UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EXPOSIÇÃO AO METILMERCÚRIO E PALMITATO DE RETINOL DESENCADEIA DISTÚRBIOS HEPÁTICOS E NEUROBIOLÓGICOS EM DIFERENTES ETAPAS DO DESENVOLVIMENTO DE RATOS WISTAR

Helen Tais da Rosa Silva

Porto Alegre, 2020

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Exposição ao metilmercúrio e palmitato de retinol desencadeia distúrbios hepáticos e neurobiológicos em diferentes etapas do desenvolvimento de ratos Wistar

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"A ciência, meu rapaz, é feita de erros, mas de erros benéficos, já que conduzem pouco a pouco à verdade."

Julio Verne – Viagem ao centro da Terra

"Venha, meu coração está com pressa
Quando a esperança está dispersa
Só a verdade me liberta
Chega de maldade e ilusão
Venha, o amor tem sempre a porta aberta
E vem chegando a primavera
Nosso futuro recomeça
Venha, que o que vem é perfeição"

Legião Urbana - Perfeição

Sumário

Resumo

O metilmercúrio (MeHg) é um derivado orgânico biocumulativo do mercúrio (Hg) que afeta o meio ambiente e representa um grave problema de saúde pública, principalmente para comunidades ribeirinhas da América do Sul. Já a vitamina A (VitA) é um nutriente essencial utilizado mundialmente na dieta normal, alimentos processados e suplementos. O MeHg é um agente neurotóxico e sua toxicidade desencadeia alterações sistêmicas e no sistema nervoso central em diferentes etapas do desenvolvimento. Nesse trabalho, foi utilizado o modelo *in vivo* a fim de compreender como a exposição ao MeHg e a VitA no período fetal e amamentação poderia afetar os efeitos de uma re-exposição ao MeHg na fase adulta dos mesmos animais. No artigo 1 desta tese foi investigado os efeitos hepáticos e neurobiológicos da exposição fetal/amamentação/fase adulta ao MeHg em ratos Wistar. No artigo 2, foi investigado parâmetros de estresse oxidativo e suscetibilidade a danos no DNA de ratos Wistar expostos ao MeHg e VitA em diferentes etapas do desenvolvimento, do feto a fase adulta. Nossos resultados mostraram que ambos os grupos tratados com MeHg apresentaram perda da memória de longo prazo, reatividade emocional e diminuição da percepção visuo-espacial. A exposição ao MeHg em diferentes períodos de exposição induz diminuição do consumo de ração padrão e consequente perda de peso. A pré-exposição fetal e durante a amamentação afeta o *clearence* de Hg no fígado, consequente acúmulo de glicogênio e hepatoxicidade. Tanto a exposição simples como dupla ao MeHg causam dano a biomoléculas via formação de ERO e não através do desequilíbrio redox nos tecidos avaliados, e a exposição fetal a VitA e VitA+MeHg reduziu a lipoperoxidação. Além disso, os danos ao DNA causados pela exposição ao MeHg em ambos períodos são irreversíveis e com potencial mutagênico. No entanto, os grupos VitA e VitA+MeHg apresentaram menor sensibilidade a danos ao DNA. MeHg modulou a via AKT / GSK3β / mTOR e a forma fosforilada da proteína Tau. Foi observado redução no imunoconteúdo de NeuN e GFAP e aumento do imunoconteúdo de Iba1 e RAGE, sugerindo um processo de neurodegeneração. Em conclusão, a exposição ao MeHg em diferentes fases da vida desencadeia efeitos crônicos no fígado e sistema nervoso central e a exposição recorrente é um agravante para desenvolvimento de patologias irreversíveis como processos neurodegenerativos. O palmitato de retinol demonstrou ter efeitos benéficos nos casos de intoxicação de MeHg a doses baixas, especialmente no sistema nervoso central. O mecanismo bioquímico dos metabolitos ativos do palmitato de retinol e sua relação com MeHg precisam ser esclarecidos em estudos futuros. Correlação com dados epidemiológicos também precisa ser investigada. De acordo com nossos resultados deve haver uma melhor e maior orientação sobre hábitos alimentares nas populações materna e infantil.

Palavras-chave: Metilmercúrio, Vitamina A, estresse oxidativo, hepatoxicidade, neurotoxicidade, neurodesenvolvimento.

Abstract
Methylmercury (MeHg) is a biocumulative organic derivative of mercury (Hg) that affects the environment and represents a serious public health problem, especially for riverside communities in South America. Vitamin A (VitA) is an essential nutrient used worldwide in the normal diet, processed foods and supplements. MeHg is a neurotoxic agent and its toxicity triggers systemic and central nervous system changes at different stages of development. In this work, the in vivo model was used in order to understand how exposure to MeHg and VitA in the fetal and breastfeeding period could affect the effects of a re-exposure to MeHg in the adult phase of the same animals. In article 1 of this thesis, the hepatic and neurobiological effects of fetal exposure / breastfeeding / adult phase to MeHg in Wistar rats were investigated. In Article 2, parameters of oxidative stress and susceptibility to DNA damage in Wistar rats exposed to MeHg and VitA in different were investigated. developmental stages, from fetus to adulthood. Our results showed that both groups treated with MeHg showed loss of long-term memory, emotional reactivity and decreased visual-spatial perception. Exposure to MeHg in different periods of exposure induces a decrease in the consumption of standard feed and consequent weight loss. Fetal and breastfeeding pre-exposure affects Hg clearance in the liver, resulting in accumulation of glycogen and hepatoxicity. Both single and double exposure to MeHg cause damage to biomolecules via ROS formation and not through redox imbalance in the evaluated tissues, and fetal exposure to VitA and VitA + MeHg reduced lipoperoxidation. In addition, DNA damage caused by exposure to MeHg in both periods is irreversible and has a mutagenic potential. However, the VitA and VitA + MeHg groups were less sensitive to DNA damage. MeHg modulated the AKT / GSK3β / mTOR pathway and the phosphorylated form of the Tau protein. A reduction in the immunocontent of NeuN and GFAP and an increase in the immunocontent of Iba1 and RAGE were observed, suggesting a process of neurodegeneration. In conclusion, exposure to MeHg at different stages of life triggers chronic effects on the liver and central nervous system and recurrent exposure is an aggravating factor for the development of irreversible pathologies such as neurodegenerative processes. Retinol palmitate has been shown to have beneficial effects in cases of low dose doses of MeHg, especially in the central nervous system. The biochemical mechanism of the active metabolites of retinol palmitate and their relationship with MeHg need to be clarified in future studies. Correlation with epidemiological data also needs to be investigated. According to our results, there should be better and greater guidance on eating habits in the maternal and child populations.

Keywords: Methylmercury, Vitamin A, oxidative stress, hepatoxicity, neurotoxicity, neurodevelopmental.

1. INTRODUÇÃO

1.1 Mercúrio e metil mercúrio

O mercúrio (Hg) é um elemento natural presente em todos os ambientes e considerado um dos metais mais reativos. A liberação ambiental do Hg na atmosfera ocorre através de fenômenos naturais e por atividades antropogênicas. De fato, quando as emissões antropogênicas de Hg chegam no ambiente aquático contribuem com grande parte do Hg circulante no ambiente (UNEP, 2013).

A diversidade de efeitos provocados pelo Hg se deve à variedade de formas que é apresentado, que pode ser na forma elementar, de compostos orgânicos ou inorgânicos. Em sua forma orgânica mais comum, o íon Hg apresenta-se ligado covalentemente a um radical orgânico, sendo o metilmercúrio (MeHg) (CH_3Hg^+) e o dimetilmercúrio ($(CH_3)_2Hg$) (CASTRO; LIMA, 2014a; WHO, 2016). Ambos são utilizados principalmente para a extração de ouro, o que leva à contaminação de mineradores e ribeirinhos por Hg metálico e da população em geral por MeHg, que é possui efeitos neurotóxicos (BARCELOS *et al.*, 2011a; BISEN-HERSH *et al.*, 2014; OLIVEIRA *et al.*, 2015; PEIXOTO *et al.*, 2003). Do grupo dos organometálicos de cadeias curtas, o MeHg é a forma mais tóxica e é responsável por efeitos nocivos para a saúde humana, incluindo doenças cardiovasculares, anemia, anormalidades do desenvolvimento, distúrbios neurocomportamentais, danos aos rins e fígado e câncer em alguns casos (SUTTON *et al.*, 2002).

Entre os episódios de exposição ambiental ao MeHg mais conhecidos estão as catástrofes ambientais que ocorreram em Minamata (1950s) e Niigata (1960s) no Japão, após a liberação no mar de sulfato de mercúrio (II), empregado como catalizador na obtenção de acetaldeído e posterior ingestão acidental do MeHg na população através de peixes contaminados (ETO; MARUMOTO; TAKEYA, 2010a, 2010b; KONDO, 1996, 2000). No entanto, apesar da natureza imprevisível dos eventos anteriores, existem atividades antropogênicas de impacto constante na contaminação do MeHg. Um processo altamente contaminante é a mineração de aluvião, que usa Hg na formação de amálgamas para a extração de ouro, e permitindo um aumento na biodisponibilidade de MeHg nos sistemas aquáticos (OLIVERO; JOHNSON; ARGUELLO, 2002; OLIVERO-VERBEL *et al.*, 2004). Além das atividades anteriores, a queima de carvão e a mineração de ouro artesanal contribuem com aproximadamente 62% das emissões anuais totais de Hg no ar, sendo América do Sul e África Subsaariana responsáveis pelo aumento dessas emissões globais (UNEP, 2013).

Atualmente, as populações mais afetadas pela contaminação por Hg são os povos indígenas da Amazônia Brasileira e Colombiana, onde a principal fonte de proteínas é o consumo de peixe (CRESPO-LÓPEZ *et al.*, 2009, 2011; GROTTO *et al.*, 2010). Na região de Mojana, no noroeste da Colômbia, onde níveis de Hg encontram-se acima do limite estabelecido pela *U.S. Environmental Protection Agency* (USEPA) e existe relação direta com a crescente atividade mineradora nos municípios de Caimito (Sucre) (OLIVERO; JOHNSON; ARGUELLO, 2002;

OLIVERO-VERBEL *et al.*, 2004), Ayapel (Córdoba) (GRACIA H.; MARRUGO N.; ALVIS R., 2010; MARRUGO; LANS; BENÍTEZ, 2007) e sul do departamento de Bolívar (MARRUGO-NEGRETE *et al.*, 2008; MARRUGO-NEGRETE; BENITEZ; OLIVERO-VERBEL, 2008; OLIVERO-VERBEL; YOUNG-CASTRO; CABALLERO-GALLARDO, 2014). Apesar da exposição clara ao MeHg dessas populações, pouco se sabe sobre possíveis efeitos tóxicos dessa exposição a longo prazo (CASTRO; LIMA, 2014b; GROTTO *et al.*, 2011; PINHEIRO *et al.*, 2008).

O MeHg é altamente persistente no ambiente devido a estabilidade da ligação carbono-Hg frente a ligação Hg-oxigênio quando o composto se encontra na água. Uma vez nos sistemas aquáticos, o MeHg é bioacumulado e biomagnificado através da cadeia alimentar, sendo as espécies carnívoras de peixe, aquelas que apresentam as maiores concentrações (OLIVERO; JOHNSON; ARGUELLO, 2002; OLIVERO-VERBEL *et al.*, 2004). O MeHg inicia-se na cadeia alimentar através da difusão e forte ligação com as proteínas da biota aquática. Isso ocorre porque organismos aquáticos metilam o Hg elementar presente na água, solo ou ar. Uma vez formado, o contaminante atinge sua concentração máxima em tecidos de peixes (LEE *et al.*, 2014; WHO, 2016). Nesses sistemas os íons de Hg são sempre encontrados ligados a moléculas como glutationa (GSH) e cisteina (Cys), devido à alta afinidade do Hg pelas biomoléculas contendo grupos tióis (BRIDGES; ZALUPS, 2005). Uma vez encontrado em nosso organismo, ao redor de 90% de todo o MeHg é absorvido no trato gastrointestinal (OLIVERO-VERBEL *et al.*, 2004).

Na célula, a alta estabilidade do enlace carbono-mercúrio no MeHg e a lipossolubilidade elevada do grupo alquil, favorece a penetração através das membranas celulares, produzindo um aumento das ligações covalentes do MeHg com o enxofre (OLIVERO-VERBEL *et al.*, 2004), especificamente com os grupos sulfidrilos (-SH) das proteínas (OGURA; TAKEUCHI; MORIMOTO, 1996). Atualmente é conhecido que a mesma associação do Hg com os grupos tióis favorece a rápida difusão transmembrana do MeHg e sua mobilização em certos tecidos, sendo sua rota de transporte nas células o sistema L de transporte de aminoácidos neutros através da formação de um complexo com L-cisteína (ASCHNER; ASCHNER, 2007; ASCHNER; CLARKSON, 1988) Na forma de complexo, o MeHg pode cruzar as barreiras hemato-encefálica (BH) e placentária (BP) como um imitador molecular da metionina (BRIDGES; ZALUPS, 2005). Uma vez nas células ocorre um processo de modificação das proteínas pela formação de ligações covalentes com MeHg, conhecido como “S-mercuriação”, e é possível que seja responsável ao menos em parte, pelos efeitos tóxicos do MeHg nas células (KANDA; SHINKAI; KUMAGAI, 2014).

Após difusão pela placenta, o MeHg pode ser armazenado no cérebro do feto em concentrações maiores que os níveis maternos. Em média, os níveis desse metal no cérebro fetal são de 5 – 7 vezes maior que no sangue materno. A meia-vida do MeHg varia de tecido para tecido, mas em média é de 45 – 70 dias (CLARKSON, 2002). Da concentração total de MeHg, cerca de 5% é encontrado na circulação sanguínea, 10% no cérebro e 20% é excretado através

das fezes, uma grande porcentagem é armazenada no fígado e tecido adiposo. Menos de 10% do MeHg é eliminado, o restante retorna através da circulação ou é desmetilado pela microbiota do intestino e sistema imunológico (DANIELSSON, 1984; YOSHIDA, 2002).

A exposição aguda ao MeHg provoca falha na coordenação motora, parestesia, ataxia, surdez, tremor muscular, constrição do campo visual, coma e até morte. Já a intoxicação crônica leva a distúrbio da sensibilidade nas extremidades, disartria, surdez, constrição do campo visual, fraqueza muscular, irritabilidade, falta de memória, ansiedade, depressão, distúrbios de coordenação e equilíbrio e sinais motores que simula esclerose lateral amiotrófica e também parece aumentar o risco para doença cardiovascular (BISEN-HERSH *et al.*, 2014; CLARKSON, 2002; GROTTO *et al.*, 2009a, 2009b).

Além da toxicidade gerada no cérebro, é interessante observar os danos gerados em outros órgãos como o fígado. Alguns autores já demonstraram que a exposição à diferentes metais pesados provocam danos ao tecido hepático sendo o fígado um órgão alvo importante para a toxicidade do MeHg (GROTTO *et al.*, 2009b). Nas células hepáticas, o MeHg forma complexos solúveis com a glutationa e a cisteína, que são excretados na bile e reabsorvidos no trato gastrointestinal, iniciando o processo de peroxidação lipídica, que pode produzir alterações nas membranas celulares e induzir a abertura do poro de transição da permeabilidade nas mitocôndrias do fígado de ratos, esse mecanismo molecular é um dos responsáveis pela morte celular (CLARKSON, 1997; CLARKSON; MAGOS; MYERS, 2003; DE OLIVEIRA; MOREIRA, 2007; GROTTO *et al.*, 2009b).

Diversos trabalhos já demonstraram a vulnerabilidade de organismos a uma variedade de insultos aplicados em idade adulta (MIZOGUCHI; YUZURIHARA; ISHIGE, 2002; SUJATHA *et al.*, 1999), ou em uma fase inicial da vida ((POGGIOLI, 2001; RAJASEKARAN, 2000; ROEGGE *et al.*, 2011). Insultos em um período inicial da vida, como a privação nutricional (PEIXOTO *et al.*, 2003, 2007; VENDITE; WOFCHUK; SOUZA, 1985), exposição a metais (PEIXOTO *et al.*, 2003; RAJASEKARAN, 2000) ou compostos orgânicos (RAJASEKARAN, 2000; ROZA *et al.*, 2005), causam déficits que podem ser irreversíveis, incluindo atividade de enzimas (RAJASEKARAN, 2000; SHARMA; SINGH; SIDDIQI, 2014; SHARMA; KUMAR; KUMAR, 2005) e alterações no comportamento (PALETZ *et al.*, 2007; PEIXOTO *et al.*, 2007; POGGIOLI, 2001; RAJASEKARAN, 2000). No entanto, estudos com exposição em períodos de vida pós-natal são escassos (PEIXOTO *et al.*, 2003, 2007). Além disso, existem evidências de que pré-exposições à um determinado composto pode modular os efeitos sobre uma nova exposição (JAISHANKAR *et al.*, 2014). Recentemente, foram encontrados mecanismos celulares e moleculares adaptativos a doses subletais de agentes oxidantes, induzindo vias de sinalização que aumentam a longevidade. GROTTO *et al.*, (2011) mostrou que ratos pré-tratados com óleo de peixe e depois com MeHg, apresentavam menor dano ao DNA do que animais sem o pré-tratamento. Mesmo assim, ainda

não há estudos suficientes esclarecendo os efeitos do mercúrio em estágios pós-natais específicos ou verificando a sensibilidades da exposição em adulto quando houve exposição fetal ou durante a amamentação.

1.2 Metil mercúrio e exposição ambiental

Após o desastre de Minamata na década de 50, houve maior atenção das agências de saúde internacionais para essa problemática. Outro caso de exposição grave ao MeHg ocorreu na zona rural do Iraque em 1970, onde agricultores e seus familiares ingeriram pão feito a partir de semente de trigo contaminado com o metal. Depois disso, examinando os cérebros das crianças afetadas, revelou-se que uma das principais alterações patológicas foi a ruptura da citoarquitetura do cérebro. Além disso, diversos neurônios ectópicos foram localizados na substância cerebral branca e agrupamentos irregulares de neurônios corticais em muitas regiões, inferindo que havia migração de neurônios corticais (CASTOLDI; COCCINI; MANZO, 2003).

Como dito anteriormente, o Hg em ambas formas químicas (orgânica e inorgânica) é neurotóxico e sua distribuição ocorre de maneira ubíqua, penetrando no SNC. Assim como o Hg, o MeHg no SNC, pode se difundir em todas as regiões cerebrais, cruzando a BH (ASCHNER; CLARKSON, 1988; ZAREBA *et al.*, 2007). No cérebro em desenvolvimento, o MeHg afeta o córtex cerebral e o cerebelo, causando degeneração de neurônios e células gliais. De acordo com CASTOLDI; COCCINI; MANZO, (2003) e LAPHAM *et al.*, (1994) a vulnerabilidade do cérebro em desenvolvimento ao MeHg ocorre porque a barreira hemato-encefálica (BH) não está totalmente desenvolvida (CASTOLDI; COCCINI; MANZO, 2003; LAPHAM *et al.*, 1994). Em humanos, os estágios de desenvolvimento do cérebro ocorrem da vida pré-natal até os 4 anos de idade (PEIXOTO *et al.*, 2003), nesse período, lesões no hipocampo e cerebelo são mais intensas, causando morte neuronal. Já em ratos, o neurodesenvolvimento ocorre de maneira mais intensa no período fetal e pós-natal, e a maturação acompanha a fase de desmame. A exposição fetal e na lactação ao MeHg causa ruptura de das vias de sinalização de crescimento celular em regiões do cerebelo e córtex e consequente disfunção cerebral (HEIMFARTH *et al.*, 2018a, 2018d).

A intoxicação ao MeHg danifica as chamadas zonas primárias do córtex cerebral de ratos, sendo o campo visual, vias auditivas, sensoriais somáticas e córtex motor as áreas mais afetadas. Vários mecanismos têm sido propostos para explicar os efeitos neurotóxicos do MeHg, incluindo a indução de estresse oxidativo, dano mitocondrial, inibição da captação de glutamato, perturbação da homeostase de Ca^{+2}, interrupção da função neurotransmissora, inibição dos receptores do ácido gama-aminobutírico (GABA) e apoptose/necrose (DO NASCIMENTO *et al.*, 2008; FARINA; ASCHNER; ROCHA, 2011a; MORETTO *et al.*, 2005; NABI, *[s. d.]*; NABI; TANVEER; GANIE, 2017; SAKAMOTO *et al.*, 1998; SHANKER; SYVERSEN; ASCHNER, 2003). Em relação a toxicidade da exposição pré-natal a MeHg, experimentos *in vitro* e *in vivo* demostraram que a exposição *in utero* ao MeHg tem influência sobre importantes parâmetros de

toxicidade (CECCATELLI; ASCHNER, 2012; FOX *et al.*, 2012; GLOVER *et al.*, 2009; HUANG *et al.*, 2008; LIANG *et al.*, 2009; YOSHIDA, 2002) De fato, doses subministradas do contaminante durante esta etapa do desenvolvimento possuem alta persistência, provocando quadros de desordens neurodegenerativas a longo prazo (CECCATELLI; ASCHNER, 2012). A fase prematura de neurodesenvolvimento é particularmente susceptível a eventos nutricionais, tóxicos e estimuladores que possam interferir com a programação neural do SNC (GALE *et al.*, 2004, 2006). Apesar de a exposição ao MeHg em crianças durante a lactação ser menor do que imediatamente depois do nascimento (K.A. *et al.*, 2003; SAKAMOTO *et al.*, 2012), estudos sobre populações amazônicas demostraram que o leite materno é uma considerável fonte de exposição pós-natal ao MeHg e ao Hg inorgânico em bebês lactantes (PINHEIRO *et al.*, 2008). Além disso, existe correlação entre menor desenvolvimento neural e o consumo do leite materno a longo prazo (JENSEN *et al.*, 2005; MARQUES *et al.*, 2013, 2016).

Por outro lado, outros autores encontraram efeitos benéficos da amamentação, sobre crianças na Amazônia Brasileira expostas ao MeHg através da dieta (MARQUES *et al.*, 2016). Apesar da controvérsia, estudos prévios com modelo animal para avaliação do efeito da lactação, mostraram que o MeHg inibe a absorção do glutamato no tecido cerebelar e tem relação com a formação de hidroperóxidos (MANFROI *et al.*, 2004), ocasionando danos na função motora nos ratos lactentes por possível diminuição dos níveis basais de tióis cerebelares (FRANCO *et al.*, 2007).

1.3 Metil mercúrio, vitamina A e estresse oxidativo

As espécies reativas de oxigênio (EROs) são espécies químicas formadas pela redução parcial do oxigênio molecular como resultado do metabolismo celular normal, e podem ser divididos em radicais livres (RL) e não radicais (BIRBEN *et al.*, 2012; RAY; HUANG; TSUJI, 2012). Os RL são qualquer espécie química (átomo, molécula, metal de transição) de existência independente, que contém um ou mais elétrons desaparelhados. Quando dois RL compartem seus elétrons desaparelhados são formados os não radicais (BIRBEN *et al.*, 2012).

