and the periphery, are still unknown. We exposed adult rats for 45 days to HgCl2, orally, to investigate the effects on oxidative biochemistry, proteomic profile, and spinal cord structures. Our results showed that exposure to this metal promoted increased levels of Hg in the medullary parenchyma, impaired oxidative biochemistry, changes in antioxidant system proteins, energy metabolism and myelin structure; as well as caused disruption in the myelin sheath and reduction in neuronal density. Thus, we conclude that prolonged exposure to HgCl2 triggers biochemical changes and in the expression of several proteins, resulting in damage to the myelin sheath and reduction of neurons in the spinal cord, which may be related to motor damage.

Keywords: Mercury, mercury chloride, spinal cord, neurotoxicology, proteomics, CNS.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ACAP	Capacidade Antioxidante Contra os Radicais Peroxil
CEUA	Comitê de Ética em Uso de Animais
ERO	Espécies Reativas do Oxigênio
GO	*Gene Ontology*
HE	Hematoxilina-Eosina
Hg	Mercúrio
Hg2+	Íon Mercúrio
$HgCl_2$	Cloreto de mercúrio
H2SO4	Ácido sulfúrico
LPO	Peroxidação lipídica
MeHg	Metilmercúrio
NM	Neurônios Motores
NIH	*National Institute of Health*
OMS	Organização Mundial da Saúde
PLGS	*Protein Lynx Global Server*
SNC	Sistema Nervoso Central
SNP	Sistema Nervoso Periférico
TEM	Microscopia Eletrônica de Transmissão

SUMÁRIO

1. INTRODUÇÃO

1.1. Mercúrio

O mercúrio (Hg) é um elemento químico metálico tóxico de caráter bioacumulativo liberado nos diversos ecossistemas, tanto naturalmente quanto por ação antropogênica, resultando em risco ao ser humano, pois a exposição ao Hg representa um importante problema de saúde pública (Marshall et al., 2018a). Estima-se que são emitidas 1400 toneladas de Hg à natureza, oriundas de mais de 70 países, levando à poluição de cerca de 37% do mundo (Ha et al., 2017; Esdaile et al., 2018). Visando reduzir os efeitos nocivos, proteção à saúde humana e ao meio ambiente desta exposição, em 2013, a Organização Mundial de Saúde (OMS) reconheceu e apoiou o tratado da Convenção Minamata (www.mercuryconvention.org), o qual atualmente é assinado por 128 países, incluindo o Brasil (OMS, 2014).

Quimicamente, o Hg apresenta-se na forma elementar (Hg^0), orgânica e inorgânica (Hg^{+1} e Hg^{+2}) (Bernhoft, 2012; Olson, 2014). Essa variedade de apresentações permite propriedades tóxicas intrínsecas e distintas aplicações na indústria e agricultura, bem como diferentes níveis de riscos aos sistemas biológicos. Fisiologicamente, nenhuma das formas de Hg têm funcionalidade nos organismos vivos, dessa forma, exposições a este metal são potencialmente tóxicas (Clarkson e Magos, 2006; Bjorklund *et al.*, 2017; J. Casarett *et al.*, 2019).

Em sua apresentação elementar, o Hg caracteriza-se como a espécie que pode ser emitida para o ambiente em forma de vapor por processos naturais como erupções vulcânicas e erosões de solo, bem como por atividades antropogênicas principalmente durante a extração artesanal do ouro (Marshall *et al.*, 2018a). Em temperatura ambiente, este elemento se apresenta na fase líquida, normalmente presente em termômetros, lâmpadas, esfigmomanômetros e demais produtos, podendo expor trabalhadores à inalação de vapores de mercúrio durante suas atividades (Clarkson e Magos, 2006; Olson, 2014).

O Hg em sua apresentação orgânica denomina-se composto organomercurial, o qual é resultante da ligação entre Hg e o carbono. Dentre os compostos orgânicos de mercúrio estão etilmercúrio, fenilmercúrio e metilmercúrio (MeHg). Devido às ações antropogênicas, como atividades industriais e mineração de ouro, os níveis de MeHg aumentaram na biosfera, poluindo, principalmente, os ecossistemas aquáticos, expondo

várias espécies aos seus efeitos tóxicos (Wood *et al.*, 1968; De Azevedo, 2003; Bocca *et al.*, 2014; Rodriguez Martin-Doimeadios *et al.*, 2014).

Além disso, a apresentação orgânica de Hg possui grande capacidade de bioacumulação e biomagnificação nas cadeias alimentares, pois além de apresentar alta capacidade de entrar nos sistemas biológicos, está amplamente distribuído no meio ambiente por causa da atividade de bactérias anaeróbias sulforredutoras, as quais desempenham importante papel na metilação da forma inorgânica, resultando em MeHg, por exemplo (Aschner e Syversen, 2005; Ke *et al.*, 2018). Embora o composto mercurial orgânico seja a forma mais tóxica, é a espécie inorgânica a mais comum emitida ao meio aquático pelas indústrias (Ribeiro *et al.*, 1996; Clarkson e Magos, 2006; De Almeida Rodrigues *et al.*, 2019).

Os compostos inorgânicos derivam da oxidação atmosférica do Hg^0, a partir da perda de um elétron (Hg^{+1}; estado mercuroso) ou perda de dois elétrons (Hg^{+2}; estado mercúrico), o que possibilita a associação do Hg com outros elementos, como o cloro, o enxofre ou o oxigênio (Barile, 2003). Os compostos resultantes são denominados sais de Hg. Excetuando o sulfeto de mercúrio (cinábrio), o qual é caracterizado pela cor vermelha, a maioria dos compostos de Hg inorgânico apresentam-se em pós ou cristais brancos, dentre os quais está o cloreto de mercúrio (Konigsberg *et al.*, 2001).

1.1.1. Cloreto de mercúrio

Cloreto de mercúrio ($HgCl_2$), denominado ainda de bicloreto de mercúrio ou dicloreto de mercúrio, composto por moléculas triatômicas lineares, as um átomo de mercúrio é ligado a dois íons, e apresenta-se comumente como cristais brancos, grânulos ou pó (Clarkson e Magos, 2006; Bridges e Zalups, 2017; J. Casarett *et al.*, 2019).

Historicamente, o $HgCl_2$ foi sintetizado pela primeira vez no século IX pelo alquimista Geber, o árabe *Jabir ibn Hayyan* considerado o pai da química primitiva, a partir da sublimação do Hg em combinação com cloreto de sódio e outros compostos. Posteriormente, em 1716, o químico alemão Johann Kunkel demonstrou como o composto poderia ser produzido mais simplesmente, baseado em sublimar uma mistura de sulfato de Hg e cloreto de sódio. Neste período, o sublimado corrosivo, como era denominado popularmente o $HgCl_2$, já era empregado como conservante para evitar a degradação de madeiras (Friberg *et al.*, 1991; De Azevedo, 2003).

Décadas depois, o $HgCl_2$ passou a ser utilizado para fins terapêuticos, quando o médico holandês Gerard Van Swieten formulou um medicamento contendo o composto

com finalidade de tratamento para a sífilis. O licor de Swieten, como era denominado, constituía-se de uma solução diluída de $HgCl_2$ em conhaque, o qual era administrado por via oral. O tratamento tornou-se popular no final do século XVIII e, atualmente, não é mais empregado. Os efeitos colaterais da intoxicação por $HgCl_2$ durante o advento deste tratamento foram atribuídos à doença (Friberg *et al.*, 1991; Ros-Vivancos *et al.*, 2018).

Nos séculos XIX e XX, o sublimado corrosivo foi largamente empregado, não apenas como conservante de madeira e para o tratamento de sífilis, mas também em diversos produtos (Friberg *et al.*, 1991; Bridges e Zalups, 2017). Topicamente, o $HgCl_2$ estava entre as formas de Hg inorgânico que foram utilizadas em bases difundidas para uma variedade de aplicação terapêutica, como antissépticos, bactericidas e fármacos de propriedades diuréticas (Clarkson, 1997). Aplicou-se, ainda, este composto mercurial em cosméticos para clareamento da pele, como sabonetes e cremes, devido à capacidade de o cátion de Hg bloquear a produção de melanina na pele (Chan, 2011).

Apesar de ser amplamente relatado o uso de $HgCl_2$ nestes produtos, as exposições ocupacionais e ambientais são as principais causas de intoxicação por Hg inorgânico atualmente. Industrialmente, ainda é empregado como catalizador de reações químicas, assim, é parcialmente despejado em efluentes de rios. Em adição, aplica-se na agronomia através da utilização agrotóxicos/pesticidas (Friberg *et al.*, 1991; J. Casarett *et al.*, 2019). Diante disso, o contato com solos contaminados e a dieta rica em peixes, pertencentes ao nível trófico superiores na cadeia alimentar, advindos de áreas contaminadas, são fontes de exposições ao Hg inorgânico (Wood *et al.*, 1968; De Azevedo, 2003; Bocca *et al.*, 2014; Rodriguez Martin-Doimeadios *et al.*, 2014).

As descrições sobre intoxicação e exposição ao mercúrio inorgânico em humanos estão documentadas principalmente em relatos de casos clínicos (Benz *et al.*, 2011; Beasley *et al.*, 2014). Comumente, após exposição aguda, os sintomas mais relatados são náusea, desconforto abdominal, insônia, agressividade, fraqueza muscular e diarreia; e como sinais, geralmente, manifesta-se febre, hipertensão arterial, hipocalcemia, distúrbios renais, taquicardia e leucocitose (Clarkson, 1997; Triunfante *et al.*, 2009; Park e Zheng, 2012).

1.2 Toxicologia do mercúrio inorgânico

1.2.1 Toxicocinética

Após exposição ao $HgCl_2$, as principais vias de absorção são a dérmica, através do contato com produtos que possuam este sal em sua formulação; e a via oral, pela qual há normalmente há maior suscetibilidade (Bocca *et al.*, 2014; Rodriguez Martin-Doimeadios *et al.*, 2014). Absorção via oral dos sais de mercúrio inorgânicos apresentam ação limitada, o que está relacionado com a sua solubilidade em água. Compostos de mercúrio inorgânicos não são lipossolúveis, porém as espécies iônicas de Hg inorgânico ligam-se facilmente a grupos sulfidrila de vários compostos, as quais contém tiol, tais como glutationa, cisteína e metalotioneína (Friberg *et al.*, 1991; Ribeiro *et al.*, 1996).

A absorção do $HgCl_2$ no trato gastrointestinal apresenta efeito corrosivo, assim, aumentando sua permeabilidade local e, consequentemente, potencializando esta etapa (Kostial *et al.*, 1979; Vazquez *et al.*, 2013). Em seguida acessa a corrente sanguínea, atingindo órgãos como os rins, fígado e o sistema nervoso central (SNC) (Teixeira *et al.*, 2014; Joshi *et al.*, 2017; Aragão, W. a. B. *et al.*, 2018), e caracteriza-se por uma meia vida em torno de quarenta dias (Magos e Clarkson, 2006).

O transporte intestinal de Hg apresenta diferentes mecanismos. Sugere-se que o transporte de Hg^{2+} através das membranas plasmáticas dos enterócitos utiliza mecanismos passivos e ativos (Andres *et al.*, 2002; Hoyle e Handy, 2005; Bridges e Zalups, 2017). Além do mais, acredita-se que o meio pelo qual o Hg^{2+} é transportado através dos enterócitos intestinais depende das espécies de Hg^{2+} apresentadas aos enterócitos (Foulkes, 2000). É relevante ressaltar que as espécies de Hg^{2+} presentes no lúmen do intestino é altamente dependente dos componentes do alimento ingerido, visto que após ingerido, o alimento frequentemente tem uma alta concentração de moléculas contendo tiol, como aminoácidos e peptídeos, que pode se ligar a Hg^{2+} (Ribeiro *et al.*, 1996). Os conjugados tiol com Hg^{2+} formados no lúmen do trato gastrointestinal podem ser similares, quando comparados em forma e tamanho a certas moléculas endógenas (aminoácidos e/ou polipeptídeos), as quais são absorvidas ao longo do intestino delgado (Bridges e Zalups, 2017). Devido tais semelhanças, baseando-se no mecanismo pelo qual os transportadores de aminoácidos e peptídeos são prevalentes nos enterócitos (Ganapathy *et al.*, 2000; Dave *et al.*, 2004), é possível que estes conjugados de $Hg2^{+}$ sejam absorvidos em enterócitos por um ou mais transportadores de aminoácidos e/ou peptídeos (Bridges e Zalups, 2017).

O duodeno, caracterizado como um dos principais sítios de absorção de aminoácidos, também é relatado como um dos locais iniciais de absorção de Hg^{2+} (Endo *et al.*, 1984). Associado a isso, o intestino também parece desempenhar um papel importante na eliminação fecal líquida de Hg^{2+} (Bridges e Zalups, 2017). Os íons de mercúrio no sangue podem cruzar os enterócitos e entrar no lúmen intestinal por mecanismos paracelulares e/ou transcelulares (Hoyle e Handy, 2005). Após absorção, é no rim que a maioria dos íons de Hg acumulam-se (Friberg *et al.*, 1991; Clarkson, 1997; Bridges e Zalups, 2017; J. Casarett *et al.*, 2019). Após ingerido é passível de interação intersticial e posterior excreção pelas fezes. A idade é um fator importante a ser levado em consideração para eliminação de mercúrio, porque após exposição ao mercúrio inorgânico, por exemplo, ratos mais jovens demonstram retenção significativamente maior. Esta diferença dependente da idade na taxa de excreção de mercúrio pode refletir diferenças nos locais de deposição de mercúrio (pêlos, glóbulos vermelhos, pele) (Nordberg *et al.*, 2011).

