UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE (ICBS)
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

"EFEITO DA CO-EXPOSIÇÃO MATERNA AO METILMERCURIO E AO PALMITATO DE RETINOL DURANTE A GESTAÇÃO E LACTAÇÃO SOBRE A TOXICIDADE EM FILHOTES EM MODELO DE RATOS WISTAR"

Pedro Juan Espitia-Pérez

Porto Alegre, 2018

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE (ICBS)
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

"Efeito da co-exposição materna ao metilmercurio e ao palmitato de retinol durante a gestação e lactação sobre a toxicidade em filhotes em modelo de ratos *Wistar*"

Pedro Juan Espitia-Pérez

Orientador: Prof. Dr. José Cláudio Fonseca Moreira

Co-orientador: Prof. Dr. Carlos Eduardo Schnorr

Dissertação de mestrado apresentada como requisito parcial para a obtenção do título de Mestre.

Banca examinadora:
Prof. Dra. Regina Pessoa Pureur (PPG Bioquímica-ICBS/UFRGS)
Prof. Dra. Maria Elisa Calcagnotto (PPG Bioquímica-ICBS/UFRGS)
Prof. Dr. Rafael Calixto Bortolin (Dpto. Ingeniería Civil y Ambiental/CUC/Colombia)

Porto Alegre, abril de 2018

CIP - Catalogação na Publicação

Espitia Pérez, Pedro Juan
Efeito da co-exposição materna ao metilmercurio e ao palmitato de retinol durante a gestação e lactação sobre a toxicidade em filhotes em modelo de ratos Wistar / Pedro Juan Espitia Pérez. -- 2018.
161 f.
Orientador: José Cláudio Fonseca Moreira.

Coorientador: Carlos Eduardo Schnorr.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2018.

1. Metilmercurio. 2. Palmitato de retinol. 3. Co-exposição. 4. Estresse oxidativo. I. Fonseca Moreira, José Cláudio, orient. II. Schnorr, Carlos Eduardo, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Este trabalho foi realizado no Centro de Estudos em Estresse Oxidativo, do Instituto de Ciências Básicas da Saúde, da Universidade Federal do Rio Grande do Sul, sendo financiado pelo Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), pela Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e pela Pró-Reitora de Pesquisa desta Universidade (PROPESQ/UFRGS).

Dedicatoria

*A **Vilma Pérez** y **Juan Espitia**, mis padres, ejemplos de lucha y sacrificio…*
*A **Juan David Espitia Pérez**, mi hermano, mi mejor amigo, Maestro de la Alegría…*
*Y de manera muy especial, a **Lyda Espitia Pérez**, mi hermana, mi Narradora de Cuentos,*
Quien siempre creyó en mí apoyando mis sueños,
Quien me enseñó el valor de estudiar y aprender…

Con un corazón humilde dedico a ustedes, mis Amados Pilares, esta conquista.
No podría pedirle a Dios un mayor amor en esta buena tierra que el de ustedes.

Gracias!

"A ciência será sempre uma busca, jamais uma descoberta. É uma viagem, nunca uma chegada"

Karl Popper

"Whatever we learn has a purpose and whatever we do affects everything and everyone else, if even in the tiniest way... And it´s much the same thing with knowledge, for whenever you learn something new, the whole world becomes that much richer"

The Princess of Pure Reason, *The Phantom Tollbooth*, Norton Juster

AGRADECIMENTOS

Definitivamente, muitas são as pessoas que quero agradecer e que me ajudaram em muitos aspectos dentro deste processo. São pessoas que fazem parte de minha história e tem um espaço no meu coração. Assim, como uma amostra de aprecio, gostaria de expressar meus sinceros agradecimentos:

- Ao professor José Claudio Fonseca Moreira, orientador deste trabalho. Obrigado pela confiança depositada em mim, ainda sem me conhecer. Obrigado por apostar nesta ideia e todos os seus ensinamentos;
- A Carlos Eduardo Schnorr, co-orientador deste trabalho. Um excelente cientista e um incredível ser humano e amigo. Obrigado por tuas imensas contribuições, tua ajuda com os animais, tuas ideias nos experimentos e apoio em tudo momento;
- A Suelen Marin Albino, a melhor *"lab partner"* que alguém possa pedir. Por toda tua ajuda na fase de biotério e de experimentos. Pelas horas de trabalho, pelas aulas de Português, pero sobretudo pela amizade. Obrigado Su!;
- A Helen da Rosa (CEO do grupo "Matança Mercosul"), uma excelente cientista, uma grande pessoa. Obrigado pelas churras e o cafezinho!;
- A toda a galera do Lab 32, mestrandos, doutorandos, pós-docs e ICs, qualquer contribuição de vocês foi muita ajuda, obrigado! De maneira especial quero agradecer a Alexandre Kleber Silveira, Maurilio da Silva Morrone, e Moara Mingori pela ajuda durante a fase de biotério, tratamentos, e as matanças. Adicionalmente agradeço a Alice Kunzler por seu conhecimento e ajuda durante os analises de retinóides;

- Ao Prof. Diogo Pompéu Moraes e sua equipe por sua contribuição durante os analises de mercúrio total. Também a Fabiano Barreto por sua ajuda em infraestrutura e brindar seu tempo e conhecimento durante a fase de analises dos retinóides;
- Aos demais professores do PPG Bioquímica e aos funcionários da Secretaria do Departamento, sendo um estrangeiro sua ajuda foi a melhor bem-vinda. Também quero agradecer ao pessoal do Biotério do Departamento de Bioquímica, aprendi muito de vocês!;
- A meus familiares, em especial a todas minhas queridas tias e seu carinho que chega desde meu pais. Obrigado por fazer parte de minha vida!;
- A meus amigos do "Laboratorio de Investigaciones Biomédicas y Biología Molecular" da Universidad del Sinú. Obrigado pelo apoio!;

Por último, e de maneira especial, gostaria de agradecer...

- A meus adorados pais, Vilma Pérez e Juan Espitia, vocês são minha força e meu impulso para continuar cada dia trabalhando pelos sonhos que Deus alimenta através de vocês. Agradeço ao Céu ter a vocês em minha existência!;
- A Juan David Espitia Pérez, meu irmão, meu melhor amigo. Obrigado por tua presença, por teu apoio incondicional e por fazer meus dias tristes mais alegres;
- E obrigado, Lyda Espitia Pérez, minha adorada irmã, obrigado por teu amor, tua vida e meu melhor presente, obrigado por apoiar meus sonhos e por acreditar em mim quando eu não posso.

Em palavras do maestro Gustavo Cerati, pronunciadas durante o último concerto de "Soda Stereo" na Argentina em março de 1997... *"Gracias... totales"*

SUMÁRIO

PARTE 1

RESUMO

Atualmente a biodisponibilidade de doses baixas de metilmercurio (MeHg) pelo consumo de peixe é muito generalizada. Por outro lado, a vitamina A (VitA) é um nutriente essencial com uma alta presença entre os consumidores globais na dieta normal, alimentos processados e suplementos. O MeHg e um conhecido neurotóxico, mesmo em doses baixas, com a capacidade de afetar diversos órgãos e produzir efeitos deletérios na saúde, especialmente em mulheres gravidas, onde pode desencadear alterações sistêmicas e no neurodesenvolvimento após o nascimento. Adicionalmente, nosso laboratório demostrou com sucesso em modelos animais, os efeitos pró-oxidantes e alterações comportamentais produzidos pela suplementação com VitA em doses consideradas seguras. No entanto, apesar de serem conhecidos os efeitos biológicos do MeHg e da VitA de forma individual, nenhum estudo da literatura avaliou seus efeitos combinados. Então, o objetivo deste trabalho foi investigar os efeitos de uma dose baixa e ambientalmente relevante de MeHg coadministrada com uma dose de suplementação de VitA em ratas gestantes e lactantes sobre o comportamento e tecidos das mães e seus filhotes. Trinta ratas *Wistar* gravidas (entre 7 e 8 por grupo) foram suplementadas oralmente com azeite mineral (controle), MeHg (0,5 mg/Kg/dia) e palmitato de retinol (7500 µg RAE/Kg/dia) individualmente ou em combinação, desde o início da gestação (GD 0) até o desmame dos filhotes (DPN 21). Nem as mães nem os filhotes apresentaram alterações morfológicas, sistêmicas, toxicológicas ou metabólicas provocadas pelo tratamento de co-exposição MeHg-VitA. No entanto, foram encontradas modulações de atividades enzimáticas nas enzimas glutationa-dependentes em forma tecido-específica, assim como efeitos deletérios/benéficos em termos da peroxidação lipídica. Por último, o tratamento MeHg-VitA foi capaz de alterar o processo de aprendizado associativo na etapa neonatal, sem modular o neurodesenvolvimento dos filhotes. Além disso, não produziu alterações comportamentais em idades posteriores, mas afetou a resposta de aprendizado de maneira sexo-dependente. Em conclusão, o tratamento MeHg-VitA sob nosso paradigma de exposição pode modular reações nocivas/benéficas em termos da toxicidade tanto em mães como filhotes, dependendo do tecido estudado, e produzir efeitos *in vivo* em combinação (MeHg+VitA) completamente diferentes dos efeitos individuais dos compostos. Nossos resultados no modelo animal mostram a importância do cuidado na alimentação de gestantes e lactantes para evitar alterações na saúde da futura criança pelo consumo inadvertido destes compostos em combinação. As respostas pró-oxidantes podem levar a efeitos a nível celular. Além disso, recomendamos mais trabalhos sobre metabolitos ativos do palmitato de retinol que podem ter efeitos benéficos durante casos de intoxicação com MeHg em doses baixas, especialmente no sistema nervoso. As implicações epidemiológicas de nossos resultados ainda são desconhecidas, por tanto são requeridos mais estudos epidemiológicos de seguimento e hábitos alimentares nas populações materna e infantil ao nível mundial.

ABSTRACT

Actually, low-dose methylmercury (MeHg) bioavailability through fish consumption is ubiquitous. In contrast, vitamin A (VitA) is an essential nutrient with a high presence among global consumers in the regular diet, processed foods, and supplements. MeHg is a well-known neurotoxin, even at low doses, capable of affecting other organs and produce deleterious effects on health, especially in pregnant women, where it can unleash systemic and neurodevelopmental alterations after birth. Additionally, our lab successfully demonstrated, in an animal model, pro-oxidant effects and behavioral disturbances produced by VitA supplementation at doses considered as safe. However, although individual biological effects of the previous compounds are well known, no study in literature evaluated their effects in combination. Thus, the aim of the present work was to investigate the effects of an environmentally relevant low-dose of MeHg co-administered with a supplementation dose of VitA in pregnant and lactating rats on maternal and offspring behavior and tissues. Thirty *Wistar* female pregnant rats were orally supplemented with mineral oil (control), MeHg (0,5 mg/Kg/day) and retinyl palmitate (7500 µg RAE/Kg/day) either individually or in combination from the beginning of gestation (GD 0) until weaning (DPN 21). Neither dams nor their offspring presented morphological, systemic, toxicological or metabolic disturbances caused by co-exposition treatment MeHg-VitA. But, showed tissue-specific modulations of enzymatic activities in glutathione-dependent enzymes, and deleterious/beneficial effects regarding lipid peroxidation. Finally, MeHg-VitA treatment was able to alter associative learning in neonatal stage, with no modulation of pup´s neurodevelopment. Also, it did not produce behavioral alterations at later age, however, affected learning response in a sex-dependent manner. In conclusion, MeHg-VitA treatment under our exposure paradigm can modulate harmful/beneficial reactions in terms of toxicity either for dams and their offspring depending of the studied tissue, and produce *in vivo* effects in combination (MeHg+VitA) completely differents from individual effects of the compounds. Our results in animal model shows the importance regarding the care in food consumption in pregnant and lactating, to avoid health disturbances in the future infant due to unadverted consumption of these compounds in combination. Pro-oxidant responses can lead to effects at cellular level. Furthermore, we recommend more studies regarding active metabolites of retinyl palmitate which can have beneficial effects during cases of low-dose MeHg intoxication, especially in the nervous system. Epidemiological implications of our results are still unknown, for that more follow-up and food habits epidemiologic studies are required among maternal and infant populations worldwide.