A presença de um ou vários elétrons desaparelhados permite que o RL seja altamente reativo, mas a reatividade química varia amplamente entre radicais (HALLIWELL; GUTTERIDGE, 2015). De fato, uma das características biológicas mais interessantes dos RL é a capacidade de sustentar reações em cadeia com um não-radical (a maioria das moléculas biológicas) mediante a remoção de elétrons desse último, e produzindo novos RLs de maneira sucessiva (HALLIWELL; GUTTERIDGE, 2015).

As defesas antioxidantes das células podem ser de dois tipos: enzimáticas e não-enzimáticas. Entre as defesas enzimáticas temos as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa-S-transferase (GST). No caso das defesas não-enzimáticas podemos incluir compostos de baixo peso molecular como as vitaminas

(vitaminas C e E), o β-caroteno, o ácido úrico e a glutationa reduzida ou GSH, um tripeptídio (L-γ-glutamil-L-cisteinil-L-glicina) que possui um grupo tiol (sulfidrila) (Birben et al., 2012).

Entre as defesas antioxidantes enzimáticas, as famílias das SODs (CuZnSOD, MnSOD, FeSOD e NiSOD) catalisam a conversão do radical ânion superóxido ($O^{2\bullet-}$) em peroxido de hidrogênio (HALLIWELL; GUTTERIDGE, 2015) . A CAT reduz o peroxido de hidrogênio a água. A GPx reduz os hidroperóxidos de lipídios a seus correspondentes álcoois. Por último, as GSTs inativam metabólitos secundários como os aldeídos insaturados, epóxidos e hidroperóxidos (BIRBEN *et al.*, 2012).

O termo estresse oxidativo denota o desequilíbrio entre a formação de espécies oxidantes (espécies reativas) e ação das defesas antioxidantes, em favor dos oxidantes (BIRBEN *et al.*, 2012) O processo leva a um dano potencial nas células conhecido como dano oxidativo que é definido como o dano biomolecular causado pelo ataque das espécies reativas sobre os constituintes dos organismos vivos (HALLIWELL; GUTTERIDGE, 2015). As consequências do estresse oxidativo nas células afetadas pelo insulto podem incluir qualquer dos seguintes efeitos de forma singular ou em combinação: proliferação incrementada, adaptação ao dano (total, parcial ou sobre proteção), dano celular, senescência e morte celular (HALLIWELL; GUTTERIDGE, 2015). O estresse oxidativo contribui a muitas condições patológicas como o câncer, desordens neurológicos, aterosclerose, hipertensão, isquemia/perfusão, diabetes, desordens pulmonares e asma (BIRBEN *et al.*, 2012)

Certas patologias ou fatores podem ser grandes estressores para um indivíduo, especialmente durante o desenvolvimento intrauterino. Condições de desnutrição/nutrição materna, exposição aos corticoides e hipóxia pré-natal são estressores intrauterinos que podem iniciar o processo de programação fetal. O estresse oxidativo pode ser o vínculo entre o insulto intrauterino e programação no feto depois do parto, fenômeno respaldado pela evidência epidemiológica que relaciona marcadores de estresse oxidativo com certas patologias desenvolvidas posteriormente por crianças nascidos sob condições de crescimento intrauterino restrito. Além disso, estudos em modelo animal suportam o fato que o estresse oxidativo induz fenótipos programados na prole adulta (THOMPSON; AL-HASAN, 2012).

Existe evidência experimental de que o MeHg é responsável pelo estresse oxidativo a nível celular, atuando diretamente nas defesas antioxidantes. A alta afinidade do MeHg pelos grupos tióis (-SH) permite sua ligação com peptídeos pequenos como a glutationa reduzida (GSH) decrescendo seus níveis e incrementando a presença de EROs a nível celular. O MeHg afeta de igual forma as atividades de enzimas antioxidantes como a SOD e CAT, o que permite a indução de produção de ($O^{2\bullet-}$) ou H_2O_2, e promovendo o estresse oxidativo e peroxidação lipídica. Outro alvo molecular da citotoxicidade induzida pelo MeHg é a GPx, a qual representa uma das principais vias de defesa contra os efeitos pró-oxidantes do MeHg *in vitro* e *in vivo* (NABI, 2014).

Em termos da neurotoxicidade conhecida do MeHg, existe uma relação entre o dano sobre o tecido neuronal durante o desenvolvimento e uma diminuição na função mitocondrial e incremento do EROs. Estudos sobre sinaptosomas em estriados dos ratos expostos ao MeHg demonstrou relação idade dependente com a produção de ERO, sendo mais frequente nas etapas pós-natais prematuras. Em relação a VitA, estudo sobre células granulares isoladas dos ratos tratados com MeHg mostrou níveis elevados do EROs e potencial de membrana diminuída em relação aos controles. Outro trabalho, encontrou aumento no dano oxidativo a proteínas, lipídeos em substância negra e estriado isolados de ratas tratadas com VitA. Além disso, o composto produz modulação das enzimas antioxidantes SOD, CAT, mas não da GPx (BELLUM *et al.*, 2007; OLIVEIRA *et al.*, 2015).

Recentemente, os efeitos tóxicos da suplementação do éster de retinol no cérebro têm sido investigados usando modelos experimentais, encontrando-se efeitos pró-oxidantes em diversas regiões do cérebro (hipocampo, estriado e córtex cerebral) (BEHR; SCHNORR; MOREIRA, 2012; GASPAROTTO *et al.*, 2015; SCHNORR *et al.*, 2015a). Apesar dos estudos sobre os efeitos do MeHg e da VitA, pouco se sabe sobre seus efeitos em conjunto. Além disso, trabalhos que investigam os efeitos, tanto do MeHg quanto da VitA, nas fases intrauterina e lactante e sua modulação frente a uma nova exposição, são desconhecidos pelo nosso grupo de pesquisa até o momento. Pensando nisso, nós investigamos a influência da exposição ao MeHg e da VitA na fase intrauterina e lactante sobre parâmetros bioquímicos e toxicológicos após uma nova exposição ao MeHg nos mesmos animais na fase adulta. No artigo 1, a seguir, nós focamos nos efeitos no fígado e no córtex occipital desse modelo de exposição somente ao MeHg. No artigo 2, utilizamos um modelo de exposição ao MeHg e a VitA e focamos nos efeitos genotóxicos do mesmo.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar parâmetros toxicológicos, neurocomportamentais e bioquímicos durante o período fetal e amamentação em ratos Wistar submetidos a suplementação materna de vitamina A e uma dose ambientalmente relevante do MeHg. Além disso, avaliar os efeitos moduladores dessa exposição em uma re-exposição, ao MeHg, na vida adulta.

2.2 Objetivos específicos

1) Avaliar parâmetros toxicológicos clássicos em fígado e córtex occipital de ratos Wistar submetidos a uma dose ambientalmente relevante do MeHg durante o período fetal e lactante sobre uma nova exposição na fase adulta ao MeHg.

2) Analisar parâmetros neurocomportamentais dos animais expostos a uma dose ambientalmente relevante do MeHg durante o desenvolvimento fetal e lactante, sobre uma nova exposição na fase adulta ao MeHg.

3) Avaliar parâmetros bioquímicos de estresse oxidativo em fígado e córtex occipital de ratos Wistar submetidos a suplementação da vitamina A e a uma dose ambientalmente relevante do MeHg durante o desenvolvimento fetal e lactante, sobre uma re-exposição na fase adulta ao MeHg.

4) Avaliar os danos ao DNA passíveis de reparo em células sanguíneas, e danos não reparáveis na medula óssea, de animais suplementados com vitamina A e a uma dose ambientalmente relevante do MeHg (durante o desenvolvimento fetal e lactante, sobre uma re-exposição na fase adulta ao MeHg.

3. RESULTADOS

3.1 *Hepatic and neurobiological effects of foetal and breastfeeding and adulthood exposure to methymercury in Wistar rats.*

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HEPATIC AND NEUROBIOLOGICAL EFFECTS OF FOETAL AND BREASTFEEDING AND ADULTHOOD EXPOSURE TO METHYLMERCURY IN WISTAR RATS

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Abstract

Methylmercury (MeHg) is an organic bioaccumulated mercury derivative that strongly affects the environment and represents a public health problem primarily to riparian communities in South America. Our objective was to investigate the hepatic and neurological effects of MeHg exposure during the phases foetal and breast-feeding and adult in Wistar rats. Wistar rats (n=10) were divided into 3 groups. Control group received mineral oil; The simple exposure (SE) group was exposed only in adulthood (0.5 mg/kg/day); and double exposure (DE) was pre-exposed to MeHg 0.5 mg/kg/day during pregnancy and breastfeeding (±40 days) and re-exposed to MeHg for 45 days from day 100. After, we evaluated possible abnormalities. behavioral and biochemical parameters in liver and occipital cortex (CO), markers of liver injury, redox and AKT/GSK3β/mTOR signaling pathway. Our results showed that both groups treated with MeHg presented significant alterations, such as decreased locomotion and exploration and impaired visuospatial perception. The rats exposed to MeHg showed severe liver damage and increased hepatic glycogen concentration. The MeHg groups showed significant impairment in redox balance and oxidative damage to liver macromolecules and CO. MeHg upregulated the AKT/GSK3β/mTOR pathway and the phosphorylated form of the Tau protein. In addition, we found a reduction in NeuN and GFAP immunocontent. These results represent the first approach to the hepatotoxic and neural effects of foetal and adult MeHg exposure.

Keyword: Methylmercury; double exposure; oxidative stress; hepatotoxicity; neurotoxicity; Akt/GSK3β/mTOR pathway.

Highlights

• MeHg exposure promotes imbalanced redox in liver and occipital cortex.

• Exposure to MeHg leads to hepatic glycogen accumulation and consequent neural damage.

• MeHg upregulated the Akt/GSK3β/mTOR pathway and Tau hyperphosphorylation in occipital cortex.

• MeHg exposure triggered processes associated with neurodegeneration and inflammation from liver damage.

1. Introduction

Mercury (Hg) is listed as one of the major World Health Organization (WHO) chemicals of great public health concern (World Health Organization, 2005). Inorganic Hg released into the environment by natural (volcanoes and ocean trenches) and anthropogenic (burning fossil fuels and mining) sources is methylated by microorganisms to generate methylmercury (MeHg). MeHg is a highly persistent Hg derivative and a well-known neurotoxin that affects the central and peripheral nervous systems. In the environment, MeHg bioaccumulates and biomagnifies through the trophic chain, and it can reach concentrations many times higher than the surrounding water (Farina et al., 2011; Lehnherr, 2014). This phenomenon represents a serious risk to human health. Dietary intake of fish is major route of exposure to MeHg. Populations with a traditionally high dietary intake of fish are the most exposed to MeHg bioaccumulation and may therefore experience deleterious effects on their health (Faial et al., 2015; USEPA, 2000). A critical example of the negative impact of MeHg occurs in the Brazilian Amazon, where gold mining activities generate a large accumulation of MeHg along the rivers and soils. In these regions, the MeHg concentration in food exceeds that authorised by Brazilian legislation, and this factor places local populations at risk (Bisen-Hersh et al., 2014; Grotto et al., 2011; Da Silva et al., 2012). Once absorbed, MeHg is rapidly transported in red blood cells, because of its increased membrane permeability, and widely distributed throughout the body, particularly the liver, kidneys and central nervous system (CNS) (Nabi, 2014). Like Hg, MeHg penetrates the placental and blood-brain barrier of the fetus, which is not fully developed, and this action increases the toxic effects and irreversible damage (Castoldi et al., 2008, 2001). Human fetuses and newborns are particularly vulnerable to MeHg-induced brain damage and are systemically sensitive to even low-level MeHg exposure (Espitia-Pérez et al., 2018b, 2018a; Grandjean and Landrigan, 2006; Grotto et al., 2009).

Several experiments demonstrated the relationship between prenatal exposure to low MeHg doses and neurodevelopmental disorders, including disrupted social behaviour, recognition memory deficits, apprehension and aversion and impaired attention and language (Debes et al., 2016; Fujimura et al., 2012; Grandjean and Landrigan, 2006; Sakamoto et al., 2018). Additionally, toxic effects in an organism are known to be related to age, concentration and frequency of exposure (Mizoguchi et al., 2002; Peixoto et al., 2003). Predatory insults, nutritional deprivation (Vendite et al., 1985), exposure to metals (Peixoto et al., 2003) and toxic organic compounds (Rajasekaran, 2000) can cause irreversible deficits at any developmental stage.

On the other hand, many types of stress, like continuous exercise, may generate an adaptation to future stressful events. This effect is probably because a small-intensity damage triggers mechanisms of cellular stress

control, which leads to a facilitated future activation and, consequently, greater tolerance of the organism to the same levels of disruption (Ristow and Schmeisser, 2014). Interestingly, some studies postulate that oxidative stress can generate this adaptive response (Ristow and Schmeisser, 2014; Stokes-Riner et al., 2011). Notably, most studies focus on the effects of occupational exposure of rats during only one stage of the life cycle, be it maternal and foetal, foetal or adult. In these experiments, the MeHg neurotoxic effects are well described by different mechanisms. Another important point is the lack of articles on systemic effects and, consequently, their relationship with neurotoxicity. Adding to this complexity, recent studies have shown that toxic metals can induce neurodegeneration due to liver dysfunction; Thus, it is possible that MeHg-induced hepatic encephalopathy may represent another neurodegenerative mechanism (Baraldi et al., 1983; Ernst and Thompson Coon, 2001; Prystupa et al., 2016; Schliess et al., 2009).

Based on these previous findings, the aim of this study was to evaluate how previous MeHg exposure during the foetal and breastfeeding period can affect the toxicity of a re-exposure to the same toxic agent during adulthood in Wistar rats, with a specific focus on connecting hepatic and neural effects.

2. Material and Methods

Animal handling followed the guidelines of the Laboratory Animal Care Principles (NRC, 2011) and the Brazilian Society of Laboratory Animal Science (SBCAL-COBEA). The Animal Experimentation Ethics Committee (CONCEA) of the Federal University of Rio Grande do Sul (UFRGS) approved the research protocol under authorization number 31795.

2.1. Animals and housing conditions

All animals were maintained under a light cycle of 12 hours (07:00 to 19:00) in a temperature-controlled room (22°C) and housed in polypropylene cages (43 cm × 30 cm x 15 cm), with access to standard pelleted ration (CR1 Lab Chow, Nuvilab, Curitiba, Brazil) and filtered water *ad libitum*. Female Wistar rats (200-250 g) from our breeding colony were monitored daily for two weeks for their estrous cycle using vaginal smear examination under a light microscope following procedures described previously (Marcondes et al., 2002). Sexually receptive females (confirmed proestrus) were caged overnight with a mature male (1F:1M). Successful mating was evaluated the following morning by the presence of viable spermatozoa in the vaginal saline smear, which marked the first gestational day (GD0).

Thirty pregnant rats were randomly divided into three groups, n = 10. The pregnant mothers were caged individually and allowed to deliver their litter naturally. Wood shaving was used as bedding material. The date of delivery was defined as postnatal day 0 (PND0). A sexually receptive, confirmed proestrus female was mated at night with a single mature male. For prenatal and breastfeeding MeHg exposure, mothers were treated from GD0 to PND21 (42 days total). The treatments were prepared daily and administered orally with a metal gastric gavage with a maximum volume of 0.5 mL. Treatments were administered in a clean and specific area, away from the colony housing room, at approximately 5:00 p.m. to ensure maximum absorption of treatments in their meals during the dark cycle. This exposure paradigm was used to avoid spillage, discomfort or pallor, and to ensure constant dosage throughout the chronic treatment. In the mothers, there was no evidence of motor alterations or general health disorders during gestation, lactation and life for any of the experimental groups.

2.2. Treatment

Methylmercury (II) chloride was obtained from Spectrum (New Brunswick, NJ, United States). A stock solution of 0.39 µg / µL MeHg was prepared in mineral oil (Embrafarma, Sao Paulo, SP, Brazil). All solutions were prepared in mineral oil. For all experiments, the whole litter (dam and their pups) were considered as the experimental unit. Details of the rationale for our approach, including the utilized doses, are fully described in our previous work (Espitia-Pérez et al., 2018b). One group of females (n = 10) was treated with MeHg via gastric probe (0.5 mg/kg body weight/day), while the other two groups (n = 10 per group) received a mineral oil probe (1 µL / g body weight/day). To avoid the litter effect, in PND 22, one male cub per mother was weaned and separated from the litter. Female offspring and / or excess were sacrificed. Adult males, n = 10 per group (250-300 g), were kept in their original groups.

All male offspring (n = 30) were kept untreated from PND22 to PND99. The group that received MeHg during the prenatal phase was treated again with 0.5 mg/kg body weight/day MeHg and termed Double Exposure (DE); another group of animals was also treated with MeHg under the same conditions and called Single Exposure (SE); the control (CN) group received only mineral oil (1 µL / g body weight / day). Both exposures were by gavage for 45 consecutive days (PND100 to PND144). The DE group underwent chronic MeHg prenatal and breastfeeding and adult exposure, while the SE group underwent chronic exposure only in adulthood (Figure 1). The MeHg doses were the same MeHg concentrations found in fish consumed by Brazilian Amazonian riverside populations (Bellinger et al., 2016). The doses used in this study are also common in MeHg-induced reproductive toxicity and other experimental studies related to MeHg toxicology (Grotto et al., 2009; Passos et al., 2008).

143

144 **2.3. Behavioural tests and procedures**

145 Behavioural tests were always performed during the light phase between 09:00 and 15:00 in specially
146 designed rooms with bright controlled light (overhead fluorescents lamps of 40 W with 90 lx light intensity).
147 Behavioural testing was always performed considering our previously randomized experimental design, whereas
148 scheduling considered the registered delivery date of dams. The latter means that several experimental groups were
149 evaluated the same day during the light phase. The behavioural experimental design is depicted in Figure 1. We
150 handled adult mothers, offspring and males to ensure minimum suffering in the animals during the test period.
151 Behavioural recording and analysis were performed using the ANY-maze video tracking system version 5.14
152 (Stoelting Co., Wood Dale, IL, USA).

153

154 **2.3.1 Open field test (OFT)**

155 The OFT consisted of a square arena (60 cm × 60 cm) surrounded by 40-cm-high white walls. Rats from each
156 experimental condition were evaluated at PND100 and PND144. The test commenced by placing a single rat in the
157 middle of the arena, and its activity was recorded for 10 min. Recordings of the test sessions were analyzed offline
158 using ANY-Maze software. The field was carefully cleaned with 10% ethanol between each rat. The parameters
159 analyzed to evaluate locomotor- and anxiety-related behaviour in the open field were the distance traveled in first 3
160 min (in m/s), center area time (in s), ratio of center distance travelled and total distance travelled, rearing time (in s)
161 and grooming time (in s; Kawai et al., 2007; Ménard et al., 2014).

162

163 **2.3.2 Elevated plus maze test (EPMT)**

164 The EPMT evaluates anxiety-like behaviour. The apparatus has two open (50 cm length × 10 cm width) and
165 two enclosed (50 cm length × 10 cm width × 40 cm height) arms that are separated by a central platform (5 cm
166 length × 5 cm width). The apparatus was placed 70 cm above the floor. ANY-Maze software was used to record the
167 parameters for 5 min. The parameters recorded included activity time (in s), entries into open arms (%), time in open
168 arms (%) and risk assessment (tries/min). The animals were evaluated only once, at PND 144. At the end of each
169 test, the apparatus was cleaned with 70% ethanol (Almeida et al., 2010; Peixoto et al., 2007).

170

171 **2.4 Tissue preparation and protein determination**

All animals were sacrificed by decapitation 24 h after MeHg administration. Blood samples were collected, and the serum was immediately separated (2000 x *g* for 10 min) for analysis of liver damage markers. OC and liver were immediately identified and dissected; a small portion of each fresh tissue was used for the comet assay. The remainder of these tissues were stored at -80°C for further analysis of redox systems and macromolecule damage. The femurs were removed to perform micronucleus analysis in erythrocytes of the bone marrow (MNPCE). For sample preparation, the tissues were homogenized in 50 mM phosphate buffer (pH 7.0) and centrifuged (3000 x *g* for 5 min) to remove the cell debris; the obtained supernatants were used for determination (Espitia-Pérez et al., 2018b). Protein concentration was measured using the method described by (Lowry, 1951)). Biochemical results were normalized to protein content using bovine serum albumin (BSA) as the standard. Western blot assays were performed only on the OC samples.

For Hg determination, a small portion of the OC and liver (130–180 mg wet tissue) was isolated and stored at −80°C for later analysis. For sample preparation, animals' OC and liver samples were weighted with an analytical balance (XS105 Excellence XS, Mettler Toledo, Switzerland) and pretreated using a wet ash digestion procedure. Sample aliquots (0.1–0.2 g) were treated with concentrated nitric acid using initial microwave-assisted heating (4 cycles of 5 s duration), and a subsequent overnight (12 h) step was applied. A final cycle of microwave-assisted heating was performed, and H_2O_2 was added before sonication using an ultrasonic bath (30 min). Finally, water was added to bring the total volume to 10 mL, and centrifugation was performed to remove solid particles from the wet ash procedure.