1.2.2. Toxicodinâmica

Os íons de Hg inorgânico apresentam baixa lipossolubilidade e, consequentemente, capacidade limitada de ultrapassar as membranas celulares (Clarkson e Magos, 2006). Porém, tais íons se caracterizam pela afinidade ao grupamento tiol (–SH) das diversas enzimas celulares e, desse modo, podem alterar a atividade enzimática, resultando em disfunções celulares/teciduais, interrupção do metabolismo e morte celular (Asano *et al.*, 2000). Além disso, podem se ligar a outras biomoléculas sem ação enzimática, mas que contenham grupamentos –SH, como glutationa, cisteína, homocisteína, N-acetilcisteína ou albumina, e até mesmo ao selênio (Bridges e Zalups, 2017).

Uma vez presente nos organismos, inúmeras são as evidências que indicam a capacidade do Hg inorgânico de alterar o equilíbrio pró e antioxidantes, como consequência do acumulo desse metal, contribuindo para a geração de espécies reativas de oxigênio (ERO's) e nitrogênio, principalmente os radicais ânion superóxido (O2-), hidroxil (OH) e o peróxido de hidrogênio (H_2O_2). Estas espécies reativas podem produzir o estresse oxidativo, o qual promove alteração no metabolismo celular, caracterizada pelo desequilíbrio entre as espécies reativas e o sistema antioxidante celular (Hercberg *et al.*, 2007).

As espécies reativas geradas pelo estresse oxidativo apresentam um alto poder de reação com diversas macromoléculas celulares, e podem promover danos à níveis metabólicos, transcriptômicos e proteômicos (Reichmann *et al.*, 2018). Assim, um dos principais danos causados pela neurotoxidade do Hg é o estresse oxidativo, alterando/inibindo enzimas antioxidantes como glutationa peroxidase, glutationa redutase, superóxido dismutase e catalase, além de causar alterações nos níveis de peroxidação lipídica, como o aumento de malondialdeído (MDA), e nitritos em região hipocampal e no tecido hematopoiético (Rizzetti *et al.*, 2016; Aragão, W. *et al.*, 2018).

Além dos danos bioquímicos, estudos prévios mostraram que o Hg inorgânico consegue modular a função neuroquímica excitatória, caracterizada por um aumento de neurotransmissores glutamatérgicos na fenda sináptica, em áreas associadas à memória e à motricidade (Teixeira *et al.*, 2018). Em suma, a exposição ao mercúrio inorgânico desencadeia um desequilíbrio na homeostase sináptica, aumentando a liberação de glutamato na fenda sináptica e, consequentemente elevação do influxo de Ca^{+2} pós-sináptico promovendo morte e disfunções de organelas celulares (Albrecht e Matyja, 1996; Hidalgo e Donoso, 2008).

Outras alterações já associadas à exposição ao Hg orgânico ou inorgânico, evidenciaram que há uma modificação do perfil proteômico, principalmente proteínas relacionadas ao estresse oxidativo, as quais são responsáveis em estabelecer o equilíbrio redox, removendo as ERO's; assim como alterações em proteínas estruturais celulares, representadas pelas modificações desta macromolécula no citoesqueleto (Wang *et al.*, 2015). Além disso, doses elevadas de MeHg em astrócitos, podem desencadear também mudanças a nível mitocondrial, que através do bloqueio de transporte de elétrons via fosforilação oxidativa altera a respiração e produção de energia mitocondrial (Shao *et al.*, 2019).

Em um estudo recente, que investigou alterações proteômicas à exposição mercurial, no tecido hipocampal, mostrou que há uma diminuição na expressão de proteínas associadas a adesão celular, enzimas antioxidantes, como a glutationa S transferase, e também redução de proteínas responsáveis pelo dobramento e desdobramento de outras proteínas (chaperonas moleculares), danos mitocôndriais associados a liberação de Ca^{+2}, morte celular po apoptose (Bittencourt *et al.*, 2019). Essas alterações, estão amplamente relacionadas com as doenças neurodegenerativas, ou seja, o mercúrio consegue reduzir a expressão de chaperonas, deixando o organismo mais susceptível à essas doenças neurodegenerativas como, Parkinson (PD), Alzheimer (AD)

e esclerose lateral amiotrófica (ELA), doenças que são resultados da quebra de homeostase do Sistema Nervoso Central (Bassi e Kersey, 2009; Barsukova *et al.*, 2011).

1.3. Sistema Nervoso Central

De modo geral, o Sistema Nervoso é o transmissor de sinais e coordenador de respostas voluntárias e involuntárias do organismo e conta com diversos órgãos para o seu desempenho (Snell, 2010). Esse sistema divide-se em duas grandes categorias Sistema Nervoso Central (SNC) e Sistema Nervoso Periférico (SNP). O SNC é a porção possui os principais órgãos responsáveis percepção de informações/estímulos, e que comandam e desencadeiam respostas no organismo, já o SNP possui a função de levar ou trazer impulsos para o SNC (Barha *et al.*, 2016).

O SNC é composto por diversas estruturas, incluído o telencéfalo, tronco encefálico, cerebelo e medula espinhal (Putz e Pabst, 2006; Snell, 2010). Especificamente, a medula espinhal (ME) não é responsável apenas pelo controle de músculos voluntários da região superior e inferior do corpo, mas também pela recepção sensorial dessas áreas do corpo (Bican *et al.*, 2013).

1.3.1Medula espinal

A medula espinhal está localizada dentro do canal vertebral, entretanto não se estende por todo este canal, limitando-se do forame magno da base do crânio até a segunda vértebra lombar (L2) (Netter e Colacino, 1989; Snell, 2010; Ganau *et al.*, 2019). Em humanos, sua extensão se aproxima de 45 cm em homens e 43 cm em mulheres, já sua largura é variada, podendo medir aproximadamente1,27 cm na região cervical e lombar e até 64 mm na região torácica (Snell, 2010).

Estruturalmente, a medula espinhal pode ser dividida em 5 regiões: cervical, torácica, lombar, sacral e coccígea (Putz e Pabst, 2006). Ao visualizar um corte transversal, a medula espinhal encontra-se perfurada centralmente pelo canal central e, redor deste canal, verifica-se a substância cinzenta em forma de H, também denominada de “H” medular. Por outro lado, sua parte mais externa é constituída por substância branca, arquitetura anatômica diferente do córtex cerebral (Snell, 2010; Ganau *et al.*, 2019).

Esta parte superficial da medula espinhal denomina-se substância branca, por possuir numerosos processos axonais dos neurônios, revestidos por mielina, o que lhe

permite uma coloração esbranquiçada e, porquanto, o nome de substância branca (Bican *et al.*, 2013). A parte mais interna, denominada substância cinzenta, constituída por neuróglia, os corpos celulares dos neuronais e fibras com menor proporção de mielina. Essa região divide-se em três porções principais: corno posterior (responsável pelo encaminhamento das propriocepções ao córtex cerebral), corno lateral (sistema nervoso autônomo), corno anterior (zona somato-motora) (Ganau *et al.*, 2019)

Essa estrutura permite a comunicação entre e cérebro e o sistema nervoso periférico. A importância da manutenção da integridade e funcionalidade da medula espinhal reflete na qualidade das atividades viscerais, somáticas, sensoriais e motoras do organismo. Os danos mais conhecidos na medula espinhal que podem causar morbidade nessas atividades são os danos mecânicos, que raramente podem causar transecção total da medula espinhal. Por outro lado, estes eventos podem causar a perda funcional completa das funções fisiológicas, o que depende do local da lesão e do tipo celular afetado (Bican *et al.*, 2013).

Os neurônios medulares presentes na substância cinzenta são denominados: neurônios radiculares, neurônios cordonais, neurônios de axônio curto (ou internunciais) (Y Cajal, 1991). Destes, os neurônios radiculares, se subdividem em neurônios viscerais (destinando-se à inervação de músculos lisos, cardíacos ou glândulas) e neurônios somáticos, também denominados de neurônios motores inferiores (inervam os músculos esqueléticos) (Cho, 2015).

Os neurônios motores são responsáveis por inervar fibra muscular dos músculos esqueléticos. Um único neurônio motor pode inervar fibras musculares distantes, pois seu axônio emite diversas ramificações que conduzem sinapses com essas fibras (Machado, 2006). Estes axônios formam raízes ventrais que, ao se juntarem com as dorsais (que transmitem impulsos sensoriais), formam os nervos espinhais mistos os quais têm sua projeção pelos espaços entre as vértebras da coluna (Bican *et al.*, 2013).

Estes neurônios motores inferiores são classificados em alfa e gama. Os neurônios alfas são mais robustos e se destinam à inervação de fibras musculares que contribuem efetivamente para a contração dos músculos, ao integrar-se à uma fibra muscular, constitui-se uma unidade motora. Enquanto os neurônios gama inervam as fibras musculares intrafusais nas duas extremidades do fuso muscular, essa constituição auxilia na contração muscular fazendo com que essas fibras se mantenham tensionadas e continuem a enviar informações ao SNC (Bican *et al.*, 2013).

Além das injúrias mecânicas, a medula espinhal pode ser suscetível a danos promovidos por diversos tipos de condições, tais como a desnutrição crônica (Quiroz-Gonzalez *et al.*, 2013), uso de abusivo de cocaína (Williamson *et al.*, 2017) e também exposição a metais, especialmente, o mercúrio (Roos e Dencker, 2012).

Os relatos expostos na literatura científica, que estudam os danos promovidos pelo mercúrio na medula espinhal são escassos. A maioria dos relatos em roedores dizem respeito à inalação (Schionning *et al.*, 1993; Roos e Dencker, 2012), bem como trabalhos mostrando apenas depósitos de mercúrios, incluído oriundo da exposição $HgCl_2$, porém sem avaliação fisiológica, funcional ou bioquímica da medula espinhal. Em relação ao cloreto de mercúrio, não há evidências científicas que descrevam seus efeitos na medula espinhal. Por outro lado, estudos apontam que existe susceptibilidade do SNC à exposição crônica a esse elemento, tais como o desbalanço da bioquímica oxidativa, morte celular, disfunção neuroquímica do glutamato, e também danos motores (Teixeira *et al.*, 2018).

Além de haver poucos relatos sobre os efeitos do $HgCl_2$ no SNC, os estudos se limitam apenas ao estudo em áreas do encéfalo como o hipocampo e o córtex motor. Estudar a medula espinhal, principal via de comunicação entre o cérebro e o organismo, é de suma importância para compreender os danos no SNC como um todo. O estudo de Schiønning et al. 1993 com ratos adultos *Wistar* aponta para uma susceptibilidade da medula espinhal ao acumulo de mercúrio (Schionning *et al.*, 1993), entretanto a literatura ainda carece de dados que mostrem os efeitos estruturais e bioquímicos do $HgCl_2$ na medula espinhal.

1.4. Delineamento da pergunta experimental

O mercúrio (Hg) é um poluente ambiental bioacumulativo, normalmente disponível ao meio ambiente através de ações da natureza, que incluem atividades vulcânicas, precipitações atmosféricas e lixiviação dos solos (Buch *et al.*, 2017; Risch *et al.*, 2017; Marshall *et al.*, 2018b). Essa disponibilidade do Hg pode ocorrer também, pela queima de combustíveis fósseis e durante o processo de garimpagem do ouro (Diringer *et al.*, 2015; Esdaile, L. J. e Chalker, J. M., 2018). Atualmente, quase 20 milhões de pessoas, em cerca de 70 países, estão expostas a este metal, representando

um problema intercontinental de saúde pública (Esdaile, L. J. e Chalker, J. M., 2018), visto que o Hg é um dos metais mais tóxicos aos sistemas biológicos, em especial ao SNC (Crespo-Lopez *et al.*, 2005; Farina *et al.*, 2013; Aschner e Carvalho, 2019).

Entre as formas existentes de Hg, o cloreto de mercúrio ($HgCl_2$) é uma espécie inorgânica utilizada em fármacos (Chan, 2011) e na agronomia compondo agrotóxicos/pesticidas (Friberg *et al.*, 1991; J. Casarett *et al.*, 2019). Outras fontes de exposição ao $HgCl_2$ podem ocorrer com solos contaminados e dieta rica em peixes advindos de áreas contaminadas pelo Hg inorgânico (Wood *et al.*, 1968; De Azevedo, 2003; Bocca *et al.*, 2014; Rodriguez Martin-Doimeadios *et al.*, 2014),

Nos últimos anos o nosso grupo tem investigado os efeitos do HgCl2 sobre o córtex motor (Teixeira *et al.*, 2014; Teixeira *et al.*, 2018; Teixeira *et al.*, 2019), uma importante região responsável pelos alto comando motor e inicialização de movimentos (Peters *et al.*, 2017; Lemke *et al.*, 2019). Estas investigações nos levaram ao entendimento que a exposição prolongada ao HgCl2 é capaz de promover aumento dos níveis de mercúrio no parênquima cortical, prejuízos na performance motora espontânea e forçada, associados a alterações neuroquímicas, desencadeamento de estresse oxidativo e indução de morte neuronal. No entanto, o controle motor não é executado apenas pelo córtex, mas por um conjunto de regiões encefálicas e vias que também participam desse funcionamento, como núcleos da base, cerebelo e tronco encefálico (Coiner *et al.*, 2019), sendo então conduzido o comando pela medula espinhal ao restante do organismo (Bican *et al.*, 2013).

A medula espinhal serve de caminho para impulsos neurofisiológicos que resultam nas atividades motoras, servindo com uma via descendente e integradora entre o alto comando motor e musculatura estriada esquelética (Bican *et al.*, 2013). Além disso, a medula espinhal está associada a funções sensitivas, servindo com uma via de aferência

entre o meio externo e regiões corticais somestésicas, além de seu envolvimento em ações de arco reflexo, um mecanismo autônomo de defesa (Cho, 2015).

Entretanto, mesmo diante das inúmeras funções motoras e sometésicas, não há relatos na literatura sobre os efeitos da exposição ao HgCl2 sobre a medula espinhal. A partir disso, propomos neste estudo investigar as possíveis repercussões toxicológicas da exposição prolongada e sistêmica ao $HgCl_2$ sobre a homeostase bioquímica oxidativa, proteômica e integridade estrutural da medula espinhal de ratos adultos.