LISTA DE ABREVIATURAS

ASGM – mineração de ouro artesanal e a pequena escala

BH – barreira hemato-encefálica

BP – barreira placentária

CAT – catalase

Cys – cisteína

GABA – ácido gama-aminobutírico

GPx – glutationa peroxidase

GSH – glutationa

GST – glutationa-S-transferase

Hg – mercúrio

JECFA – Joint WHO/FAO Expert Committee on Food Additives

LOAEL – menor dose teratogênica

MeHg – metilmercúrio

NAC – N-acetil cisteína

OFT – teste de campo aberto

ORT – teste de reconhecimento de objetos

RBP – proteínas ligantes de retinol

RDA – consumo diário recomendado

RfD – dose de referencia

SNC – sistema nervoso central

USEPA – U.S. Environmental Protection Agency

VitA – vitamina A

LISTA DE FIGURAS

INTRODUÇÃO

CAPITULO II

DISCUSSÃO

LISTA DE TABELAS

CAPITULO I

CAPITULO II

1. INTRODUÇÃO

1.1. Mercúrio e Metilmercúrio

O mercúrio (Hg) é um elemento metálico (número atômico 80 na Tabela Periódica), que é liquido a condições standard de temperatura e pressão. Possui um ponto de fusão de -38.83°C e ponto de ebulição de 356.73°C (Nabi, 2014d). Na natureza, Hg de fontes naturais como antropogênicas pode existir quimicamente na forma elemental (Hg^0), inorgânica (com estados de oxidação 1+ e 2+) e organometálica (Crespo-López et al., 2009). Nos compostos organometálicos, o Hg se encontra ligado covalentemente a um ou dois átomos de carbono formando compostos do tipo RHgX e RHgR' respectivamente. R e R' são grupos orgânicos, e X pode ser um grupo cloreto, brometo, nitrato ou acetato. Como exemplo, nós temos os sais de metilmercúrio (CH_3HgX), fenilmercúrio (C_6H_5HgX), metoxietilmercúrio ($CH_3OC_2H_4HgX$) e dimetilmercúrio ($(CH_3)_2HgX$) (Olivero-Verbel e Johnson-Restrepo, 2002). Do grupo dos organometálicos de cadeias curtas, o metilmercúrio (MeHg) é a forma mais tóxica (WHO, 1996), 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).

Hg se encontra ligado covalentemente ao resíduo de carbono do MeHg (CH_3Hg^+). Nessa forma química de cátion não ligado e livre, o MeHg existe em uma baixa concentração em sistemas biológicos (Hughes, 1957). Na realidade, nesses sistemas os ions de mercúrio são sempre encontrados ligados a moléculas como glutationa (GSH) e cisteina

(Cys), devido à alta afinidade do Hg pelas biomoléculas contendo grupos tióis (Bridges e Zalups, 2005). Uma vez encontrado em nosso organismo, ao redor de 90% de todo o MeHg é absorbido através do trato gastrointestinal. Logo após sua absorção, uma grande parte do composto presente no plasma é acumulado pelos glóbulos vermelhos (eritrócitos) em uma relação 300 a 1 (Olivero-Verbel e Johnson-Restrepo, 2002).

Na célula, a alta estabilidade da ligação 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 e Johnson-Restrepo, 2002), especificamente com os grupos sulfidrilos (-SH) das proteínas (Ogura et al., 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 e Aschner, 1990). Na forma de complexo, o MeHg pode cruzar as barreiras hemato-encefálica (BH) e placentária (BP) como um imitador molecular da metionina (Bridges e 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, dos efeitos tóxicos do MeHg nas células, devido a que constitui uma perturbação na homeostase biológica (Kanda et al., 2014).

1.2. Fenômenos de contaminação ambiental com metilmercúrio

A liberação ambiental do Hg na atmosfera pode ser por fenômenos naturais (70%) o pela contribuição antropogênica (30%). De fato, quando as emissões antropogênicas de Hg chegam no ambiente aquático contribuem com centos de toneladas na quantidade de Hg circulante no ambiente. O MeHg é principalmente formado nesses sistemas aquáticos, quando o Hg (elemental ou inorgânico) liberado na atmosfera e armazenado nos sedimentos e corpos de água, sendo biotransformado por processos naturais microbianos a MeHg (UNEP, 2013).

O MeHg é altamente persistente no ambiente devido à estabilidade da ligação carbono-mercúrio frente a ligação mercúrio-oxigênio quando o composto se encontra na água (Fergusson, 1990). Uma vez nos sistemas aquáticos, o MeHg é bioacumulado e biomagnificado através da cadeia alimentícia, sendo as espécies carnívoras de peixe, encontradas no nível trófico superior, aquelas que registram as maiores concentrações (Olivero-Verbel e Johnson-Restrepo, 2002). De fato, hoje é conhecido que 84% do mercúrio que ingressa ao nosso organismo, é ingerido mediante alimentação com peixes contaminados (WHO, 1996). Entre os episódios de exposição ambiental ao MeHg mais conhecidos se encontra a catástrofe ambiental da Minamata, Japão (1950s) e Niigata (1960s) pela liberação no mar de sulfato de mercúrio (II), empregado como catalizador na obtenção de acetaldeído e posterior ingestão acidental do MeHg na população pelo peixe contaminado (Kondo, 1996, Eto, 2000b, Kondo, 2000).

No entanto, apesar da natureza imprevisível dos eventos anteriores, atualmente 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-Verbel e Johnson-Restrepo, 2002). De fato, junto com a queima de carvão, a mineração de ouro artesanal e a pequena escala (ASGM) contribui aproximadamente com 62% das emissões anuais totais de Hg no ar, sendo América do Sul e África Subsaariana responsáveis pelo incremento nas emissões globais (UNEP, 2013).

O fenômeno de aumento na ASGM em América do Sul mostrou evidencias de afetação em populações a nível ambiental. Os casos mais graves de contaminação por mercúrio se encontram a exposição pelo consumo de peixe dos povos indígenas da Amazônia Brasileira, onde o MeHg é gerado pela atividade de mineração (Crespo-López et al., 2009, Grotto et al., 2010, Crespo-Lopez et al., 2011). Existem outros reportes para o caso da região da Mojana, no noroeste da Colômbia, onde níveis de mercúrio por encima do limite estabelecido pela USEPA (*U.S. Environmental Protection Agency*) foram encontrados em população, vegetação, sedimentos e peixes; apresentando relação direita com a crescente atividade minera artesanal desenvolvida nos corpos de agua nos municípios do Caimito (Sucre) (Olivero et al., 2002, Olivero-Verbel et al., 2004), Ayapel (Córdoba) (Marrugo et al., 2007, Gracia et al., 2010) e sul do departamento do Bolívar (Marrugo-Negrete et al., 2008a, Marrugo-Negrete et al., 2008b, Olivero-Verbel et al., 2011).

1.3. Neurotoxicidade do metilmercúrio

O mercúrio em ambas formas químicas (orgânica e inorgânica) é neurotóxico. A extensão e diversificação dos estudos sobre relação do MeHg e patologias neurológicas ocorre com a catástrofe ambiental da Minamata e Niigata, pois os indivíduos expostos no MeHg mostrarem formas severas nas doenças neurológicas como desbalces cognitivos, sensoriais e motores (Nabi, 2014a).

De fato, depois de sua absorção, o MeHg e distribuído de maneira ubíqua e penetra o sistema nervoso central (SNC) tendo afinidade particular pelo tecido cerebral. É aqui onde o MeHg pode se difundir em todas as regiões cerebrais, cruzando a barreira hemato-encefálica (BH) (Aschner e Clarkson, 1988, Zareba et al., 2007). 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 (Wakabayashi et al., 1995, Shanker et al., 2002, Moretto et al., 2005, Aschner et al., 2007, do Nascimento et al., 2008, Belle et al., 2009, Ferraro et al., 2009, Nabi, 2014a).

1.4. Efeito do metilmercúrio durante o desenvolvimento do sistema nervoso central

O efeito do MeHg sobre o sistema nervoso durante o desenvolvimento foi reconhecido no desastre do Minamata como uma forma congênita da doença (Takeuchi et al., 1979, Eto, 2000), e posteriormente estudos nas populações humanas e modelos animais demostraram que uma exposição a níveis tóxicos do MeHg durante fases pré e pós-natal

causa anormalidades neurológicas, dano cognitivo e uma mudança nos parâmetros comportamentais (Steuerwald et al., 2000, Cordier et al., 2002). Acordo vários autores como Castoldi et al. (2001) e Lepham et al. (1995) a vulnerabilidade do cérebro em desenvolvimento ao MeHg é devida à capacidade de este composto lipofílico para cruzar a placenta e poder concentrar-se no SNC em quanto a BH não está desenvolvida a totalidade durante o período pré-natal. No SNC o MeHg está relacionado com uma grande quantidade de efeitos tóxicos relacionados ou não relacionados uns aos outros, como afetação do córtex cerebelar, sendo as células granulares as mais afetadas. Outros efeitos são a demielinização, inflamação neuronal e destruição astrocitaria. A complexa rede de eventos celulares inicia uma sequência de novos eventos que levam ultimamente a morte celular (Nabi, 2014c). Outros efeitos a nível celular produzidos pelo MeHg são perturbação da homeostase de Ca^{2+}, incrementado sua concentração e afeitando o normal desenvolvimento neural e função sináptica (Nabi, 2014a). Por último, o MeHg ataca as catecolaminas, incluindo a dopamina, assim como os sistemas GABA, dois neurotransmissores implicados nos processos de reforço por aprendizagem e afeitados durante exposição no desenvolvimento (Nabi, 2014b).

Em relação a exposição pré-natal a MeHg, evidencias experimentais em modelos *in vitro* e *in vivo* demostraram que a exposição *in utero* a MeHg tem influência sobre parâmetros toxicológicos (Glover et al., 2009, Liang et al., 2009, Huang et al., 2011, Yoshida et al., 2011, Fox et al., 2012, Jayashankar et al., 2012, Ceccatelli et al., 2013). De fato, doses subministradas do contaminante durante esta etapa do desenvolvimento possuem alta persistência, provocando quadros de desordens neurodegenerativas a longo prazo (Ceccatelli et al., 2013). Por outro lado, a relação da exposição pré-natal a MeHg

com parâmetros comportamentais foi demostrada em modelos animais de exposição efetuados por Gandhi et al. (2013) e Bisen-Hersh et al. (2014).

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, Dorea et al., 2012). Apesar de a exposição a MeHg em crianças durante a lactação ser menor do que imediatamente depois do nascimento (Bjornberg et al., 2005, Sakamoto et al., 2012), estudos sobre populações amazónicas demostraram que o leite materno é uma considerável fonte de exposição pós-natal ao MeHg e ao Hg inorgânico em bebes lactantes (Vieira et al., 2013). No entanto, parece que a lactação tem efeitos favoráveis ou desfavoráveis sobre a exposição ao MeHg. Trabalhos sobre populações humanas expostas a MeHg pelo consumo conjunto de peixe demostraram uma correlação entre diminuição no desenvolvimento neural e consumo do leite materno a logo prazo (Jensen et al., 2005, Marques et al., 2009, Marques et al., 2013), mas evidencia recente encontrou que a lactação, junto ao nível de educação materno e a dieta normal na região são importantes fatores atenuantes das sequelas atribuídas ao MeHg na população infantil da Amazônia Brasileira, exposta ao MeHg pelo consumo de peixe (Marques et al., 2016). Apesar da controvérsia, estudos prévios sobre modelo animal para avaliação do efeito da lactação, mostraram que o MeHg inibe a absorção do glutamato no tecido cerebelar e tem relação com níveis altos do hidroperóxido (Manfroi et al., 2004), ocasionando danos na função motora nos ratos lactantes por possível decrescimento nos níveis basais de tióis cerebelares (Franco et al., 2006).

1.5. Recomendações

Atualmente é conhecido que uma exposição a doses altas do MeHg durante o desenvolvimento produz sinais de paralisia cerebral e de retardo mental, no entanto, alterações na função cognitiva ou intelectual aparecem com exposições a doses baixas deste poluente durante episódios de exposição *in utero*. O anterior é suportado por uma grande quantidade de evidencia epidemiológica obtida em populações com consumo regular de peixe ao nível mundial (Nabi, 2014b, Sheehan et al., 2014). O panorama atual de exposição ao MeHg é variável ao redor do mundo, predominando uma tendência informacional entre mulheres gravidas suportada por certos autores e entidades de saúde pública, sobre o benefício no consumo de certos tipos de peixe e mariscos ricos em selênio e micronutrientes essenciais como os ácidos graxos poli-insaturados (PUFA), junto com um consumo prudente evitando sobre exposição ao Hg (USDA, 2015, Gribble et al., 2016). Como exemplo desta tendência, estudos recentes demostraram que uma grande maioria de mulheres gestantes na União Europeia possuem níveis moderados de Hg abaixo dos valores sugeridos pela USEPA para cabelo (1 µg/g) e sangue (5,8 µg/L) (Castaño et al., 2015, Taylor et al., 2016). Apesar da filosofia conservadora, recente evidencia sugere risco no desenvolvimento de doenças neurocognitivas, comportamentais e cardiovasculares ainda em valores de Hg abaixo dos mostrados anteriormente (Karagas et al., 2012).

Baseadas em evidencia cientifica e para a proteção no feto em desenvolvimento, várias agências internacionais estabeleceram as *doses consideradas seguras* de consumo materno do MeHg. A dose de referência do MeHg (RfD) definida pela USEPA é 0,1 µg MeHg/kg/dia (Rice et al., 2000). No 2003, a JECFA (*Joint WHO/FAO Expert Committee on Food Additives*) recomendou uma dose tolerável semanal para MeHg, o *Provisional*

Tolerable Weekly Intake (pTWI), de 1,6 µg MeHg/kg/semana (equivalente a 0,23 µg MeHg/Kg/dia) (WHO, 2003).