2.5 Hg determination in occipital cortex and liver samples

Hg quantification followed the steps of chemical vapor generation coupled to atomic absorption spectrometry (CVG-AAS), described previously by Espitia-Pérez et al. (2018b). Hg measurements were performed with an atomic absorption spectrometer (AAnalyst 200, Perkin-Elmer, USA) equipped with a quartz T-cell (160 mm length, 7 mm inner diameter [i.d.]) at 100°C. A Hg electrodeless discharge lamp (EDL) at 220 mA was used as the radiation source with deuterium lamp as the continuum source for background correction. The wavelength was set to 253.7 nm and spectral bandpass to 2.7/1.05 nm (width and height, respectively). All measurements were made in integrated absorbance (peak area). Sample injection was performed via a flow injection analysis system (FIAS 100, Perkin-

199 Elmer, USA) with a 500 µL sampling loop. A 1 mol L^{-1} hydrochloric acid solution at a flow rate of 4.8 mL min^{-1} and
200 0.75% (m/v) sodium tetra hydroborate at a flow rate of 1.8 mL min^{-1} was used as the carrier and reductant,
201 respectively.
202 Argon was used as the carrier gas at a flow rate of 50 mL min^{-1}. The FIAS system arrangement was
203 performed using polytetrafluorethylene (PTFE) tubes (1.0 mm i.d.) and a 200 mm (reaction coil) to transport the
204 solutions to a gas-liquid separator. Milli-Q water (18.2 MΩ cm; Millipore, USA) and analytical-grade reagents were
205 used throughout the analysis. Standard solutions were prepared on the day of use by serial dilution of 1000 mg L^{-1}
206 stock Hg solution (Fluka Analytical, Sigma-Aldrich, USA). These standard stock solutions were also used for the
207 analyte recovery tests. The calibration solutions were prepared in 10% (v/v) nitric acid in the range of 2.5 – 20 µg L^{-1}
208 for Hg determination by CVG-AAS. All laboratory materials, such as glasses and polypropylene vials, were cleaned
209 before use by soaking in a 10% (v/v) nitric acid bath for seven days (Espitia-Pérez et al., 2018b; Kaercher et al.,
210 2005; Segade and Tyson, 2003). Data expressed using limit of detection (LOD# = <0.05 µg g-1).
211
212 **2.6 Redox and damage profile of occipital cortex (OC) and liver**
213 **2.6.1 Cellular oxidative damage**
214 Total reduced thiol (SH) content was examined according to the protocol described by Draper and Hadley
215 (1990). Briefly, SH content was estimated through cellular overall redox status and results were expressed in µmol
216 SH/mg protein. Carbonyl groups were used as a parameter for protein oxidative damage, based on the reaction with
217 dinitrophenylhydrazine (DNPH), as previously described by Levine et al. (1994) and adaptations by Schnorr et al.,
218 (2011). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and re-solubilized in DNPH.
219 Next, the absorbance was read at 370 nm with a spectrophotometer. Results are expressed as ηmol carbonyl/mg
220 protein.
221 Malondialdehyde (MDA) formation measurement was performed as described by Draper and Hadley (1990),
222 Ellman (1959) and Levine ebt al. (1990). MDA estimated an index of lipid peroxidation, and results are expressed as
223 ηmol MDA/mg protein. This analysis was performed in liver and OC samples.
224
225 **2.6.2 Enzymatic activities**

The enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) in liver and OC were analyzed in tissue homogenized with 50 mM phosphate buffered saline (PBS, KH_2PO_4 and K_2HPO_4, pH 7.4) according to methods described by (Aebi, 1984; Misra and Fridovich, 1972; Wendel, 1981), respectively.

The glutathione (GSH) reaction method based on the fluorophore o-phthalaldehyde (OPT) was performed according to Browne and Armstrong (1998), and the results are expressed in nmol GSH/mg protein. Additionally, the ratio between SOD, CAT and GPx was analyzed for better comprehension of effects upon the enzymatic antioxidant system, since these enzymes all work in pathways to convert the superoxide anion into water (Schnorr et al., 2014). Results are expressed as the ratio [U SOD / (U CAT + GPx)] / mg protein.

2.6.3 Comet assay

The alkaline comet assay was performed according to Collins (2015) and Singh et al. (1988) and adaptations by Da Silva et al. (2013;). Blood samples were homogenized in a 0.7% low-melting-point agarose solution, placed on a pre-coated slide with 1.5% agarose and contacted with a lysis solution. After lysis, the slides were placed in a horizontal flask with alkaline buffer, and after 20 min the DNA was then developed by electrophoresis (25 V and 300 mA) for a further 15 min. The slides were neutralized, fixed and stained with silver nitrate. The analysis was performed using conventional optical microscopy and applying the analytical criteria described in the literature. To calculate a damage index (ID), the nucleoids were classified according to tail size in relation to the comet's head (no damage = 0 to maximum damage = 4). The damage index (ID) of the group can range from 0 (no damage = 100 cells completely X 0) to 400 (maximum damage = 100 cells X 4). Data are expressed as % DNA damage index.

2.6.4 Micronucleus test in polychromatic erythrocytes

A micronucleus assay was performed according to methodology previously described by Horta et al. (2016) and the OECD guidelines (OECD, 2014). The bone marrow was extracted, and the smears were prepared directly on slides, two per animal, with one drop of fetal bovine serum. The slides were stained with 10% Giemsa for 5 min, air dried and encoded for blind analysis. To avoid false-negative results (and as a measure of bone marrow toxicity), polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) were classified in 2000 cells. The frequency of micronuclei was observed in 2000 PCE (MNPCE) for each animal (i.e., 1000 for each of the two slides prepared from the duplicate) using conventional light microscopy.

256 **2.7 Damage in liver and glycogen determination**

257 Aspartate transaminase (AST) and alanine transaminase (ALT) serum levels were determined by standard
258 biological kits supplied by Labtest® (Labtest, MG, Brazil). ALT and AST activities were expressed in U/dL. Glycogen
259 determination was conducted according to methods previously described by Espitia-Pérez et al. (2018b) and
260 expressed in percentage (%) related to control.

261

262 **2.8 Western blot assay**

263 The OC homogenate (30 µg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis
264 (SDS-PAGE) and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at
265 20 V in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes
266 were washed for 10 min in Tris-buffered saline with 0.1% Tween-20 (TTBS; 0.5 M NaCl, 20 mM Tris, 0.1% Tween-
267 20, pH 7.5), followed by 1 h incubation in blocking solution (TTBS plus 5% fat-free dried milk). After incubation, the
268 blots were washed three times for 15 min with TTBS and incubated overnight at 4°C in blocking solution with the
269 following monoclonal antibodies diluted 1:1000: anti-GFAP (Sigma G4546), anti- NeuN (Cell Signaling 12943), anti-
270 phosphoGSK3β (Ser9) (Cell Signaling 5558S), anti-GSK3β (Cell Signaling 9315S), anti-AKT (Cell Signaling 9272S),
271 anti-phosphoAkt (Thr308) (Cell Signaling 13038S), anti-PI3K (85 and 110 kDa) (Cell Signaling 4257S and 4249S),
272 anti-mTOR (Cell Signaling 4517S), anti-phospho-mTOR (Cell Signaling 2971S), anti-IBA1 (Sigma SAB2500041),
273 anti-RAGE (Santa Cruz Biotechnology 4527), anti-Tau (Cell Signaling 4019), anti-phosphoTau (ser396) (Thermo
274 Fisher 355300) and anti-actin (Sigma 5441). The blots were then washed three times for 15 min with TTBS and
275 incubated for 2 h in blocking solution that contained peroxidase-conjugated anti-mouse IgG diluted 1:2000. The blots
276 were washed twice again for 15 min with TTBS and twice for 15 min with TBS. The blots were then developed using
277 a chemiluminescent substrate. Blots were quantified, and optical density values were obtained for the studied
278 proteins.

279

280 **2.9 Statistical analysis**

281 All results are expressed as mean ± standard error of the mean (SEM), and differences were considered
282 significant when $p \leq 0.05$. For behavioural testing, normality was confirmed by the D´Agostino-Pearson test.
283 Differences among treatments were analyzed by one-way analysis of variance (ANOVA) and post hoc analysis to
284 detect differences between experimental groups and their respective controls. Non-parametric analyses were

285 performed when needed (Kruskal-Wallis test with Dunn's multiple comparisons). Two-way ANOVA using treatment
286 used to assess differences in OFT testing and redox profile parameters from the liver and OC. Post hoc comparisons
287 were performed using Bonferroni and Sidak tests, and one-way ANOVA with Tukey's comparisons, primarily to
288 evaluate differences among oxidative stress parameters between experimental groups and their controls, and
289 similarly for object exploration in animals. Comparisons of Hg content were analyzed with unpaired Student's t test.
290 One-way ANOVA was conducted, followed by Tukey's post hoc, for the comet assay and MNPCE results. Data
291 analysis and graphics were performed using GraphPad Prism version 7.01 software (GraphPad Software Inc., San
292 Diego, CA, USA).
293
294 **3. RESULTS**
295 **3.1 MeHg exposure decreased weight and consumption food**
296 The body weight and consumption food of the MeHg-exposed animals decreased after the fourth week of the
297 experiment compared to the control group. For this parameters, pre-exposure to MeHg (DE group) did not influence
298 in systemic toxicity. The results are described in Supplementary Material (Table S1).
299 **3.2 Total Hg content**
300 The total Hg contents in liver and OC are presented in Supplementary Material (Table S2). Both liver and OC
301 Hg levels in MeHg-treated rats were significantly higher than in controls, which were below the limit of detection
302 (LOD). Additionally, in both tissues, the DE group presented a higher Hg content (%) relative to the SE, and for both
303 groups, the highest Hg contents were in the liver.
304
305 **3.3 Liver damage and glycogen content**
306 MeHg-exposed rats exhibited increased AST and ALT activity. Additionally, MeHg treatment significantly
307 increased hepatic glycogen content. AST and glycogen content markers were higher for the DE compared to the SE
308 group. These findings suggest the occurrence of liver damage that was cumulative. The results are presented in
309 Table 1.

3.4 Changes in oxidative stress biomarkers

3.4.1 Liver

The effects of MeHg treatment on redox parameters and oxidative damage determined in the liver are shown in Table 2. MeHg exposure increased SOD activity, did not alter CAT activity and significantly decreased GPx activity. The ratio of SOD, CAT and GPx [SOD/ (CAT + GPx)] was lower in MeHg-exposed animals. GST activity and GSH levels were higher in MeHg-exposed animals. Regarding the oxidative damage markers, MDA and carbonyl levels were increased in the MeHg-exposed groups. There was a decrease in SH levels in the animals exposed to MeHg.

3.4.2 Occipital cortex (OC)

OC results are also presented in Table 2. MeHg decreased both SOD and CAT activities for both exposed groups. Additionally, we did not observe alterations in GPx and GST activity or GSH levels. However, as opposed to what was found in liver samples, the SOD/ (CAT + GPx) ratio was increased in the OC of the MeHg-treated rats as compared to the control group. The MDA and carbonyl contents were increased, while the SH content was decreased.

3.4.3 Comet assay and micronucleus

The DNA damage index evidenced by the comet assay in liver and OC cells and the micronucleus frequency are plotted in Figure 2. MeHg-exposed rats exhibited a significant increase in DNA damage in both tissues, regardless of the intensity of the exposure (Figure 2A and B). Further, MNPCE frequency in red bone marrow cells was increased in MeHg-exposed animals when compared to the controls. Notably, there was enhanced MNPCE frequency in the DE compared to the SE group (Figure 2C).

3.5 The effects of MeHg exposure on protein expression

The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/glycogen synthase kinase 3β (GSK3β) signaling pathway was analyzed in the OC (Figure 3). While PI3K was not altered (Figure 3A-B), Akt (Figure 3C) was activated in the DE- and SE-group OC. Additionally, the ratio of phosphorylated to total GSK3β (Ser9; Figure 3D) and phosphorylated to total mTOR (Figure 3E) was increased. MeHg also significantly increased phosphorylated Tau protein (Figure 3F). MeHg treatment decreased the immunocontent of neuronal and astrocytic proteins, NeuN (Figure 4A) and GFAP, respectively (Figure 4B), and

increased Iba1 (Figure 4C). The receptor for advanced glycation end products (RAGE) level was increased in the OC of MeHg-exposed rats. Notably, there were no differences for any protein levels between the SE and DE rats.

3.6 Evaluation of behaviour in MeHg-exposed animals

The effect of the intensity of MeHg exposure on the motor and emotional behaviour of the rats was evaluated using the OFT at PND100 (OFT1) and PND144 (OFT2) and EPMT at PND144 (Figure 5). For OFT2, the MeHg-exposed animals (for both DE and SE groups) walked longer distances in the apparatus (Figure 5A) and spent less time in the central area (Figure 5B). Interestingly, the DE group spent significantly less time in the central area compared to the SE group (Figure 5C). MeHg treatment also decreased the ratio between the centre distance and total distance traveled at OFT2 (Figure 5D). There were no other differences between the DE and SE groups for the remaining analysed OFT parameters (Figure 5D-E). For EPMT at PND144, the MeHg-exposed rats showed less activity in the apparatus (Figure 5F), lower number of entrances (Figure 5G) and less time spent in the open arms of the apparatus (Figure 5H). Additionally, the MeHg-exposed animals presented more episodes of risk assessment (Figure 5I).

4. DISCUSSION

The literature clearly indicates that fish consumption, especially during pregnancy, is the main route of MeHg exposure and contamination. This intrauterine and adult exposure to MeHg is responsible for irreversible toxic effects such as systemic toxicity, neuronal death and cognitive and motor deficits (Cusack et al., 2017; USEPA, 2000). However, some researchers postulate that continuous exposure (including toxic agents) may be beneficial and allow adaptation to future stressful events (Ristow and Schmeisser, 2014; Stokes-Riner et al., 2011). With this proposal in mind, we exposed one group of animals to MeHg during the intrauterine phase and breastfeeding and repeated the exposure during adulthood (DE group); another group was exposed only during adulthood (SE group). This design allowed us to assess whether there was an adaptive pattern in response to the second exposure by assessing markers of hepatic and neural damage.

In this study, we concentrated our analyses of MeHg-mediated damage on the liver and OC, since a large portion of MeHg deposits occur in these tissues (Farina et al., 2011; Roegge et al., 2011, 2004). We observed physical signs of MeHg toxicity in the rats, namely reduced standard feed consumption and consequent weight loss. Both effects were previously described by Rice et al. (2014) and are associated with the lack of palatability and

367 irritation of the intestinal mucosa caused by MeHg. There were no significant differences between both groups DE
368 and SE with regards to this parameter.

369 Hg quantification in the liver is reliable, since it provides the most stable Hg contents as compared to the rapid
370 Hg clearance in the brain (Newland et al., 2006, 2004; Newland and Reile, 1999). We found statistically higher
371 concentrations of Hg in DE compared to the SE group in both tissues. It is known that the levels of Hg absorbed in
372 the uterus accumulate with the new exposure in adult life. In addition, the DE group had a relatively higher
373 concentration of Hg in CO compared to SE, indicating that Hg clearence in this tissue may have been affected
374 (Roegge et al., 2011; Stringari et al., 2008)

375 Liver function has also been evaluated to elucidate some of the processes involved in MeHg hepatotoxicity.
376 According to Berger et al. (2009), glycogen accumulation suggests effects on the reprogramming of glucose
377 metabolism in animals. We used this analysis and observed a significantly higher glycogen deposition, especially in
378 the DE group. A strong indication of metabolism and consequent glycogen deposition was affected by double
379 exposure to MeHg and that MeHg clearance in this tissue was impaired. The elevated hepatic enzyme activities
380 (AST and ALT) in the blood suggest hepatic damage in both DE and SE groups (Gelain et al., 2012; Huang et al.,
381 2006; Mortensen et al., 2014). One of the mechanisms proposed to induce MeHg-mediated damage is the
382 generation of reactive oxygen species (ROS) and redox homeostasis imbalance (Rasinger et al., 2017; Sarbassov
383 and Sabatini, 2005; Sharma et al., 2005). Our results are consistent with these studies, since changes in the
384 activities of antioxidant and detoxifying enzymes were observed in MeHg-exposed rat liver and OC. Low MeHg
385 doses can generate oxidative damage that leads to cellular rupture and cognitive impairment (Heimfarth et al., 2018,
386 2017). Protein damage detected by carbonyl and sulfhydryl levels indicates a possible impairment of cell signaling
387 processes and energy metabolism regulation.

388 Increased protein carbonylation, in both tissues, may also induce the formation of high-molecular-mass
389 clusters with high cytotoxic potential. In liver, the increased oxidation of thiol (-SH) groups may induce excessive
390 formation of disulfide bonds between proteins, a phenomenon that affects the coiling of proteins and induces protein
391 aggregation and degradation (Cumming et al., 2004; Sitia and Molteni, 2004; Winterbourn and Metodiewa, 1999).
392 Notably, just like our work, some studies show that low MeHg doses generate high lipid peroxidation in several
393 tissues. This effect is probably an indirect consequence of the pro-oxidative effects of MeHg (Carvalho et al., 2008;
394 Joshi et al., 2014; Roegge et al., 2006; Ruszkiewicz et al., 2016).

395 Furthermore, our data, in both tissues, did not show an imbalance in antioxidant enzyme activities (Table 2),
396 suggesting that it is a direct oxidizing effect of MeHg and not an effect of it on enzyme antioxidants. MeHg rats

showed increased levels of TBARS and carbonylated proteins in OC and liver, and decreased thiol content. Protein carbonylation is a product of the irreversible oxidation of various amino acid residues, their accumulation can disrupt signaling cells and promote the generation of highly cytotoxic substances (Halliwell, 2006; Winterbourn and Metodiewa, 1999). The decrease in SH groups can also result in excessive disulfide bond generation, protein folding, aggregation and degradation and even lead to cell death (Cumming et al., 2004; Schnorr et al., 2015; Winterbourn and Metodiewa, 1999).

In vitro (Crespo-López et al., 2007) and *in vivo* (Amorim et al., 2000; Betti et al., 1993; Espitia-Pérez et al., 2018b) studies demonstrated that MeHg can act as a clastogenic agent. MeHg can inhibit the activity of poly (ADP-ribose) polymerase-1 (PARP-1), which repairs DNA strand breaks (Pieper et al., 2014). On the other hand, the DNA damage observed in this work was probably generated by a pro-oxidant pathway. MeHg-exposed rats presented increased MNPCE frequencies when compared to control. Increasing the MNPEC frequency in adult rats is related to DNA repair inefficiency or apoptosis induction in cells (Crespo-López et al., 2007; Amorim et al., 2000; Espitia-Pérez et al., 2018b). DE group presented a higher MNCT frequency compared to SE. This is because few studies have confirmed the genotoxic and mutagenic potential of mercury exposure. However, MeHg leads to the formation of ROS that can induce conformational changes in proteins responsible for DNA repair, mitotic spindle and chromosomal segregation (Tchounwou et al., 2012).

Previous works by our group and others (Espitia-Pérez et al., 2018b, 2018a; Heimfarth et al., 2018, 2017; Vicente et al., 2004) revealed that MeHg exposure has complex effects on cellular biological processes, which may exhibit multiphasic responses due to the vulnerability of each brain structure (as well as the dose and time of exposure). The OC is one of the most sensitive brain structures with regards to MeHg exposure (Haykal-Coates et al., 1998; Heimfarth et al., 2018). Furthermore, several molecular targets and complex mechanisms are involved in MeHg-mediated neurotoxicity and behavioural impairment effects (Heimfarth et al., 2017). Gestational MeHg exposure (Haykal-Coates et al., 1998; Heimfarth et al., 2018) also alters the activity of survival/death signaling cascades, changes that could be associated with impaired brain development. Considering this possibility, we evaluated the immunocontent of the active enzymes that participate in some signaling survival pathways in the motor OC of MeHg-exposed rats. We observed upregulation of the PI3K/Akt/mTOR signaling pathway and inactivation of GSK3β (Ser9). The PI3K/Akt pathway promotes cell growth, differentiation and survival by downregulating apoptotic signals (Endo et al., 2006). Several studies demonstrated that oxidative stress regulates Akt activation, and MeHg activates this pathway by increasing ROS production (Song et al., 2008; Uranga et al., 2013; Yu and Cui, 2016; Zhang and Yang, 2013). Through PI3K, Akt is recruited to the plasma membrane and activated by protein kinases.

Akt activation phosphorylates several downstream substrate proteins that are critical in cell cycle progression and survival (Lawlor and Alessi, 2001).

Several studies describe the Akt pathway as an important molecular target for MeHg in the CNS, an interaction that promotes cellular damage and neurotoxicity (Endo et al., 2006; Heimfarth et al., 2018). GSK3β is important for CNS development, since it controls neurogenesis, neuronal polarization and axonal growth. Neuronal GSK3β activity is under the control of multiple mechanisms. GSK3β downregulation neural plasticity mechanisms during development (Seira and Del Rio, 2014). GSK3β is an important protein kinase that is downstream of Akt. Akt phosphorylates it at serine 9, which inhibits its activity. Namely, in previous studies, observed that PI3K / Akt / mTOR pathway upregulation and GSK3β (Ser9) downregulation could indicate that these changes are associated with neural damage in the MeHg-exposed animals (Gasparotto et al., 2018; Heimfarth et al., 2018, 2017; Seira and Del Rio, 2014). In our study observed this disruption of GSK3β in adult rats exposed to MeHg. Therefore, the imbalance of GSK3β (Ser9) activity, seen in animals exposed to MeHg, may be involved in neural damage.

Neurological changes present in neurodegenerative diseases are known to be associated with Tau protein deregulation (Gasparotto et al., 2018; Heimfarth et al., 2018). Elevated Tau protein (Ser396) destabilizes microtubules, and Tau accumulation leads to the formation of tangles associated with abnormal intracellular structures, phenomena that promote neuronal damage and death. In this line, the Tau hyperphosphorylation observed in animals exposed to MeHg could be one of the mechanisms responsible for neuronal damage in OC; it could cause neural and astrocytic death and microglial activation through cytoskeletal rupture. It is important to note that structural changes in the cytoskeleton are relevant characteristics present in neurodegenerative diseases, such as Alzheimer's disease and neurotoxin intoxication (Heimfarth et al., 2017; Maqbool et al., 2016).

Despite PI3K/Akt/mTOR pathway upregulation, we observed a decrease in the immunocontent of both NeuN (neuronal density) and GFAP levels in the OC of MeHg-treated rats. Additionally, the increased microglial activation, denoted through increased Iba1 protein, suggests a local inflammation modulates astrocytic and microglial dysfunction in the OC of MeHg-exposed animals (Gasparotto et al., 2018; Hol and Pekny, 2015; Rasinger et al., 2017; Sarbassov and Sabatini, 2005; Takei and Nawa, 2014; Xu et al., 2015). We also observed increased RAGE levels. The increase in RAGE is associated with inflammation, and where it is expressed becomes very relevant to understanding the physiological state of the tissue (Gasparotto et al., 2018). Thus, neural damage in the OC generated by MeHg exposure could play an important role in susceptibility to neurodegenerative diseases and may be associated with the behavioural impairment observed in MeHg-exposed animals.

The OFT and EPMT results revealed that MeHg induced behavioral changes, results that indicate the presence of cognitive impairment already described in previous studies from our group (Espitia-Pérez et al., 2018b, 2018a). Male offspring of MeHg-exposed mothers are more sensitive than female offspring; in these affected animals, the synaptic circuit is apparently affected during MeHg exposure (Espitia-Pérez et al., 2018a). Additionally, MeHg-treated rats exhibited a longer uptake response within 3 min of OFT, data that suggest the existence of a possible long-term memory disorder. A similar result was observed in the MeHg-exposed mothers (Espitia-Pérez et al., 2018b). Intrauterine MeHg exposure did not attenuate the behavioural deficiency generated by the new adulthood exposure (DE group). Behavioural results indicate that MeHg-treated animals exhibited anxious behaviour, such as aversion to the central area of the device. This finding suggests that MeHg induces changes in emotional reactivity. Our data is consistent with findings in human populations that consume high levels of MeHg-contaminated fish. The children exposed to MeHg in the uterus present a lower intelligence quotient (IQ) and impaired memory, attention, language and visual perception (Bisen-Hersh et al., 2014; Grandjean and Landrigan, 2006; Roegge et al., 2011).