2. OBJETIVOS

2.1. Objetivo geral

Investigar os efeitos bioquímicos, proteômicos e estruturais da exposição prolongada ao cloreto de mercúrio sobre a medula espinhal de ratos

2.2. Objetivos específicos

- ✓ Determinar se há aumento das concentrações de mercúrio no parênquima medular após período de exposição proposto;
- ✓ Avaliar se exposição ao $HgCl_2$, mesmo em baixas doses é capaz de promover alteração no balaço oxidativo da medula espinhal;
- ✓ Caracterizar o perfil proteômico da medula espinhal dos ratos expostos, traçando comparações quanto aos animais controle;
- ✓ Verificar se este modelo de exposição é capaz de provocar desorganização da bainha de mielina;
- ✓ Averiguar se a ingestão de uma dose menor que a de exposição ambiental pode promover redução na densidade de neurônios motores e na população total de neurônios na medula espinhal

3. CORPO DO ARTIGO

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Title: **Ultrastructural and molecular damages underlying spinal cord neurodegeneration after inorganic mercury long-term exposure in adult rats**

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Highlights:

- Long-term $HgCl_2$ exposure leads to protein expression alterations
- Mercury chloride disrupts myelin sheath in spinal cord axons
- $HgCl_2$ reduced motor neurons number in spinal cord

Abstract

Mercury chloride (HgCl2) is a chemical pollutant widely found in the environment. This form of mercury is able to promote several damages to the Central Nervous System (CNS), including changes to the motor cortex, an area related to the planning and execution of motor activity. However, the effects of HgCl2 on the spinal cord, an important pathway for the communication between the CNS and the periphery, are still unknown. We administered HgCl2 to adult rats for 45 days, orally, to investigate the effects on oxidative biochemistry, proteomic profile, and spinal cord structures. Our results showed that exposure to this metal promoted increased levels of Hg in the medullary parenchyma, impaired oxidative biochemistry, changes in antioxidant system proteins, energy metabolism and myelin structure; as well as caused disruption in the myelin sheath and reduction in neuronal density. Despite the low dose, we conclude that prolonged exposure to HgCl2 triggers biochemical changes and in the expression of several proteins, resulting in damage to the myelin sheath and reduction of neurons in the spinal cord.

Keywords: Mercury, mercury chloride, spinal cord, neurotoxicology, proteomics, CNS.

1. Introduction

The mercurial exposure is a reality faced by several people around the world (Diringer et al., 2015; Esdaile and Chalker, 2018), due the routes of exposure to the metal be associated with the used in pharmaceuticals products (Chan, 2011), use of cosmetics, (Copan et al., 2015) in agronomy composing pesticides (Friberg et al., 1991; J. Casarett et al., 2019). Other sources of $HgCl_2$ exposure may occur with contaminated soil and fish-rich diet (Wood et al., 1968; DE AZEVEDO, 2003; Bocca et al., 2014; Rodriguez Martin-Doimeadios et al., 2014). In endemic regions, high levels of mercurial compound, including $HgCl_2$, are found in commercial fishes used in diet of riverine populations in Amazon (Rodriguez Martin-Doimeadios et al., 2014). After the ingestion, the biggest portion of the metal is absorbed by the intestine (Bridges & Zalups), however, it is important to highlight that $HgCl_2$ presents lower absorption and consequent lower bioavailability, when compared to organomercurial compounds (Vazquez et al., 2013). Even with low lipossolubility, it gets into the blood circulation and is distributed to biological tissues, including the CNS (Teixeira et al., 2014; Aragao et al., 2018; Teixeira et al., 2019).

In the last years our group has been investigating the effects of $HgCl_2$ over the motor córtex (Teixeira et al., 2014; Teixeira et al., 2018, Teixeira et al., 2019), an important region responsible for high motor command and movement initialization (Peters et al., 2017; Lemke et al., 2019). These investigations led us to understand that prolonged exposure to $HgCl_2$ is capable of promoting increased Hg levels in the cortical parenchyma, impaired spontaneous and forced motor performance, associated with neurochemical changes, triggering oxidative stress and induction of neuronal death. However, motor control is not only performed by the cortex, but by a set of brain regions and pathways that also participate in this function, such as basal nuclei, cerebellum and

brainstem nuclei (Coiner et al., 2019), conducted then, to the spinal cord command and afterwards, to the rest of the organism (Bican et al., 2013)

The spinal cord acts as a pathway for neurophysiological impulses that result in motor activity, serving as a downward and integrating pathway between high motor command and skeletal striated musculature (Bican et al., 2013). In addition, the spinal cord is involvement in reflex arc actions, an autonomous defense mechanism (Cho, 2015), is associated with somesthetic functions, serving as an afferent pathway between the external environment and somesthetic cortical regions. Furthermore, spinal cord a pathway for axonal substances directly to CNS, such as neuromotor junction Mercury through retrograde transport (Pamphlett and Kum Jew, 2013).

Thus, considering that spinal cord is a CNS region involved with somesthetic and motor pathways, we aimed to investigate the $HgCl_2$ long-term exposure and its possible effects on neurochemical, proteomic, and morphologic integrity of rat spinal cord.

2. Methods

2.1. Animals and design study

Fifty male *Wistar* rats (*Rattus norvegicus*), with 90 days old, weighing 150-200g, were housed at Federal University of Pará (UFPA) vivarium under a 12h light/dark cycle (lights on 7:00 AM) and a climate-controlled room (25±2°C). The animals were kept in collective cages (four animals per cage) and received food and water *ad libitum.* Experiments followed the protocol approved by the Ethics Committee on the use of animals (CEUA – UFPA), under the number BIO139-13. Besides, all procedures following the NIH Guide for the Care and Use of Laboratory Animals (Council et al., 2011).

The experimental animals were equal and randomly allocated according to exposure protocol. Distilled water ($H_2O_{dist.}$) or mercury chloride (0.375 mg/kg/day $HgCl_2$) was orally administered (by gavage), during a period of 45 days. The exposure protocol to inorganic mercury was according to previous studies (Teixeira et al., 2014; Aragao et al., 2017; Aragao et al., 2018; Teixeira et al., 2018; Teixeira et al., 2019). Weekly, the animals were weighed for dose adjustment. The methodological steps were summarized in the Figure 1.

INSERT FIGURE 1

After the experimental period, eight animals per group were euthanized by cervical dislocation, and their spinal cord were immediately removed and equally sectioned by longitudinal cutting (antero-posterior). Thus, the animals used to

biochemical assays were the same collected to mercury measurements using hemi section of the rat spinal cord by test. While others eight animals per group were also euthanized by cervical dislocation and their spinal cord were fully removed to proteomic analysis. The remaining animals were anesthetized and perfused to histological and immunohistochemistry analysis (eight animals per group); and evaluation of microscopic findings by transmission electron microscopy (one animal per group).

2.2. Mercury measurements

The rat spinal cords were removed and submitted to freezing until analysis. Total mercury (Hg) content in the samples was estimated by digestion, reduction, and atomic absorption spectrometry with cold vapor (CVAAS) using a semi-automated Mercury Analyzer (model Hg-201, Sanso Seisakusho Co. Ltd.,Tokyo, Japan), according to protocol described in our studies (Teixeira et al., 2014; Aragão et al., 2018; Bittencourt et al., 2019; Freire et al., 2019; Santana et al., 2019). The results of the samples analyses were expressed in ppb.

2.3. Proteomic analyses

This analysis was performed exactly as described in protocols previously described (Bittencourt et al., 2017; Dionizio et al., 2018; Bittencourt et al., 2019). The proteomics analysis was performed by sample homogenization, protein extraction, reduction, alkylation, digestion, desalination and purification. The reading and identification of the peptides were performed on a nanoAcquity UPLC-Xevo QTof MS system (Waters, Mancester, UK), using the Protein Lynx Global Server (PLGS), as previously described (Lima Leite et al., 2014; Bittencourt et al., 2017; Dionizio et al., 2018; Bittencourt et al., 2019). The proteins identification was performed by downloading

Uniprot databases. After, the bioinformatics analyses were performed using Cytoscape (3.6.1 version, Java®) with ClusterMarker plugin for protein-interaction network, and for determination of biological processes groups we used ClueGO plugin, based on Gene Ontologies (GO) annotations (Bindea et al., 2009).

2.4. Oxidative biochemistry assays

For these analyses, samples were thawed and resuspended in 20mM Tris-HCl buffer (pH 7.4, at 4°C) for sonic disintegration (~ 1 g/mL). The avoid extensive alterations to proteins and lipids was carried out the homogenization in ice-cold buffer for a few seconds each time. All biochemical tests were performed in triplicate and utilized the supernatant after centrifugation, with time and rotation variable according to the following protocols described.

The parameters of antioxidant capacity against peroxyl radicals (ACAP) was evaluated through reactive oxygen species (ROS) determination in tissue samples treated or not with a peroxyl radical generator, by fluorimeter (Amado et al., 2009); lipid peroxidation (LPO), using malondialdehyde (MDA) as an indicator (Esterbauer and Cheeseman, 1990); and nitrite levels (an indirect marker of nitric oxide production) by spectrophotometry (Green et al., 1982). Furthermore, we evaluated the total protein, following the method proposed by Bradford (1976). Thus, after correcting the protein concentration values, the results of the inverse area (relative difference between the ROS areas with and without ABAP), MDA concentration and nitrite levels were expressed as percentages of the control groups.

2. 5. Perfusion procedure, histological and immunohistochemistry analysis

The animals were deeply anesthetized with ketamine hydrochloride (90 mg/kg) and xylazin hydrochloride (10mg/kg) solution and transcardially perfused with heparinized phosphate-buffered saline (PBS, 0.1 M) followed by paraformaldehyde (4%). The spinal cords and postfixed for 12 hours in formaldehyde (10%) solution. Then, these tissues were dehydrated in increasing EtOH solutions, diaphanized in xylol and embedded in Paraplast® (MCCormick). Cross sections of 5μm thickness were obtained by traversal cuts in microtome and then put on microscopy slides.

Microscopic findings by transmission electron microscopy (TEM)

For this investigate the ultrastructural, the animals were perfused following 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M Cacodylate plus 2.5% sucrose, pH 7.4, for 1 at room temperature. After perfusion, the spinal cords were washed twice for 10 min with 0.1 M cacodylate buffer pH 7.2 added with 5nM $CaCl_2$ and post-fixed with 1% osmium tetroxide (OsO_4) 5nM 0.8% $CaCl_2$ potassium ferrocyanide in 0.1 M Cacodylate buffer; pH 7.2 for 1h at 4 ° C. For block contrast then the samples were washed in 0.1 M cacodylate buffer pH 7.4 for 10 minutes twice with distilled water for the same time period and then the material was immersed in uranyl acetate solution. 1% in 25% acetone for 1 h at 4 °C. For dehydration, the acetone was used at room temperature in increasing concentrations and tissue was infiltrated with acetone-Epon mixture and polymerized at 60ºC for 48h. Sections were made using an ultramicrotome, mounted in mesh nets and subsequently impregnated with uranyl acetate and lead nitrate. These grids were viewed under a transmission electron microscope (LEO 960 E) at 60000x. For qualitative analysis, pathological findings regarding myelin and axon structures were considered. Myelin rupture including myelin vacuolation, loss of compaction, vacuoles and white matter vacuolization were analyzed in the samples.

Histological evaluation

For motor neurons (NM) analysis, five micrometers sections were stained with routine Hematoxylin-Eosin (HE). Photomicrographs of two slides and three sections per slide/animals were obtained (10x and 40x magnification - Leica Microscope DM500 and IC50 HD Camera, Leica Microsystems, Wild AG, Heerbrugg, Switzerland, SWI). Number of MN were considered for cervical, thoracic, and lumbar regions using cell counter function of NIH ImageJ version 1.52 (NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). Motor neurons identification was performed considering localization, size, staining characteristics, and morphology. MN are large basophilic multipolar cells (Three to 5 concave sides with 1 or 2 visible dendritic stems owning a large, central, and pale nucleus with scarcely condensed chromatin (euchromatin); size of 18-67µm; and localization in ventral horn of the spinal cord (Ferrucci et al., 2018).

Immunohistochemistry

This analysis was performed as described by our studies (Lima et al., 2016; Freire et al., 2019; Lamarao-Vieira et al., 2019; Santana et al., 2019), with adaptions to spinal cords tissue. The immunohistochemistry analysis was realized by endogenous peroxidase activity using anti-NeuN (1:100, Chemicon®) and the fixed tissue sections was revealed with 3,3′diaminobenzidine (DAB).

The number of $NeuN^+$ cells were analyzed by light microscopy (Leica Microscope DM500 and IC50 HD Camera, Leica Microsystems, Wild AG, Heerbrugg, Switzerland, SWI) using objective lens of 4x, 10x, and 40x. For analyses of anti-NeuN immunostaining, we first obtained the photomicrographs using the microscope Leica DM500 with IC50 HD® attached to it. The photomicrographs were analyzed using ImageJ software with “Color Deconvolution plugin" (Gabriel Landini, http://www.dentistry.bham.ac.uk/landinig/software/software.html) from ImageJ software version 1.52 (NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). Positive cells

for anti-NeuN immunostaining were segmented using Threshold and Binary function, and then numerically counted by ImageJ software. For total cell count, the medullary H region was measured in μm^2 and the number of total cells per area (μm^2) was considered for immunohistochemical analysis.

2.6. Statistical analysis

All the results were tabulated after data collection and analyzed by GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, USA). To verification of normality, the Shapiro-Wilk method was utilized. The weight curve was evaluated with two-way ANOVA followed the Tukey post hoc test and the other data performed by Student's t-test. The results were expressed in mean ± standard error of the mean (SEM) and values of $p \leq 0.05$ were considered statistically significant. In proteomic analyses, the PLGS software, was used to obtain the difference of protein expression between the groups, applying the Monte-Carlo algorithm ($p<0.05$ for down-regulated proteins and $1\text{-}p>0.95$ for up-regulated proteins).