1.6. Vitamina A

A vitamina A (VitA) é um micronutriente essencial que pertence na família de vitaminas lipossolúveis. A VitA é por definição *all-trans*-retinol, mas é um termo genérico para descrever a todos os metabolitos do retinol (Figura 1) que incluem suas formas para armazenamento (ésteres de retinil como o palmitato de retinol) e formas biologicamente ativas (retinal e ácido retinóico) (Blaner et al., 2016). A VitA não pode ser sintetizada *de novo* em nosso organismo, é por isso que deve ser obtida através da dieta onde pode ser encontrada em duas formas: vitamina A pré-formada (retinol ou ésteres de retinil) e carotenoides pró-vitamina A (Ross, 2010, Napoli, 2012, Oliveira, 2015). Altos conteúdos de vitamina A pré-formada podem ser encontrados em fígado e peixe; enquanto vegetais verdes, laranjas e amarelos são as formas dietárias mais comuns de pró-vitamina A, sendo o *β*-caroteno (Figura 1) o carotenoide pró-vitamina A mais importante (Ross, 2010). Os dois tipos de compostos podem ser convertidos a VitA pelo organismo posteriormente (Bellovino et al., 2003, Napoli, 2012, Oliveira, 2015).

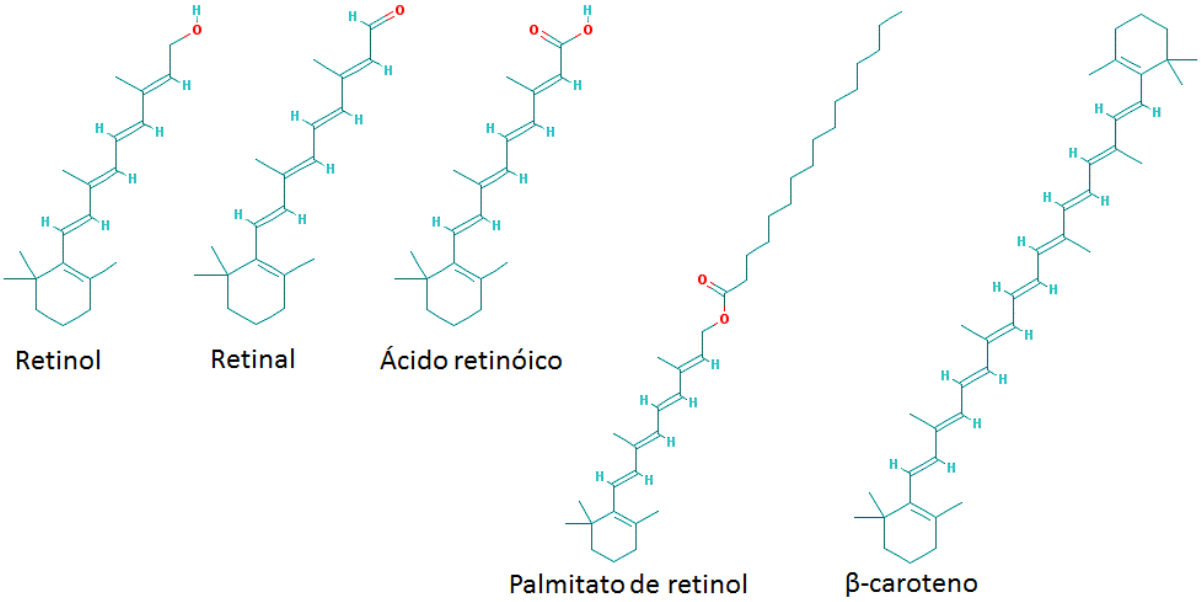

Figura 1. Estrutura molecular dos metabolitos da vitamina A.
As estruturas foram obtidas da PubChem Open Chemistry Database (https://pubchem.ncbi.nlm.nih.gov/search/), 2017.

A vitamina A é requerida para o correto funcionamento de um conjunto de atividades metabólicas e fisiológicas como a visão, hematopoiese, desenvolvimento embriogênico, diferenciação de células epiteliais, imunocompetência e transcrição genica (Chapman, 2012). Uma vez encontradas em nosso organismo, as diversas formas de VitA são solubilizadas dentro das micelas do lúmen intestinal e absorbidas pelas células mucosas do duodeno (IOM, 2001). Tanto a vitamina A pré-formada como a pró-vitamina A são transformadas a retinol que é posteriormente oxidado a retinal e ácido retinóico nas células, mas uma grande quantidade da VitA adquirida pelo organismo é armazenada no fígado na forma de ésteres de retinil (NIH, 2016). No plasma, o transporte de virtualmente toda a VitA presente no sangue é efetuado pela *retinol-binding protein* (RBP) mediante a formação de um complexo 1:1 com o retinol (holo-RBP). A captação do retinol nas células e nos tecidos é mediada pelo receptor transmembrana STRA6, de alta afinidade com o

complexo holo-RBP, mas o mecanismo de transporte exato ainda se encontra em discussão (Kawaguchi et al., 2015).

A VitA e os retinóides (compostos naturais ou sintéticos com similaridade estrutural ou propriedades similares a VitA) são importantes durante a fase de desenvolvimento fetal. De fato, o embrião tem como fonte de VitA os retinóides como retinol, ésteres de retinil e *β*-caroteno, que circulam na torrente sanguínea materna (Spiegler et al., 2012). Entretanto, os mecanismos de transferência de retinóides pelo embrião através da placenta se encontram em discussão. Atualmente, é sabido que, na maioria de vertebrados, a RBP materna não consegue cruzar a placenta (Quadro et al., 2005), mas a captação do retinol materno e posterior transferência ao embrião pode ocorrer na superfície endodérmica do saco vitelino, que possui uma alta síntese de RBP e STRA6 (Spiegler et al., 2012). Ao nível celular, o retinol pode ser transferido ao feto por três possíveis mecanismos: 1) transferência direta do complexo RBP-retinol envolvendo captação celular e liberação do complexo proteína-ligando; 2) transferência de retinol livre; e 3) captação celular de retinol por seu receptor especifico STRA6 (Spiegler et al., 2012).

A rota do RBP-retinol materno é o principal contribuinte para o desenvolvimento fetal. Entretanto, a rota dos ésteres de retinil permite o acumulo das reservas de retinóides no feto, ainda em condições de depleção da RBP materna (Quadro et al., 2005). Os ésteres de retinil presentes em quilomicrones e remanentes, são os maiores retinóides da circulação materna durante o estado alimentado (Wassef e Quadro, 2011). De acordo a estudos de Wassef e Quadro (2011) os retinóides pós-prandiais podem ser transferidos em direção do embrião, através da RBP expressa no tecido placentário.

Considerando o anterior, os níveis de retinóides na circulação materna, determinado pelas quantidades de reserva e retinóides recentemente consumidos, determinam o status materno de VitA e, portanto, as quantidades disponíveis de VitA circulante ao feto através da placenta. Entretanto, depois do parto, a lactação constitui a única fonte disponível de VitA para o neonato. De fato, a transferência de VitA é 60 vezes maior durante os primeiros meses de lactação do que durante a gestação (Stoltzfus e Underwood, 1995).

Quando é descrita em términos de nutrição, há vários anos o termo unidades internacionais (IU em inglês) foi usado para expressar o aporte nutricional de VitA. Atualmente é usada uma unidade global, conhecida como miligramas de equivalentes de atividade de retinol (µg RAE). Os µg RAE permitem a expressão dos aportes de VitA, da dieta e suplementos, em uma unidade universal. De fato, 1 µg RAE (3,33 IU) é equivalente a 1 µg de retinol, 2 µg de β-caroteno dos suplementos, 12 µg de β-caroteno da dieta e 24 µg de outros carotenoides como α-caroteno ou β-criptoxantina (NIH, 2016).

1.7. Neurotoxicidade da vitamina A

A VitA é um composto de importância para o desenvolvimento do feto (Clagett-Dame e Knutson, 2011), e para o SNC em desenvolvimento, contribui nos processos de diferenciação celular e apoptose e regula sua homeostase durante a vida adulta (McCaffery et al., 2003, Lane e Bailey, 2005, McCaffery et al., 2006). No entanto, evidencia experimental suporta a neurotoxicidade dose-dependente da VitA.

Os casos de ingesta excessiva de VitA são raros e devido a isso a toxicidade de doses altas do composto é difícil de avaliar de acordo a certos autores (Oliveira, 2015).

Contudo, certas patologias cerebrais como *pseudotumor cerebri*, além de alterações comportamentais como confusão, irritabilidade, ansiedade, depressão e ideias suicidas, têm sido documentadas em episódios de hipervitaminose A (Snodgrass, 1992).

Devido a sua importância no neurodesenvolvimento do feto, a deficiência de VitA tende a ser evitada em muitos países. Além disso, não se tem reportes sólidos que demostrem neurotoxicidade com o uso de doses baixas. No entanto, evidencias em modelos animais demostraram decréscimo na locomoção e exploração no teste de campo aberto em ratas Wistar adultas expostas a doses terapêuticas de VitA (300 – 750 µg RAE/Kg/dia) (de Oliveira et al., 2007a). Adicionalmente, na forma de palmitato de retinil, a VitA em doses de suplementação (750, 3750 e 7500 µg RAE/Kg/dia) induz ansiedade em mães expostas, além de alterações nos filhotes (Schnorr et al., 2011b).

1.8. Recomendações

A dose diária recomendada de VitA para homens adultos é de 900 µg RAE e para mulheres adultas é de 700 µg RAE; sendo a dose máxima tolerada de 3000 µg RAE (Chapman, 2012, NIH, 2016). Durante a engravides, os requerimentos de VitA na mulher são: o basal de 370 µg RAE/dia, a dose máxima de 3000 µg RAE/dia e a dose diária recomendada de 770 µg RAE/dia (McCauley et al., 2015).

Evidencias dos efeitos do excesso/deficiência da VitA têm sido amplamente referenciados. O excesso da VitA produzida em casos não tão raros, possui a capacidade teratogênica. O padrão dos defeitos (síndrome do ácido retinóico) inclui malformações do SNC, craniofaciais, cardiovasculares e tímicas (Azaïs-Braesco e Pascal, 2000). Entretanto,

a deficiência subclínica da VitA (VitAD) incrementa taxas de mortalidade materna, fetal e infantil e afeta países desenvolvidos e industrializados (Quadro et al., 2005). Para proteção das populações com alta incidência de VitAD, a organização mundial da saúde (WHO) sugere como doses diárias provavelmente seguras as de até 3000 µg RAE (equivalentes a 10000 IU) ou 7500 µg RAE (equivalentes a 25000 IU) na semana depois do dia 60 de gestação (WHO, 2009, McCauley et al., 2015).

1.9. Efeitos da exposição a misturas químicas

Em vez de enfocar-se nos efeitos tóxicos produzidos a substâncias químicas individuais, atualmente mais estudos levam em conta que muitas doenças em humanos estão relacionadas a exposição ambiental a misturas químicas em uma base diária (CDC, 2009, Kienzler et al., 2016). De acordo aos toxicólogos, os efeitos das misturas químicas podem ser aditivos, antagónicos, potenciais, sinérgicos e sequenciais (Zeliger, 2011).

Os efeitos **aditivos** são produzidos quando duas o mais substancias com a mesma toxicidade são encontradas juntas e seu efeito global é a somatória dos efeitos das substancias individuais. O **antagonismo** ocorre quando dois químicos interferem com os efeitos produzidos por cada um; mas no caso dos efeitos **potenciais**, um composto químico aumenta o efeito do outro quando atuam como misturas químicas. Por outro lado, o termo **sinergismo** e relacionado com o fato do que o efeito da exposição a uma mistura produze uma resposta muito maior do efeito aditivo (o que é conhecido como resposta quantitativa) ou afetando um órgão que não foi previsto (resposta qualitativa). Por último, os efeitos **sequenciais** são produzidos em uma base temporal, quando um primer composto absorbido

permite a absorção em transcurso do tempo de um segundo, resultando em efeitos globais diferentes dos compostos individuais da mistura química.

Entretanto, existem outro tipo de efeitos das misturas químicas conhecidos como **não esperados** pela literatura. De acordo com Zelinger (2003), nesse caso as misturas estão formadas pelo menos por um químico lipofílico e outro hidrofílico. O composto lipofílico facilita a absorção da espécie hidrofílica através das membranas mucosas em nosso organismo, em quantidades maiores do que seria absorvido se o lipofílico não estivera presente. Esses efeitos seriam agudos ou crônicos. Atualmente, esta teoria explica os efeitos tóxicos sequenciais de misturas como contaminantes orgânicos persistentes (POPs), de natureza lipofílica e absorbidos no soro, e compostos de natureza hidrofílica como ftalatos, além de sua possível relação com doenças crónicas neurocognitivas, cardiovasculares e diabetes (Zeliger, 2013a, Zeliger, 2013b, Zeliger, 2013c).