It is important to note that besides the oxidative damage that occurs in the nervous system because of the MeHg poisoning, another path of neurotoxicity may mediate the liver damage. Many authors have shown that liver injury, as observed in our results (Table 1), can lead to encephalopathies with neurodegenerative consequences (Braissant et al., 2019; Cudalbu and Taylor-Robinson, 2019). This may be associated with hepatic glycogen deposition in animals exposed to MeHg, especially in the DE group. Other authors, have found similar results, followed by severe hypoglycemia and weight loss in MeHg-treated postnatal rats (Snell et al., 1977) as chronic MeHg dosing increases constant liver Hg saturation (Salvaterra et al., 1975). Along with weight loss, evidenced in both groups exposed to MeHg, we found a low food intake by these animals (probably due to loss of palatability). This poor food intake is responsible for low serum glucose levels and increased AST and ALT activity in the organs (Moriyama et al., 2008). In addition, recent evidence shows that MeHg exposure affects hepatic proteome, especially glucose metabolism, possibly by disruption of antioxidant defense (Yadetie et al., 2016).

Glycogen accumulation in the liver and low endogenous glucose availability predispose to brain damage. Epidemiological results from pediatric cohort studies have shown brain damage, probably caused by recurrent severe hypoglycemia, in patients with Type I Glycogen Storage Disease (GSDI) (Melis et al., 2004). GSDI patients have impaired glucose-6-phosphatase (G6P) activity, which is curiously a molecular target for MeHg inhibition (Snell et al., 1977). Decreased G6P activity is related to low endogenous glucose content after the post-absorption state, increases the accumulation of glycogen and glucose-6-phosphate in the liver, which increases organ dysfunction

(Cohn and Ohri, 2017; Winnick et al., 2016). The following hypoglycemia may induce brain damage in vivo due to lack of metabolic fuel (Cryer, 2007). Finally, glycogen phosphorylase (GP) is another molecular target of irreversible MeHg inhibition. GP is an enzyme that limits the rate in the initial pathway of glycogenolysis. Previous work has found increased glycogen deposition in the tissue of animals exposed to MeHg following in vivo GP imbalance (Rao and Sharma, 2001; Xu et al., 2014).

Our central hypothesis is based on neurotoxicity associated with hepatitoxicity problematically by an excess of toxic metabolites such as ammonia (hyperammonemia). Hyperammonemia can cause irreparable damage to the developing brain, presenting symptoms such as cognitive impairment (mental retardation), seizures and cerebral palsy. The neurotoxic effect of ammonia can be confirmed by neuronal death in our study and associated with hepatotoxic damage. In rats exposed to DE, anxiety-like behavior was found. Therefore, that increased liver damage, glycogen deposition and subsequent hypoglycemia favored this stereotyped behavior (Haack et al., 2014; Hertz et al., 2017).

Previous work has shown that during in vivo hypoglycemic stress, MeHg induced hyperthyroid status and low dopamine synthesis due to renal failure in rats (Kabuto, 1991). Low dopamine levels are related to the physiological response of anxiety (Zarrindast and Khakpai, 2015). However, other behavioral phenotypes are encountered during hypoglycemia-induced brain damage, which also promotes learning and spatial memory deficits (Puente et al., 2010). Our increase in AST and ALT may be responsible for concomitant liver damage with endogenous low glucose. However, a recent in vivo multimodal model showed locomotive impairment during early hepatic encephalopathy in rats, which correlated with increased ammonium and glutamine transport and a decrease in antioxidant levels (Braissant et al., 2019)The latter may suggest the importance of considering other metabolites of importance during liver injury and their relationship to brain injury, especially in future studies.

Our results show the importance of considering the systemic effects of MeHg exposure, especially in new exposure scenarios, where increased MeHg bioavailability may expose young infants to various doses of MeHg over a lifetime with unknown affects.

5. CONCLUSION

In conclusion, low MeHg doses during the foetal period and breastfeeding do not condition the best or an adaptive response against new adulthood MeHg exposure. But yes, they increase liver glycogen accumulation, redox imbalance and neural damage, alterations that promote behavioral deficits manifested in adulthood. It is important to note that the damage caused to biomolecules by MeHg exposure were mutagenic and affected

hematopoiesis. MeHg exposure also caused irreversible hepatic and neural damage, and we suppose that liver damage may be associated with brain and behavioral damage observed in MeHg-treated rats.

Even with these results, further studies are needed to confirm our findings and elucidate molecular mechanisms, such as the mitogen activated protein kinase (MAPK) cascade and the study of proinflammatory markers associated with neurodegeneration. The epidemiological implications of our results also still need to be determined. To the best of our knowledge, our results represent the first insight into the possible consequences of double exposure to MeHg (foetal and breastfeeding and adult life) and cumulative damages.

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Compliance with ethical standards

Ethical standards - This manuscript does not contain clinical studies of patients performed by any of the authors.

Ethical approval - All applicable international, national, and institutional guidelines for the care and use of the animals were followed. The Animal Experimentation Ethics Committee (CONCEA) of the Universidade Federal do Rio Grande do Sul (UFRGS) approved the research protocol under authorization number 31795.

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

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TABLES

Table 1. Markers of hepatic glycogen content and damage in rats.

	Groups		
	Control	**DE**	**SE**
Number animals	10	10	10
AST concentration (U AST/dL)	3.5±0.43	**11.2±0.36*#**	**9.6±0.47***
ALT concentration (U ALT/dL)	17.3±1.11	**38.5+2.49***	**35.7±1.84***
% Hepatic glycogen content	107.1±4.6	**139.5±8.5*#**	**126.9±9.1***

Data are represented as mean ± SEM;
Bold for statistically significant difference;
*Represents a significant difference of Control group ($p \leq 0.05$);
#Represents a significant difference between groups ($p \leq 0.05$);
Control (n = 10), Double – exposure group: DE (n = 10), Single – exposure: SE (n = 10).

Table 2. Effects of MeHg exposure on redox parameters in liver and cortex.

	Groups		
	Control	**DE**	**SE**
N° of animals	10	10	10
Liver			
SOD activity (U SOD/mg protein)	36.84±0.84	**40.50±1.07***	**40.70±0.90***
CAT activity (U CAT/ mg protein)	40.41±3.92	36.65±2.44	36.37±2.63
GPx activity (U GPx/mg protein)	47.95±1.29	**32.62±1.26***	**35.61±1.05***
SOD/(GPx+CAT) ratio (arbitrary units)	0.94±0.20	1.19±0.36	1.22±0.31
GST activity (U GST/mg protein)	12.94±0.40	**19.53±0.70***	**17.95±0.6***
GSH activity (ηmol GSH/mg protein)	6.90±0.44	**20.77±0.21***	**17.84±0.26***
MDA content (ηmol MDA/mg protein)	0.021±0.1	**0.028±0.02***	**0.030±0.02***
Total thiol content (ηmol SH/mg protein)	20.51+1.65	**14.77±0.47***	**16.22±0.72***
Total carbonyl content (μmol carbonyl/mg protein)	2.60±0.27	**3.62±0.23***	**3.91±0.35***
Occipital cortex			
SOD activity (U SOD/mg protein)	45.12±6.91	**36.07±5.51***	**38.76±3.68***
CAT activity (U CAT/ mg protein)	1.86±0.24	**0.62±0.15*#**	**0.80±0.07***
GPx activity (U GPx/mg protein)	42.41±2.64	36.86±5.71	38.53±1.96
SOD/(GPx+CAT) ratio (arbitrary units)	1.02±0.15	0.97±0.17	0.98±0.09
GST activity (U GST/mg protein)	67.54±7.44	59.81±8.28	66.28±6.91
GSH activity (ηmol GSH/mg protein)	6.51±2.45	7.36±1.08	7.01±2.61
MDA content (ηmol MDA/mg protein)	0.55±0.11	**2.35±0.91***	**2.61±0.33***
Total thiol content (ηmol SH/mg protein)	16.13±1.30	**12.86±1.66***	**13.13±1.05***
Total carbonyl content (μmol carbonyl/mg protein)	2.40±0.64	**3.48±0.55***	**3.9±0.78***

Data are represented as mean ± SEM;
Bold for statistically significant difference;
*Represents a significant difference of Control group ($p \leq 0.05$);
#Represents a significant difference between groups ($p \leq 0.05$);
Control (n = 10), Double – exposure group: DE (n = 10), Single – exposure: SE (n = 10).

FIGURES AND CAPTIONS

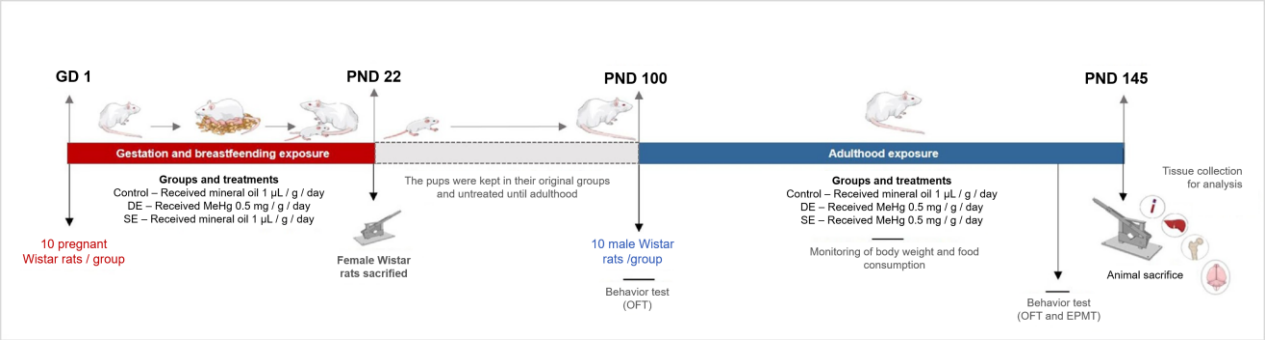


Figure 1. Treatment scheme: Nine female Wistar rats (200-250 g), with regular reproductive cycles, presenting sperm in the vaginal smear were in gestational day zero (GD0). In GD 1 females were divided randomically in three groups: The Control group and single exposure (SE) group, received mineral oil 1 µL / kg body weight / day; double exposure (DE) received 0.5 mg / kg of body weight / day of MeHg. Throughout the gestational period until the weaning of the pups, on the postnatal day (PND) 22, totalizing 42 days of treatment. In PND 22, the male offspring were weaned, and the females were sacrificed. The offspring were maintained untreated from PND 22 to PND 99. The animals, male adults (n = 30) were kept in their source groups (DE group, SE group and Control). The DE group and SE group received 0.5 mg / kg of body weight / day of MeHg and the control received 1 µL of mineral oil / kg body weight / day by gavage for 45 consecutive days (7 weeks). During adult life exposure period, average food intake and individual body weight were weekly collected. At the end of the treatment, all animals were sacrificed, and tissues were collected for analysis.

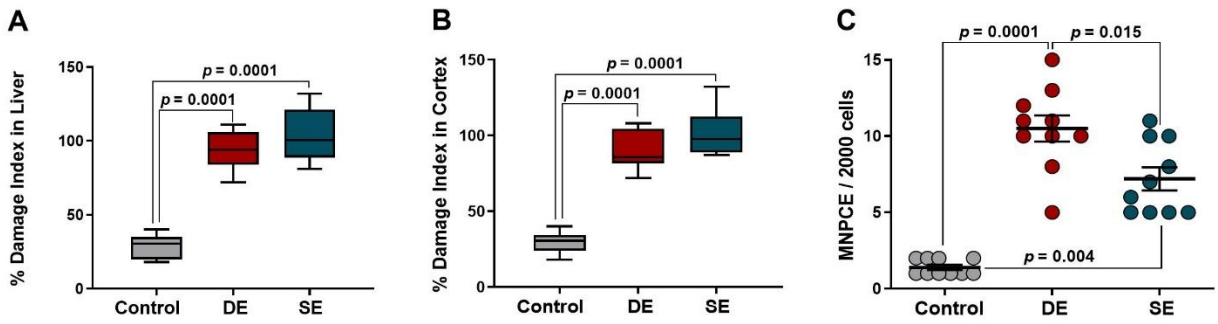


Figure 2. DNA damage index and MNPCE frequencies. 2A) DNA damage index analyzed from the hepatic cells. 2B) DNA damage index analyzed from the frontal cortex cells. C) The frequency of micronuclei per 2000 cells analyzed from the animal's femur. The results were expressed as a mean ± standard error (S.E.M.). Data were evaluated by Two-way ANOVA followed by Tukey's multiple comparisons test. *p* values are embedded in the figure. Dashed lines show the groups that were compared. Control (n = 10) – gray bars; Double – exposure group: DE (n = 10) – red bars; Single – exposure: SE (n = 10) – green bars.

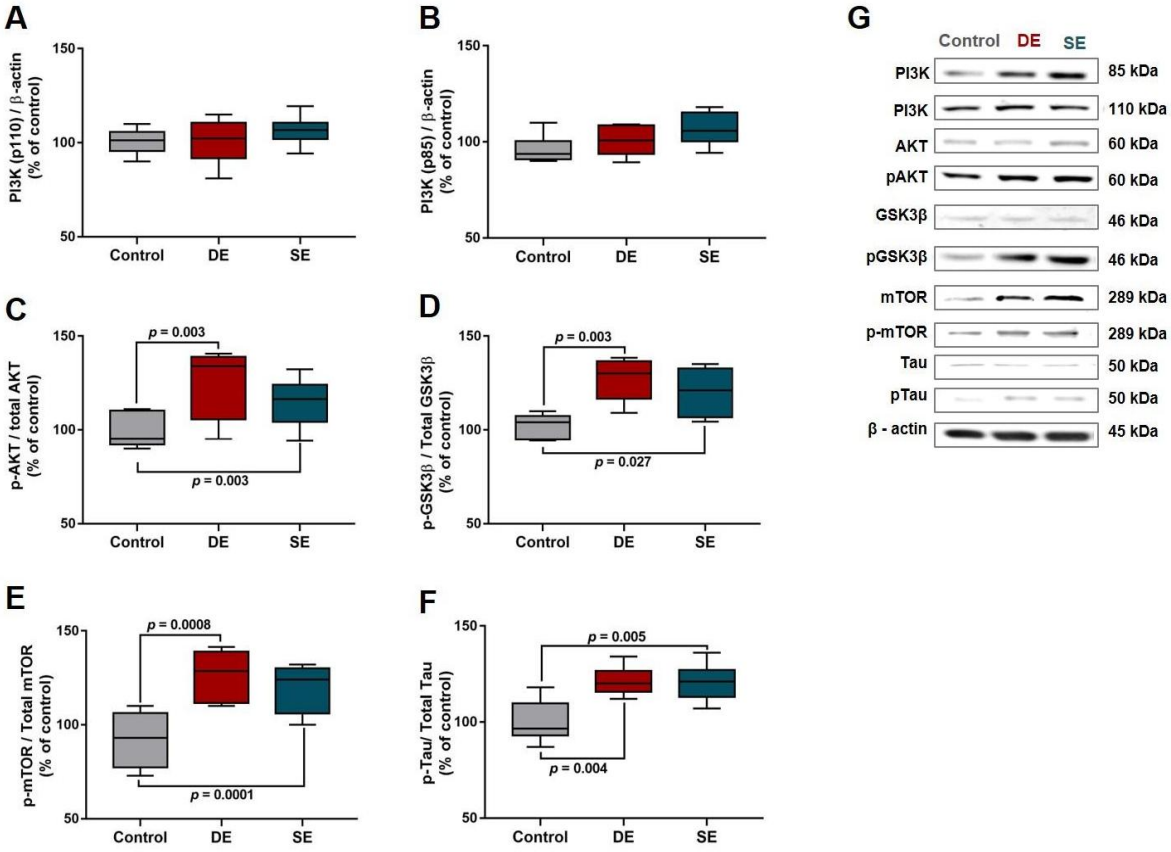


Figure 3. Effect of adult exposure to MeHg on the signaling AKT/GSK3β/mTOR pathway in the frontal cortex of the rats at 145 days. Western blot assays were carried out with the specific antibodies and dilutions described in "Materials and methods": anti-Pi3K (110 kDa) (Fig 5A) catalytic subunit antibody; anti-Pi3K (85 kDa) (Fig 5B) regulatory subunit antibody; anti-total and anti-phosphorylated AKT/PDK antibodies expressed as total/phosphorylated ratio (Fig 5C). Anti-total and anti-phosphorylated GSK3β antibodies expressed as total/phosphorylated ratio (Fig 5D); anti-total and anti-phosphorylated mTOR antibodies expressed as total/phosphorylated ratio (Fig 5E); anti-total and anti-phosphorylated Tau antibodies expressed as total/phosphorylated ratio (Fig 5F). Representative blots are shown in G. Data are reported as mean ± SEM for ten animals and expressed as percent of control. Statistically significant differences from control group, as determined by *T* test. Dashed lines show the groups that were compared. Control (n = 10) – gray bars; Double – exposure group: DE (n = 10) – red bars; Single – exposure: SE (n = 10) – green bars.

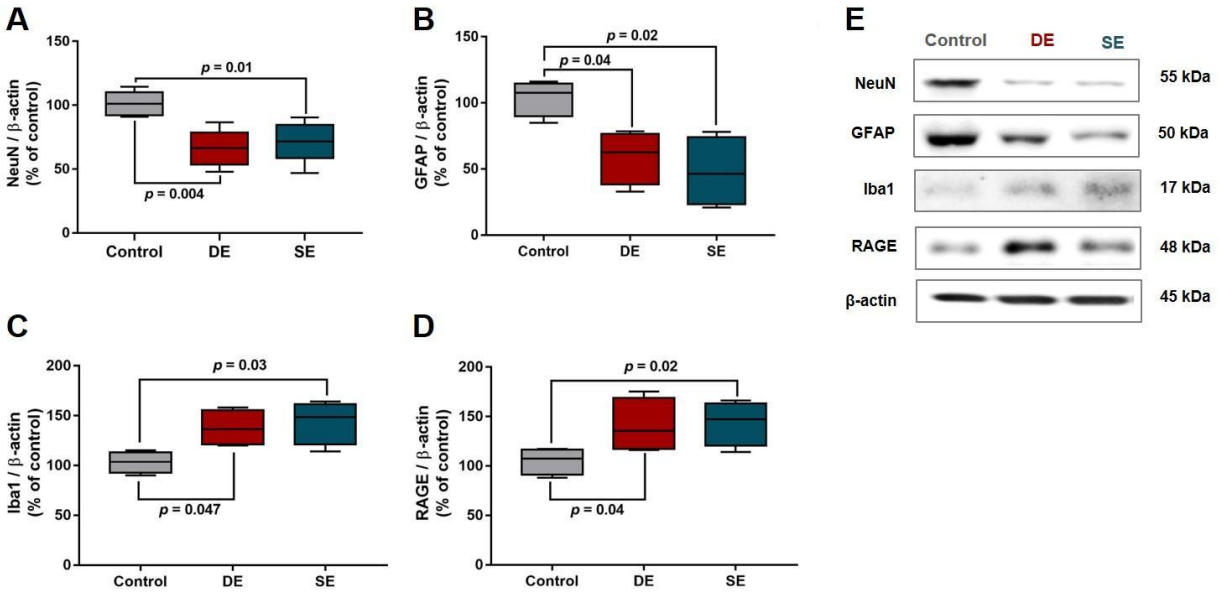


Figure 4. Effect of adult exposure to MeHg on the specific markers of neural. Western blot assay of NeuN (Fig 6A), GFAP (Fig 6B), Iba1 (Fig 6C), RAGE (Fig 6D), representative blots are shown in Fig 6E. was carried out as described in "Materials and methods." Representative blots are shown (c). Data are reported as mean ± SEM for ten male animals and expressed as percent of control. Statistically significant differences from control group, as determined by *T* test**.** Data are reported as mean ± SEM for ten animals and expressed as percent of control. Statistically significant differences from control group, as determined by *T* test. Dashed lines show the groups that were compared. Control (n = 10) – gray bars; Double – exposure group: DE (n = 10) – red bars; Single – exposure: SE (n = 10) – green bars.