3. Results

3.1. Effects of exposure to $HgCl_2$ on body weight of rats during 45 days

The animals of control group and the exposed to HgCl2 had gained weight ($p<0.0001$). At the end of the experiment, the animals of both the groups did not show body weight difference ($p>0.05$, Figure 2).

INSERT FIGURE 2

3.2. Analysis of total Hg levels revealed the higher levels of metal in the rats' spinal cord after exposure to $HgCl_2$

The Hg levels in the spinal cord of control animals (0.28 ± 0.08 ppm) differ significantly to exposed animals (0.98 ± 0.07 ppm; $p<0.05$), as observed in the Figure 3.

INSERT FIGURE 3

3.3. HgCl2 induces oxidative biochemistry changes in rats' spinal cord

The $HgCl_2$-exposed animals reduced their parameters of ACAP. Thus, the ACAP concentration was less in exposed group (64.63 ± 4.97%) than in control group (100 ± 6.75%; $p < 0.005$, Figure 4A).

Besides that, pro-oxidant factors were found to be statistically increased in the $HgCl_2$ group. The exposure to $HgCl_2$ during the period proposed was able to modulate oxidative biochemistry by increasing LPO levels in the rats' spinal cord (Control group:100 ± 8.95% versus $HgCl_2$ group: 220 ± 43.23%; $p < 0.05$, Figure 4B).

When observed the indirect marker of nitric oxide production, $HgCl_2$ induced increases in nitrite levels also ($HgCl_2$ group: 188.6 ± 23.01%) compared to control group (100 ± 25.85%; $p < 0.05$, Figure 4C).

INSERT FIGURE 4

3.4 The HgCl2 deeply affects the whole proteomic profile of rats' spinal cord, impairing several biological processes and cellular components based on GO analyses of the proteome

The proteomic profile analysis revealed the modulation of 234 proteins, in which 2 were up-regulated and 142 down-regulated. Moreover, 23 were exclusively found in exposed group, while 65 in control group. In table 1, we highlighted a few proteins found in this investigation that contributed in discussion of $HgCl_2$ damages over spinal cord.

INSERT TABLE 1

Performing the GO analyses, a total of 27 biological processes showed to be related to the effects of HgCl2 over the spinal cord of adult rats and, evaluating the most impaired biological processes of spinal cord of rats exposed to HgCl2, the top 5 processes affected were axon development (17%), ADP metabolic process (12,6%), cerebellum development (5,7%), sodium: potassium-exchanging ATPase activity (5,7%) and hippocampus development (5,7%), as shown in Figure 5 .

INSERT FIGURE 5

Also, the GO analyses of cellular components of rats' spinal cord exposed to $HgCl_2$ revealed 22 processes involved. The top 5 of cellular components involved were mitochondrial membrane (20%), microtubule (19,3%), stress fiber (5,5%), terminal bouton (4,8%) and mitochondrial outer membrane (4,8%), as observed in Figure 5 and 6.

INSERT FIGURE 6

The proteome networks created by ClustMarker analyses showed several proteins found in our investigation interacting with each other, creating a functional network that demonstrated how HgCl2 is able of impairing the cellular and molecular pathway cycles, as shown in Figures 7 and 8.

INSERT FIGURE 7

INSERT FIGURE 8

3.5. Exposure to HgCl2 promotes disorganization of the myelin sheath in spinal cord

TEM photomicrographs in qualitative analysis showed some pathological characteristics of axons. Vacuolated white matter was present in exposed HgCl2 axons as well as periaxonal space, such as intramyelinic and extramyelinic vacuoles as seen in Figure 9.

INSERT FIGURE 9

3.6. Exposure to HgCl2 promotes decreased of density cell in spinal cord

Considering number of MNs in photomicrographs analysis, animals exposed to $HgCl_2$ presented a lower number of MNs in cervical (Control group: 19.63 ± 1.41; $HgCl_2$ group: 13.5 ± 0.56; $p < 0.05$), thoracic (Control group: 11.6 ± 0.34; $HgCl_2$ group: 10.43 ± 0.20; $p < 0.05$), and lumbar spinal cord regions (Control group: 15.78 ± 0.74; $HgCl_2$ group: 13 ± 0.40; $p < 0.03$).

Immunoistochemistry essays for anti-$NeuN^+$ antibody demonstrated lower number of mature cells.μm^{-1} for cervical (Control group: 100 ± 6.63; $HgCl_2$ group: 78.76 ± 4.65; $p < 0.05$), thoracic (Control group: 100 ± 10.17; $HgCl_2$ group: 64.29 ± 3.37; $p < 0.05$), and lumbar photomicrographs (Control group: 100 ± 11.44; $HgCl_2$ group: 64 ± 5.39; $p < 0.05$).

INSERT FIGURE 10

INSERT FIGURE 11

4. Discussion

In this study, we show for the first time the multiple mechanisms of damage that long-term exposure to $HgCl_2$ can cause to spinal cord. Underlying the damages, the unprecedented investigation of the proteomic profile of the spinal cord revealed effects over the energy metabolism, neuroplasticity mechanisms and neuroglial function. In addition, we also evidenced nitrosative and oxidative stress, which resulted in death of total neurons and motoneurons in the cervical, thoracic and lumbar regions, as well as ultrastructural changes to the myelin sheath. In this way, our study brings the translational appeal in elucidating important spinal cord damage mechanisms that populations in endemic regions of mercurial exposure may be susceptible.

The literature presents lacks of evidence when comes to values of inorganic Hg levels in human populations living in endemic areas (for review see Berzas-Nevado et al.,

2010). However, Mantín-Doimeadios et al. (2014) investigated the speciation of Hg species in commercial fishes in legal Amazonia, an endemic area of mercurial exposure, and pointed out that although inorganic Hg levels could reach 0.3μg/g of fish muscle, no difference was observed between endemic and non-endemic areas of Hg exposure. It is important to highlight that is hard to consider the intake of only one Hg specie by food or water consumption, in this way, Martín-Doimeadios et al. (2014) also described the total Hg content in the samples could reach 8.71μg/g of fish muscle.

Additionally, during the Hg toxicokinetic, some studies highlight the capacity of methylation and demethylation of inorganic and organic forms, respectively in gut microbiota and in CNS, representing an important point in the dynamic of Hg distribution along the body (Shapiro and Chan, 2008; Martin-Doimeadios et al., 2017). Following the toxicokinetic, other studies suggest that Hg distribution may occurs through retrograde axonal transport from neuromuscular junctions (Arvidson, 1994; Pamphlett and Waley, 1996). Thus, in addition to the systemic distribution in the blood, Hg may also be conducted to the spinal cord by the fact that this organ is an axonal afferent center (Pamphlett and Kum Jew, 2013). In this way, our results of Hg levels in spinal cord reinforce that although inorganic Hg has lower liposolubility, it may cross biological barriers and reach the spinal cord.

After reaching the spinal cord parenchyma, Hg was able of triggering oxidative/nitrosative stress. This event was observed by the reduction of ACAP, which increases the overproduction of ROS and consequently, drove to an increase of LPO levels. When an overproduction of ROS occurs and the antioxidant competence is impaired, illustrated herein by ACAP reduction, the LPO is an event that occurs, featuring an oxidative stress state, and in our investigation, we observed a substantial increase of it, characterizing a neurodegeneration induced by $HgCl_2$ long-term exposure.

Additionally, nitrosative stress is an important event that occurs in spinal cord injury, as well reported in literature as a key-factor for progression of neurodegeneration processes in spinal cord due to energetic metabolism failure related to DNA disorders and peroxinitrite anions (Iadecola, 1997; Tardivo et al., 2015). In this way, our observations suggest that the increase of LPO and nitrosative stress may be associated to the neurodegeneration triggered by $HgCl_2$ in spinal cord of rats. Underlying this issue, our proteomic approach pointed the down-regulation of *peroxiredoxin-1* (Q63716), *2* (P35704) and *5* (Q9R063), and exclusive regulation of *peroxiredoxin-4* (Q9Z0V5) in control group, that acts protecting cells against oxidative stress mediated by ROS (Rhee, 2006). In the other hand, enzymes involved in this cellular protection as superoxide dismutase (Fukai and Ushio-Fukai, 2011), was also found down-regulated (*Superoxide dismutase [Mn], mitochondrial*; P07895). Some studies have suggested that *Carbonic anhydrase 3* (P14141) is associated with the protection against oxidative stress after hydrogen peroxide and hypoxic insults (Roy et al., 2010; Shi et al., 2017), and interestingly, we found it negatively regulated in exposed group.

In this way, the overwhelming of ROS production may drive to mitochondrial dysfunction, once it leads to oxidation of DNA, proteins and lipids from mitochondria, possibly culminating into a reduced mitochondrial biogenesis (Beckman and Ames, 1999; Bhatti et al., 2017). Surrounding this metabolic impairment, our proteomic analysis showed down-regulation of *ADP/ATP translocase* subunits *1* (Q05962) and 2 (Q09073), besides *ATP synthase* subunits alpha (P15999) and beta (P10719) and citrate synthase (Q8VHF5) down-regulation, that are directly associated to energetic metabolism in mitochondria, participating in citric acid cycle in isocitrate synthesis (Shepherd and Garland, 1969), catalyzes the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane and integrating the ATP formation (Meyer et

al., 2007). Moreover, the mitochondrial failure also plays a significant role in cellular aging by down-regulation of cytochrome c oxidase down-regulation (Zhang et al., 2002), and our findings suggest that $HgCl_2$ acts in this pathway by down-regulating the subunits 4 (P10888) and 5A (P11240) of cytochrome c oxidase.

Increased oxidative stress may promote cellular death processes (Finkel and Holbrook, 2000) and can be associated with the findings of reduced number of motoneurons and total mature neurons in our investigations. However, when it comes to Hg and nervous system, another factor must be considered: the excitotoxicity, which is another pathway of cell death intrinsically associated with Hg exposure (Albrecht and Matyja, 1996; Aschner et al., 2000; Xu et al., 2012; Teixeira et al., 2019). The $HgCl_2$ have been reported as capable of inhibiting glutamate reuptake and glutamine synthetase activity (Allen et al., 2001), besides cause damages over microtubule structures (Xu et al., 2012). Interestingly, our proteome showed down-regulation of several tubulin subunits affected by $HgCl_2$ (see Supplementary Material, Table 1) and also revealed the down-regulation of actin filaments proteins types (P68035, P68136, P62738, P60711, P63259, P63269) that are essentials for vesicle trafficking in pre and post synaptic terminals - For review see (Cingolani and Goda, 2008). Moreover, our findings also pointed the negative regulation of *Clathrin heavy chain 1* (P11442), and *Excitatory amino acid transporter 2* (P31596), associated with vesicle trafficking and excitatory synapse (Sakurai et al., 2015). The cytoarchitecture organization is essential not only for development of neurons and glial cells, but for promoting an efficient synaptic transmission (Schubert and Dotti, 2007; Stavoe and Colon-Ramos, 2012), in this way, we hypothesize that $HgCl_2$ may contribute to a synaptic misbalance and further cell death by excitotoxicity, besides decrease on spinal cord neurofunctions.

In this perspective, other factors contribute for neuroplasticity besides the synaptic and cytoskeleton organization. Our proteomic approach revealed down-regulation of *glial fibrillary acidic protein* (GFAP; P47819), which can be associated with a reduction of astrocyte number and consequent lower astrocyte reactivity to an insult (Brahmachari et al., 2006). Corroborating to this fact, the *protein S100-B* (P04631) that is considered a glial and neurotrophic substance (Selinfreund et al., 1991; Pena et al., 1995) was found down-regulated, suggesting a reduced stimulus to neuro and gliogenesis. However, the other face of this protein lives in the fact that it is considered an early biomarker of excitotoxicity when is found over expressed, even in spinal cord (Mazzone and Nistri, 2014), due the ability of stimulating the glial response in order to protect and repair tissues from damages. In fact, a recent report with human populations exposed to methylmercury demonstrated that the increased expression of this protein detected in blood is associated with the higher levels of the metal (de Paula Fonseca Arrifano et al., 2018).

The astrocyte and microglia interplay in neurodegeneration in fact has a dual role, while in one hand it is necessary to tissue response against threats/injuries, in the other, it can exacerbate the damage and impair the neuroplasticity (Pekny et al., 2007). In this way, the spinal cord of rats exposed to $HgCl_2$ may try to avoid more severe damages by the down-regulation of the proteins mentioned above, besides *vimentin* (P31000) negative regulation and *CD81 antigen* (Q62745) exclusive expression in control group (Wilhelmsson et al., 2004), which in a reactive state, contribute for reactive gliosis and glial scar after injury. Moreover, the complement system also plays an in neurodegenerative processes due the participation in tissue regeneration besides its the detrimental role (Pekny et al., 2007), and in the proteome we found down-regulation of *Complement C3* (P01026), suggesting a possible mechanism of defense, once this protein is related to the increase of brain injuries damages(Alawieh et al., 2018).

Following in this biochemical and morphological issue in spinal cord of rats exposed to $HgCl_2$, it is important to highlight the substantial role that myelin plays for nervous system in neurotransmission - For review see (Nave and Werner, 2014). The MBP (P02688) is responsible for the adhesion of the cytosolic surfaces of multilayered compact myelin (Boggs, 2006) and was found down-regulated in this study. In addition, *myelin proteolipid protein* (P06907) that is the most abundant protein and maintains the structural and functional integrity of myelin and *myelin protein P0 (P06907)* were also found down-regulated (Greer and Pender, 2008). Also, it is important to highlight that CD81 protein (Q62745), found exclusive in control group, and CD9 (*CD9 antigen;* P40241), found down-regulated are also important components of myelin sheath. Then, our results suggest that this proteomic modulation is responsible of the disorganization of myelin sheath observed by TEM.