1.10. Espécies reativas de oxigênio

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

Os radicais livres são descritos como a espécie química seguida de um ponto sobrescrito, para indicar o elétron desaparelhado. Como exemplos, o radical livre mais simples, o hidrogênio atômico ($H^{\bullet}$) (Halliwell, 2006, Halliwell e Gutteridge, 2015). As ROS de maior importância fisiológica são o radical libre ânion superóxido ($O_2^{\bullet -}$), o radical hidroxil ($OH^{\bullet}$) e o peróxido de hidrogênio (H_2O_2) (Birben et al., 2012).

A presença de um ou vários elétrons desaparelhados permite que o radical livre seja altamente reativo, mas a reatividade química varia amplamente entre radicais (Halliwell e Gutteridge, 2015). De fato, uma das características biológicas mais interessantes dos radicais livres 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 radicais livres de maneira sucessiva (Halliwell e Gutteridge, 2015).

1.11. Defesas antioxidantes

As defesas antioxidantes das células podem ser de dois tipos: enzimáticas e não-enzimáticas. Entre as defesas enzimáticas temos a enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa-S-transferase (GST). No caso das defesas enzimáticas 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 família das SODs (CuZnSOD, MnSOD, FeSOD e NiSOD) catalisam a reação de obtenção de peroxido de hidrogênio

através do radical ânion superóxido ($O_2^{\bullet -}$), expressada na equação química: $O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ (Halliwell e Gutteridge, 2015). O peróxido de hidrogênio produzido pela ação das SODs, ou as oxidases (como a xantine oxidase) é reduzido a agua pela CAT e a GPx, processo representado pela equação química: $2H_2O_2 \rightarrow 2H_2O + O_2$. No entanto, a GPx também 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).

1.12. Estresse oxidativo

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 e 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 e 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/sobre nutrição materna, exposição ao 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 evidencia 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 e Al-Hasan, 2012).

1.13. Estresse oxidativo gerado pelo metilmercúrio e vitamina A

Existe evidencia experimental suportando o fato que o MeHg, além das espécies de Hg são responsáveis de gerar estresse oxidativo a nível celular afetando as defesas antioxidantes. A alta afinidade do Hg pelos grupos tióis (-SH) permite sua ligação com peptídeos pequenos como a glutationa reduzida (GSH) decrescendo seus níveis e incrementando a presença de ROS a nível celular. O Hg 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, 2014d)

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 ROS. Estudos sobre sinaptosomas estriados dos ratos expostos ao MeHg demostrou uma relação idade dependente com a produção dos ROS, sendo mais frequente nas etapas pós-natais prematuras (Dreiem et al., 2005). No outro estudo sobre células granulares isoladas dos ratos tratados com MeHg mostraram níveis elevados do ROS e potencial de membrana diminuída respeito a controles (Bellum et al., 2007).

Uma importante quantidade de evidencia experimental suporta o fato que a doses que excedem as requeridas para o funcionamento normal das células, a VitA produz efeitos deletérios no ambiente celular como o desequilíbrio redox. Modelos animais demostraram incremento no dano oxidativo a proteínas, lipídeos em substantia nigra e striatum isolados de ratas tratadas com VitA. Além disso, o composto produz modulação das enzimas antioxidantes SOD, CAT pero não GPx (Oliveira review 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 et al., 2012, Gasparotto et al., 2015, Schnorr et al., 2015). No entanto, certos estudos no modelo animal de desenvolvimento mostraram que durante a gestação e lactação, doses debaixo do *Lowest Observed Adverse Effect Level* (LOAEL), e sugeridas pelos comitês mundiais para suplementação materna do retinol palmitato, incrementaram a proporção superóxido dismutase/catalase (SOD/CAT), dano oxidativo em estriado e hipocampo (Schnorr et al., 2011a), e dano multi-tecidual das mães e os filhotes (Schnorr et al., 2011b).

2. OBJETIVOS

2.1. Objetivo geral

Avaliar parâmetros toxicológicos, neurocomportamentais e bioquímicos em um modelo animal utilizando ratas *Wistar* gravidas durante o desenvolvimento pré-natal (gestação) e pós-natal (lactação) submetidas a suplementação da vitamina A (7500 µg RAE/Kg/dia) e uma dose ambientalmente relevante do MeHg (0,5 mg/Kg/dia).

2.2. Objetivos específicos

1) Avaliar parâmetros toxicológicos clássicos nos filhotes e nas mães em modelos animais de ratos *Wistar* submetidos a suplementação da vitamina A (7500 µg RAE/Kg/dia) e a uma dose ambientalmente relevante do MeHg (0,5 mg/Kg/dia) durante o desenvolvimento pré-natal (gestação) e pós-natal (lactação)

2) Analisar parâmetros neurocomportamentais dos filhotes e das mães em modelos animais de ratos *Wistar* submetidos a suplementação da vitamina A e uma dose ambientalmente relevante do MeHg durante o desenvolvimento pré-natal (gestação) e pós-natal (lactação)

3) Avaliar parâmetros bioquímicos de estresse oxidativo nos filhotes e nas mães em modelos animais de ratos *Wistar* submetidos a suplementação da vitamina A (7500 µg RAE/Kg/dia) e a uma dose ambientalmente relevante do MeHg (0,5 mg/Kg/dia) durante o desenvolvimento pré-natal (gestação) e pós-natal (lactação)

4) Comparar parâmetros toxicológicos, neurocomportamentais e bioquímicos em ratos machos e fêmeas da linhagem *Wistar* nascidos de mães submetidas a suplementação com vitamina A (7500 µg RAE/Kg/dia) e uma dose ambientalmente relevante do MeHg (0,5 mg/Kg/dia) durante a gestação e lactação.

PARTE 2

3. MATERIAIS, MÉTODOS E RESULTADOS

Na presente dissertação, os MATERIAIS E MÉTODOS, assim como os RESULTADOS estão dirigidos em forma detalhada na forma de dois artigos científicos a ser submetidos (Capítulo I e Capitulo II).

CAPITULO I - EFFECTS OF METHYLMERCURY AND RETINOL PALMITATE CO-ADMINISTRATION IN RATS DURING PREGNANCY AND BREASTFEEDING: METABOLIC AND REDOX PARAMETERS IN DAMS AND THEIR OFFSPRING

Artigo a ser submetido no periódico: **Ecotoxicology and Environmental Safety**

“EFFECTS OF METHYLMERCURY AND RETINOL PALMITATE CO-ADMINISTRATION IN RATS DURING PREGNANCY AND BREASTFEEDING: METABOLIC AND REDOX PARAMETERS IN DAMS AND THEIR OFFSPRING”

Pedro Espitia-Pérez[1,*], Suelen Marin Albino[1], Helen da Rosa[1], Alexander Kleber Silveira[1], Lyda Espitia-Pérez[2], Hugo Brango[3], Diogo Pompéu Moraes[4], Paolla Silva Hermann[4], Moara Mingori[1], Fabiano Barreto[5], Alice Kunzler[1], Daniel Pens Gelain[1], Carlos Eduardo Schnorr[6] and José Cláudio Fonseca Moreira[1,**]

[1] Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600, Anexo Depto. Bioquímica, Lab 32, CEP 90035-003, Porto Alegre, Rio Grande do Sul, Brazil.

[2] Facultad de Ciencias de la Salud, Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Calle 38 Carrera 1W, Barrio Juan XXIII, Montería, Córdoba, Colombia.

[3] Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil.

[4] Instituto de Química, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brazil.

[5] Laboratório de Análise de Resíduos de Pesticidas e Medicamentos Veterinários (RPM), Laboratório Nacional Agropecuário RS, Estrada da Ponta Grossa 3036, CEP: 91780-580, Porto Alegre, Rio Grande do Sul, Brazil.

[6] Departamento de Civil y Ambiental, Programa de Ingeniería Ambiental, Universidad de la Costa, Calle 58 #55– 66, Barranquilla, Atlántico, Colombia.

For correspondence with the author:
*E-mail address: pjespitia@gmail.com (P. Espitia-Pérez)

** E-mail address: 00006866@ufrgs.br (J. C. Fonseca Moreira)

Abstract

Ubiquitous low-dose methylmercury (MeHg) exposure through an increased fish consumption represents a global public health problem, especially among pregnant women. A plethora of micronutrients presented in fish affects MeHg uptake/distribution, but limited data is available. Vitamin A (VitA), another fish micronutrient is used in nutritional supplementation, especially during pregnancy. However there is no information about the health effects arising from their combined exposure. Therefore, the aim of the present study was to examine the effects of both MeHg and retinyl palmitate administered on pregnant and lactating rats in metabolic and redox paramaters from dams and their offspring. Thirty *Wistar* female rats were orally supplemented with MeHg (0,5 mg/Kg/day) and retinyl palmitate (7500 µg RAE/Kg/day) via gavage, either individually or in combination from the gestational day 0 to weaning. For dams (150 days old) and their offspring (31 days old), glycogen accumulation (hepatic and cardiac) and retinoid contents (plasma and liver) were analyzed. Hg deposition in liver tissue was quantified. Redox parameters (liver, kidney and heart) were evaluated for both animals. Cytogenetic damage was analyzed with micronucleus test. Our results showed no general toxic or metabolic alterations in dams and their offspring by MeHg-VitA co-administration during pregnancy and lactation. However, increased lipoperoxidation in maternal liver and a disrupted pro-oxidant response in the heart of male pups was encountered, with apparently no particular effects in the antioxidant response in female offspring. MeHg-VitA treatment affected GST activity in dam kidney, leading to possible redox disruption of this tissue, with no alterations in offspring. Finally, genomic damage was exacerbated in both male and female pups from MeHg-VitA group. In conclusion, low-dose MeHg exposure and retinyl palmitate supplementation during gestation and lactation produced a potentiated pro-oxidant effect, which was tissue-specific. Although this is a pre-clinical approach, we recommend precaution for pregnant women in terms of food consumption, and we encourage more epidemiological studies to assess possible modulations effects of MeHg-VitA co-administration at safe or unadvertely used doses in humans, which may be related to certain pathologies in mothers and their children.

Keywords: Methylmercury, Vitamin A, Pregnancy, Co-exposure, Oxidative stress

1. Introduction

Methylmercury (MeHg) is a well-documented ubiquitous contaminant [1, 2]. In the environment, MeHg bioaccumulates and biomagnifies through the food web, reaching concentrations many times higher than the levels in the surrounding water [3] representing a serious risk to human health. As dietary fish intake is also a major MeHg source to fish consumers [4, 5], populations with a traditionally high dietary intake of fish are the most exposed to MeHg bioaccumulation and may consequently experience its deleterious effects on human health [4, 6]. Once absorbed, MeHg is rapidly transported in red blood cells due to its increased membrane permeability, and widely distributed in the body, particularly in the central nervous system (CNS); however, MeHg conversion to inorganic Hg and posterior storage is mostly carried in the liver and kidney [7]. Like Hg, MeHg penetrates the placental and the blood-brain barrier of the fetus, which is not completely developed, increasing its exposure [8, 9]. Effects observed following *in utero* exposure in poisoning events have included impaired motor development, spasticity, blindness, abnormal reflexes, deafness, seizures and deficiencies in memory, learning and psychological parameters, particularly in cases of low dose chronic exposure [10-13]. Therefore, as a particularly susceptible group, the effect of MeHg exposure on pregnant women remains an important issue for elucidation, especially in populations where fish is the main source of animal proteins [14].

Maternal fish consumption during pregnancy exposes the fetus simultaneously to other substances and nutrients present in fish such as n-3 fatty acids, minerals and vitamins, including vitamin A (VitA), a fat-soluble retinoid also present in eggs and dairy products [15]. VitA may be obtained from both vegetal (provitamin A) and animal (preformed) diet [16, 17]. High contents of preformed VitA can be encountered especially in liver and fish [18]. Several important metabolic and physiologic processes in the organisms require VitA, such as vision, hematopoiesis, embryonic development, cell differentiation, immunocompetence and gene transcription [19]. In fact, in pregnant women, VitA contributes to early fetal development, especially in the CNS [20-23] and VitA supplementation is recommended as safe in doses of 3000 or 7500 μg RAE/daily, independently of previous VitA consumption [24]. Both MeHg and VitA may have pro-oxidant characteristics. Reactive oxygen species (ROS) generation has been linked to MeHg- induced toxicity both *in vivo* and in *vitro* systems. Evidence suggests that MeHg exposure causes production of ROS [25, 26], depletion of glutathione (GSH) [27], excessive accumulation of calcium (Ca^{2+}) [28], apoptosis/necrosis [29] and a decrease in mitochondrial membrane potential in nervous [30] and immune [29] systems. On the other

hand, despite its importance in the healthy development of the fetus and the newborn, recent evidence supports that VitA is a redox-active molecule capable of inducing pro-oxidant effects in an animal model at safe/therapeutical doses [31-35]. In rats, VitA supplementation at therapeutic doses resulted in impairment of liver and kidney redox balance in mothers and their offspring [36]. VitA also possesses teratogenic effects at higher doses (retinoic acid syndrome), originating a pattern of birth defects including craniofacial, cardiovascular and thymic affectations [37]. Thus, during the first weeks of embryogenesis, supplementation with VitA must be carefully managed in order to avoid congenital malformations caused by either deficiency or excessive intake [38, 39].