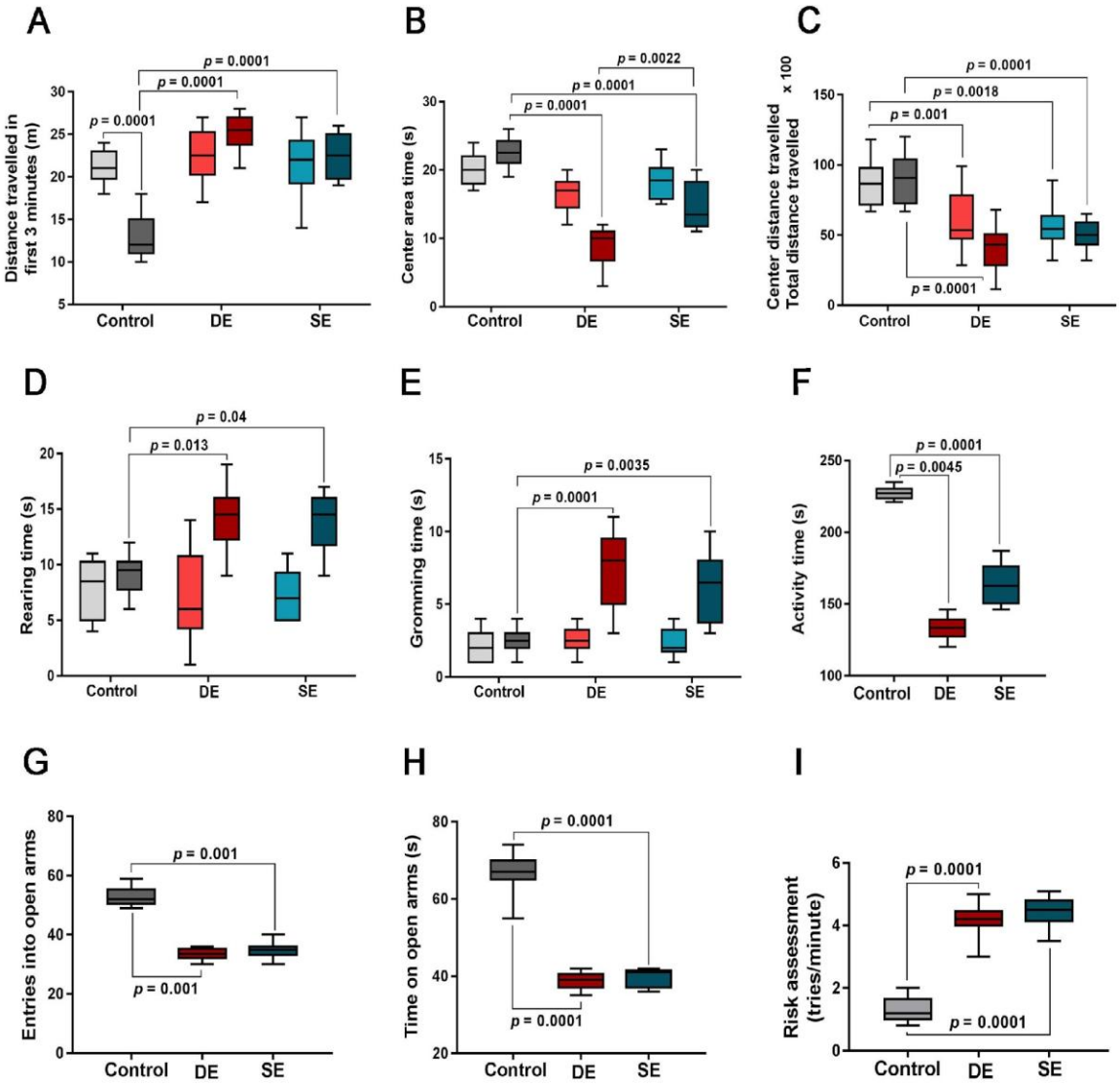
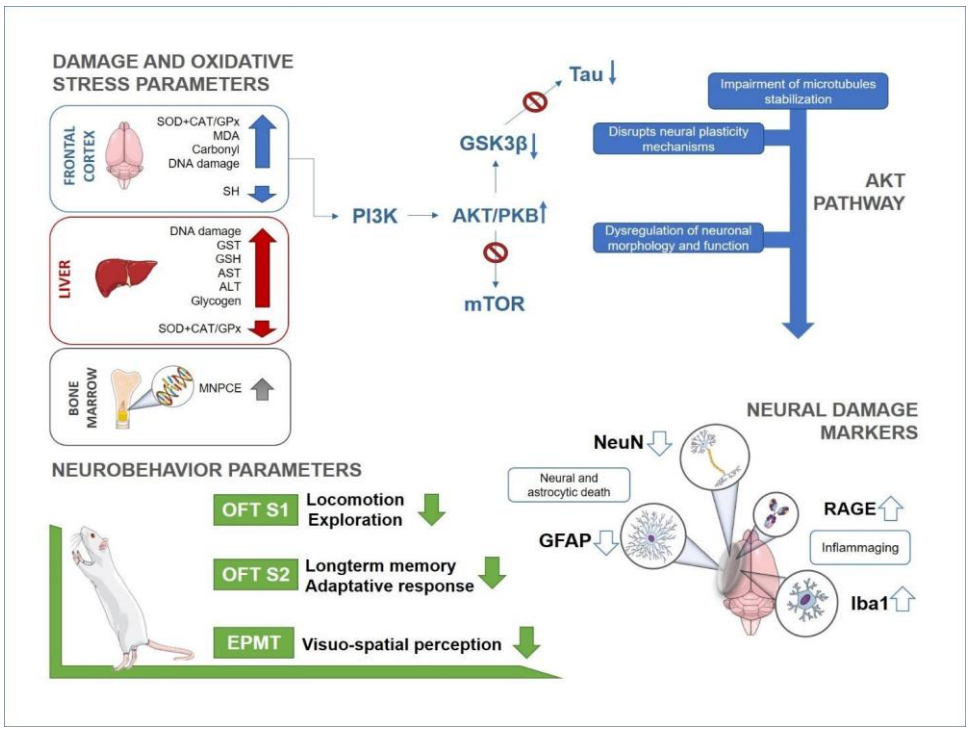


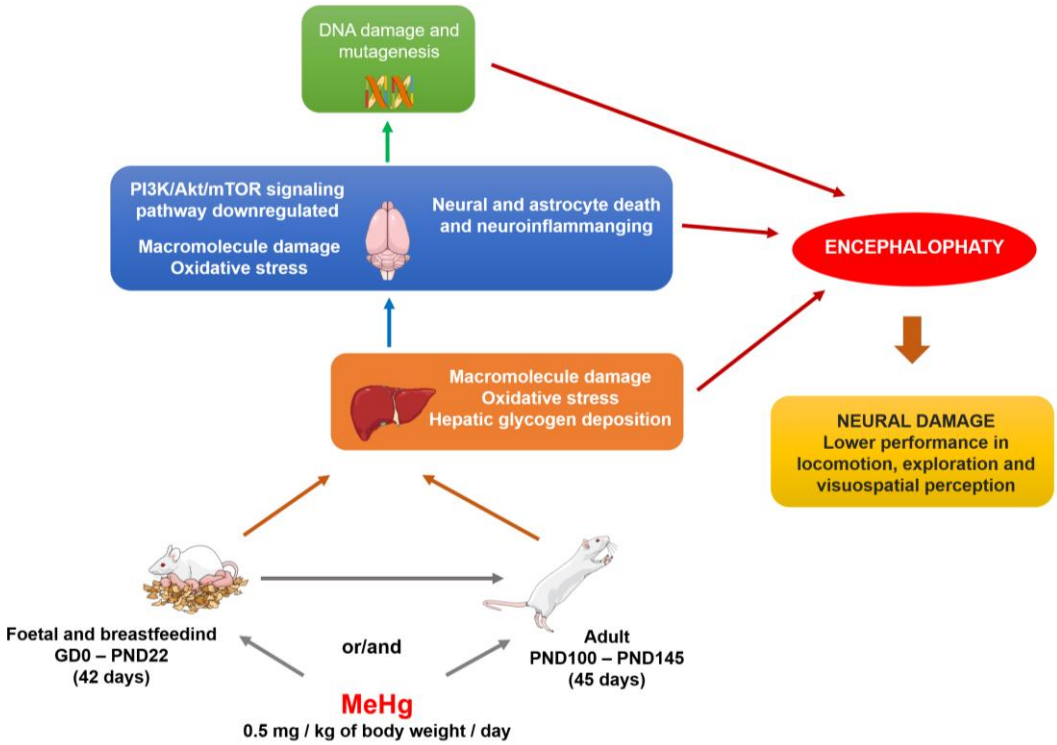
Figure 5. Effect of adult exposure to MeHg on anxiety-like behavior at days 99, OFT session 1, and 145, EPMT and OFT session 2. In OFT each animal freely explored the open field test apparatus for 10 min in two sessions. The total distance traveled in first 3 minutes (Fig 2A), center area time (Fig 2B), ration of center distance travelled, and total distance travelled (Fig 2C), rearing (Fig 2D) and grooming (Fig 2E) time. In EPMT, activity time (Fig 2F), entries into open arms (Fig 2G), time on open arms (Fig 2H) and the risk assessment (Fig 2I). Were collected and recorded with ANY-Maze video tracking Software. Results are expressed as mean ± SEM for ten male animals. Statistically significant differences from control group, by one-way ANOVA following by Tukey's post hoc test. Dashed lines show the groups that were compared. Control (n = 10) – gray bars (light gray bars: test in PND 100; dark gray bars: test in PND 145). Double – exposure group: DE (n = 10) – red bars (light red bars: test in PND 100; dark red bars: test in PND 145). Single – exposure: SE (n = 10) – green bars (light green bars: test in PND 100; dark green bars: test in PND 145).

GRAPHICAL ABSTRACT

previous graphical abstract:



updated graphical abstract - ARTWORK



3.2 *Effects of foetal and breastfeeding exposure to methylmercury (MeHg) and retinol palmitate (Vitamin A) in rats: Redox parameters and susceptibility to DNA damage in liver.*

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Effects of foetal and breastfeeding exposure to methylmercury (MeHg) and retinol palmitate (Vitamin A) in rats: redox parameters and susceptibility to DNA damage in liver.

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Abstract

Methylmercury (MeHg) is known to be a chemical that poses a risk to public health. Exposure to MeHg and vitamin A (VitA) occurs through the ingestion of fish, present in the diet of most pregnant women. The absorption of these elements generates oxidative stress and can generate adaptations for future stressful events. Here, we assessed how exposure to VitA and/or MeHg during the fetal and breastfeeding period modulates the toxicity of MeHg reexposure in adulthood. We focus on redox systems and repairing DNA damage. Male rats (n=50), were divided into 5 groups. Control received mineral oil; The VitA group received VitA during pregnancy, during breastfeeding and was exposed to MeHg in adulthood; VitA+MeHg received VitA and MeHg during pregnancy and breastfeeding and was exposed to MeHg in adulthood. The single exposure group (SE) was exposed to MeHg only in adulthood; and the MeHg group was pre-exposed to MeHg during pregnancy and breastfeeding and re-exposed to MeHg in adulthood. After treating the animals, we evaluated the redox status and the level of DNA damage in all rats. The results revealed that MeHg significantly decreased the activity of glutathione peroxidase (GPx) and sulfhydryl levels and increased the activity of superoxide dismutase (SOD), glutathione transferase, glutathione and carbonyl in all exposed groups. These results suggest that the second exposure to MeHg directly altered the effects of oxidation and that there were no specific effects associated with exposure during the fetal and breastfeeding periods. In addition, our findings indicate that MDA levels increased in MeHg and SE levels and no differences in MDA levels were observed between the VitA and MeHg+VitA groups. We also observed that animals pretreated exclusively with VitA showed residual damage similar to the control's DNA, while the other groups showed statistically higher levels of damage. In conclusion, low doses of MeHg and VitA during fetal and breastfeeding periods were unable to condition an adaptive response to subsequent exposure to MeHg in adulthood in relation to the observed levels of oxidative damage assessed after exposure.

Keywords: Methylmercury, vitamin A, DNA-damage, oxidative stress, DNA-repair

1. Introduction

Mercury (Hg) has been identified as a chemical of major concern to public health by the World Health Organization (WHO) [1]. In the environment, methylmercury (MeHg) bioaccumulates and biomagnifies throughout the food web, enabling it to reach concentrations within fish and other organisms that are many times higher than their surrounding environments. This phenomenon produces serious health concerns for humans [2,3]. Dietary intake of fish is major route of human exposure to MeHg. Populations with a traditionally high dietary fish intakes are the most susceptible to accumulating harmful levels of MeHg and are most likely to experience deleterious health effects associated with exposure [4–6]. Populations within the Brazilian Amazon, where gold mining activities generate high levels of MeHg contamination along rivers and within the soil, serves as a critical example of the negative impact of MeHg, In the region, MeHg concentrations in food exceed that which has been deemed acceptable according to Brazilian legislation, and has placed the local population at risk [7–10].

Once absorbed, MeHg is rapidly transported via red blood cells throughout the body since it is highly permeable to membranes. The chemical is distributed widely, particularly to the liver, kidneys and central nervous system (CNS); however, MeHg conversion to inorganic Hg and its subsequent storage is predominantly carried out in the liver and kidneys [10]. Like Hg, MeHg can penetrate the placental and blood-brain barriers of the foetus. The foetus, which is not fully developed, is sensitive to exposure to MeHg and toxic effects are increased relative to adult exposure and can cause irreversible damage [11,12]. Human foetuses and newborns are particularly vulnerable to MeHg-induced brain damage and are systemically sensitive to even low-levels of MeHg exposure. Therefore, as a particularly susceptible group, dangers of MeHg exposure to pregnant women, especially in populations where fish is the main source of animal proteins, require further elucidation [13–15].

Maternal fish consumption during pregnancy exposes the foetus to substances and nutrients within the fish simultaneously. These include n-3 fatty acids, minerals, and vitamins, including vitamin A (VitA), a fat-soluble retinoid also presents in eggs and dairy products VitA may be obtained from both a vegetal and animal diet [16]. High levels of preformed VitA are found in liver and fish [16–18]. Several important metabolic and physiologic processes of the organisms require VitA including vision, hematopoiesis, embryonic development, cell differentiation, immunocompetence and gene transcription [19,20]. In pregnant women, VitA contributes to early foetal development, especially within the CNS [21–23], and VitA supplementation is recommended independently of previous VitA consumption in safe doses between 3,000 and 7,500 µg RAE daily [24]. Both MeHg and VitA have pro-oxidant characteristics. Reactive oxygen species (ROS) generation has been linked to MeHg- induced toxicity within both in vivo and in vitro systems. Evidence suggests that MeHg exposure causes the production of ROS [25,26], depletion of glutathione (GSH) [27], excessive accumulation of calcium (Ca^{2+}) [28], apoptosis/necrosis [29], hepatotoxicity and can lead to neural damage [15] and decreased mitochondrial membrane potential in nervous [30]and immune systems [29]. In rats, VitA supplementation at therapeutic doses resulted in liver impairment and kidney redox imbalance in mothers and their offspring [31–33]. VitA also possesses

teratogenic effects at higher doses (retinoic acid syndrome), which produce an array of birth defects including craniofacial, cardiovascular and thymic abnormalities [34,35]. Thus, during the first weeks of embryogenesis, supplementation with VitA must be carefully managed to avoid congenital malformations caused by either deficiency or excessive intake of the chemical [36,37]. Nevertheless, the nutritional requirements of women increase during pregnancy, especially with respect to fish and shellfish, which are the primary source of long-chain polyunsaturated fatty acids [38]. Despite recommendations from global committees in regard of MeHg exposure, fish consumption in pregnant women has exclusively increased in the US [38,39]. The increase creates a possible scenario in which MeHg and VitA bioavailability is increased, which poses unknown risks to the expectant mother and her foetus.

Many types of stress, including continuous exercise, have the capacity to generate an adaptation to future stressful events. This effect is likely caused as a result of small-intensity damage triggering mechanisms of cellular stress control, which can lead to the facilitation of their future activation and, consequently, greater tolerance of the organism to similar levels of disruption [39]. Interestingly, some studies have shown that oxidative stress can generate this type of adaptive response [40,41]. Notably, most studies have focused on the effects of occupational exposure in rats during one stage of their life cycle, such as maternal and foetal, foetal or adult stage rats. Individual effects of MeHg and VitA exposure are well documented. However, there is a lack of information regarding the impact of combined types of exposure and how previous exposure affects the physiological response to subsequent MeHg exposure [15,42,43].

This study aimed to assess how previous exposure to VitA and MeHg and co-exposure to both agents during the fetal and breastfeeding period affecting MeHg toxicity when reexposed during adulthood. The experiments were carried out on blood, liver and bone marrow of Wistar rats, focused on redox systems and DNA damage repair mechanisms.

2. Material and methods

Animal handling followed guidelines specified in Laboratory Animal Care Principles and the Brazilian Society of Laboratory Animal Science (SBCAL-COBEA) [44]. The Animal Experimentation Ethics Committee (CONCEA) of the Federal University of Rio Grande do Sul (UFRGS) approved the research protocol (UFRGS authorisation number 31795).

2.1. Animals and housing conditions

All animals were maintained under a light cycle of 12 h (07:00 to 19:00) in a temperature-controlled room (20°C) and housed in polypropylene cages (43 cm x 30 cm x 15 cm), with access to standard pelleted ration (CR1 Lab Chow, Nuvilab, Curitiba, Brazil) and filtered water ad libitum. Female Wistar rats (200 - 250 g) from our breeding colony were monitored daily for two weeks for their estrous cycle using vaginal smear examination under a light microscope following procedures described previously [13,45]. Sexually receptive

females (confirmed proestrus) were caged overnight with a mature male (1F:1 M). Successful mating was evaluated the following morning by the presence of viable spermatozoa in the vaginal saline smear, which marked the first gestational day (GD0) [45].

Then, the rats were randomly divided into five groups, n = 50 and kept in cages (n = 10 animals/group). For both groups, the duration of exposure ranged from the beginning of gestation (GD0) to the end of the puppy breastfeeding period, which corresponded to postnatal day 21 (PND21), and totaled an average of 42 d.

Treatments were prepared daily and administered orally with a metallic metal gavage at a maximum volume of 0.5 mL. Treatment administration was performed in a specific clean area, away from the accommodation room of the colonies, at approximately 17:00 to ensure the maximum absorption of food from meals during the dark cycle. In our previous work, we did not observe motor changes or general health disorders during pregnancy, lactation or the adult life of any of the rats used in experiments [13–15].

2.2 Treatment and experimental design

Methylmercury (II) chloride from Spectrum (New Brunswick, NJ, United States) was used. A 0.39 μg × μL^{-1} stock solution of MeHg was prepared in mineral oil (Embrafarma, São Paulo, SP, Brazil). One group of females (n = 10) were treated with MeHg using a gastric tube (0.5 mg × kg^{-1} body weight daily). VitA, a final dose of 7,500 μg RAE × kg^{-1} daily (retinyl palmitate as the sole source of VitA) was administered using dams. Treatment was based on a 10-fold uncertainty factor of interspecific variation and a 10-fold factor of intraspecific variation, according to USEPA guidelines for assessing maternal development and toxicity [5]. Our palmitate retinyl (RAE) dosage was previously used in animal models, with no general effects of toxicity observed in mothers and pups used to assess VitA supplementation [13,40,41]. A dose of VitA is considered safe for supplementation in pregnant women that had not previously consumed the micronutrient [46,47]. It was scaled for animal testing and the final dosage used was well below the lowest teratogenic dose (LOAEL) of rat-RAE (48.900 μg RAE × kg^{-1} daily) that had been previously established [47]. Co-exposure treatment (MeHg + VitA) was prepared by diluting an 8 mL retinol stock to a final volume of 60 mL in mineral oil and finally dissolving 23.4 mg methylmercury (II) chloride within the solution. Two groups (n = 10 / group) received only mineral oil (1 μL × g^{-1} body weight daily). On PND21, pups were weaned, and the offspring and/or mothers and extra rats were sacrificed. Adult males, n = 10 / group (250–300 g), were kept in their original groups and were not exposed to treatment from PND22 to PND99. All groups that had previously received treatment during a prenatal phase were also treated in adulthood (PND100) with 0.5 mg MeHg × kg^{-1} body weight daily. VitA, MeHg and VitA + MeHg remained. the Single Exposure (SE) group, which had previously received mineral oil, was treated with 0.5 mg MeHg × kg^{-1} body weight daily. The control group (CN) received only mineral oil (1 μL × g^{-1} body weight daily). Both groups were held for 45 consecutive days (PND100 to PND145). The representation of the treatment scheme can be seen in Figure 1.

2.3 Tissue preparation and protein determination

All animals were sacrificed by decapitation 24 h after the administration on their last treatment. Liver samples were collected and stored at -80°C to assess redox systems and macromolecular damage. Femurs were removed to perform bone marrow erythrocyte micronucleus analyses (MNPCE). To prepare samples, tissues were homogenised in 50 mM phosphate buffer (pH 7.0) and centrifuged (3,000 x g for 5 min) to remove cell debris [13,15]. Blood was used to quantify DNA damage via comet assay. Protein quantification within liver samples was measured using the method described by Lowry in 1951 [48]. Biochemical results were normalised to protein content using bovine serum albumin (BSA) as a standard. In this research, we do not quantify the levels of Hg and VitA. However, in previous works [13–15], these levels, in similar treatment regimens, were presented.

2.4 Redox and damage profiles

2.4.1 Enzymatic activities in liver

The enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) in the liver were analysed using tissues homogenised with 50 mM phosphate buffered saline (PBS, KH_2PO_4 and K_2HPO_4, pH 7.4) according to methods described previously [49–52], respectively. The GSH method, which assessed emission from the o-phthalaldehyde (OPT) fluorophore, was performed according to methods previously described by Browne and Armstrong (1998) [52], and the results were expressed in nmol GSH × mg^{-1} protein. In addition, the SOD, CAT and GPx ratios were evaluated in relation to the total protein values and compared to the control group for a better understanding of the treatment effects on the enzymatic antioxidant system, since the enzymes work within pathways that convert superoxide anions in water [53]. The results were expressed as the ratio of each enzyme to the total protein [U SOD × (U CAT + GPx) $^{-1}$] / mg of protein.

2.4.2 Cellular oxidative damage liver

These analyses were performed using liver samples. Total reduced thiol (SH) content was examined according to a protocol described previously [54]. Briefly, SH content was estimated through evaluating the overall cellular redox status and results were expressed in μmol SH × mg^{-1} protein. Carbonyl groups were used as a parameter for assessing oxidative damage in proteins and were based on reactions with dinitrophenylhydrazine (DNPH), as previously described by Levine et. al. [55,56]. Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and re-solubilised in DNPH. Next, the absorbance was read at 370 nm with a spectrophotometer. Results are expressed as ηmol carbonyl × mg^{-1} protein.

Measurements assessing the formation of malondialdehyde (MDA) were performed as described previously. MDA was estimated via its index of lipid peroxidation, and results are expressed as ηmol MDA × mg^{-1} protein [54,55,57].

2.4.3 Alkaline comet assay in blood

The alkaline comet assay was performed according to Collins et. al. [58] and Singh et. al. [59] and adaptations by Da Silva et. al. [60], were incorporated. Blood samples were homogenised in a 0.7% low-melting-point agarose solution, placed on a slide pre-treated with 1.5% agarose and exposed to lysis solution. After lysis, the slides were placed in a horisontal flask containing alkaline buffer, and after 20 min DNA was then separated via electrophoresis (25 V and 300 mA) for 15 min. The slides were neutralised, fixed and stained with silver nitrate. The analysis was performed using conventional optical microscopy and analytical criteria described in the literature were applied. To calculate the damage index (ID) of samples, the nucleoids were classified according to tail size in relation to the size of the comet's head (no damage = 0 to maximum damage = 4). The damage index (ID) of the group ranged from 0 (no damage = 100 cells completely × 0) to 400 (maximum damage = 100 cells × 4). Data were expressed as damage Index values, which ranged from 0 to 400.

2.4.4 Comet assay to assess susceptibility to exogenous DNA damage

Using the comet assay, we evaluated the assess susceptibility to exogenous DNA damage. After making the slides at time t0', the remaining sample was treated with H_2O_2 (75 μM) for 30 min at 37 °C, in an incubator with 5% CO_2. After this treatment, the sample was washed with PBS, in order to remove H_2O_2, and then the slides were made with the damage insult after 30 minutes (t30') of the t0' slides. The sample is incubated again at 37 °C in an incubator with CO_2, for 1h. Induced damages are expected to be repaired during this period. After that time, slides t90'are made, or 90 minutes after t0'. Again, the sample is incubated at 37 °C for 1h, and then the t150'slides are made (150 minutes after baseline). Then, the slides are placed in a lysis solution and the remaining steps follow the original protocol of the comet assay described in section 2.4.3. The percentage of residual damage (RD) induced with H_2O_2 to DNA after 150 minutes (t150') was calculated using the value determined at t30' as 100% DNA damage [60].

2.4.5 Micronucleus test in polychromatic erythrocytes of bone marrow

A micronucleus assay was performed according to methodology previously described by Horta et. al. [61] in accordance with Organization for Economic Co-operation and Development (OECD) guidelines [62]. Bone marrow was extracted, and the smears were prepared directly on slides, two per animal, with one drop of

foetal bovine serum. The slides were stained with 10% Giemsa for 5 min, air dried and encoded for blind analysis. To avoid false-negative results (and to measure bone marrow toxicity), polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) were classified using 2,000 cells. The frequency of micronuclei observed in the 2,000 PCE (MNPCE) of each animal (i.e., 1,000 from each of the duplicate slides prepared) using conventional light microscopy.