The demyelination is as consequence of a failure on remyelination process, often associated with oligodendrocytes death and consequent axonal degeneration (Wang et al., 2017), while the loss of the compacted form, as observed here in the spinal cord of animals exposed do $HgCl_2$, is associated with the loss of proteins that compose the structural part of compact myelin, as mentioned above (Jahn et al., 2009). In addition, (Wang et al., 2017) reviewed that there are two kinds of injury to spinal cord: primary that occurs immediately after the injury that only is featured by neuron and axonal degeneration; and secondary, that also presents oxidative stress, neuroinflammation and glial scar. In this way, for the first time we can conclude that $HgCl_2$ deeply affects myelin sheath by a secondary injury that drove to loss of compaction and might be intrinsically associated with the motor impairments already observed by our group in previous studies (Teixeira et al., 2018; Teixeira et al., 2019).

Motor neurons are the main motility-related cells, so damage to these cell populations culminates in motor damage (Teixeira et al., 2014). In our morphological analyses, we observed that the $HgCl_2$ intoxication was able to reduce the number of motor neurons and total neuronal density, which includes motor, sensory, and inter neurons in the cervical, thoracic, and lumbar segments. This indicates that spinal cord is also a susceptible organ to $HgCl_2$ damages, causing neuron death, as also described in others CNS areas, as hippocampus (Teixeira et al., 2014; Aragao et al., 2018). There are several mechanisms that can drive to cell death caused by Hg exposure, including excitotoxicity and apoptosis. (Aragao et al., 2018; Teixeira et al., 2018) possible pathways that may have caused a reduction in neuronal density in the spinal cord of our animals. These results highlights concern about long-term $HgCl_2$ exposure effects on spinal cord and may lead for a better knowledge to motor damage related to inorganic mercury.

Conclusions

Considering all molecular and structural findings from our investigations, we conclude that the the increase of Hg levels in neural parenchyma, nitrosative/oxidative stress, with consequent proteomic misbalance and ultrastructural impairments may lead to medullary function disorders and motor damage after long-term HgCl2 exposure.

6. Conflicts of interest

The authors declare no conflicts of interest in the study.

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Table 1. Proteins identified with significantly expression in the spinal cord of significantly of control (C) *vs.* $HgCl_2$ (Hg) groups

			Fold change
[a]Access Number	**Protein Description**	***PLGS Score***	**Hg**
P68035	Actin, alpha cardiac muscle 1	13315	-0,787
P68136	Actin, alpha skeletal muscle	13788	-0,779
P62738	Actin, aortic smooth muscle	13302	-0,763
P60711	Actin, cytoplasmic 1	15445	-0,835
P63259	Actin, cytoplasmic 2	15445	-0,844
P63269	Actin, gamma-enteric smooth muscle	13302	-0,763
Q05962	ADP/ATP translocase 1	917,06	-0,651
Q09073	ADP/ATP translocase 2	732,14	-0,651
P15999	ATP synthase subunit alpha, mitochondrial	1820,8	-0,852
P10719	ATP synthase subunit beta, mitochondrial	4597,8	-0,835
P14141	Carbonic anhydrase 3	801,5	-0,167
Q8VHF5	Citrate synthase, mitochondrial	103,05	-0,698
P11442	Clathrin heavy chain 1	271,19	-0,741
P10888	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	150,03	-0,811
P11240	Cytochrome c oxidase subunit 5A, mitochondrial	910,09	-0,779
P31596	Excitatory amino acid transporter 2	301,95	-0,795
P47819	Glial fibrillary acidic protein	18590	-0,811
P02688	Myelin basic protein	34181	-0,763
P06907	Myelin protein P0	5905,2	-0,379
P60203	Myelin proteolipid protein	12631	-0,763
Q63716	Peroxiredoxin-1	155,39	-0,719
P35704	Peroxiredoxin-2	789,55	-0,861
Q9R063	Peroxiredoxin-5, mitochondrial	871,47	-0,763
P04631	Protein S100-B	4248	-0,691
P07895	Superoxide dismutase [Mn], mitochondrial	231,54	-0,835
P31000	Vimentin	471,74	-0,719
Q62745	CD81 antigen	208,8	-
P01026	Complement C3	34,93	-
Q9Z0V5	Peroxiredoxin-4	79,67	-
+ 203 proteíns with com diferente regulatory status			

Figures

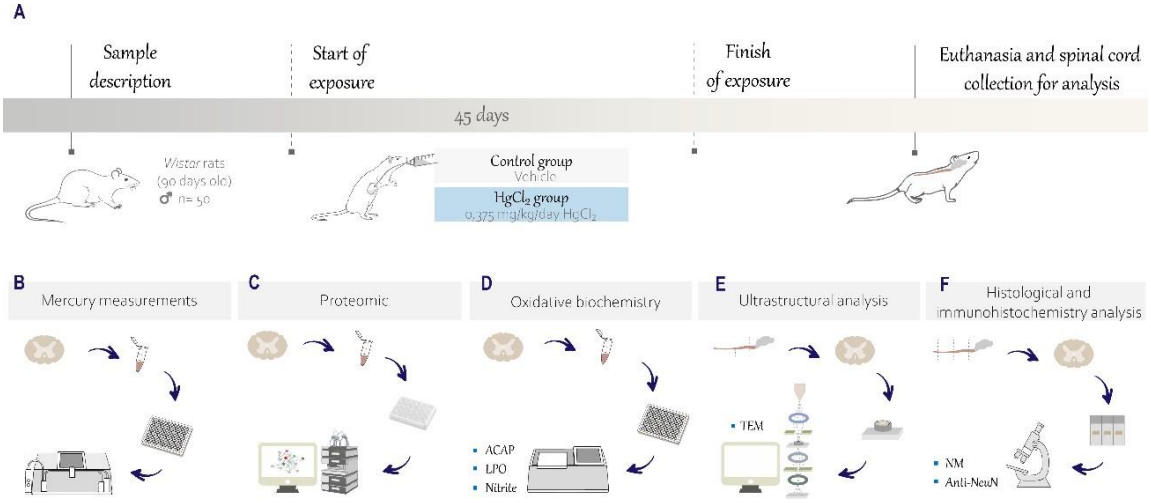

Figure 1. Sample description and experimental stages. (A) Description of the sample and model of exposure to HgCl2; division of experimental groups and animal destinations for each stage of analysis: (B) total Hg measurement assay, (C) proteomics analysis; (D) oxidative balance assays by antioxidant capacity against peroxyl radicals (ACAP), Lipid Peroxidation (LPO) and Nitrite levels (Nitrite); (E) transmission electron microscopy (TED) and (F) immunohistochemistry analysis by motor neurons (MN), anti-NeuN.

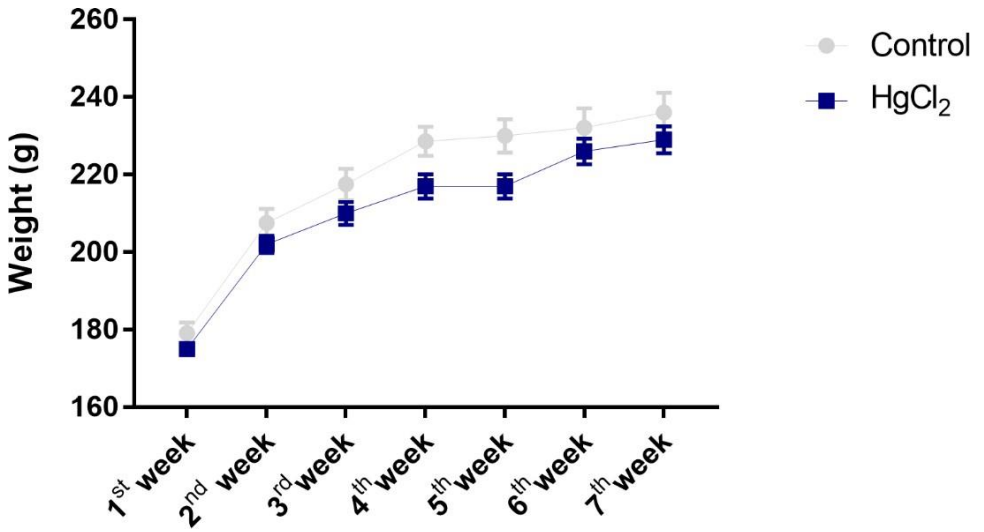

Figure 2. Effects of exposure to $HgCl_2$, during 45 days, on body weight gain (g) of the Wistar rats. Results were expressed as mean ± standard error of mean. Two-way ANOVA and Tukey's post-hoc test, $p<0.05$.

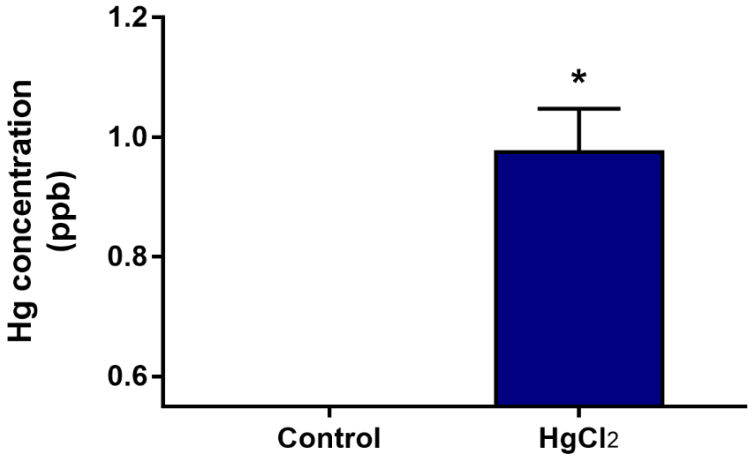

Figure 3. The total Hg levels after exposure to $HgCl_2$, during 45 days, on spinal cord of the Wistar rats. Results are expressed as mean ± standard error of mean. Student's t-test, $p<0.05$. *Statistical difference in relation the control group.

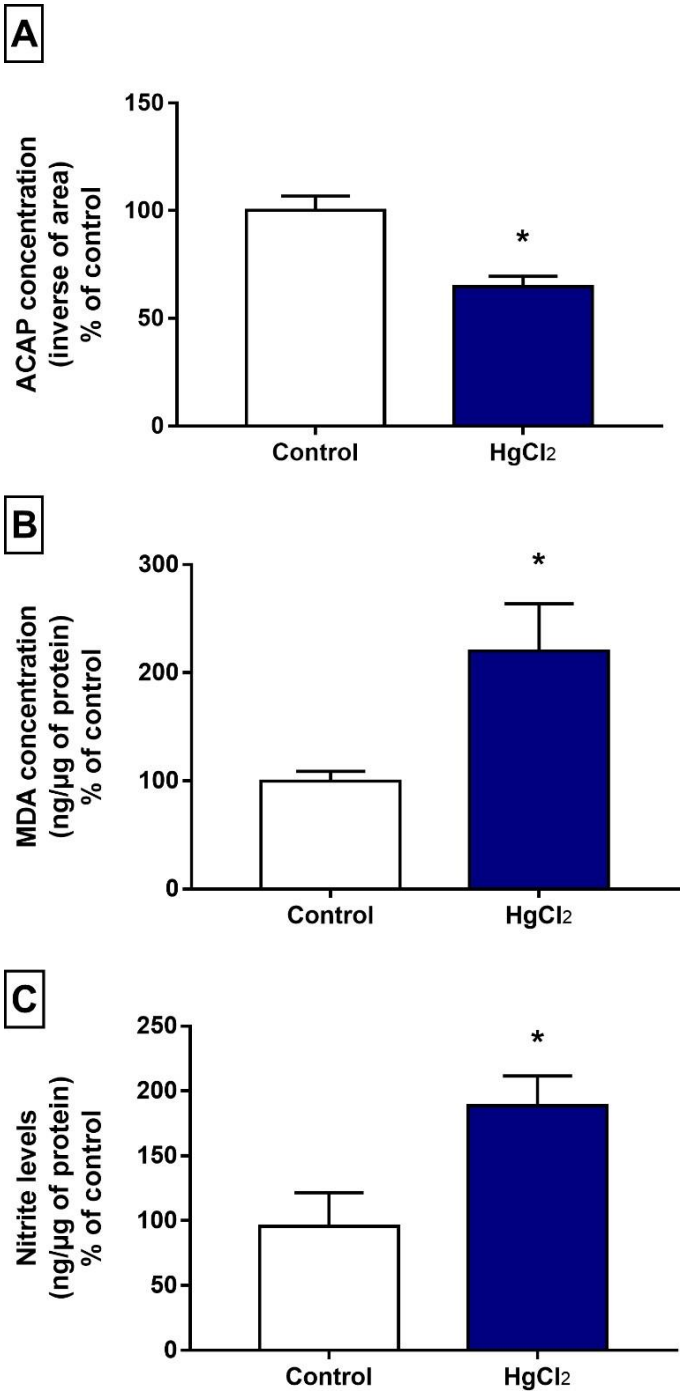

Figure 4. Effects of exposure to $HgCl_2$, during 45 days, on oxidative balance in spinal cord of *Wistar* rats. (A) Antioxidant capacity against peroxyl radicals (ACAP); (B) percentages of milligram malondialdehyde per protein in relation to the control group, to analyses of the lipid peroxidation (LPO) and (C) percentages of nitrite per milligram of protein in relation to the control group. Results are expressed as mean ± standard error of mean. Student's t-test, $p<0.05$. *Statistical difference in relation the control group.