Individual effects of MeHg and VitA exposure are well documented. However, there is a lack of information about the effects arising from their combined exposure. The particular nutritional requirements in women increases during pregnancy, especially for fish and shellfish, which are the primary source of long-chain polyunsaturated fatty acids [40]. Despite recommendations from global committees in regard of MeHg exposure, only in the US, during the last years, fish consumption has increased among pregnant women [40, 41], creating a possible scenario of increasing MeHg and VitA bioavailability, imposing unknown risks to the expectant mother and her fetus. The aim of the present study was to examine the effects of both MeHg and VitA administered on pregnant and lactating rats. Thus, we assess redox state, mercury deposition, metabolic liver function and genomic damage in adult rats and their offspring, in order to evaluate possible additive/synergistic/antagonistic interactions between these compounds *in vivo*.

2. Materials and methods

Experimental procedures were conducted in accordance with the Principles of Laboratory Animal Care (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, revised 2011) [42], and were in compliance with recommendations of the Brazilian Society for Science in Laboratory Animals (SBCAL-COBEA). The Ethical Committee for Animal Experimentation (CONCEA) ascribed to Federal University of Rio Grande do Sul (UFRGS) approved the research protocol under authorization number 31672.

2.1. Animal and housing conditions

Male and female Wistar rats (*Rattus novergicus*) were obtained from our breeding colony and housed in groups of four animals under a 12 h light-dark cycle (7:00 – 19:00h) at constant

temperature (21 ± 1°C). Standard commercial food (CR1 Lab Chow, Nuvilab, Curitiba, Brazil; 3600 µg RAE/Kg) and filtered water were provided *ad libitum*. Female nulliparous rats (200-250g) with 120 days were checked daily for their estrous cycle using direct vaginal smear evaluation under light microscopy, as a previously described protocol [43]. Estrous cycle monitoring was performed for two weeks, and through a later period of a week, in order to obtain a maximum number of females in proestrous as possible in each reproductive cycle. Sexually receptive females (confirmed proestrous) were caged overnight with a single mature male (1F:1M). The next morning the presence of viable sperm in the vaginal smear was considered as successful mating and designated as gestation day 0 (GD0). Pregnant dams were caged individually and allowed to litter naturally. The date of delivery was defined as postnatal day 0 (PND0).

Restriction stress in late pregnancy (GD17-21) affects the dam and influences somatic growth and weight gain in offspring [44]. In order to avoid stress-related effects on offspring, dams were minimally handled during a 4-day period (comprising GD20 and PND2) and pups were only manipulated and registered on PND2. However, gavage continued during the restriction period performed by the same operant, in order to avoid additional stress in the dam. Pre-culled litter size possibly have influence on further development in pups from culled litters, due to maternal and offspring mutual adjustment to genetically defined litter sizes. The latter may increase variability in pup´s weight, as treatment-related mortality progresses in culled litters [45]. For that reason, we wanted to assure a maximum number of live pups. Consequently, pups were not culled to avoid possible interferences in our results, and full-sized litters were maintained with the dam until weaning (PND21). Pups were separated from the dam and immediately sexed on PND21. Separated litters were co-housed in groups of the same sex to eliminate interferences on behavior related to sexual hormones [46]. No further treatment was applied to male and female pups, which continued in the same room conditions as their parents with standard food and filtered water *ad libitum*.

2.2. Treatment

Methylmercury (II) chloride was obtained from Spectrum (Spectrum, New Brunswick, NJ, United States). A stock solution of 0,39 μg/μL of MeHg was prepared in mineral oil. Retinol palmitate (a commercial form of vitamin A dissolved in mineral oil) was purchased from Embrafarma, (Embrafarma, Sao Paulo, SP, Brazil) as a stock solution of 45000 µg RAE. Thirty pregnant dams were randomly divided into four experimental groups as described:

Group 1 (control), where rats were treated with mineral oil; Group 2 (MeHg), where rats received methylmercury (II) chloride at a dose of 0,5 mg/Kg/day; Group 3 (VitA), where rats received retinol palmitate at a dose of 7500 µg RAE/Kg/day; Group 4 (MeHg-VitA): where rats received methylmercury (II) chloride (0,5 mg/Kg/day) and retinyl palmitate (7500 µg RAE/Kg/day) both mixed. All solutions were prepared in mineral oil.

All treatments were prepared daily and administrated orally with a metallic gastric tube (via gavage) in a maximum volume of 0,5 mL. Pregnant rats were treated once a day from GD0 until weaning at PND21. In dams, no evidence of toxicity, motor alterations, or general health disturbances were observed during gestation and lactation in either of the experimental groups.

2.3. Reproductive success data

During experiments, dams were monitored daily for treatment-related symptoms. Body weight was assessed during gestation (GD0, 7, 14, 20 and 21) and lactation (PND2, 7, 14 and 21) and total gestational length from females were scored. In order to avoid stress-related effects on weight, dams were minimally handled from GD20 to PND2 and pups from each litter were counted and weighted starting from PND2 [44]. The weight of the offspring was assessed during lactation (PND2, 7, 14 and 21). During nursing period, pups were examined on a daily basis for clinical signs, mortality and/or frequency of morphological abnormalities. For dams, implantation sites were confirmed and counted in a posterior necropsy. Gestation and lactation weight gains, delivery index, sex ratio and viability indexes were calculated on PNDs 2, 7, 14 and 21 as previously described by Schnorr et al. [34, 47].

2.4. Tissues extraction and sample preparation

All animals were sacrificed by decapitation. Dams were scheduled for sacrifice 24h after the last treatment at PND21, whereas male and female pups were set on PND31. Livers, hearts, and kidneys from all animals were dissected in ice and stored in -80°C for posterior analysis. Two fractions of fresh liver (100-200 mg of wet tissue) were isolated and employed to determine glycogen, retinol and retinyl palmitate levels. Additionally, the second portion of fresh heart samples (100 mg) was used to obtain cardiac glycogen, and a third portion of the liver (130-180 mg of wet tissue) was isolated and stored in -80°C for posterior mercury quantification. Two femurs from each animal (dams and pups) were also dissected and cut to obtain bone marrow samples. Blood samples from all animals were collected, and the plasma was separated immediately to obtain serum retinol concentrations.

For biochemical analyses, tissues were homogenized in 50 mM phosphate buffer (pH 7.0) and centrifuged (3000 *x g*, 5 min) to remove cellular debris. Supernatants were employed for all biochemical analyses described forward. Results were normalized by protein content according to Lowry technique using bovine albumin as standard [48].

2.5 Biochemical analyses

2.5.1 Glycogen determination

Glycogen determination was performed using a slight modification of the method described by Hassid and Abraham [49]. Briefly, freshly collected liver and heart samples (100-150 mg) were digested in boiling bath with 30% potassium hydroxide (KOH) for 20 min and posterior heating with 70% ethanol at 70°C for extraction. Extracted glycogen was solubilized with potassium iodide/iodine (KI/I_2) and colored products were measured spectrophotometrically at 460 nm.

2.5.2 Extraction of retinol, retinyl acetate and quantification by HPLC in serum and liver samples

Sample preparation and extraction were performed as described in previous methodology [50, 51], with slight modifications. Briefly, plasma (100 uL) was extracted using two-cycle vortex with 150 uL acetonitrile and centrifugated (13000 rpm, 10 min). A 200 uL aliquot was transferred for injection to HPLC system. For liver, pre-weighted samples were homogenized in ethyl acetate (medium speed, cycles of 10 s), vortexed for 10 min (180 rpm) and centrifugated (4000 rpm, 10 min). Homogenate samples were evaporated to dryness using a Tecnal TE-0194 evaporator system with nitrogen as carrier gas. Finally, the residue was re-dissolved in methanol and vortexed. A 200 uL aliquot was transferred for injection to HPLC system.

Analysis was carried out using an Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled a triple quadrupole mass spectrometer SCIEX 5500 QTRAP (Foster City, CA, USA). The separation was achieved by an Agilent (Agilent Technologies, Palo Alto, CA, USA) Zorbax Eclipse XDB-C18 column (C18 3.5 µm, 100 mm × 2.1mm) coupled to a C18 guard column (Phenomenex, CA, USA). Injection volume was 2 µL, and mobile phase composition in gradient elution was water with 2 mM ammonium formate (A) and 2 mM ammonium formate acetonitrile both containing 0.1% formic acid (B). The flow rate was 500 µl min-1, and a three-minute equilibrate time was applied. The gradient starts with 95% of A

decreasing to 5% at 1 minutes, kept for 8 minutes and returns to initial conditions at 9.5 minutes. A diverter valve was employed to reduce the introduction of matrix components in the spectrometer.

Quantification was performed by relating peak area of analites obtained via determination, versus peaks from retinol and retinyl acetate standards and expressed as μg/g wet weight for liver samples and μg/mL for plasma samples. To avoid photodecomposition of analites, all extraction procedures, handling of homogenates and quantifications were performed under light-controlled areas.

2.6 Mercury determination by chemical vapor generation coupled to atomic absorption spectrometry (CVG-AAS)

Liver samples from mothers and their offspring were weighted in an analytical balance (XS105 Excellence XS, Mettler Toledo, Switzerland) and pretreated by using a wet ash digestion procedure. Sample aliquots (0.1 to 0.2 g) were treated with concentrated HNO_3 using an initial microwave-assisted heating (4 cycles of 5 s duration) with a subsequent overnight (12 h) step. A final cycle of heating assisted by microwave was performed and sequentially H_2O_2 was added before sonication step using an ultrasonic bath (30 min). Finally, total volume was completed to 10 mL with water and centrifugation was performed to remove solid particles from wet ash procedure. Pretreated samples were injected via flow injection analysis (FIAS 100, Perkin-Elmer, USA) using a sampling loop of 500 μL. The solution of 1 mol L^{-1} hydrochloric acid at flow rate of 4.8 mL min^{-1} and 0.75% (m/v) sodium tetrahydroborate at flow rate of 1.8 mL min^{-1} were used as carrier and reductant, respectively. Argon was used as carrier gas at a flow rate of 50 ml min^{-1}. The arrangement of FIAS system was carried out using a polytetrafluoroethylene (PTFE) tubes 1.0 mm i.d. and 200 mm (reaction coil) to transport the solutions to a gas-liquid separator. Milli-Q water (18.2 MΩ cm, Millipore, USA) and analytical-grade reagents were used throughout the analysis. Standards solutions were prepared on the day of use by serial dilution of 1000 mg L-1 stock solution of Hg (Fluka Analytical, Sigma-Aldrich, USA). These standard stock solutions were also used for the analytes recoveries tests. The calibration solutions were prepared in 10% (v/v) HNO_3 in the range of 2.5 - 20 μg L^{-1} for Hg determination by CVG-AAS.

Mercury measurements were carried out with an atomic absorption spectrometer (Analyst 200, Perkin Elmer, USA) equipped with a quartz T-cell (160 mm length, 7 mm i.d.) at 100 ºC. A mercury electrodeless discharge lamp (EDL) at 220 mA was used as radiation source with

deuterium lamp as continuum source for background correction. 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). All laboratory materials such as glasses and polypropylene vials were cleaned by soaking in an acid bath (10% v/v HNO_3) for seven days.

2.7 Redox profile

2.7.1 Cellular oxidative damage

Quantification of thiobarbituric acid reactive species (TBARS) was performed according to the protocol described by Draper and Hadley [52]. Briefly, protein from homogenized samples was extracted using 15% trichloroacetic acid (TCA) and supernatants were treated with 0,67% thiobarbituric acid and heated at 100°C on a microplate dry bath for 25 min. TBARS content was determined spectrophotometrically at 532 nm. Results were expressed as nanomoles of TBARS per milligrams of protein.

Total reduced thiol content in samples was quantified using the protocol described by Ellman [53]. Briefly, homogenized samples were treated with 5,5-dithiobis-2-nitrobenzoic acid (0,01 M) in ethanol. The developed yellow color was measured spectrophotometrically at 412 nm after 60 min. Protein and non-protein fractions were obtained using 15% TCA for separation. Results were expressed as micromoles of sulfhydryl per milligrams of protein.

2.7.2 Enzymatic antioxidant activities

Superoxide dismutase (SOD) activity was determined according to Misra and Fridovich [54] by using the quantification of adrenaline-dependent inhibition of superoxide auto-oxidation in a spectrophotometer at 480 nm. Results were expressed as units of SOD activity per milligram of protein.