2.5 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM), and differences were considered significant when $p \leq 0.05$. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) and post hoc analyses were used to detect differences between experimental groups and their respective controls. One-way ANOVA was conducted, followed by Tukey's post hoc test, to assess data from the comet assay and MNPCE. Data analysis was performed, and graphics were created using GraphPad Prism version 8.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. RESULTS

3.1 Changes in redox and detox systems

The effects of MeHg-exposure throughout foetal and breastfeeding and adult stages on redox parameters and detox systems in the liver are shown in Figure 2. Both exposure during both periods significantly increased SOD activity (Fig. 2A). CAT activity was not altered by exposure (Fig. 2B), while GPx activity decreased (Fig. 2C). An increase in the proportion of SOD, CAT, and GPx [SOD/ (CAT + GPx)] was observed in all groups that had been exposed (Fig.2D). GST activity and GSH levels were also increased in all exposed groups. In addition, the VitA group had significantly less GSH content compared to the other groups.

3.2 Oxidative damage markers

Regarding oxidative damage markers, MDA levels were increased in MeHg and SE groups (Fig. 3A). No difference was observed in MDA levels of ViTA and MeHg+VitA groups. Moreover, there was a reduction in SH levels in all groups (Fig. 3B) and the opposite trends were observed for levels of carbonyl groups observed in all exposed animals (Fig. 3C).

3.3 DNA damage

Figure 4 contains the basal damage (or t0 ') to DNA and the ability to repair after insulting DNA with H_2O_2 treatment. The rate of DNA damage in the control group increased after 30 minutes of H_2O_2 treatment when compared to t0 'in the same group (***$p \leq 0.0001$) and decreased after 90 minutes but remained higher than

t0' (**$p \leq$ 0.001). The total repair of DNA damage in the control group occurs at t150'. There was no statistically significant difference at t0' and t30' between the groups. However, all treated groups showed a higher damage index at t90' (MeHg, MeHg + VitA and SE: *** $p \leq$ 0.0001; VitA: **$p \leq$ 0.001) and at t150' (MeHg: ***$p \leq$ 0.0001; MeHg + VitA and SE: **$p \leq$ 0.001; VitA: *$p \leq$ 0.05). The damage not repaired after t150 'is called "residual damage" or RD-DNA. To calculate this parameter, we consider that the DNA damage at t30 'is 100% for each group; and at 150 'it is analyzed which% of damage (at 100%) was not repaired. It was observed that the animals pre-treated with VitA (mean of 44.10%) presented levels of RD-DNA similar to the controls (mean of 27.71%), while the other groups presented statistically higher mean values (MeHg 100%, MeHg + VitA 76.42% and SE 89.97%), indicating that less RD is associated with the repair process, while the increase indicates greater susceptibility to induced damage.

Data on the frequency of micronuclei in polychromatic erythrocytes (2000 PCE) and the ratio between normochromatic and polychromatic erythrocytes are shown in Table 1. Regarding the frequency MNPCE, all exposed groups showed increases in this parameter and no statistical differences was found between treated groups. In addition, we identified a decrease in the PCE:NCE ratio for MeHg ($p \leq$ 0.002) and SE ($p \leq$ 0.009) groups.

4. DISCUSSION

Several previous studies have suggested that antioxidant supplementation may provide substantial protective effects against various oxidative stress-related human diseases [15,63–65]. Moreover, it has been postulated that oxidative stress can generate an adaptive response to different oxidants [40,41]. Some antioxidants such as vitamin E [66,67] and vitamin C [68,69] have been shown to induce protective effects against damage to MeHg in various models. VitA has redox properties and is also known for its antioxidant effects in various test systems However, Espitia-Perez et. al. showed that even at low doses of MeHg co-administered with VitA supplementation during pregnancy and lactation, it promotes changes in redox status in mothers and children [13]. Co-administration of VitA in the fetal and breastfeeding period has no protective effect on adult MeHg exposure [13,14].

Regarding VitA dose and rationale used in this study, we can highlight some interesting facts. Our administrated dose for dams can be translated as a human daily dose of 1216,22 μg RAE, using the body surface area (BSA) approach, as previously described [91]. This supplementation is well above the daily Recommended Dietary Allowance (RDA) for VitA considering only retinyl palmitate as a dietary source, and a healthy VitA consumption, since we exclude β-carotene supplements, α-carotene or β- cryptoxanthin. However, our supplementation dose is also below the daily Tolerable Upper Intake Level (UL) for VitA (3000 μg RAE) [92]. Coincidently, an intake resembling our model is the RDA for women in reproductive age during pregnancy and primarily through the lactation period (1300 μg RAE), were VitA demand increases [92]. Considering our results suggesting the protective role of VitA in MeHg adulthood exposure,

we recommend future studies of dietary vigilance on the effects of VitA supplementation, especially in Hg influenced areas, particularly in childbearing women as a more susceptible group. Although our work is merely a pre-clinical approach, we encourage to elucidate whether possible MeHg-VitA modulations can persist through a lifespan assuming a protective role.

Our analysis of liver tissue revealed an imbalance in the antioxidant enzyme activity of all groups (Figure 2), in which SOD activity was increased and GPx activity was reduced. This suggested a direct effect of the second MeHg exposure and did not indicate that specific effects were produced as a result of foetal and breastfeeding exposure. No changes in CAT activity were observed between the groups exposed to MeHg. Previous work by our group also showed that exhibitions do not alter CAT activity [70,71]. SOD/ (GPx + CAT) ratios increased, altering their response to oxidative stress as a result of excess H_2O_2 production in all groups [72]. Our results are in accordance with other studies that have shown that H_2O_2 plays a central role in MeHg oxidation mechanisms [73,74] functioning mainly by changing glutathione metabolism [75,76].

Our results also revealed increases in carbonylated proteins and decreases in thiol content, which indicated that protein-protein interactions and protein folding were misregulated. These effects likely contributed to MeHg toxicity. Protein carbonation is a product of the irreversible oxidation of various amino acid residues, and the frequency of the modification can disrupt cell signaling and promote the generation of highly cytotoxic substances [33,76,77]. Reduction of SH groups also can lead to the excessive generation of dissolved contacts, altered protein folding, aggregation, degradation and even cell death [78]. No differences were observed between SE, VitA and VitA+MeHg groups, indicating a direct effect of oxidation status on protein disruption, which occurred as a result of the second MeHg exposure. Accumulating evidence continues to indicate that adult MeHg exposure can disrupt redox homeostasis and contribute to the development of several illnesses and the accumulation of oxidative damage. Kirkpatrick et al. showed that MeHg exposure can alter levels of biomarkers of oxidative stress, especially with respect to GPX and thioredoxin reductase levels [77]. Further, the compound alters CAT and SOD activities [79] and disrupts protein and lipid organisation [13,33,53]. These results seem to indicate that the second exposure to a toxicant probably has a direct effect on the antioxidant defense system or may promote carbonylation and reduce thiol content [13,33,53].

Interestingly, only animals treated in both phases of life with MeHg (MeHg group) and the group exposed to MeHg only in adulthood (SE group) showed increased levels of thiobarbituric acid reactive substances (TBARS). In addition, the VitA group has lower levels of GSH compared to other groups. This result suggested that fetal exposure and breastfeeding to VitA, even in co-exposure to MeHg, had a modulating / protective effect against subsequent exposure to MeHg. VitA has redox properties and is also known for its antioxidant effects [80-82]. These effects provide additional protection from lipids and membranes that persists throughout adult life. In addition, previous results indicated that the co-administration of vitamins A

and C can provide protective effects, such as the modulation of glutathione metabolism (also seen in our results) against oxidative-antioxidant imbalances induced by a xenobiotic [70,80,81].

However, as discussed earlier, these effects depend on the concentration. In addition, previous studies have shown that VitA and its derivatives have the ability to protect against lipid peroxidation, DNA damage and impaired cell redox status [83-86].Regarding DNA damage (Figure 4), several *in vitro* [86,87] and *in vivo* studies [13–15] have already demonstrated the genotoxic and clastogenic effects of MeHg. Damage observed in the comet assay is indicative of recent, repairable DNA damage associated with adult exposure (MeHg). H_2O_2-induced susceptibility (75μM) to DNA damage was measured at 30, 90 and 150 minutes after treatment. Using the control group as a standard, Figure 4 contains data used to assess susceptibility to H_2O_2-induced DNA damage (75 μM) in treated animals. These analyzes were observed at times t30', t90' and t150'. DNA damage at t30' is considered 100% DNA damage for all groups. At t30', DNA damage is high in all groups, including control. After 1 hour of t30', at t90', the damage is expected to be reduced, as occurred with the control group. However, only the VitA and MeHg + VitA groups had this damage reduced, while the MeHg and SE groups showed an increase in this damage. After 2 hours and 30 minutes (t150'), in the control group there was almost complete repair of DNA damage induced by H_2O_2. The exposed groups, on the other hand, show late DNA repair, which is high when compared to the control group at the same time. Residual damage is calculated using t30 'as 100% damage, and at t150' it is observed how much (in %) residual damage remains. Only the VitA group showed residual damage (44.4%) like the control group (27.51%).

The groups pre-exposed and / or co-exposed with MeHg show late DNA repair, on the other hand, the repair of DNA damage in the ViT group is similar to the control. Residual damage is calculated using t30 'as 100% damage, and at t150' it is observed how much (in%) residual damage remains. Again, Only the VitA group showed residual damage (44.4%) like the control group (27.51%).

This indicated that the groups pretreated with MeHg or MeHg and VitA, and then exposed only to MeHg, are more highly susceptible to exogenous damage, than the group that was exposed in the fetal phase and breastfeeding only VitA (VitA group). It is possible that foetal and breastfeeding exposure to VitA played a protective role in inducing oxidative damage by MeHg following H_2O_2 challenge. Exposure to MeHg is known to promote the formation of ROS, which can induce conformational changes in proteins responsible for DNA repair, mitotic spindle formation and chromosomal segregation [78] by the inhibition of poly (ADP-ribose) polymerase-1 (PARP-1) activity, which repairs DNA strand breaks [38,76,88]. The DNA damage observed here was probably generated via a pro-oxidant pathway. All exposed animals possessed increased MNPCE frequencies when compared controls, Table 1. Increased MNPCE frequency in adult rats is related to inefficient DNA repair or the induction of cell apoptosis [80,81]. Interestingly, groups that were not exposed to VitA during foetal or breastfeeding periods (MeHg and SE) had lower PCE:NCE ratios than controls, indicating increased effects of toxicity in these animals. In general, frequent exposure to heavy

metals such as mercury derivatives, can lead to reactions that generate ROS [68,89,90]. Our results highlight the importance of analyzing the effects of frequent oxidative damage, which is caused by repeated MeHg exposure throughout life, especially in scenarios in which new exposure takes place. Further, increased MeHg and VitA bioavailability may increase instances in which multiple-dose MeHg exposures occur over their lifetimes and produce unknown effects. New studies will be necessary to clarify the molecular basis of MeHg exposure-related injuries that occur as a result of exposure during developmental periods and in adult life.

5. CONCLUSION

In conclusion, low doses of MeHg and VitA during foetal and breastfeeding periods do not condition an adaptive response to subsequent MeHg exposure in adulthood with respect to levels oxidative damage are assessed. However, the presence of Vitamin A, both in pure administration during fetal exposure and breastfeeding, and in co-exposure with MeHg, seems to have a protective role on the effects of MeHg exposure in adulthood in markers of antioxidant activity, damage to macromolecules and susceptibility to DNA damage.

In short, exposure to MeHg in adulthood causes an increased redox imbalance and damage to macromolecules, such as DNA, are observed in exposed animals. Importantly, damage to biomolecules from MeHg exposure was mutagenic and affected hematopoiesis. However, the toxic effect on hematopoiesis (Ratio PCE: NCE) was not observed in animals pre-exposed in the fetal phase and breastfeeding with Vitamin A (VitA and MeHg + VitA groups), suggesting that Vitamin A has a modulating and protective effect about that mechanism. Obviously, further studies are needed to confirm findings presented here and to further elucidate molecular mechanisms involved in DNA signaling and repair in animals exposed to MeHg and VitA. To date, there has been few studies that have investigated the direct effects of MeHg and VitA on responding to and repairing DNA damage.

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Compliance with ethical standards

This manuscript does not contain clinical studies involving patients. All applicable international, national, and institutional guidelines for the care and use of the animals were followed. The Animal Experimentation

Ethics Committee (CONCEA) of the Universidade Federal do Rio Grande do Sul (UFRGS) approved the research protocol under authorisation number 31795.

Conflict of interest

The authors declare that they have no conflict of interest.

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Graphical Abstract - MUTGEN-D-20-00013R3

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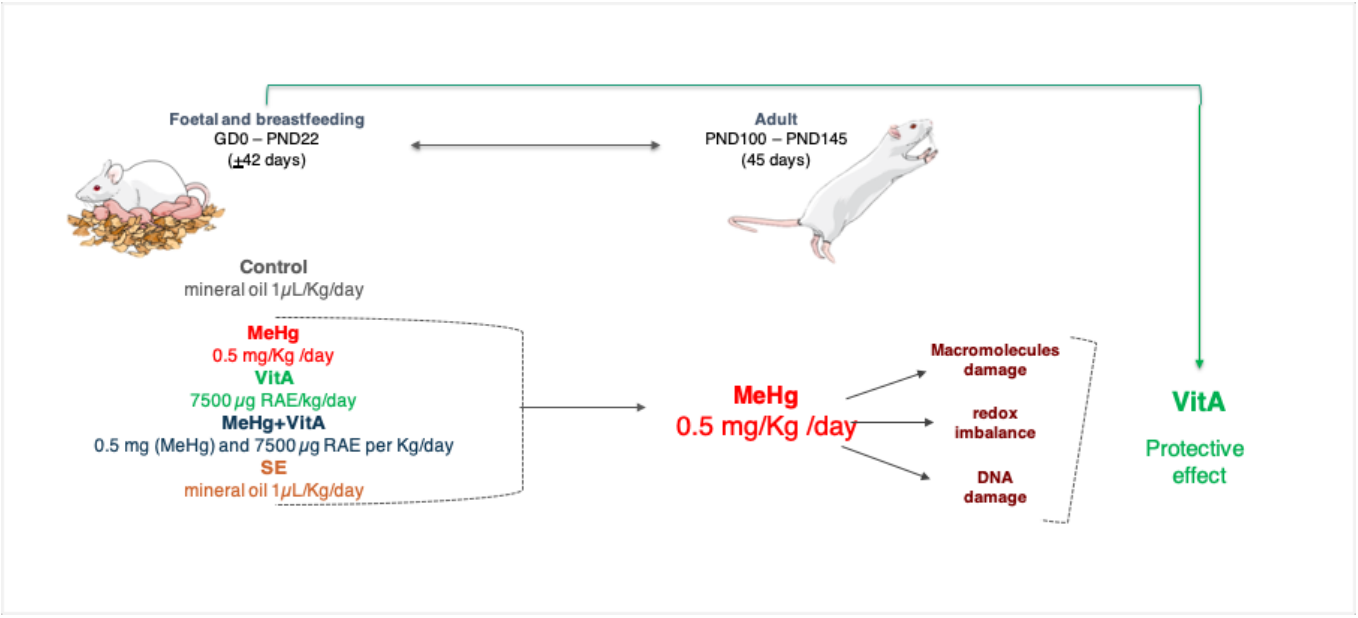


FIGURES

Figure 1.

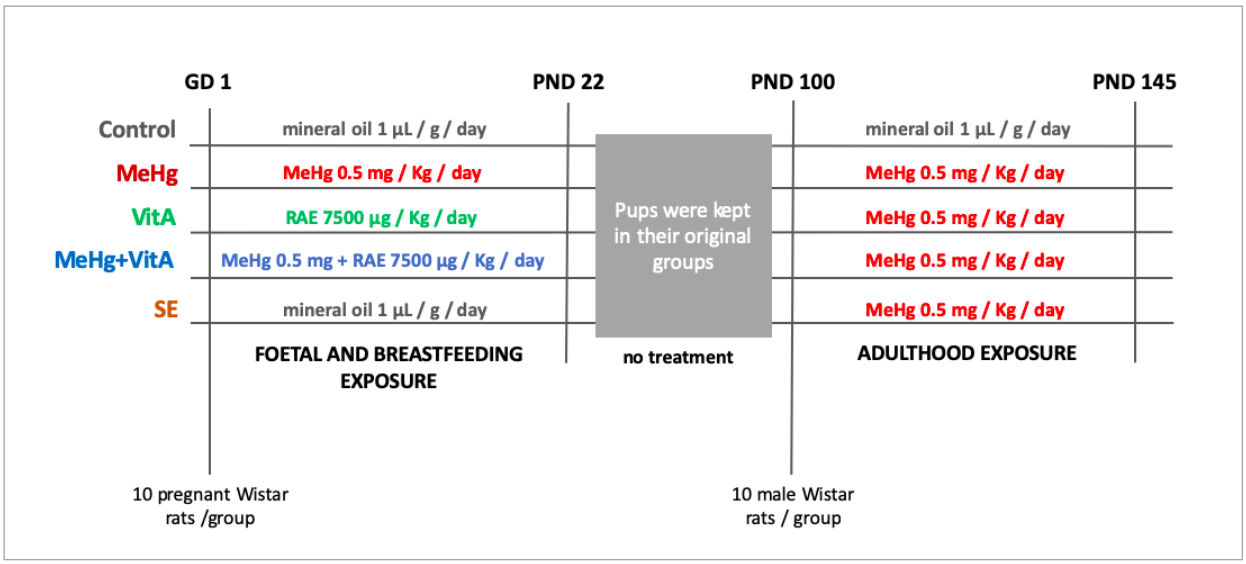


Figure 2.

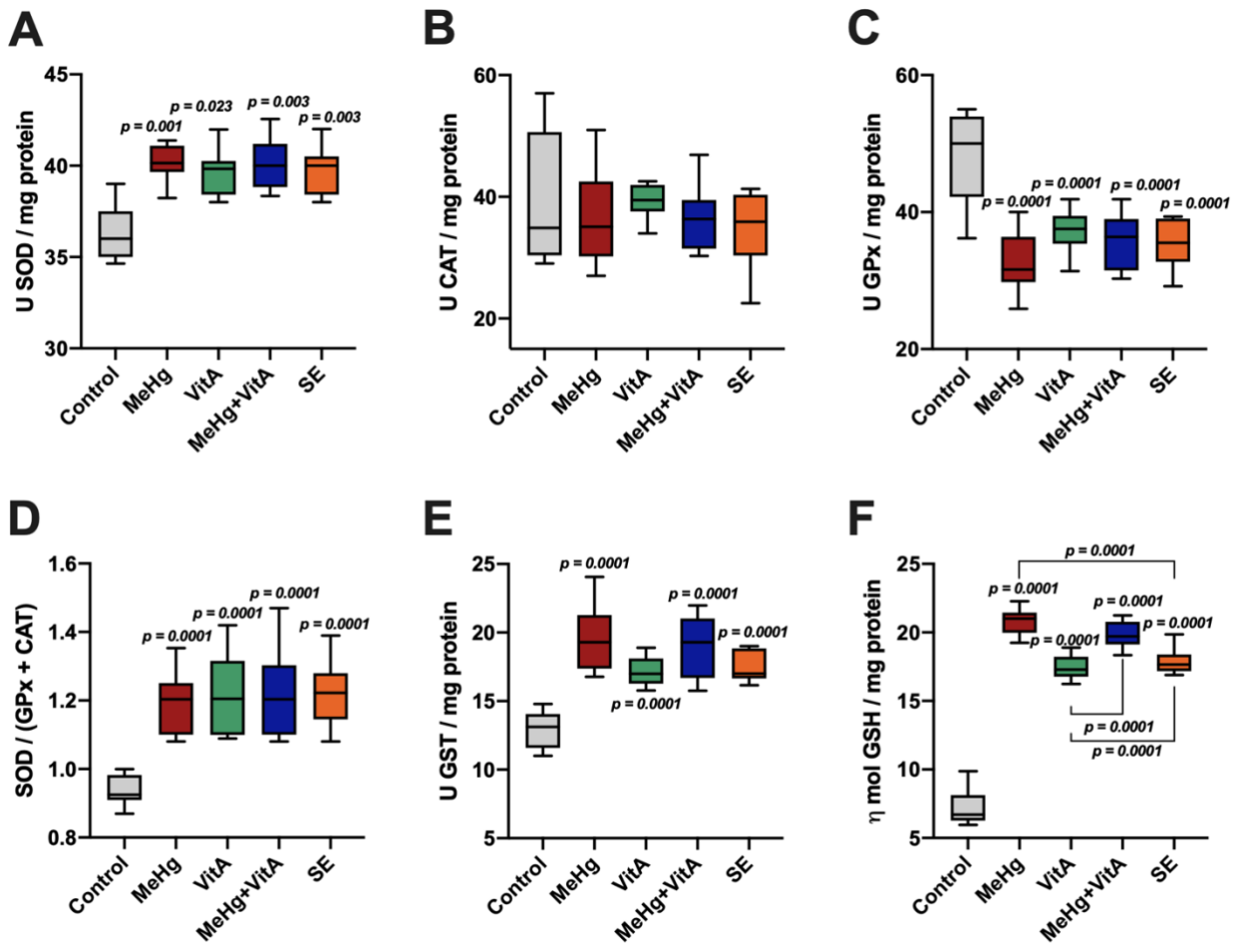
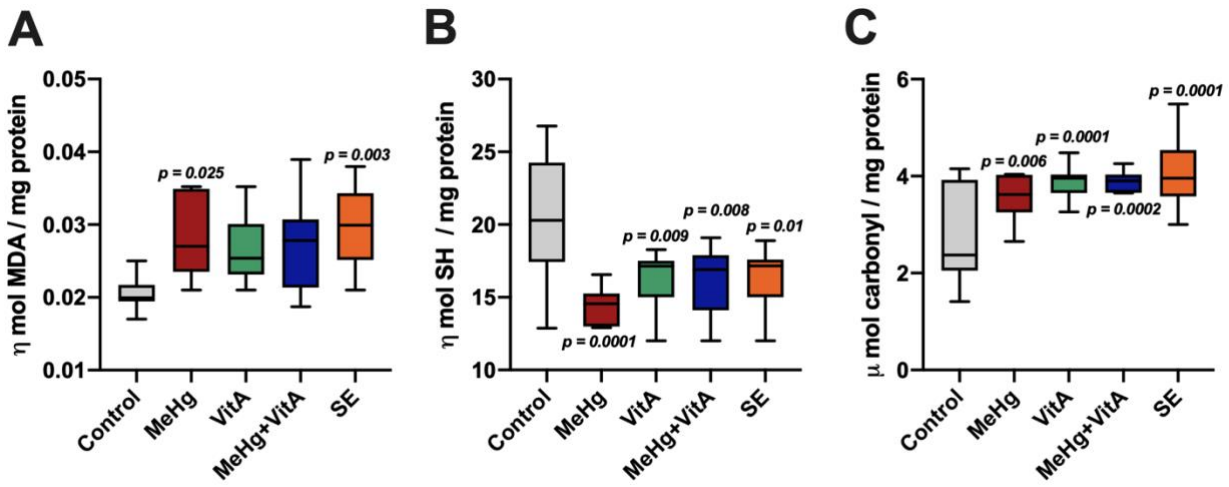
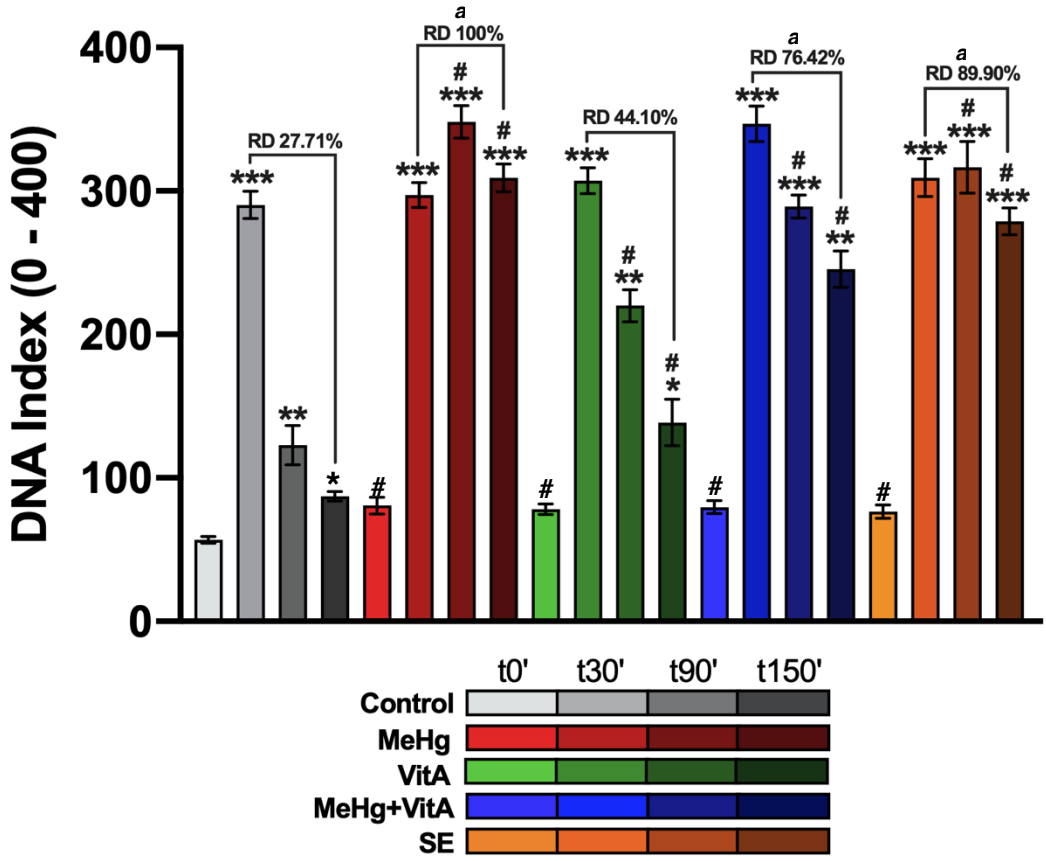


Figure 3.