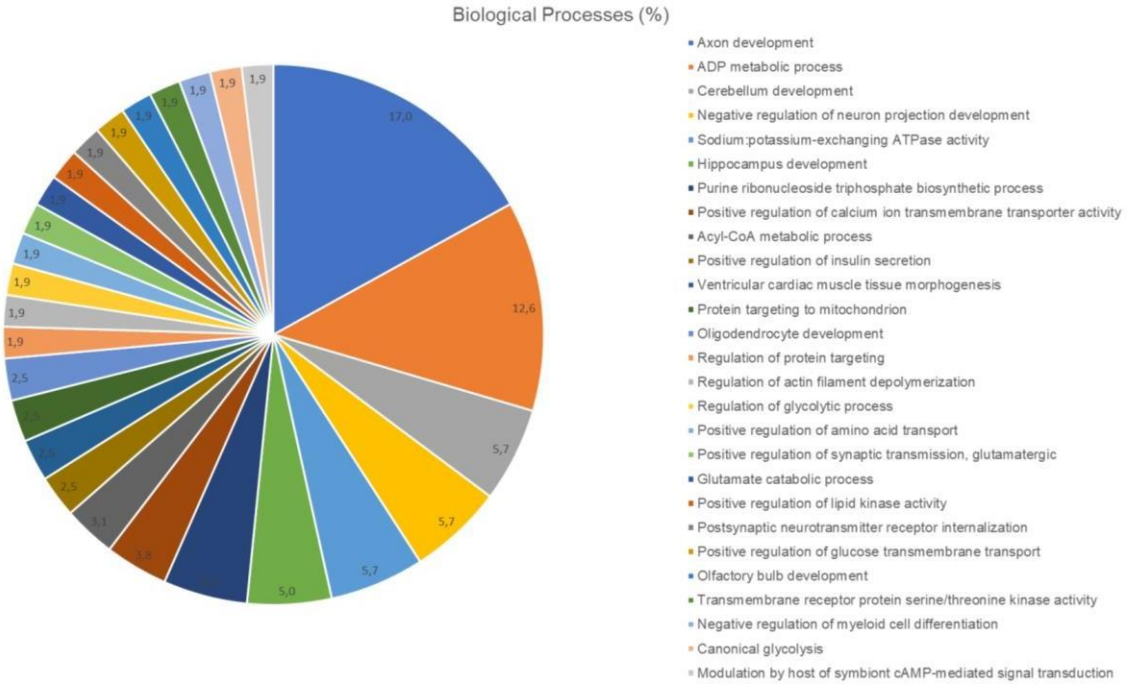

Figure 5. Functional distribution of proteins identified with differential expression in spinal cord of rats exposed to $HgCl_2$ vs control group. Categories of proteins based on Gene Ontology annotation of biological process. Terms significant (Kappa Score = 0.4) and distribution according to percentage of number of genes. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® plugin of Cytoscape® software 3.7.1.

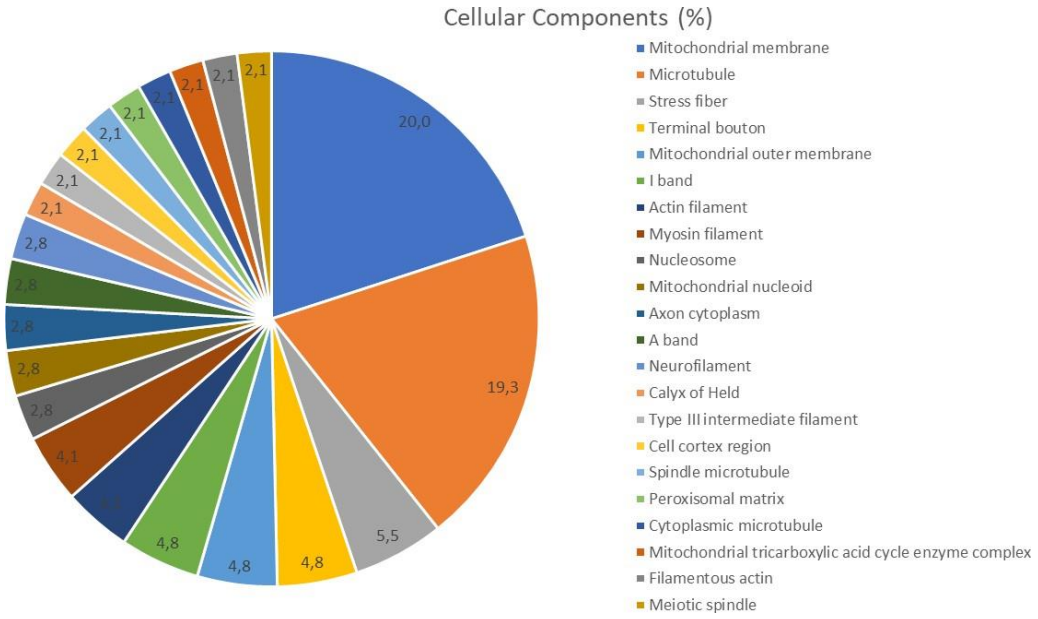

Figure 6. Functional distribution of proteins identified with differential expression in spinal cord of rats exposed to HgCl2 vs control group. Categories of proteins based on Gene Ontology annotation of cellular component. Terms significant (Kappa Score = 0.4) and distribution according to percentage of number of genes. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® plugin of Cytoscape® software 3.7.1.

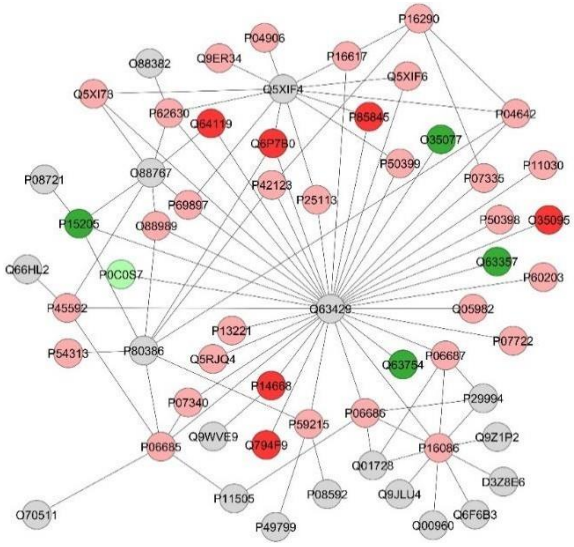

Figure 7. Subnetworks clustered by ClusterMarker app to determinate the interaction among identified proteins of spinal cord with different expression on $HgCl_2$ group vs. control group. The node colors indicate different status of expression of the respective protein, named by its accession ID from Uniprot. The dark red and dark green indicate proteins unique/exclusive of control and exposed groups, respectively. Light green and

pink indicate upregulated and downregulated proteins, respectively. Gray nodes represent those proteins that were not identified in our study, but interacted on the network by importation from public databases. *Phosphoglycerate mutase 2 (P16290); L-lactate dehydrogenase A chain (P04642); Creatine kinase B-type (P07335); Acyl-CoA-binding protein (P11030); Rab GDP dissociation inhibitor alpha (P50398); Neurochondrin (O35095); Unconventional myosin-Id (Q63357); Myelin proteolipid protein (P60203); Nucleoside diphosphate kinase A (Q05982) Myelin-associated glycoprotein (P07722) Sodium/potassium-transporting ATPase subunit alpha-3 (P06687); Beta-synuclein (Q63754); Inositol 1,4,5-trisphosphate receptor type 1 (P29994); Alpha-actinin-1 (Q9Z1P2); Calmodulin-regulated spectrin-associated protein 1 (D3Z8E6); Protein TANC1 (Q6F6B3); Glutamate receptor ionotropic, NMDA 2B (Q00960); SH3 and multiple ankyrin repeat domains protein 3 (Q9JLU4); Sodium/calcium exchanger 1 (Q01728); Spectrin alpha chain, non-erythrocytic 1 (P16086); Sodium/potassium-transporting ATPase subunit alpha-2 (P06686); Guanine nucleotide-binding protein G(o) subunit alpha (P59215); Amyloid-beta A4 protein (P08592); Regulator of G-protein signaling 4 (P49799); Guanine nucleotide-binding protein G(o) subunit alpha (P59215); 4F2 cell-surface antigen heavy chain (Q794F9); Plasma membrane calcium-transporting ATPase 1 (P11505); 4F2 cell-surface antigen heavy chain (Q794F9); Annexin A5 (P14668); Intersectin-1 (Q9WVE9); Sodium/potassium-transporting ATPase subunit alpha-1 (P06685); odium/potassium-transporting ATPase subunit beta-1 (P07340); NAD-dependent protein deacetylase sirtuin-2 (Q5RJQ4); Aspartate aminotransferase, cytoplasmic (P13221); 5'-AMP-activated protein kinase subunit beta-1 (P80386); Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (P54313); Cofilin-1 (P45592); Ankyrin-3 (O70511); Src substrate cortactin (Q66HL2); Histone H2A.Z (P0C0S7); Microtubule-associated protein 1B (P15205); Osteopontin (P08721); Malate dehydrogenase, cytoplasmic (O88989); Protein/nucleic acid deglycase DJ-1 (O88767); Rho GDP-dissociation inhibitor 1 (Q5XI73); Elongation factor 1-alpha 1 (P62630); Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 2 (O88382); Tubulin beta-5 chain (P69897); Myosin light polypeptide 6 (Q64119); Aconitate hydratase, mitochondrial (Q9ER34); Glutathione S-transferase P (P04906); Small ubiquitin-related modifier 3 (Q5XIF4); Tryptophan--tRNA ligase, cytoplasmic (Q6P7B0); L-lactate dehydrogenase B chain (P42123); Phosphoglycerate mutase 1 (P25113); Phosphoglycerate kinase 1 (P16617); Tubulin alpha-4A chain (Q5XIF6); Fascin (P85845); Rab GDP dissociation inhibitor beta (P50399); Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic (O35077) and Polyubiquitin-C (Q63429).*

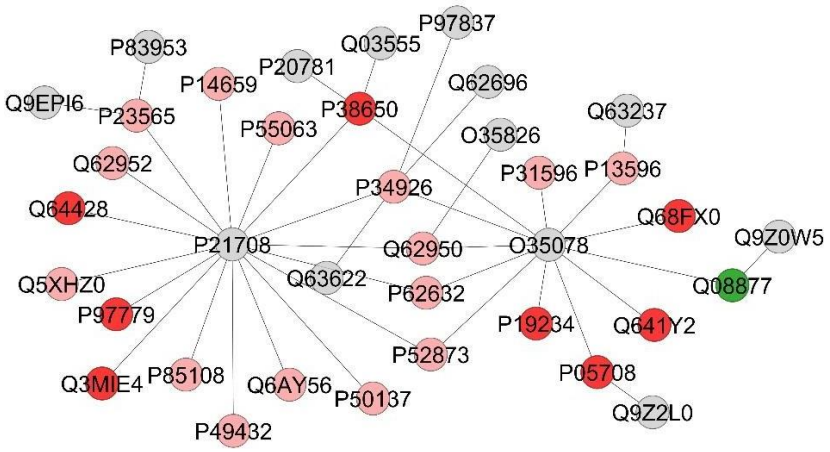

Figure 8. Subnetworks clustered by ClusterMarker app to determinate the interaction among identified proteins of spinal cord with different expression on $HgCl_2$ group vs. control group. The node colors indicate different status of expression of the respective protein, named by its accession ID from Uniprot. The dark red and dark green indicate proteins unique/exclusive of control and exposed groups, respectively. Pink nodes indicate downregulated proteins, respectively. Gray nodes represent those proteins that were not identified on our study, but interacted on the network by importation from public databases. *Penicillin G acylase (P31956); Neural cell adhesion molecule 1 (P13596); Heparin-binding fibroblast growth factor receptor 2 (Q63237); Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial (Q68FX0); Protein kinase C and casein kinase substrate in neurons protein 1 (Q9Z0W5); Dynamin-3 (Q08877); NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial (Q641Y2); Voltage-dependent anion-selective channel protein 1 (Q9Z2L0); Hexokinase-1 (P05708); NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial (P19234); D-amino-acid oxidase (O35078); Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (O35826); Disks large homolog 2 (Q63622); Disks large homolog 1 (Q62696); Disks large-associated protein 2 (P97837); Gephyrin (Q03555); Glycine receptor subunit beta (P20781); Cytoplasmic dynein 1 heavy chain 1 (P38650); Microtubule-associated protein 1A (P34926); Dihydropyrimidinase-related protein 1 (Q62950); Elongation factor 1-alpha 2 (P62632); Pyruvate carboxylase, mitochondrial (P52873); Transketolase (P50137); Tubulin alpha-8 chain (Q6AY56); Pyruvate dehydrogenase E1 component subunit beta, mitochondria (P49432); Tubulin beta-2A chain (P85108); Synaptic vesicle membrane protein VAT-1 homolog (Q3MIE4); Hyaluronan-mediated motility receptor (P97779); Heat shock protein 75 kDa, mitochondrial (Q5XHZ0); Trifunctional enzyme subunit alpha, mitochondrial (Q64428); Dihydropyrimidinase-related protein 3 (Q62952); Alpha-internexin (P23565); Hypothetical transmembrane protein (Q92PI6); Importin subunit alpha-5 (P83953); Heat shock-related 70 kDa protein 2 (P14659); Heat shock 70 kDa protein 1-like (P55063) and Mitogen-activated protein kinase 3 (P21708).*