Catalase (CAT) activity was measured by the method of Aebi [55]. Briefly, using hydrogen peroxide (H_2O_2) as substrate, the decrease in absorbance due to H_2O_2 degradation was monitored spectrophotometrically at 240 nm for 1 min. Results were expressed as units of CAT per milligrams of protein.

Glutathione peroxidase (GPx) activity was estimated using the method described by Wendel [56], by monitoring the oxidation of NADPH in a spectrophotometer at 340 nm. Results were expressed as units of GPx per mg of protein.

Glutathione-S-transferase (GST) activity was determined using the method described by Habig and Jakoby [57] by the increase in conjugation rate of glutathione with 1-cloro-2,4-

dinitrobenzene measured spectrophotometrically as absorbance for 3 minutes at 340 nm. Results were expressed as units of GST per milligrams of protein.

2.8 Micronucleus test in polychromatic erythrocytes

Micronucleus assay was carried out according to methodology previously described by Horta et al. [58] and the OECD guidelines [59]. Bone marrow smears were prepared for dams and their offspring. The bone marrow was extracted from the two femurs, and the smears were prepared directly on slides, two per animal, with a drop of fetal calf serum. The slides were stained with 10% Giemsa for 5 min, air-dried and coded for blinded analysis. To avoid false negative results and as a measure of toxicity in bone marrow, the polychromatic erythrocytes: normochromatic erythrocytes (PCE/NCE) ratio was scored in 1,000 cells. The incidence of micronuclei was observed in 2,000 PCE for each animal (i.e., 1,000 from each of the two slides prepared from the duplicate), using bright-field optical microscopy at a magnification of 200–1000. Pup test groups were compared to the respective negative controls by sex, separately and in combination.

2.9 Statistical analysis

Data analysis and graphics were performed using the software GraphPad Prism version 7.01 (GraphPad Software Inc., San Diego, CA, USA). Specific statistical testing was carried out using software R version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria) and the Statistical Package for the Social Sciences software version 18.0 (SPSS Inc., Chicago, IL, USA — SPSS version 18.0) software. All results are expressed as mean ± SEM and differences were considered significant when $p \leq 0{,}05$. D´Agostino-Pearson omnibus test was used to confirmed the data normality. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) and *post hoc* analysis by Holm-Sidak test to detect differences between experimental groups and their respective controls. Non-parametric analyses were performed when needed (Kruskal-Wallis test with Dunn´s multiple comparisons). Two-way ANOVA using treatment and sex as factors was used to assess differences in redox profile parameters ($\alpha = 0{,}05$). When needed, *post hoc* comparisons were performed using Bonferroni and Sidak tests. Mercury contents between experimental groups were analyzed with unpaired *t* student´s test.

3. Results

3.1 Reproductive data

The effects of MeHg, VitA and co-administration treatments on reproductive parameters of dams and offspring are depicted in Table 1. No treatment-related symptoms were evidenced in the dams throughout the experiment period and no gross malformations were observed in pups at PND0. Only one of the dams from the co-exposure group presented a delayed delivery and gave birth to two pups (male and female). The female pup presented low weight and a shorter tail. The following necropsy performed in euthanized dams and pups showed no gross lesions. As shown in Table 1, no treatment-related changes were observed in dams for any of the analyzed reproductive parameters. No treatment-related symptoms were neither evidenced in pups throughout the lactation period. Additionally, no alterations in viability index or pup body weight were observed.

3.2 Glycogen content in liver and heart

Results of glycogen quantification are summarized in Table 2. Glycogen levels on maternal liver and heart showed no differences among experimental groups. Similar results were obtained for both male and female pups among experimental groups when compared to its respective controls.

3.3 Liver and serum retinoid contents

Retinoid contents in liver (retinol, retinyl acetate) and serum retinol are displayed in Table 3 for both dams and their offspring. Differences were encountered only for liver retinol contents in dams ($F_{[3,\ 26]}$ = 6,915, p = 0,001). When compared to controls, *post hoc* Holm Sidak test revealed that VitA treated dams showed an increase on retinol content in liver (adjusted p value = 0,009), followed by dams from the MeHg-VitA group (adjusted p value = 0,039). Finally, in offspring, no significant differences were encountered in liver retinol contents among any of the treatments when compared to its respective controls.

3.4 Total mercury concentration

Results for total Hg contents in maternal and offspring liver are represented in Table 4. No differences between Hg content in liver were encountered for dams treated with MeHg and MeHg-VitA co-administration. Also, Hg content in controls were below limit of detection of the analysis (LOD).

In offspring, no significant differences between total Hg contents in liver were evidenced for MeHg and MeHg-VitA treated male pups when comparing both experimental groups. Also, no significant differences were encountered for total Hg contents in liver for female pups when comparing both MeHg and MeHg-VitA groups.

3.5 Redox parameters in mothers and offspring

3.5.1 Liver

The effects of MeHg, VitA and co-administration treatments on redox parameters determined in the maternal liver are shown in Table 5. MeHg-VitA co-exposed dams showed increased TBARS values compared to control group ($F_{[3, 26]}$ = 2,544, p = 0,005). Regarding total thiol or non-protein thiol contents, no changes were evidenced among experimental groups. Compared to the control group, CAT activity decreased for dams in the MeHg treated group ($F_{[3, 56]}$ = 3,109, p = 0,034). On the other hand, GST activity showed a decrease only with the VitA treatment ($F_{[3, 56]}$ = 3,037, p = 0,037). Finally, no changes in SOD, GPx or SOD to GPx plus CAT ratio (SOD/(GPx + CAT)) values were found among treatments. Different results were obtained when we analyzed the redox parameters in the male and female offspring (Table 6). In general, no changes on lipoperoxidation or total thiol and non-protein thiol contents were evidenced among treatments compared to control. SOD, CAT and GPx activities showed no differences between experimental groups in either male or female pups. However, GST activity increased only for VitA treated males ($F_{[3, 56]}$ = 3,650, p = 0,018) but not in females. The ratio of the activities of antioxidant enzymes SOD/(GPx + CAT), was increased in male and female pups exposed to MeHg suggesting a possible increased peroxide production in the liver. However, as revealed by the non-parametric Kruskal-Wallis test, this effect was significant only in female pups (K = 10,07, p = 0,018).

3.5.2 Kidney

Results from redox profile in maternal kidney were also analyzed and presented in Table 5. An increase in GST activity was encountered for both MeHg and MeHg-VitA treated dams ($F_{[3, 56]}$ = 27,39, p < 0,0001). VitA treatment decreased the total thiol content as revealed by the *post hoc* Dunnett test analysis ($p \leq 0,05$). When compared to controls, an increase in total non-protein thiol content was encountered in dams from the MeHg and MeHg-VitA groups respectively ($F_{[3, 26]}$ = 15,19, p < 0,0001). No changes on lipoperoxidation were evidenced among treatments. Additionally, no differences among CAT, SOD or GPx activities were found in dams. Finally, SOD/(GPx + CAT) ratio showed no differences between experimental groups.

The same parameters were analyzed in the male and female offspring and are summarized in Table 7. Compared to controls, in MeHg male pups, CAT activity decreased significantly ($F_{[3, 43]}$ = 4,022, p = 0,013) while total thiol content presented an increase ($F_{[3, 56]}$ = 4,236, p = 0,009). Additionally for male pups, all treatments produced a decrease in total non-protein thiol content. ($F_{[3, 24]}$ = 7,248, p = 0,0013). None of the others parameters analyzed showed differences related to the experimental treatments. SOD/(GPx + CAT) showed no differences in both male and female offspring when compared to its respective controls.

3.5.3 Heart

Most analyzed redox parameters showed no significant differences among treatments in the maternal heart (Table 5). However, total thiol content decreased for MeHg treated dams ($F_{[3, 55]}$ = 3,516, p = 0,021). Redox profile in the heart of offspring is displayed in Table 8. In general, no changes on lipoperoxidation were evidenced between male and female pups for any of the experimental groups. No differences among CAT, SOD, GST or GPx activities were encountered among offspring. Only female pups treated with VitA, showed a decrease in total thiol content ($F_{[3, 36]}$ = 2,897, p = 0,048). Male pups treated with the co-exposure treatment, showed a significant SOD/(GPx + CAT) ratio increase (K = 9,20, p = 0,027).

3.6 Micronucleus frequencies in polychromatic erythrocytes

Micronuclei frequency in polychromatic erythrocytes (MNPCE) for dams and their offspring (2,000 PCE) are summarized in Table 9. For dams, MNPCE increased in MeHg (adjusted p-value = 0,045), VitA (adjusted p-value = 0,0002) and MeHg-VitA (adjusted p-value = 0,012) treated rats. An increase in total MNPCE frequency in offspring was also found (K = 22,99, p < 0,0001) in MeHg (p <0,0001) and MeHg-VitA (p < 0,05) treated groups. Two-way ANOVA analysis showed a highly significant effect of treatments ($F_{[3, 48]}$ = 52,61, p < 0,0001) and sex ($F_{[1, 48]}$ = 12,94, p = 0,0008) on MNPCE frequencies. However, *post hoc* Sidak´s test did not reveal differences when comparing male and females from the same experimental group. A further discrimination analysis among male and females using the Kruskal-Wallis test, revealed that increases in MNPCE for males were caused by MeHg (p < 0,01) and MeHg-VitA (p < 0,01) treatments. Female rats treated with MeHg-VitA also showed increased MNPCE frequencies (p < 0,001), but a highly significant increase in MNPCE was encountered for the MeHg treatment alone (p < 0,0001).

4. Discussion

Pregnant women are encouraged to eat fish because is a nutrient-rich food with low content of saturated fat and high levels of proteins and polyunsaturated fatty acids [60]. However maternal fish consumption during pregnancy exposes the fetus simultaneously to other substances and nutrients present in fish such as MeHg and VitA. MeHg has adverse health effects, especially on the cognitive development of fetuses [61] and VitA also possesses teratogenic effects at high doses, originating a pattern of birth defects including craniofacial, cardiovascular and thymic affectations [37]. During the last years, fish consumption has increased among pregnant women [40, 41], creating a possible scenario of increasing MeHg and VitA bioavailability, imposing unknown risks to the expectant mother and her fetus. Hence, we examined the effects of both MeHg and VitA administered on pregnant and lactating rats to assess redox state, mercury deposition, metabolic liver and heart function and genomic damage in adult rats and their offspring, and evaluate possible additive/synergistic/antagonistic interactions between these compounds *in vivo*.

Under our experimental conditions, no treatment-related signs of maternal and offspring toxicity and/or gross lesions were found. Additionally, reproductive and developmental toxicity endpoints neither showed treatment-related effects in mothers and offspring. Similar results have been described in several studies involving pregnant rats and pups exposed to Hg and MeHg [47, 62, 63], demonstrating that physiological states such as pregnancy and lactation may exert a protective effect in the mother and their offspring against MeHg during the supplementation phase. In this regard, previous findings have suggested that some substances like metallothionein that appear highly increased in rodents during pregnancy [64] could act as detoxifying agents against Hg ions [65].

Further, qualitative examination of the main tissues in dams and their offspring (liver, kidney and heart) showed no visible alterations or malformations. However, we decided to extend our scope by analyzing any subtle alterations in tissue metabolite deposition produced by our treatments. During *in utero* MeHg exposure, rapid transference and high concentrations of this contaminant are encountered in liver [66]. Therefore, our study focalized in hepatic function in terms of metabolic processes, possibly attributed to the hepatotoxic nature of MeHg [67].

Analysis of glycogen deposition allows assessing the effects in metabolic programming produced by certain chemical compounds in animal models, especially during episodes of exposure both *in vivo* [68] and *in utero* [69]. We decide to analyze total liver glycogen content in freshly isolated liver samples for both dams and their offspring. However, our results showed

no differences in glycogen deposition when comparing particularly MeHg and MeHg-VitA dams and their offspring to its respective controls. We can attribute this results to our low-dose MeHg dosing paradigm, since other studies in murine models used high MeHg doses during chronic exposure, which maintained a constant Hg saturation in tissues [70], and therefore affecting glycogen deposition [71]. On the other hand, altered retinoid metabolism and concomitant dysregulation of retinoid signaling in the liver contribute to the development of hepatic pathologies [72]. Retinoid uptake is a well-known parameter for assessment of metabolic processes in the liver, widely used in certain models *in vitro* [73] and *in vivo* [74]. We determined retinoid contents in both liver and plasma samples collected from dams and their offspring in order to assess the efficiency of our treatments. As expected, dams from the VitA and MeHg-VitA treated groups showed increased retinol and retinyl acetate accumulation in the liver, with no differences in retinoid contents in plasma, indicating a disruption in the VitA homeostasis due to retinyl palmitate supplementation. However, no differences in retinol contents both in liver and plasma samples were encountered for male and female offspring. Finally, dams and offspring confirmed Hg deposition in the liver, probably due to MeHg demethylation in the liver of dams, followed by rapid inorganic Hg transference into offspring liver via placenta [75, 76] and posteriorly through lactation [77]. However, MeHg and MeHg-VitA treated animals showed no differences in Hg deposition. Conjunctively, under our experimental conditions, our results showed no overall metabolic disturbances and no alterations in Hg uptake/transference in liver when analyzing both dams and their offspring.