A
η mol MDA / mg protein
0.05
0.04
0.03
0.02
0.01
p = 0.025
p = 0.003
Control
MeHg
VitA
MeHg+VitA
SE
B
η mol SH / mg protein
30
25
20
15
10
p = 0.0001
p = 0.009
p = 0.008
p = 0.01
Control
MeHg
VitA
MeHg+VitA
SE
C
μ mol carbonyl / mg protein
6
4
2
0
p = 0.006
p = 0.0001
p = 0.0002
p = 0.0001
Control
MeHg
VitA
MeHg+VitA
SE

*

Figure 4.


DNA Index (0 - 400)
400
300
200
100
0
RD 27.71%
a
RD 100%
RD 44.10%
a
RD 76.42%
a
RD 89.90%
t0'
t30'
t90'
t150'
Control
MeHg
VitA
MeHg+VitA
SE

CAPTIONS

Figure 1. Figure 1. A treatment scheme for female Wistar rats (200–250 g) is shown. Regular reproductive cycles in which sperm were observed in the vaginal smear were used to identify gestational d zero (GD0). In GD 1, females were randomly divided into five groups, n = 10/group, and kept in cages. For both groups, the exposure periods for rats ranged from the beginning of gestation (GD0) through the completion of pup breastfeeding, postnatal d 21 (PND21), and totaled an average of 42 d. Throughout the gestational period until the weaning of the pups, which occurred on PND 22, which resulted in a 42 d treatment period. In PND 22, male offspring were weaned, and the females were sacrificed. Offspring were not treated from PND 22 to PND 99. Animals, male adults (n = 10/group) were kept in their source groups (MeHg, VitA, MeHg + VitA, SE and Control groups). All groups that had previously received treatment during a prenatal phase were treated again, now in adulthood (PND100), with 0.5 mg × kg^{-1} MeHg daily. Thus, the VitA, MeHg and VitA + MeHg groups remained. The SE group, which had previously received mineral oil, received the same treatment 0.5 × kg^{-1} MeHg daily. The control group (CN) received only mineral oil (1 µL × g^{-1} daily). All animals were held for 45 consecutive d (PND100 to PND145). Throughout the adult-exposure period, average food intake and individual body weight measurements were weekly collected. At the end of the treatment period, all animals were sacrificed, and tissues blood, liver and femurs were collected for further analysis.

Figure 2. Effects of MeHg exposure on redox parameters in the liver. 2A) SOD activity (U SOD × mg^{-1} protein). 2B) CAT activity (U CAT × mg^{-1} protein). 2C) GPx activity (U GPx × mg^{-1} protein). 2D) SOD × $(GPx + CAT)^{-1}$, in arbitrary units. 2E) GST activity (U GST × mg^{-1} protein). 2F) GSH activity (mol GSH^{-1} per mg protein). Results are expressed as a mean ± standard error (S.E.M.). Data were evaluated by two-way ANOVA followed by use of the Tukey's multiple comparisons test. *p* values are embedded in the figure. Dashed lines show the groups that were compared. The control group (n = 10) is indicated using gray bars; the MeHg group (n = 10) is indicated using red bars; the VitA group (n = 10) is indicated using green bars; the MeHg+VitA group (n = 10) is indicated with blue bars; and the SE group (n = 10) is shown using orange bars.

Figure 3. Effect of adult exposure to MeHg on production of damage markers. A) MDA content (ηmol MDA × mg^{-1} protein); B) Total thiol content (ηmol SH/mg protein); and C) Total carbonyl content (µmol carbonyl × mg^{-1} protein). Results are expressed as a mean ± standard error (S.E.M.). Data were evaluated by two-way ANOVA followed by use of the Tukey's multiple comparisons test. *p* values are embedded in the figure. Dashed lines indicate groups that were compared. The control group (n = 10) is indicated using gray bars; the MeHg group (n = 10) is indicated using red bars; the VitA group (n = 10) is indicated using green bars; the MeHg+VitA group (n = 10) is indicated with blue bars; and the SE group (n = 10) is shown using orange bars.

Figure 4. DNA damage and repair capacity detected by comet assay. Basal damage (t0') and damages in treatments times t30', t90'and t150'(minutes) post H_2O_2 treatment. Results are expressed as a mean ± standard error (S.E.M.). Data were evaluated by two-way ANOVA followed by use of the Tukey's multiple comparisons test. The color scale represents the treatment time: lightest tone from basal or t0'to the darkest, final time t150'and between the ends t30' and t90'. *Represents the statistical difference between baseline or t0 with the other times (t30', t90' and t150') in the same group (*$p \leq 0.05$, ** $p \leq 0.001$, ***$p \leq 0.0001$). **RD**: indicates % residual damage at t150'. [a]Represents the statistical difference of residual damage found between the groups. The mean value of the group in percentage (%) is shown in the image. ([a]$p \leq 0.05$). The control group (n = 10) is indicated using grayscale bars; the MeHg group (n = 10) is indicated using red scale bars; the VitA group (n = 10) is indicated using green scale bars; the MeHg+VitA group (n = 10) is indicated with blue scale bars; and the SE group (n = 10) is shown using orange scale bars.

Table 1. Detection of micronucleus in bone marrow polychromatic erythrocytes (MNPCE) and ratio PCE:NCE in prenatal and breastfeeding exposed and controls.

Groups (n = 10)	MNPCE in 2000 cells	Ratio (PCE: NCE)
Control	1.21 ± 0.35	1.35 ± 0.12
MeHg	**11.70 ± 1.32**	**0.84 ± 0.23***
VitA	**8.60 ± 0.61**	1.073 ± 1.01
MeHg+VitA	**10.81 ± 1.71**	1.51 ± 0.43
SE	**7.51 ± 1.12**	**0.90 ± 0.56***

Data are represented as mean ± standard error of the mean (SEM); Statistical analysis was performed using one-way ANOVA with a Tukey's multiple comparison test. **Bold** was used to indicate statistically significant differences between the group indicated and the control group ($p < 0.0001$); *Significant differences between a group compared to the control group (MeHg: $p < 0.002$ and SE: $p < 0.009$). Groups tested include control (n = 10); pre-exposed with MeHg (MeHg; n = 10); pre-exposed with VitA (VitA; n = 10); pre-exposed to MeHg and VitA (MeHg+VitA; n = 10) and single exposure tp MeHg (SE; n = 10).

4. DISCUSSÃO

Estudos recentes mostram que a contaminação pelo mercúrio (Hg) já atingiu escalas globais devido a sua biodisponibilidade no ambiente. Atualmente, o consumo de peixes representa 60-80% da via de contaminação por Hg e ele tem aumentado cerca de 20 Kg a cada ano (CISNEROS-MONTEMAYOR *et al.*, 2016). Em países subdesenvolvidos, o consumo de peixe é até 15 vezes maior em relação a populações de países desenvolvidos (AL-SALEH *et al.*, 2016; DEBES *et al.*, 2006; HSI *et al.*, 2016).

A exposição materna ao Hg e derivados, como o MeHg, no período fetal pode afetar o desenvolvimento de funções motoras, linguagem, atenção e memória de crianças e adolescentes (ESPITIA-PÉREZ *et al.*, 2018a; FUJIMURA; CHENG; ZHAO, 2012; SOKOLOWSKI *et al.*, 2013). A exposição crônica ou aguda ao MeHg produz efeitos inibitórios sobre a neurogênese hipocampal (ESPITIA-PÉREZ *et al.*, 2018a; NEWLAND; REED; RASMUSSEN, 2015). Estudos do nosso grupo já mostraram que a dose de 0.5 mg/Kg/dia em modelos animais durante a gestação produz efeito sutil, porém irreversível nos filhotes após a idade adulta (GANDHI, D. DHULL DINESH, 2014; GANDHI; PANCHAL; DHULL, 2013; NEWLAND; REED; RASMUSSEN, 2015; VITALONE *et al.*, 2008). A mesma dose já foi descrita por outros autores (OBIORAH *et al.*, 2015).

Em relação a suplementação com VitA, a dose utilizada é semelhante a encontrada na dieta da população (SCHNORR *et al.*, 2011a, 2011b). Utilizamos a concentração de 7500 µg RAE/Kg/dia (25000 UI/Kg/dia). Essa dose já foi utilizada em trabalhos do nosso grupo de pesquisa, sendo mais bem descrita por (SCHNORR *et al.*, 2015b). A dose é baseada em um fator de incerteza de 10 vezes para diferentes espécies, e é considerada uma dose segura para suplementação de VitA em mulheres grávidas sem pré consumo deste micronutriente (SCHNORR *et al.*, 2015b). Além disso, a dose utilizada se encontra abaixo da menor dose teratogênica (LOAEL) de palmitato de retinil em ratas (48900 µg RAE/Kg/dia), garantindo uma absorção estável de VitA pelo feto ao longo da suplementação (SCHNORR *et al.*, 2011b). Apesar da toxicidade, alguns pesquisadores postulam que a exposição contínua (incluindo agentes tóxicos) pode ser benéfica e permitir a adaptação ou modular futuros eventos tóxicos (RISTOW; SCHMEISSER, 2014; STOKES-RINER *et al.*, 2011).

No primeiro artigo expusemos um grupo de animais ao MeHg durante a fase intra-uterina e a amamentação e repetimos a exposição durante a vida adulta (grupo DE); outro grupo foi exposto apenas durante a idade adulta (grupo SE) e ambos grupos foram comparados ao grupo exposto apenas ao óleo mineral (grupo controle). Esse desenho experimental permitiu avaliar se havia um padrão adaptativo ou modulador em resposta à segunda exposição ao MeHg, avaliando marcadores de dano hepático e neural, uma vez que os danos causados pelo MeHg se concentram nesses tecidos (FARINA; ROCHA;

ASCHNER, 2011; ROEGGE *et al.*, 2004, 2011). Observamos sinais físicos de toxicidade do MeHg nos animais, como a redução do consumo padrão de ração e consequente perda de peso. Ambos os efeitos foram descritos anteriormente por (RICE *et al.*, 2014) e estão associados à falta de palatabilidade e irritação da mucosa intestinal causada por MeHg. Não houve diferenças significativas entre os grupos DE e SE em relação a este parâmetro.

A quantificação de Hg no fígado é confiável, pois fornece o conteúdo mais estável de Hg em comparação com a rápida depuração de Hg no cérebro (NEWLAND; REED; RASMUSSEN, 2015; NEWLAND; REILE; LANGSTON, 2004a, 2004b). Encontramos concentrações estatisticamente maiores de Hg no grupo DE (expostos no período fetal e na fase adulta) comparado ao grupo SE (exposto somente na fase adulta) nos dois tecidos. Sabe-se que os níveis de Hg absorvidos no útero se acumulam à nova exposição na vida adulta. Além disso, o grupo DE apresentou uma concentração relativamente mais alta de Hg no córtex occipital (CO) comparado ao SE, indicando que a liberação de Hg nesse tecido pode ter sido afetada (ROEGGE *et al.*, 2011; STRINGARI *et al.*, 2008)

Ainda, avaliamos marcadores de função hepática a fim de elucidar processos envolvidos na hepatotoxicidade por MeHg. Quando avaliamos o conteúdo de glicogênio, esse foi bem superior nos animas expostos ao MeHg. De acordo com BERGER *et al.*, 2009, o acúmulo de glicogênio sugere efeitos na reprogramação do metabolismo da glicose em animais. Além disso, o conteúdo de glicogênio no grupo DE foi superior ao grupo SE, sugerindo que o metabolismo e consequente deposição de glicogênio foi afetado pela dupla exposição ao MeHg e que a depuração do MeHg nesse tecido foi prejudicada.

Encontramos atividade elevada das duas enzimas hepáticas avaliadas (Aspartato aminotransferase e Alanina aminotransferase, AST e ALT, respectivamente), sendo um indicativo de dano hepático para ambos grupos expostos ao MeHg (GELAIN *et al.*, 2012; HUANG *et al.*, 2008, 2012; MORTENSEN *et al.*, 2014). Um dos mecanismos propostos para induzir danos mediados por MeHg é a geração de EROs e o desequilíbrio da homeostase redox (RASINGER *et al.*, 2017; SARBASSOV; SABATINI, 2005; SHARMA; KUMAR; KUMAR, 2005). Nossos resultados são consistentes com esses estudos, pois também observamos alterações nas atividades das enzimas antioxidantes e detoxificantes de fígado e CO de ratos expostos ao MeHg. Doses baixas de MeHg podem gerar dano oxidativo que leva à ruptura celular e prejuízo cognitivo (HEIMFARTH *et al.*, 2018a, 2018b). Os danos às proteínas detectados pelos níveis de carbonila e sulfidrila indicam diminuição dos processos de sinalização celular e da regulação do metabolismo energético.

Além disso, nossos dados, em ambos os tecidos, não mostraram um desequilíbrio nas atividades das enzimas antioxidantes (Tabela 2, Artigo 1), sugerindo que é um efeito oxidante direto do MeHg e não um efeito via antioxidantes enzimáticos. Os ratos MeHg apresentaram níveis aumentados de TBARS e proteínas carboniladas no CO e no fígado e

diminuição do teor de tiol. O aumento da carbonilação de proteínas, em ambos os tecidos, também pode induzir a formação de aglomerados de alta massa molecular e alto potencial citotóxico. No fígado, o aumento da oxidação dos grupos tiol (-SH) pode induzir a formação excessiva de ligações dissulfeto entre proteínas, um fenômeno que afeta o enrolamento de proteínas e induz a agregação e degradação de proteínas (CUMMING *et al.*, 2004; SITIA; MOLTENI, 2004; WINTERBOURN; METODIEWA, 1999). Notavelmente, assim como nosso trabalho, alguns estudos mostram que doses baixas de MeHg geram alta peroxidação lipídica em vários tecidos. Esse efeito é provavelmente uma consequência indireta dos efeitos pró-oxidativos do MeHg (CARVALHO *et al.*, 2008; JOSHI *et al.*, 2014; ROEGGE *et al.*, 2011; RUSZKIEWICZ *et al.*, 2016).

Estudos *in vitro* (CRESPO-LÓPEZ *et al.*, 2007; CRESPO-LOPEZ *et al.*, 2016) e *in vivo* (AMORIM *et al.*, 2000; BETTI; BARALE; POOL-ZOBEL, 1993; ESPITIA-PÉREZ *et al.*, 2018a, 2018c) demonstraram que o MeHg pode atuar como agente clastogênico. O MeHg pode inibir a atividade da poli (ADP-ribose) polimerase-1 (PARP-1), que repara as quebras da fita de DNA (PIEPER *et al.*, 2014). Por outro lado, o dano ao DNA observado neste trabalho provavelmente foi gerado por uma via pró-oxidante. Ratos expostos a MeHg apresentaram frequências aumentadas de MNPCE quando comparados ao controle. O aumento da frequência de MNPCE em ratos adultos está relacionado à ineficiência no reparo do DNA ou indução de apoptose nas células (AMORIM *et al.*, 2000; CRESPO-LÓPEZ *et al.*, 2007; ESPITIA-PÉREZ *et al.*, 2018a). O grupo DE apresentou maior frequência de MNPCE em relação ao SE. Poucos estudos confirmaram o potencial genotóxico e mutagênico da exposição ao mercúrio. No entanto, o MeHg leva à formação de ROS que podem induzir alterações conformacionais nas proteínas responsáveis pelo reparo do DNA, fuso mitótico e segregação cromossômica (TCHOUNWOU *et al.*, 2012).

Trabalhos anteriores (ESPITIA-PÉREZ *et al.*, 2018a, 2018c; HEIMFARTH *et al.*, 2018a, 2018d; PESSOA-PUREUR; HEIMFARTH; ROCHA, 2014; VICENTE *et al.*, 2004) revelaram que a exposição ao MeHg tem efeitos complexos nos processos biológicos celulares, que podem exibir respostas multifásicas devido à vulnerabilidade de cada estrutura cerebral (bem como a dose e o tempo de exposição). O CO é uma das estruturas cerebrais mais sensíveis em relação à exposição ao MeHg (HAYKAL-COATES *et al.*, 1998; HEIMFARTH *et al.*, 2018a). Além disso, vários alvos moleculares e mecanismos complexos estão envolvidos nos efeitos da neurotoxicidade e do comprometimento comportamental mediados por MeHg (HEIMFARTH *et al.*, 2018a).

A exposição gestacional ao MeHg também altera a atividade das cascatas de sinalização celular de sobrevivência / morte, alterações que podem estar associadas ao comprometimento do desenvolvimento cerebral (HAYKAL-COATES *et al.*, 1998; HEIMFARTH *et al.*, 2018a). Considerando essa possibilidade, avaliamos o imunoconteúdo

das enzimas ativas que participam de algumas vias de sobrevivência de sinalização no CO motor de ratos expostos a MeHg. Observamos uma regulação positiva da via de sinalização PI3K / Akt / mTOR e inativação da GSK3β (Ser9). A via PI3K / Akt promove o crescimento, diferenciação e sobrevivência celular, através da regulação negativa de sinais apoptóticos (ENDO *et al.*, 2006). Vários estudos demonstraram que o estresse oxidativo regula a ativação de Akt, e o MeHg ativa essa via aumentando a produção de ROS (SONG; KANTHASAMY; KANTHASAMY, 2011; URANGA; KATZ; SALVADOR, 2013; YU; CUI, 2016; ZHANG; YANG, 2013). Através do PI3K, Akt é recrutada para a membrana plasmática e ativada pelas proteínas cinases. A ativação da Akt fosforila várias proteínas do substrato a jusante que são críticas na progressão e sobrevivência do ciclo celular (LAWLOR; ALESSI, 2001).

A via Akt é um importante alvo molecular para MeHg no SNC, uma interação que promove danos celulares e neurotoxicidade (ENDO *et al.*, 2006; HEIMFARTH *et al.*, 2018c). A GSK3β é importante para o desenvolvimento do SNC, pois controla a neurogênese, a polarização neuronal e o crescimento axonal. A atividade neuronal da GSK3β está sob o controle de múltiplos mecanismos. Mecanismos de plasticidade neural de regulação negativa de GSK3β durante o desenvolvimento (SEIRA; DEL RIO, 2014). A GSK3β é uma importante proteína cinase que está a jusante da Akt. Em estudos anteriores, observou-se que a regulação positiva da via PI3K/Akt/ mTOR e a regulação negativa da GSK3β (Ser9) poderiam indicar que essas alterações estão associadas a danos neurais nos animais expostos ao MeHg (GASPAROTTO *et al.*, 2018; HEIMFARTH *et al.*, 2018a, 2018d; SEIRA; DEL RIO, 2014) Em nosso estudo, observamos essa interrupção da GSK3β em ratos adultos expostos a MeHg. Portanto, o desequilíbrio da atividade da GSK3β (Ser9), observado em animais expostos ao MeHg, pode estar associado ao dano neural.