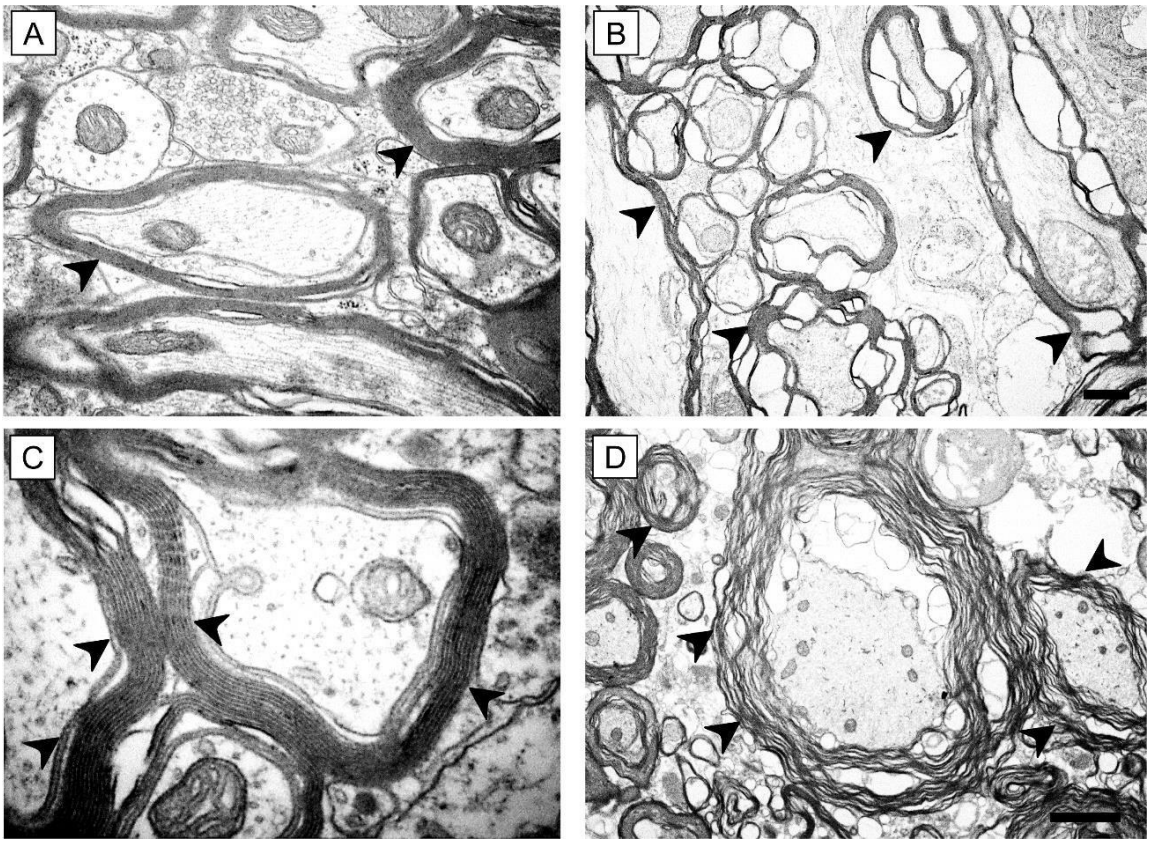

Figure 9. Effects of exposure to $HgCl_2$, during 45 days, on myelin sheath (arrowhead) in spinal cord of Wistar rats. Representative photomicrographs of axon of the (A and C) control group and (B and D) $HgCl_2$ group. Scale bar: 2 µm (A – B) and 5 µm (C – D).

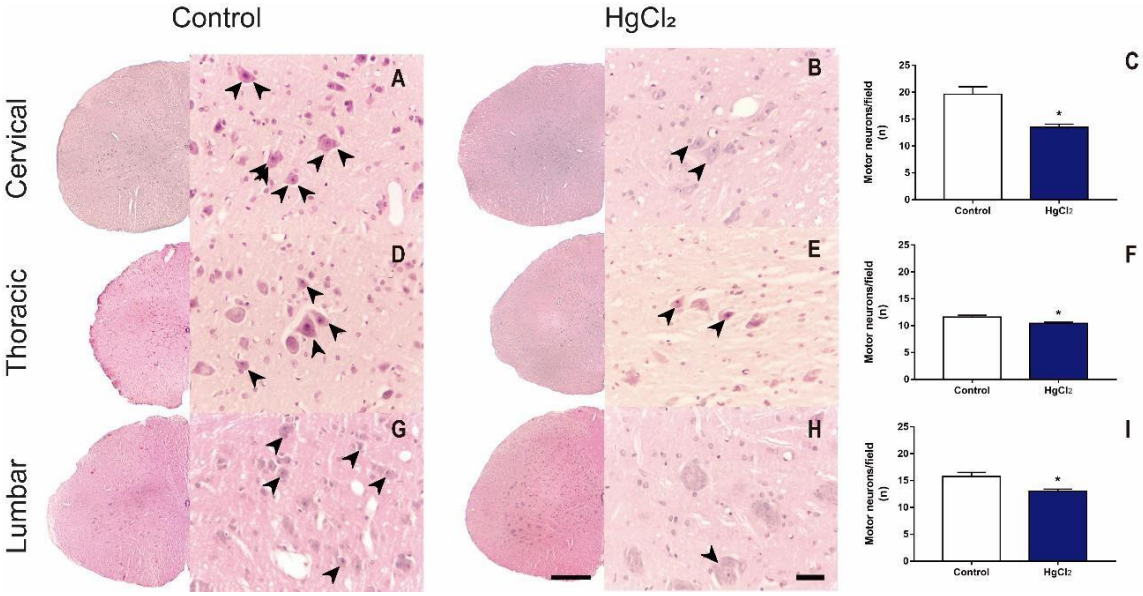

Figure 10. Effects of exposure to $HgCl_2$, during 45 days, on motor neurons (arrowhead) in spinal cord of *Wistar* rats. Sections were stained with hematoxylin and eosin (HE). Representative photomicrographs of the (A, D and G) control group and (B, E and H) $HgCl_2$ group. Results are expressed as mean ± standard error of mean (C, F and I). Student's t-test, $p<0.05$. *Statistical difference in relation the control group. Scale bar: 20 µm and 100 µm.

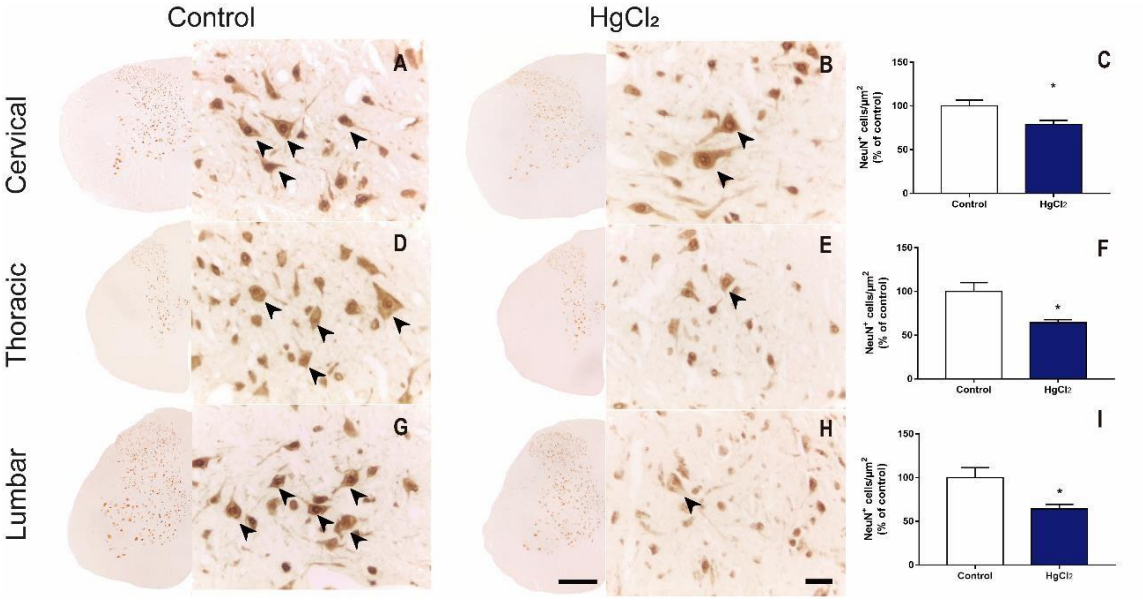

Figure 11. Effects of exposure to $HgCl_2$, during 45 days, on $NeuN^+$ cells (arrowhead) in spinal cord of *Wistar* rats. Representative photomicrographs of the (A, D and G) control group and (B, E and H) $HgCl_2$ group. Results are expressed as mean ± standard error of mean (C, F and I). Student's t-test, $p<0.05$. *Statistical difference in relation the control group. Scale bar: 20 µm and 100 µm.

S1. Proteins identified with significantly expression in the spinal cord of significantly of control (C) *vs.* $HgCl_2$ (Hg) groups

			Fold change
[a]Número de Acesso	**Descrição da Proteína**	***PLGS Score***	**Hg**
P63102	14-3-3 protein zeta/delta	1925,59	-0,914
P13233	2',3'-cyclic-nucleotide 3'-phosphodiesterase	25135,83	-0,795
P50554	4-aminobutyrate aminotransferase, mitochondrial	296,19	-0,631
P63039	60 kDa heat shock protein, mitochondrial	13-0,76	-0,705
Q9ER34	Aconitate hydratase, mitochondrial	267,38	-0,763
P68035	Actin, alpha cardiac muscle 1	13315,24	-0,787
P68136	Actin, alpha skeletal muscle	13787,81	-0,779
P62738	Actin, aortic smooth muscle	13301,87	-0,763
P60711	Actin, cytoplasmic 1	15444,71	-0,835
P63259	Actin, cytoplasmic 2	15444,71	-0,844
P63269	Actin, gamma-enteric smooth muscle	13301,87	-0,763
P11030	Acyl-CoA-binding protein	1877,46	-0,763
Q05962	ADP/ATP translocase 1	917,06	-0,651
Q09073	ADP/ATP translocase 2	732,14	-0,651
P04764	Alpha-enolase	2115,13	-0,771
P23565	Alpha-internexin	2107,98	-0,835
P13221	Aspartate aminotransferase, cytoplasmic	984,56	-0,852
P00507	Aspartate aminotransferase, mitochondrial	967,52	-0,869
P15999	ATP synthase subunit alpha, mitochondrial	1820,81	-0,852
P10719	ATP synthase subunit beta, mitochondrial	4597,84	-0,835
P15429	Beta-enolase	1128,03	-0,835
P0DP29	Calmodulin-1	915,62	-0,677
P0DP30	Calmodulin-2	929,68	-0,684
P0DP31	Calmodulin-3	915,62	-0,684
P14141	Carbonic anhydrase 3	801,5	-0,167
P40241	CD9 antigen	460,62	-0,549
Q4V7C8	Centrosomal protein of 55 kDa	43,8	-0,56
Q8VHF5	Citrate synthase, mitochondrial	103,05	-0,698
P11442	Clathrin heavy chain 1	271,19	-0,741
P45592	Cofilin-1	5306,13	-0,748
P07335	Creatine kinase B-type	4962,74	-0,741
P00564	Creatine kinase M-type	2483,48	-0,372
P25809	Creatine kinase U-type, mitochondrial	557,95	-0,719
Q68FY0	Cytochrome b-c1 complex subunit 1, mitochondrial	154,44	-0,733
P10888	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	150,03	-0,811
P11240	Cytochrome c oxidase subunit 5A, mitochondrial	910,09	-0,779
P48675	Desmin	330,4	-0,741

P11348	Dihydropteridine reductase	406,85	-0,835
Q62950	Dihydropyrimidinase-related protein 1	429,71	-0,733
P47942	Dihydropyrimidinase-related protein 2	3885	-0,827
Q62952	Dihydropyrimidinase-related protein 3	388,31	-0,811
P62630	Elongation factor 1-alpha 1	752,62	-0,852
P62632	Elongation factor 1-alpha 2	1412,27	-0,844
P06761	Endoplasmic reticulum chaperone BiP	160,22	-0,852
P31596	Excitatory amino acid transporter 2	301,95	-0,795
P05065	Fructose-bisphosphate aldolase A	2961	-0,698
P09117	Fructose-bisphosphate aldolase C	1575,53	-0,803
Q5M964	Fumarate hydratase, mitochondrial	88,17	-0,691
P07323	Gamma-enolase	2327,9	-0,861
P47819	Glial fibrillary acidic protein	18589,55	-0,811
Q6P6V0	Glucose-6-phosphate isomerase	192,79	-0,719
P10860	Glutamate dehydrogenase 1, mitochondrial	356,01	-0,914
P09606	Glutamine synthetase	327,69	-0,827
P04906	Glutathione S-transferase P	1507,31	-0,811
P04797	Glyceraldehyde-3-phosphate dehydrogenase	18893,41	-0,748
Q9ESV6	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	1561,49	-0,67
P09811	Glycogen phosphorylase, liver form	81,73	-0,726
P00489	Glycogen phosphorylase, muscle form	211,5	-0,771
P09812	Glycogen phosphorylase, muscle form	233,87	-0,664
P54311	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	218,56	-0,733
P54313	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	131,39	-0,726
P59215	Guanine nucleotide-binding protein G(o) subunit alpha	743,6	-0,852
P0DMW0	Heat shock 70 kDa protein 1A	278,72	-0,878
P0DMW1	Heat shock 70 kDa protein 1B	280,76	-0,887
P55063	Heat shock 70 kDa protein 1-like	276,01	-0,878
P63018	Heat shock cognate 71 kDa protein	1966,28	-0,819
Q5XHZ0	Heat shock protein 75 kDa, mitochondrial	136,26	-0,835
P82995	Heat shock protein HSP 90-alpha	327,1	-0,795
P14659	Heat shock-related 70 kDa protein 2	698,07	-0,779
P01946	Hemoglobin subunit alpha-1/2	32929,94	-0,869
P02091	Hemoglobin subunit beta-1	6719,31	-0,787
P11517	Hemoglobin subunit beta-2	2840,06	-0,811
P0C0S7	Histone H2A.Z	261,31	1,3231
Q00715	Histone H2B type 1	3123,47	-0,733
Q00729	Histone H2B type 1-A	511,88	-0,771
P62804	Histone H4	1153,81	-0,763
P04642	L-lactate dehydrogenase A chain	623,67	-0,67
P42123	L-lactate dehydrogenase B chain	1364,67	-0,869
O88989	Malate dehydrogenase, cytoplasmic	1725,82	-0,887
P04636	Malate dehydrogenase, mitochondrial	3622,4	-0,811
P34926	Microtubule-associated protein 1A	28,84	-0,771
P19332	Microtubule-associated protein tau	69,31	-0,383