Considering that ROS generation and oxidative stress have been suggested to play a preponderant role in the appearance of pregnancy complications, such as preeclampsia, spontaneous abortion, pregnancy loss [78] and, congenital defects [79], we have proposed the redox evaluation of several enzymatic and non-enzymatic parameters in dams and their offspring. Considering their central role in MeHg and VitA biotransformation, storage and/or elimination, redox parameters were analyzed in livers, kidneys, and hearts [80] for both dams and their offspring.

A differential effect of MeHg exposure on the activity of CAT in liver was observed mainly in exposed dams. In our study, CAT activity decreased significantly in the MeHg treated groups, suggesting the direct inhibition of the enzyme [81], the excessive production of H_2O_2 [7, 82] and/or excess of superoxide anion (O_2^-). However, previous studies using isolates of rat liver mitochondria shows a high O_2^- production with no further H_2O_2 generation following MeHg treatment, demonstrating that during MeHg challenge, liver has a relatively high ability to suppress ROS generation and promote ROS elimination [83]. However, in MeHg-VitA

treated dams, there was an increase in TBARS content, indicating increase lipoperoxidation in the liver. Evidence suggests that supplementation with antioxidants have a protective effect in various human diseases related to free radicals [84, 85]. Potent antioxidants like vitamin E [86, 87] and vitamin C [88] have shown to induce a protective effect against MeHg damage in several models. VitA possesses redox properties and is also known for its powerful antioxidant effects in several test systems [89, 90]. However, as discussed before, this behavior depends on its concentration. Previous reports have confirmed the potential of VitA and derivatives to induce lipid peroxidation, DNA damage, and impaired the cellular redox state [91-93]. In our case, in the co-exposed group, 7500 μg RAE/Kg/day of VitA induced lipoperoxidation in the liver, which is consistent with previous reports in a murine exposure model, where a high dose *β*-carotene supplementation following MeHg exposure increased lipid peroxidation in the liver, probably due to the pro-oxidant effect of *β*-carotene [94]. Additionally, the pro-oxidant effects of retinyl palmitate at the same dose regimen has been previously demonstrated in our laboratory [34]. We can therefore hypothesize that, under our experimental conditions, the increase in lipoperoxidation evidenced in the liver of dams can be related to a combined pro-oxidant effect of MeHg and VitA.

Glutathione (GSH) constitutes almost 90% of intracellular non-protein thiols in mammalian cells [95, 96], and due to its high affinity to SH groups, nearly all MeHg can be found in the body bound to glutathione [97]. Our results of kidney redox state in dams showed a significant increase in the total non-protein thiol content in both MeHg and MeHg-VitA treated rats, and therefore may indicate an increment in GSH. On the other hand, only male offspring showed a significant decrease in total non-protein thiols, which was evidenced for all experimental groups. The differences herein observed may be explained in terms of two enzymes with profound effects in renal MeHg uptake/excretion. Renal γ-glutamylcysteine synthetase (γ-GTS) is a rate-determining enzyme crucial in GSH biosynthesis and previous work from literature demonstrated an increase in renal γ-GTS activity following episodes of acute MeHg intoxication [98]. The same GSH increase in kidney has been evidenced in rats following chronic MeHg exposure, even at low doses [99]. In the case of MeHg and MeHg-VitA dams, the increase in the activity of γ-GTS would possibly explain the increase in renal GSH, as a response to MeHg toxicity in this tissue. However, previous work have suggested that MeHg can be excreted via MeHg-GSH complexes in the kidney, and taken up by renal cells to degradation of the GSH moiety, a process catalyzed by the γ-glutamyltranspeptidase (γ-GTP) [100]. The activation of the GSH-γ-GTP pathway is responsible for Hg clearance in the kidney [101]. The activity of renal γ-GTP is under hormonal control, possibly by testosterone [102-

105]. Hormonal control explains the better Hg clearance of periadolescent males when compared to females (approximately 28 days postnatal old), a process which improves at latter developmental stages [101]. Although no measures of Hg deposition in kidney were performed, we evidenced a decrease in total non-protein thiol content in male offspring, suggesting MeHg degradation. We therefore hypothesize that sex differential response herein described may also be related to hormones, since our pups were in the periadolescent period. Remarkably, we encountered a significant increase in GST activity when analyzing kidney samples of dams. Recent evidence supports that GST participates in MeHg elimination through the formation of a readily transported MeHg-GSH conjugate [106]. We may hypothesize that during the chronic MeHg and MeHg-VitA co-exposure, MeHg elimination in kidney was dramatically increased, which may explain the concomitant elevation in GSH concentrations and GST activity, both evidenced in MeHg and MeHg-VitA treated rats. However, modification in GST activity may be related to a redox disruption in the tissue, therefore inducing a signaling cascade and dysregulation of kinase pathways [107, 108]. Summarizing our results in kidney, we can affirm that MeHg-VitA co-administration in the kidney might affect redox balance in dams, but not in their offspring, possibly via hormonal activation of GSH-γ-GTP pathway, with no disruption in other redox parameters.

Despite no evidence of effects in heart glycogen deposition, surprisingly, SOD/GPx + CAT ratio increased in the heart of male pups treated during perinatal period with MeHg-VitA co-administrated doses, hence indicating an oxidative stress response due to excess H_2O_2. Active oxygen species including H_2O_2 play a major role in ischemia-reperfusion injury in the heart [109, 110], since excess H_2O_2 in the heart reduces molecular contractile response and enhances cardiomyocyte Ca^{2+} release [111]. Extensive epidemiological evidence supports the relationship between MeHg exposure and heart diseases among human populations [112-116] and a recent *in vitro* approach demonstrated MeHg-induced cytotoxicity in human cardiomyocytes [117]. Interestingly, although our MeHg dose did not affect H_2O_2 availability, our results suggest that VitA co-administration induced a potentialized pro-oxidant effect in heart tissue, which may increase susceptibility to oxidative damage injury in the heart of male pups produced by excess H_2O_2.

In contrast, female pups from the MeHg perinatal exposure group showed the classical effects related to decreased CAT activity and increased SOD/GPx + CAT ratio, resulting in free-hydrogen peroxide in liver, but no other effects in the antioxidant defenses of kidney and heart, different to male pups. We can attribute this sex-dependent antioxidant response to hormonal control, which particularly protects females against cardiovascular injury [118]. In

fact, oxidative stress response is modulated by estrogen as demonstrated *in vivo* [119]. Our female pups were at postnatal day 31 until the end of our experiments, meaning the periadolescence period (vaginal opening and sexual maturity) [120]. We cannot exclude the possibility that their estrous cycle affected their response to our treatments, possibly in a protective way.

MeHg is both a spindle poison and a clastogen, predominant clastogenic effect, like previously evidenced in both *in vitro* [121, 122] and *in vivo* [123, 124] studies. Recent work demonstrated that MeHg intoxication inhibits the activity of the poly (ADP-ribose) polymerase-1 enzyme (PARP-1), which repairs breaks in the DNA strand [125]. On the other hand, recent studies support that high doses of provitamin A carotenoids can induce DNA damage [126] probably via a pro-oxidant pathway [127]. Our results for dams with MeHg-VitA co-administration showed an increase in MNPCE frequencies when compared with control. However, in offspring, total genomic damage was exacerbated in both males and females when compared to its respective controls. The fact that offspring presented higher MNPCE frequencies may be attributed to maternal transference of these pollutants. However, the exact mechanism of the combined effect produced by MeHg and VitA in offspring, related to DNA damage, remains to be elucidated in future studies.

In summary, our results may indicate that, although no toxic or metabolic alterations were evidenced in dams and their offspring by the co-administration of low-dose MeHg and retinyl palmitate supplementation during pregnancy and lactation, other subtle alterations indicate an increased oxidative stress response in the maternal liver, which surpasses the natural defenses of this organ. Additionally, a disrupted pro-oxidant response in the heart of male pups, with apparently no particular effects in female offspring. Finally, genomic damage in offspring is exacerbated in a large proportion possibly via maternal exposure.

5. Conclusions

Based on our data, we can suggest that low-dose MeHg in co-administration with retinyl palmitate supplementation during pregnancy and lactation can induce oxidative stress in maternal liver and promote a pro-oxidant response in the heart, especially for male offspring, possibly increasing cardiac tissue susceptibility to oxidative damage injury. Additionally, GST activity in dam liver was altered leading to possible redox disruption of this tissue with no alterations in offspring. Finally, genomic damage in offspring is exacerbated in a large proportion via maternal exposure. However, further studies are needed to confirm our findings and elucidate the molecular mechanisms of MeHg-VitA modulation. Although this is a pre-

clinical approach using an *in vivo* model, we also recommend more epidemiological studies in order to assess the true extent of fish consumption during pregnancy and possible modulatory effects with retinyl palmitate supplementation at safe or inadvertently used doses in humans, which may be related to certain pathologies in mothers and their children.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS), Brazil.

REFERENCES

1. Sheehan, M.C., et al., *Global methylmercury exposure from seafood consumption and risk of developmental neurotoxicity: a systematic review.* Bulletin of the World Health Organization, 2014. **92**(4): p. 254-269F.
2. Sunderland, E.M., M. Li, and K. Bullard, *Decadal Changes in the Edible Supply of Seafood and Methylmercury Exposure in the United States.* Environ Health Perspect, 2018. **126**(1): p. 017006.
3. Miklavčič, A., et al., *Mercury, arsenic and selenium exposure levels in relation to fish consumption in the Mediterranean area.* Environmental research, 2013. **120**: p. 7-17.
4. Faial, K., et al., *Mercury levels assessment in hair of riverside inhabitants of the Tapajós River, Pará State, Amazon, Brazil: Fish consumption as a possible route of exposure.* Journal of Trace Elements in Medicine and Biology, 2015. **30**: p. 66-76.
5. USEPA, *Guidance for assessing chemical contaminant data for use in fish advisories: Volume II, risk assessment and fish consumption limits*, D. EPA 823-B-97-009. Washington, Editor. 1997.
6. EPA, *Guidance for assessing chemical contaminant data for use in fish advisories. Volume 2: Risk assessment and fish consumption limits. Third Edition*. 2000, Washington, DC: Office of Science and Technology. Office of Water. U.S. Environmental Protection Agency.
7. Nabi, S., *Toxic Effects of Mercury*. 2014: Springer India.
8. Castoldi, A.F., et al., *Neurotoxicity and molecular effects of methylmercury.* Brain Res Bull, 2001. **55**(2): p. 197-203.
9. Lepham, L., et al., *An analysis of autopsy of brain tissue from infants prematurely exposed to methylmercury.* Neurotoxicology, 1995. **16**: p. 689–704.
10. Bellinger, D.C., et al., *Country-specific estimates of the incidence of intellectual disability associated with prenatal exposure to methylmercury*. Environmental Research, 2016. **147**: p. 159-163.
11. Debes, F., P. Weihe, and P. Grandjean, *Cognitive deficits at age 22 years associated with prenatal exposure to methylmercury.* Cortex, 2016. **74**: p. 358-69.