Sabe-se que as alterações neurológicas presentes em doenças neurodegenerativas estão associadas à desregulação da proteína Tau (GASPAROTTO *et al.*, 2018; HEIMFARTH *et al.*, 2018a, 2018d). A super expressão da proteína Tau (Ser396) desestabiliza os microtúbulos e seu acúmulo leva à formação de emaranhados associados a estruturas intracelulares anormais, fenômenos que promovem dano neuronal e morte. Nesta linha, a hiperfosforilação de Tau observada em animais expostos ao MeHg pode ser um dos mecanismos responsáveis pelos danos neuronais no CO, intensificando a morte neuronal e astrocitária e ativação microglial. Novamente é importante notar que as alterações estruturais no citoesqueleto são características relevantes presentes nas doenças neurodegenerativas, como a doença de Alzheimer e a intoxicação por neurotoxinas (HEIMFARTH *et al.*, 2018ª; (MAQBOOL; MOBASHIR; HODA, 2016)

Apesar da regulação positiva da via PI3K / Akt / mTOR, observamos uma diminuição no imunoconteúdo de NeuN (densidade neuronal) e de GFAP (astrócitos) em CO dos ratos

tratados com de MeHg. Além disso, o aumento da ativação microglial, através do aumento do imunoconteúdo da proteína Iba1, sugere que uma inflamação local module a disfunção astroglial e microglial no CO de animais expostos ao MeHg (GASPAROTTO *et al.*, 2018; HOL; PEKNY, 2015; RASINGER *et al.*, 2017; SARBASSOV; SABATINI, 2005; TAKEI; NAWA, 2014; XU *et al.*, 2015) Também observamos maior imunoconteúdo de RAGE nos grupos tratados comparado ao controle. O aumento do RAGE está associado à inflamação, e onde é expresso se torna muito relevante para a compreensão do estado fisiológico do tecido (GASPAROTTO *et al.*, 2018). Assim, o dano neural no CO gerado pela exposição ao MeHg pode desempenhar um papel importante na suscetibilidade a doenças neurodegenerativas e pode estar associado ao comprometimento comportamental observado nos animais expostos ao MeHg.

Os resultados dos testes comportamentais OFT e EPMT revelaram que o MeHg gerou alterações comportamentais, resultados que indicam comprometimento cognitivo já descrito em estudos anteriores do nosso grupo (ESPITIA-PÉREZ *et al.*, 2018a, 2018b). Os filhotes machos de mães expostas a MeHg são mais sensíveis do que filhotes fêmeas; nesses animais afetados, o circuito sináptico é aparentemente afetado durante a exposição ao MeHg (ESPITIA-PÉREZ *et al.*, 2018ª). Além disso, ratos tratados com MeHg exibiram uma resposta de adaptação mais longa, dentro de 3 minutos no OFT, dados que sugerem a existência de um possível distúrbio da memória a longo prazo. Resultado semelhante foi observado nas mães expostas ao MeHg em trabalhos anteriores do nosso grupo (ESPITIA-PÉREZ *et al.*, 2018a, 2018b). A exposição intra-uterina ao MeHg não atenuou a deficiência comportamental gerada pela nova exposição na idade adulta (grupo DE).

Os resultados comportamentais indicam que os animais tratados com MeHg exibiram comportamento ansioso, como aversão à área central do dispositivo. Esse achado sugere que o MeHg induz mudanças na reatividade emocional. Nossos dados são consistentes com os achados em populações humanas que consomem grandes quantidades de peixes contaminado com MeHg. As crianças expostas intra-utero ao MeHg apresentam menor quociente de inteligência (QI) e comprometimento da memória, atenção, linguagem e percepção visual (BISEN-HERSH *et al.*, 2014; GRANDJEAN; LANDRIGAN, 2006; ROEGGE *et al.*, 2011).

É importante notar que, além do dano oxidativo que ocorre no sistema nervoso pela exposição ao MeHg, outra via de neurotoxicidade pode ser através do dano hepático. Muitos autores demonstraram que a lesão hepática, conforme observado em nossos resultados (Tabela 1), pode levar a encefalopatias com consequências neurodegenerativas (BRAISSANT *et al.*, 2019a; CUDALBU; TAYLOR-ROBINSON, 2019). Isso pode estar associado à deposição hepática de glicogênio em animais expostos a MeHg, principalmente no grupo DE. Outros autores encontraram resultados semelhantes, seguidos por

hipoglicemia grave e perda de peso em ratos tratados com MeHg, já que a exposição crônica ao MeHg aumenta a concentração de Hg no fígado (SALVATERRA *et al.*, 1975; SNELL; ASHBY; BARTON, 1977). Juntamente com a perda de peso, evidenciada em ambos os grupos expostos ao MeHg, encontramos uma baixa ingestão alimentar por esses animais (provavelmente devido à perda de palatabilidade). Essa baixa ingestão de alimentos é responsável por baixos níveis séricos de glicose e aumento da atividade de AST e ALT nos órgãos (MORIYAMA *et al.*, 2008). Além disso, evidências recentes mostram que a exposição ao MeHg afeta o proteoma hepático, especialmente as proteínas associadas ao metabolismo da glicose, possivelmente pela interrupção da defesa antioxidante (YADETIE *et al.*, 2016).

O acúmulo de glicogênio no fígado e a baixa disponibilidade endógena de glicose predispõem a danos cerebrais. Resultados epidemiológicos de estudos de coorte pediátricos mostraram danos cerebrais, provavelmente causados por hipoglicemia grave recorrente, em pacientes com Doença de Armazenamento de Glicogênio Tipo I (GSDI) (MELIS *et al.*, 2004). Pacientes com GSDI apresentam atividade prejudicada de glicose-6-fosfatase (G6P), que é curiosamente um alvo molecular para do MeHg (SNELL; ASHBY; BARTON, 1977). A diminuição da atividade da G6P está relacionada ao baixo conteúdo endógeno de glicose após o estado pós-absorção, aumenta o acúmulo de glicogênio e glicose-6-fosfato no fígado, o que aumenta a disfunção orgânica (COHN; OHRI, 2017; WINNICK *et al.*, 2016). A hipoglicemia a seguir pode induzir dano cerebral *in vivo* devido à falta de combustível metabólico (CRYER, 2005). Finalmente, a glicogênio fosforilase (GP) é outro alvo molecular da inibição irreversível da MeHg. GP é uma enzima que limita a taxa na via inicial de glicogenólise. Trabalhos anteriores encontraram aumento da deposição de glicogênio no tecido de animais expostos a MeHg após desequilíbrio GP in vivo (RAO; SHARMA, 2001; XU *et al.*, 2015).

Nossa hipótese central é baseada na neurotoxicidade associada à hepatoxicidade por excesso de metabólitos tóxicos, como amônia (hiperamonemia). A hiperamonemia pode causar danos irreparáveis ao cérebro em desenvolvimento, apresentando sintomas como comprometimento cognitivo (retardo mental), convulsões e paralisia cerebral. O efeito neurotóxico da amônia pode ser associado à morte neuronal a partir do dano hepatotóxico visto em nosso estudo. Em animais do grupo DE, foi encontrado comportamento indicativo de ansiedade. Portanto, esse aumento do dano hepático, a deposição de glicogênio e a hipoglicemia subsequente favoreceram esse comportamento estereotipado (HERTZ *et al.*, 2017).

Trabalhos anteriores mostraram que, durante o estresse hipoglicêmico *in vivo*, o MeHg induzia um estado hipertireoidiano e baixa síntese de dopamina devido à insuficiência renal (KABUTO, 1991). Baixos níveis de dopamina estão relacionados à resposta fisiológica da

ansiedade (ZARRINDAST; KHAKPAI, 2015). No entanto, outros fenótipos comportamentais são encontrados durante os danos cerebrais induzidos pela hipoglicemia, que também promovem déficits de aprendizado e memória espacial (PUENTE *et al.*, 2010). O aumento de AST e ALT observado pode ser indicativo de danos ao fígado devido a baixa glicose endógena.

No entanto, um recente modelo multimodal *in vivo* mostrou comprometimento da locomoção durante a encefalopatia hepática precoce em ratos, que se correlacionou com o aumento do transporte de amônia e glutamina e uma diminuição nas atividades de complexos antioxidantes (BRAISSANT *et al.*, 2019b). Este último pode sugerir que se faz necessário considerar outros metabólitos de importância durante lesão hepática e sua relação com lesão cerebral, especialmente em estudos futuros. Nossos resultados mostram a importância de considerar os efeitos sistêmicos da exposição ao MeHg, especialmente em novos cenários de exposição, nos quais o aumento da biodisponibilidade do MeHg pode expor o indivíduo ao MeHg ao longo da vida com efeitos desconhecidos.

No artigo 2, novamente estudamos como a exposição fetal à determinado agente modularia uma nova exposição na idade adulta. Trabalhos anteriores do nosso grupo sugerem que a suplementação com antioxidantes pode fornecer efeitos protetores substanciais contra várias doenças humanas relacionadas ao estresse oxidativo (VERLECAR; JENA; CHAINY, 2008; ZIMMERMANN *et al.*, 2013). Além disso, foi postulado que o estresse oxidativo pode gerar uma resposta adaptativa a diferentes oxidantes (LEMIRE *et al.*, 2010; MORRONE *et al.*, 2016). Alguns antioxidantes, como a vitamina E (FARINA; ASCHNER; ROCHA, 2011b; STRINGARI *et al.*, 2008) e a vitamina C (KHAN; BLACK, 2003), demonstraram induzir efeitos protetores contra danos ao MeHg em vários modelos. VitA tem propriedades redox e também é conhecida por seus efeitos antioxidantes em vários sistemas de teste (GRANDJEAN *et al.*, 1998; KANDA; SHINKAI; KUMAGAI, 2014). No entanto, ESPITIA-PÉREZ *et al.*, (2018c) mostraram que, mesmo em baixas doses de MeHg co-administradas com suplementação de VitA durante a gravidez e lactação, promove alterações no estado redox em mães e crianças. A administração concomitante de VitA no período fetal e de amamentação não tem efeito protetor na exposição a MeHg em adultos.

Nossa análise do tecido hepático revelou um desequilíbrio na atividade das enzimas antioxidantes de todos os grupos (Figura 2 do Artigo 2), na qual a atividade da SOD foi aumentada e a atividade da GPx reduzida. Isso sugeriu um efeito direto da segunda exposição ao MeHg e não indicou que efeitos específicos foram produzidos como resultado da exposição ao feto e à amamentação. Não foram observadas alterações na atividade da CAT entre os grupos expostos ao MeHg. Trabalhos anteriores do nosso grupo também mostraram que as exposições não alteram a atividade do CAT (VERLECAR; JENA; CHAINY,

2008; ZIMMERMANN *et al.*, 2013). As relações SOD / (GPx + CAT) aumentaram, alterando sua resposta ao estresse oxidativo como resultado do excesso de produção de H_2O_2 em todos os grupos (UNG *et al.*, 2010) Nossos resultados estão de acordo com outros estudos que demonstraram que o H_2O_2 desempenha um papel central nos mecanismos de oxidação de MeHg (ANTUNES DOS SANTOS *et al.*, 2016; HALLIWELL, 2006b), funcionando principalmente pela alteração do metabolismo da glutationa (CUMMING *et al.*, 2004; WINTERBOURN; METODIEWA, 1999).

Nossos resultados também revelaram aumento na carbonilação de proteínas e diminuição nos níveis tióis reduzidos, o que indicou danos oxidativos a proteínas com possíveis erros nas interações proteína-proteína e a dobramentos mal regulados de proteínas. Esses efeitos provavelmente contribuíram para a toxicidade do MeHg. A carbonilação de proteínas é um produto da oxidação irreversível de vários resíduos de aminoácidos, e a frequência da modificação pode interromper a sinalização celular e promover a geração de substâncias altamente citotóxicas (CUMMING *et al.*, 2004; KIRKPATRICK *et al.*, 2015; SCHNORR *et al.*, 2015a).

A redução dos grupos SH também pode levar à geração excessiva de interações dissociadas de proteínas alterados, agregação, degradação e até morte celular (DE FREITAS *et al.*, 2009). Não foram observadas diferenças entre os grupos SE, VitA e VitA + MeHg, indicando um efeito direto do estado de oxidação ou na ruptura de proteínas, como resultado da segunda exposição ao MeHg. Essa evidência continua a indicar que a exposição a MeHg em adultos pode interromper a homeostase redox e contribuir para o desenvolvimento de várias doenças e o acúmulo de danos oxidativos. KIRKPATRICK *et al.* (2015) mostraram que a exposição ao MeHg pode alterar os níveis de biomarcadores de estresse oxidativo, especialmente com relação aos níveis de GPX e tioredoxina redutase. Além disso, o composto altera as atividades de CAT e SOD (DE CARVALHO *et al.*, 2017) e interrompe a organização de proteínas e lipídios (DE OLIVEIRA *et al.*, 2007; HOSSEINI OMSHI *et al.*, 2018; OLIVEIRA *et al.*, 2015). Esses resultados indicam que a segunda exposição a uma substância tóxica tem um efeito direto no sistema de defesa antioxidante ou pode promover a carbonilação e reduzir o conteúdo de tiol.

Curiosamente, apenas os animais dos grupos MeHg e SE apresentaram níveis aumentados de substâncias reativas ao ácido tiobarbitúrico (TBARS). Esse resultado sugeriu que a exposição fetal e na amamentação à VitA, mesmo com a co-exposição ao MeHg, teve um efeito protetor contra a exposição subsequente ao MeHg. VitA possui propriedades redox e também é conhecido por seus efeitos antioxidantes (KLAMT *et al.*, 2000, 2008). Esses efeitos fornecem proteção adicional de lipídios e membranas que persiste por toda a vida adulta. Além disso, resultados anteriores indicaram que a coadministração de vitaminas A e C pode fornecer efeitos protetores (HOSSEINI OMSHI *et*

al., 2018) contra desequilíbrios oxidantes-antioxidantes induzidos por um xenobiótico (KLAMT *et al.*, 2000, 2008; MURATA; KAWANISHI, 2000). No entanto, como discutido anteriormente, esses efeitos dependem da concentração. Além disso, estudos anteriores mostraram que o VitA e seus derivados têm a capacidade de proteger contra a peroxidação lipídica, danos ao DNA e estado redox celular prejudicado (CRESPO-LÓPEZ *et al.*, 2007; LINE; KARRI; KUMAR, 2017).

Em relação aos danos no DNA (Figura 4, artigo 2), estudos *in vitro* (AMORIM *et al.*, 2000; BETTI; BARALE; POOL-ZOBEL, 1993) e *in vivo* (ESPITIA-PÉREZ *et al.*, 2018c; TCHOUNWOU *et al.*, 2012) já demonstraram os efeitos genotóxicos e clastogênicos do MeHg. Os danos observados no ensaio do cometa são indicativos de danos recentes e passíveis de reparo no DNA, associados à exposição ao MeHg na fase adulta.

A suscetibilidade ao dano no DNA induzida por H_2O_2 (75 µM) foi mensurada 30, 90 e 150 minutos após o tratamento. Usando o grupo controle como padrão, a Figura 4 do artigo 2 mostra que o índice de danos ao DNA do grupo controle aumentou 30 minutos após o tratamento com H_2O_2 e diminuiu após 150 minutos após a exposição. Nos grupos tratados, o dano induzido ao DNA permaneceu alto 150 minutos após o tratamento. Para investigar essas diferenças, calculamos alterações no índice de danos 150 minutos após o tratamento em comparação com o índice de danos 30 minutos após a exposição para cada grupo.

Esse parâmetro, chamado dano residual ao DNA, revelou que os níveis de dano no DNA detectados 150 minutos após o tratamento nos controles diminuíram em relação aos níveis determinados 30 minutos após o tratamento. Isso pode indicar que um processo de reparo do DNA está ocorrendo. Por outro lado, os valores residuais dos grupos tratados, com exceção do grupo pré-exposto ao VitA, permaneceram altos. Isso indicou que os grupos tratados são mais altamente suscetíveis a danos exógenos. É possível que a exposição fetal e da amamentação à VitA tenha desempenhado um papel protetor na indução de dano oxidativo pelo MeHg após o desafio com H_2O_2. Sabe-se que a exposição ao MeHg promove a formação de EROs, que pode induzir alterações conformacionais nas proteínas responsáveis pelo reparo do DNA, formação de fuso mitótico e segregação cromossômica (PIEPER *et al.*, 2014) via inibição da atividade da polimerase-poli (ADP-ribose) (PARP-1), que repara quebras na fita de DNA (AMORIM *et al.*, 2000; CRESPO-LÓPEZ *et al.*, 2007; ESPITIA-PÉREZ *et al.*, 2018c).

O dano ao DNA observado aqui foi provavelmente gerado por uma via pró-oxidante. Todos os animais expostos possuíam frequências aumentadas de MNPCE quando comparados aos controles. O aumento da frequência de MNPCE em ratos adultos está relacionado ao reparo ineficiente do DNA ou à indução de apoptose celular (BEYERSMANN; HARTWIG, 2008; FRANKE *et al.*, 2018; JOMOVA; VALKO, 2011). Curiosamente, os grupos que não foram expostos a VitA durante os períodos fetais ou de amamentação (MeHg e SE)

apresentaram menor razão de PCE: NCE do que os controles, indicando aumento dos efeitos da toxicidade nesses animais.

Em geral, a exposição frequente a metais pesados, como derivados de mercúrio, pode levar a reações que geram ERO (JOMOVA; VALKO, 2011; POPRAC *et al.*, 2017; TCHOUNWOU *et al.*, 2012). Nossos resultados destacam a importância de analisar os efeitos de danos oxidativos frequentes, causados pela exposição repetida ao MeHg ao longo da vida, especialmente em cenários em que a nova exposição ocorre. Além disso, o aumento da biodisponibilidade de MeHg e VitA pode aumentar os casos em que exposições a doses múltiplas de MeHg ocorrem ao longo da vida e produzem efeitos desconhecidos. Novos estudos serão necessários para esclarecer a base molecular das lesões relacionadas à exposição ao MeHg que ocorrem como resultado da exposição durante os períodos de desenvolvimento e na vida adulta.

5. CONCLUSÕES

De acordo com resultados apresentados nessa tese, podemos concluir os seguintes pontos:

1) A exposição a baixas doses de MeHg durante o período fetal e a amamentação não condicionam a melhor ou uma resposta adaptativa à nova exposição à MeHg na idade adulta. Bem como a exposição e a co-exposição a uma dose baixa e ambientalmente relevante de MeHg junto com uma dose de suplementação com VitA (dose considerada como segura) durante o período fetal e amamentação (Artigos 1 e 2);

2) A exposição ao MeHg em ambos períodos de exposição gera toxicidade sistêmica, como diminuição do consumo de ração padrão e consequente perda de peso (Artigo 1);

3) A concentração de Hg no grupo DE foi superior a encontrada no grupo SE (Artigo 1), indicando que a exposição na fase fetal e amamentação é capaz de alterar o *clearence* de Hg e consequente acúmulo desse metal (Artigo 1);

4) A exposição ao MeHg gera hepatoxicidade e sua intensidade está relacionada aos períodos de exposição a esse metal e ao acúmulo de glicogênio (Artigo 1);

5) A exposição simples ou dupla ao MeHg causa dano a biomoléculas via formação de EROs e não através do desequilíbrio redox no córtex occipital (Artigo 1) e fígado (Artigos 1 e 2); No entanto, a exposição fetal a VitA, bem como sua co-exposição ao MeHg, apresentou menores níveis de TBARS (Artigo 2), sugerindo que a pré exposição a VitA possa ter efeito protetor sobre as biomoléculas desse marcador;

6) Os danos ao DNA causados pela exposição ao MeHg em ambos períodos são irreversíveis e com potencial mutagênico (Artigos 1 e 2). Além disso, animais pré-expostos com MeHg (MeHg e SE) apresentam maior suscetibilidade a danos ao DNA, enquanto os

animais pré-expostos a VitA (grupos VitA e VitA+MeHg) apresentaram menor dano residual ao DNA após o desafio com H_2O_2 (Artigo 2). Ainda, animais pré-expostos a VitA apresentaram maior razão PCE:NCE, indicando que a exposição ao MeHg na fase adulta gerou menos efeitos tóxicos (Artigo 2);

7) Além disso, o MeHg gera maior regulação da via de sinalização PI3K/Akt/mTOR e consequente inibição de GSK3ß que está associada ao dano neural (Artigo 1);

8) O MeHg em ambos períodos de exposição gera hiperfosforilação da proteína Tau e provavelmente morte celular neuronal e neuroinflamação, através da análise do imunoconteúdo de NeuN, GFAP, Iba1 e RAGE (Artigo 1);

9) As alterações comportamentais encontradas foram associadas a perda de memória de longo prazo e reatividade emocional (Artigo 1).

A contaminação ambiental por MeHg e semelhantes é considerada uma epidemia toxicológica pelas agências ambientais regulatórias. A principal fonte de contaminação por MeHg ocorre através da ingestão de peixes contaminados e as populações mais atingidas são as ribeirinhas da Amazônia. Grupos de pesquisa que atuam nessa Região mostraram que mais de 80% do metal presente e identificado na água e alimentos estava em sua forma orgânica, o MeHg. Essa é a forma mais tóxica de mercúrio, capaz de driblar o sistema de defesa do organismo e atingir o cérebro. Nesses casos, pode causar uma síndrome neurodegenerativa grave chamada doença de Minamata, caracterizada por tremores, problemas de coordenação, distúrbios sensoriais, comprometimento da visão e da audição e, em casos extremos, morte. Muito se fala sobre os efeitos diretos sobre o cérebro, no entanto, quando se analisa os efeitos sistêmicos causados pela exposição ao MeHg em conjunto com os neurais, como fizemos nesse trabalho, vê-se que o MeHg pode apresentar efeitos ainda mais catastróficos sobre a saúde da comunidade exposta.

Apesar de estudos já terem quantificado altos níveis de MeHg no organismo da população atingida, nenhum dos ribeirinhos foi diagnosticado com qualquer problema de saúde associado à intoxicação por MeHg, por sinais clínicos associados à contaminação por esse metal. De fato, existe total descaso por parte de governos e agências regulatórias com essa problemática, sendo dessas a responsabilidade pelo risco que essas populações correm. Maior controle e diminuição do extrativismo, mineração, instalação de represas, além de mais investimento em pesquisas, como a deste trabalho, seriam medidas mitigatórias viáveis para conter os efeitos a longo prazo da intoxicação por MeHg.

6. PERSPECTIVAS

A partir dos resultados dessa tese pode-se estabelecer algumas perspectivas para refinamento desses resultados, através da utilização de outras técnicas ou modelos biológicos a fim de elucidar mecanismos moleculares sobre a exposição em diferentes fases do desenvolvimento ao MeHg e a VitA. Abaixo algumas propostas para a continuação desse trabalho:

1) Utilizar um modelo *in vitro* focando em linhagem celulares neuronais como neuroblastoma derivado de humano (SH-SY5Y) ou células-tronco neurais (NSC) as quais são amplamente utilizadas para avaliação dos efeitos neurotóxicos do MeHg, a fim de avaliar os metabólitos ativos do palmitato de retinol como o ácido retinóico ou retinol;

2) Estudar outros parâmetros de neurodesenvolvimento em modelos *in vitro* ou *in vivo* que permitam avaliar a neurogênese durante as fases de co-exposição a MeHg-VitA em áreas como o hipocampo (como o *gyrus dentatus*);

3) Em modelo *in vivo* avaliar parâmetros emocionais mais específicos, como medo, dor e depressão após a co-exposição a MeHg-VitA.

4) Avaliar mecanismos moleculares *in vitro* ou *in vivo*, como a cascata da proteína quinase MAPK e o estudo de marcadores pró-inflamatórios associados à neurodegeneração e ao reparo de danos no DNA.

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