P02688	Myelin basic protein	3418-0,88	-0,763
P06907	Myelin protein P0	5905,22	-0,379
P60203	Myelin proteolipid protein	12631,25	-0,763
P07722	Myelin-associated glycoprotein	269,57	-0,811
Q63345	Myelin-oligodendrocyte glycoprotein	1352,59	-0,852
P02600	Myosin light chain 1/3, skeletal muscle isoform	1052,26	-0,32
P12847	Myosin-3	469,9	-0,278
Q29RW1	Myosin-4	706,99	-0,223
P02563	Myosin-6	474,11	-0,375
P02564	Myosin-7	530,9	-0,239
Q5RJQ4	NAD-dependent protein deacetylase sirtuin-2	438,7	-0,795
P13596	Neural cell adhesion molecule 1	101,02	-0,684
P16884	Neurofilament heavy polypeptide	1274,82	-0,787
P19527	Neurofilament light polypeptide	8158,63	-0,719
P12839	Neurofilament medium polypeptide	4331,87	-0,748
Q05982	Nucleoside diphosphate kinase A	1392,81	-0,763
P19804	Nucleoside diphosphate kinase B	1383,43	-0,763
P10111	Peptidyl-prolyl cis-trans isomerase A	1837,49	-0,748
Q63716	Peroxiredoxin-1	155,39	-0,719
P35704	Peroxiredoxin-2	789,55	-0,861
Q9R063	Peroxiredoxin-5, mitochondrial	871,47	-0,763
P31044	Phosphatidylethanolamine-binding protein 1	3269,54	-0,914
P16617	Phosphoglycerate kinase 1	481,94	-0,803
P25113	Phosphoglycerate mutase 1	1022,66	-0,844
P16290	Phosphoglycerate mutase 2	61,82	-0,436
P09626	Potassium-transporting ATPase alpha chain 1	195,88	-0,827
P54708	Potassium-transporting ATPase alpha chain 2	470,99	-0,852
P04631	Protein S100-B	4247,96	-0,691
P52873	Pyruvate carboxylase, mitochondrial	80,29	-0,589
P49432	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	344,7	-0,756
P12928	Pyruvate kinase PKLR	924,18	-0,835
P11980	Pyruvate kinase PKM	3560,94	-0,763
P50398	Rab GDP dissociation inhibitor alpha	756,66	-0,748
P50399	Rab GDP dissociation inhibitor beta	457,15	-0,741
Q5XI73	Rho GDP-dissociation inhibitor 1	1040,02	-0,771
P02770	Serum albumin	1851,79	-0,705
P06685	Sodium/potassium-transporting ATPase subunit alpha-1	1313,36	-0,914
P06686	Sodium/potassium-transporting ATPase subunit alpha-2	1605,96	-0,878
P06687	Sodium/potassium-transporting ATPase subunit alpha-3	2061,77	-0,869
Q64541	Sodium/potassium-transporting ATPase subunit alpha-4	523,87	-0,861
P07340	Sodium/potassium-transporting ATPase subunit beta-1	2943,18	-0,844
P16086	Spectrin alpha chain, non-erythrocytic 1	50,8	-0,741
P61765	Spectrin alpha chain, non-erythrocytic 1	660,04	-0,869
P07895	Superoxide dismutase [Mn], mitochondrial	231,54	-0,835

P46462	Transitional endoplasmic reticulum ATPase	145,12	-0,554
P50137	Transketolase	84,77	-0,787
P48500	Triosephosphate isomerase	3752,73	-0,733
F1M0Z1	Triple functional domain protein	31,85	1,2461
P68370	Tubulin alpha-1A chain	12481,32	-0,787
Q6P9V9	Tubulin alpha-1B chain	14403,96	-0,787
Q6AYZ1	Tubulin alpha-1C chain	10696,78	-0,763
Q68FR8	Tubulin alpha-3 chain	9254,94	-0,803
Q5XIF6	Tubulin alpha-4A chain	11408,91	-0,803
Q6AY56	Tubulin alpha-8 chain	6486,25	-0,779
P85108	Tubulin beta-2A chain	18476,78	-0,733
Q3KRE8	Tubulin beta-2B chain	18323,48	-0,733
Q4QRB4	Tubulin beta-3 chain	12206,53	-0,726
Q6P9T8	Tubulin beta-4B chain	15698,49	-0,726
P69897	Tubulin beta-5 chain	16882,94	-0,719
Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1	4105,59	-0,771
Q9QUL6	Vesicle-fusing ATPase	131,56	-0,819
P31000	Vimentin	471,74	-0,719
P26772	10 kDa heat shock protein, mitochondrial	866,48	Controle
Q5XI78	2-oxoglutarate dehydrogenase, mitochondrial	243,38	Controle
Q794F9	4F2 cell-surface antigen heavy chain	113,28	Controle
O35552	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3	55,4	Controle
Q5XI22	Acetyl-CoA acetyltransferase, cytosolic	178,19	Controle
P39069	Adenylate kinase isoenzyme 1	270,97	Controle
O88923	Adhesion G protein-coupled receptor L2	35,2	Controle
P00330	Alcohol dehydrogenase 1	5383,04	Controle
P06238	Alpha-2-macroglobulin	32,96	Tratado
Q5BJT1	Ankyrin repeat domain-containing protein 34A	113,53	Controle
P14668	Annexin A5	138,3	Controle
P31399	ATP synthase subunit d, mitochondrial	266,86	Controle
P47860	ATP-dependent 6-phosphofructokinase, platelet type	168,58	Controle
Q63754	Beta-synuclein	399,82	Tratado
O35112	CD166 antigen	54,51	Controle
Q62745	CD81 antigen	208,8	Controle
P01026	Complement C3	34,93	Controle
Q63198	Contactin-1	120,22	Controle
P97528	Contactin-6	28,68	Controle
Q0V8T3	Contactin-associated protein like 5-4	44,94	Tratado
O89046	Coronin-1B	50,37	Tratado
P09605	Creatine kinase S-type, mitochondrial	73,1	Controle
P32551	Cytochrome b-c1 complex subunit 2, mitochondrial	64,73	Controle
P20814	Cytochrome P450 2C13, male-specific	42,43	Controle
P38650	Cytoplasmic dynein 1 heavy chain 1	29,32	Controle
P39052	Dynamin-2	34,19	Tratado

Q08877	Dynamin-3	34,19	Tratado
F1LP64	E3 ubiquitin-protein ligase TRIP12	31,62	Controle
Q6P6T4	Echinoderm microtubule-associated protein-like 2	85,71	Controle
Q6ED65	Echinoderm microtubule-associated protein-like 5	31,37	Controle
Q811U3	ELKS/Rab6-interacting/CAST family member 1	39,42	Controle
Q76MT4	ESF1 homolog	33,93	Tratado
P85845	Fascin	85,38	Controle
Q5MJ12	F-box/LRR-repeat protein 16	49,39	Tratado
O35077	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	111,48	Tratado
P05708	Hexokinase-1	49,83	Controle
P62959	Histidine triad nucleotide-binding protein 1	1009,92	Controle
P97779	Hyaluronan-mediated motility receptor	48,03	Controle
P20760	Ig gamma-2A chain C region	84,96	Tratado
P20761	Ig gamma-2B chain C region	314,06	Controle
P15693	Intestinal-type alkaline phosphatase 1	73,98	Controle
Q99NA5	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	221,3	Controle
Q68FX0	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	117,92	Controle
P41565	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	99,62	Controle
Q4FZU2	Keratin, type II cytoskeletal 6A	131,75	Tratado
Q2PQA9	Kinesin-1 heavy chain	42,46	Controle
Q5M9H1	Leucine-rich repeat-containing protein 41	45,27	Controle
Q62813	Limbic system-associated membrane protein	119,67	Controle
D3ZEN0	MICAL-like protein 2	44,47	Tratado
F1LRL9	Microtubule-associated protein 1B	15,7	Tratado
P15205	Microtubule-associated protein 1B	20,22	Tratado
Q8CG09	Multidrug resistance-associated protein 1	33,31	Controle
Q6IE52	Murinoglobulin-2	51,7	Controle
P16409	Myosin light chain 3	59,91	Controle
Q64119	Myosin light polypeptide 6	61,16	Controle
P04466	Myosin regulatory light chain 2, skeletal muscle isoform	966,3	Controle
P19234	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	194,2	Controle
Q641Y2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	47,26	Controle
O35095	Neurochondrin	55,38	Controle
Q9JIL9	Nibrin	61,24	Controle
Q03351	NT-3 growth factor receptor	24,81	Tratado
Q6AYD9	Nucleoside diphosphate-linked moiety X motif 19	51,08	Tratado
Q63425	Periaxin	92,48	Controle
P21807	Peripherin	96,75	Controle
Q9Z0V5	Peroxiredoxin-4	79,67	Controle
Q63448	Peroxisomal acyl-coenzyme A oxidase 3	73,34	Controle
P16036	Phosphate carrier protein, mitochondrial	216,19	Controle
Q5U309	Probable inactive glycosyltransferase 25 family member 3	98,92	Controle
P67779	Prohibitin	80,84	Controle
P05943	Protein S100-A10	245,46	Controle

P26284	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	112,57	Controle
Q9ES67	Rho guanine nucleotide exchange factor 11	27,8	Tratado
P02769	Serum albumin	62,6	Controle
Q6AYB5	Signal recognition particle 54 kDa protein	50,07	Tratado
Q63553	SNF-related serine/threonine-protein kinase	36,82	Tratado
P13638	Sodium/potassium-transporting ATPase subunit beta-2	117,28	Controle
Q8K4Y7	Soluble calcium-activated nucleotidase 1	56,6	Controle
P09951	Synapsin-1	43,76	Tratado
Q3MIE4	Synaptic vesicle membrane protein VAT-1 homolog	214,19	Controle
Q5XIM9	T-complex protein 1 subunit beta	58,32	Controle
Q05546	Tenascin-R	30,06	Controle
P63029	Translationally-controlled tumor protein	149,89	Tratado
Q64428	Trifunctional enzyme subunit alpha, mitochondrial	75,6	Controle
Q7TNK6	tRNA (guanine(10)-N2)-methyltransferase homolog	81,35	Tratado
Q6P7B0	Tryptophan--tRNA ligase, cytoplasmic	156,9	Controle
F1LNJ2	U5 small nuclear ribonucleoprotein 200 kDa helicase	30,06	Controle
Q63357	Unconventional myosin-Id	45,9	Tratado
P62483	Voltage-gated potassium channel subunit beta-2	185,05	Controle

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ANEXO 1 – APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS

PARECER BIO139-13

Projeto: Avaliação das alterações em glândulas salivares e sistema nervoso de ratos intoxicados **cronicamente com cloreto de mercúrio e metilmercúrio: análise motora, tecidual e de** marcadores do estresse oxidativo

Coordenador: Prof. Dr. Rafael Rodrigues Lima

Área Temática: Neurociências

Vigência: 06/2013 a 07/2015

Nº no CEPAE-UFPA: 139-13

O projeto acima identificado foi avaliado pelo Comitê de Ética Em Pesquisa Com Animais de Experimentação da Universidade Federal do Pará (CEPAE). O tema eleito para a investigação e de alto teor científico justificando a utilização do modelo animal proposto. Os procedimentos experimentais utilizados seguem as normas locais e internacionais para tratamento e manipulação de animais de experimentação. Portanto, o CEPAE, através de seu presidente, no uso das atribuições delegadas pela portaria Nº 3988/2011 do Reitor da Universidade Federal do Pará, resolve **APROVAR** a utilização de animais de experimentação nas atividades do projeto em questão, no período de vigência estabelecido. As atividades experimentais fora do período de vigência devem receber nova autorização deste comitê.

Belém, 21 de junho de 2013

Prof. Dr. Walace Gomes Leal
Presidente do CEPAE-UFPA

CHEMOSPHERE

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DESCRIPTION

Chemosphere is an international journal designed for the publication of original communications and review articles. As a multidisciplinary journal, Chemosphere offers broad and impactful dissemination of investigations related to all aspects of environmental science and engineering.

Chemosphere will publish:

- Original communications (research papers) describing important new discoveries or further developments in important fields of investigation related to the environment and human health
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- Discussion papers
- Letters to the editor
- Short communications
- Special themed issues on relevant topics.

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Specific topics of interest include:

- Emerging contaminants, such as pharmaceuticals, pesticides, flame retardants, other industrial chemicals, POPs, endocrine disruptors, etc.
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- Transformation and mineralisation ofchemicals, e.g. bybio- and photodegradation, redoxprocesses and hydrolysis
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- Effects of nanoparticles, nanocomposites and microplastics in the environment
- Endocrine disruption
- High-throughput screening
- Mechanistic toxicology
- Fish toxicology
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- Phytotoxicity

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This section focuses on technologies that manage and/or reduce environmental contaminants, including reuse and recycling processes. The technology must be beyond a basic laboratory study or have obvious implications for current or potential treatment or remediation technologies. As an example, manuscripts focusing on fundamental (bio)adsorption studies or metal extraction by plant species should be submitted to a more suitable journal. The results of studies of a routine nature should not be submitted for review. For example, for oxidation processes, the intermediates and/or the extent of mineralization of the targeted compound(s) and wastes must be quantified in addition to target compound attenuation.

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- Hazardous waste industrial chemicals
- Hydraulic fracturing and produced water
- Electrochemical methods for water and solids treatment
- Nanotechnology
- Advanced oxidation processes
- Photolysis and photocatalysis
- Natural treatment systems (riverbank filtration, aquifer recharge and recovery)
- Characterization and fate of natural and effluent organic matter

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AUDIENCE

Environmental scientists, chemical engineers, biologists, toxicologists.

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