12. Llop, S., et al., *Prenatal exposure to mercury and neuropsychological development in young children: the role of fish consumption.* Int J Epidemiol, 2016.
13. Yorifuji, T., et al., *Intrauterine Exposure to Methylmercury and Neurocognitive Functions: Minamata Disease.* Arch Environ Occup Health, 2015. **70**(5): p. 297-302.
14. Stokes-Riner, A., et al., *A longitudinal analysis of prenatal exposure to methylmercury and fatty acids in the Seychelles.* Neurotoxicology and Teratology, 2011. **33**(2): p. 325-328.
15. Reifen, R. and K. Ghebremeskel, *Vitamin a during Pregnancy.* Nutrition and Health, 2001. **15**(3-4): p. 237-243.
16. Napoli, J.L., *Physiological insights into all-trans-retinoic acid biosynthesis.* Biochim Biophys Acta, 2012. **1821**(1): p. 152-67.
17. Oliveira, M.R., *The neurotoxic effects of vitamin A and retinoids.* An Acad Bras Cienc, 2015. **87**(2 Suppl): p. 1361-73.
18. Ross, C.A., ed. *Vitamin A.* 2 ed. Encyclopedia of Dietary Supplements 2nd Edition, ed. P.M. Coates, et al. 2010, Informa Healthcare: New York. 778-91.
19. Chapman, M.S., *Vitamin a: history, current uses, and controversies.* Semin Cutan Med Surg, 2012. **31**(1): p. 11-6.
20. (WHO), W.H.O., *WHO Guidelines Approved by the Guidelines Review Committee*, in *Guideline: Use of Multiple Micronutrient Powders for Point-of-Use Fortification of Foods Consumed by Pregnant Women*. 2016, World Health Organization Copyright (c) World Health Organization 2016.: Geneva.
21. Lane, M.A. and S.J. Bailey, *Role of retinoid signalling in the adult brain.* Prog Neurobiol, 2005. **75**(4): p. 275-93.
22. McCaffery, P., J. Zhang, and J.E. Crandall, *Retinoic acid signaling and function in the adult hippocampus.* J Neurobiol, 2006. **66**(7): p. 780-91.
23. McCaffery, P., et al., *Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen.* Eur J Neurosci, 2003. **18**(3): p. 457-72.
24. (WHO), W.H.O., *Global Prevalence of Vitamin A deficiency in Populations at Risk 1995-2005. WHO Global Database on Vitamin A Deficiency. Geneva. World Health Organization.* 2009.
25. Aschner, M., et al., *Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity.* Braz J Med Biol Res, 2007. **40**(3): p. 285-91.
26. Ishihara, Y., et al., *Involvement of reactive oxygen species derived from mitochondria in neuronal injury elicited by methylmercury.* Journal of Clinical Biochemistry and Nutrition, 2016. **59**(3): p. 182-190.
27. Yin, Z., et al., *Methylmercury induces oxidative injury, alterations in permeability and glutamine transport in cultured astrocytes.* Brain Res, 2007. **1131**(1): p. 1-10.
28. Hare, M.F., K.M. McGinnis, and W.D. Atchison, *Methylmercury increases intracellular concentrations of Ca++ and heavy metals in NG108-15 cells.* J Pharmacol Exp Ther, 1993. **266**(3): p. 1626-35.
29. Shenker, B.J., T.L. Guo, and I.M. Shapiro, *Low-level methylmercury exposure causes human T-cells to undergo apoptosis: evidence of mitochondrial dysfunction.* Environ Res, 1998. **77**(2): p. 149-59.
30. Limke, T.L. and W.D. Atchison, *Acute exposure to methylmercury opens the mitochondrial permeability transition pore in rat cerebellar granule cells.* Toxicol Appl Pharmacol, 2002. **178**(1): p. 52-61.
31. Behr, G.A., et al., *Increased cerebral oxidative damage and decreased antioxidant defenses in ovariectomized and sham-operated rats supplemented with vitamin A.* Cell Biol Toxicol, 2012. **28**(5): p. 317-30.
32. Gasparotto, J., et al., *Supplementation with vitamin A enhances oxidative stress in the lungs of rats submitted to aerobic exercise.* Appl Physiol Nutr Metab, 2015. **40**(12): p. 1253-61.

33. Schnorr, C.E., et al., *Chronic retinyl palmitate supplementation to middle-aged Wistar rats disrupts the brain redox homeostasis and induces changes in emotional behavior.* Mol Nutr Food Res, 2015. **59**(5): p. 979-90.
34. Schnorr, C.E., et al., *The effects of vitamin A supplementation to rats during gestation and lactation upon redox parameters: increased oxidative stress and redox modulation in mothers and their offspring.* Food Chem Toxicol, 2011. **49**(10): p. 2645-54.
35. Schnorr, C.E., et al., *Supplementation of Adult Rats with Moderate Amounts of β-Carotene Modulates the Redox Status in Plasma without Exerting Pro-Oxidant Effects in the Brain: A Safer Alternative to Food Fortification with Vitamin A?* Nutrients, 2014. **6**(12): p. 5572-5582.
36. Schnorr, C.E., et al., *The effects of vitamin A supplementation to rats during gestation and lactation upon redox parameters: Increased oxidative stress and redox modulation in mothers and their offspring.* Food and Chemical Toxicology, 2011. **49**(10): p. 2645-2654.
37. Azaïs-Braesco, V. and G. Pascal, *Vitamin A in pregnancy: requirements and safety limits.* The American Journal of Clinical Nutrition, 2000. **71**(5): p. 1325s-1333s.
38. West Jr, K.P., *Extent of vitamin A deficiency among preschool children and women of reproductive age.* The Journal of nutrition, 2002. **132**(9): p. 2857S-2866S.
39. Verma, A.R., et al., *Maternal vitamin A status in intrauterine growth retarded babies.* Asian Journal of Medical Sciences, 2017. **8**(3): p. 30-34.
40. Razzaghi, H. and S.C. Tinker, *Seafood consumption among pregnant and non-pregnant women of childbearing age in the United States, NHANES 1999–2006.* Food & Nutrition Research, 2014. **58**: p. 10.3402/fnr.v58.23287.
41. Cusack, L.K., et al., *Regional and temporal trends in blood mercury concentrations and fish consumption in women of child bearing Age in the united states using NHANES data from 1999–2010.* Environmental Health, 2017. **16**: p. 10.
42. NIH, *Guide for the Care and Use of Laboratory Animals: Eighth Edition. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals*. 2011: National Academies Press.
43. Marcondes, F.K., F.J. Bianchi, and A.P. Tanno, *Determination of the estrous cycle phases of rats: some helpful considerations.* Braz J Biol, 2002. **62**(4a): p. 609-14.
44. Amugongo, S.K. and L.J. Hlusko, *Impact of Maternal Prenatal Stress on Growth of the Offspring.* Aging and Disease, 2014. **5**(1): p. 1-16.
45. Chahoud, I. and F.J.R. Paumgartten, *Influence of litter size on the postnatal growth of rat pups: Is there a rationale for litter-size standardization in toxicity studies?* Environmental Research, 2009. **109**(8): p. 1021-1027.
46. Dridi, I., et al., *Dietary early-life exposure to contaminated eels does not impair spatial cognitive performances in adult offspring mice as assessed in the Y-maze and the Morris water maze.* Nutrition Research, 2014. **34**(12): p. 1075-1084.
47. Schnorr, C.E., et al., *Vitamin A supplementation in rats under pregnancy and nursing induces behavioral changes and oxidative stress upon striatum and hippocampus of dams and their offspring.* Brain Res, 2011. **1369**: p. 60-73.
48. Lowry, O.H., et al., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 265-75.
49. Hassid, W.Z. and S. Abraham, *[7] Chemical procedures for analysis of polysaccharides*, in *Methods in Enzymology*. 1957, Academic Press. p. 34-50.
50. Kim, Y.K. and L. Quadro, *Reverse-phase high-performance liquid chromatography (HPLC) analysis of retinol and retinyl esters in mouse serum and tissues.* Methods Mol Biol, 2010. **652**: p. 263-75.
51. Schaffer, M.W., et al., *Qualitative and quantitative analysis of retinol, retinyl esters, tocopherols and selected carotenoids out of various internal organs form different species by HPLC.* Anal Methods, 2010. **2**: p. 1320-1332.

52. Draper, H.H. and M. Hadley, *Malondialdehyde determination as index of lipid peroxidation.* Methods Enzymol, 1990. **186**: p. 421-31.
53. Ellman, G.L., *Tissue sulfhydryl groups.* Archives of Biochemistry and Biophysics, 1959. **82**(1): p. 70-77.
54. Misra, H.P. and I. Fridovich, *The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase.* J Biol Chem, 1972. **247**(10): p. 3170-5.
55. Aebi, H., *Catalase in vitro.* Methods Enzymol, 1984. **105**: p. 121-6.
56. Wendel, A., *Glutathione peroxidase.* Methods Enzymol, 1981. **77**: p. 325-33.
57. Habig, W.H. and W.B. Jakoby, *Assays for differentiation of glutathione S-transferases.* Methods Enzymol, 1981. **77**: p. 398-405.
58. Horta, R.N., et al., *Protective effects of acerola juice on genotoxicity induced by iron in vivo.* Genet Mol Biol, 2016. **39**(1): p. 122-8.
59. OECD, *Test No. 474: Mammalian Erythrocyte Micronucleus Test*. 2016: OECD Publishing.
60. Lando, A.M., S.B. Fein, and C.J. Choinière, *Awareness of methylmercury in fish and fish consumption among pregnant and postpartum women and women of childbearing age in the United States.* Environmental Research, 2012. **116**: p. 85-92.
61. USEPA. *U.S. Environmental Protection Agency. Integrated Risk Information System (IRIS). Methylmercury*. 2001 08-05-2012]; Available from: www.epa.gov/iris/subst/0073.htm.
62. Oliveira, C.S., et al., *Biochemical parameters of pregnant rats and their offspring exposed to different doses of inorganic mercury in drinking water.* Food and Chemical Toxicology, 2012. **50**(7): p. 2382-2387.
63. Oliveira, V.A., et al., *Acute mercury exposition of virgin, pregnant, and lactating rats: Histopathological kidney and liver evaluations.* Environ Toxicol, 2017. **32**(5): p. 1500-1512.
64. Yoshida, M., et al., *Maternal-to-fetus transfer of mercury in metallothionein-null pregnant mice after exposure to mercury vapor.* Toxicology, 2002. **175**(1-3): p. 215-222.
65. Klaassen, C.D., J. Liu, and S. Choudhuri, *Metallothionein: An intracellular protein to protect against cadmium toxicity*, in *Annual Review of Pharmacology and Toxicology*. 1999. p. 267-294.
66. Snell, K., S.L. Ashby, and S.J. Barton, *Disturbances of perinatal carbohydrate metabolism in rats exposed to methylmercury in utero.* Toxicology, 1977. **8**(3): p. 277-280.
67. Ashour, H., M. Abdel-Rahman, and A. Khodair, *The mechanism of methyl mercury toxicity in isolated rat hepatocytes.* Toxicology Letters, 1993. **69**(1): p. 87-96.
68. Beger, R.D., et al., *Single valproic acid treatment inhibits glycogen and RNA ribose turnover while disrupting glucose-derived cholesterol synthesis in liver as revealed by the [U-13C6]-d-glucose tracer in mice.* Metabolomics, 2009. **5**(3): p. 336.
69. Maranghi, F., et al., *In utero exposure to di-(2-ethylhexyl) phthalate affects liver morphology and metabolism in post-natal CD-1 mice.* Reprod Toxicol, 2010. **29**(4): p. 427-32.
70. Salvaterra, P., et al., *Time-dependent tissue/organ uptake and distribution of 203Hg in mice exposed to multiple sublethal doses of methyl mercury.* Toxicology and Applied Pharmacology, 1975. **32**(2): p. 432-442.
71. Verschuuren, H.G., et al., *Toxicity of methylmercury chloride in rats I. Short-term study.* Toxicology, 1976. **6**(1): p. 85-96.
72. Shirakami, Y., et al., *HEPATIC METABOLISM OF RETINOIDS AND DISEASE ASSOCIATIONS.* Biochimica et biophysica acta, 2012. **1821**(1): p. 124-136.
73. Vicente, C.P., et al., *Retinol uptake and metabolism, and cellular retinol binding protein expression in an in vitro model of hepatic stellate cells.* Mol Cell Biochem, 1998. **187**(1-2): p. 11-21.
74. Hendriks, H.F.J., et al., *Uptake and processing of [3h]retinoids in rat liver studied by electron microscopic autoradiography.* Hepatology, 1988. **8**(2): p. 276-285.
75. Dock, L., R.-L. Rissanen, and M. Vahter, *Demethylation and placental transfer of methyl mercury in the pregnant hamster.* Toxicology, 1994. **94**(1): p. 131-142.

76. Nordenhall, K., L. Dock, and M. Vahter, *Transplacental and lactational exposure to mercury in hamster pups after maternal administration of methyl mercury in late gestation.* Pharmacol Toxicol, 1995. **77**(2): p. 130-5.
77. Nordenhall, K., L. Dock, and M. Vahter, *Cross-fostering study of methyl mercury retention, demethylation and excretion in the neonatal hamster.* Pharmacol Toxicol, 1998. **82**(3): p. 132-6.
78. Agarwal, A., et al., *The effects of oxidative stress on female reproduction: a review.* Reproductive Biology and Endocrinology : RB&E, 2012. **10**: p. 49-49.
79. Hansen, J.M., B.R. Jacob, and T.B. Piorczynski, *Oxidative stress during development: Chemical-induced teratogenesis.* Current Opinion in Toxicology, 2018. **7**: p. 110-115.
80. Sonne, C., et al., *Are liver and renal lesions in East Greenland polar bears (Ursus maritimus) associated with high mercury levels?* Environ Health, 2007. **6**: p. 11.
81. Abdel-Hamid, H.A., F.C. Fahmy, and I.A. Sharaf, *Influence of free radicals on cardiovascular risk due to occupational exposure to mercury.* The Journal of the Egyptian Public Health Association, 2001. **76**(1-2): p. 53-69.
82. Allen, J.W., L.A. Mutkus, and M. Aschner, *Methylmercury-mediated inhibition of 3H-d-aspartate transport in cultured astrocytes is reversed by the antioxidant catalase.* Brain Research, 2001. **902**(1): p. 92-100.
83. Mori, N., A. Yasutake, and K. Hirayama, *Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity.* Archives of Toxicology, 2007. **81**(11): p. 769-776.
84. Vendemiale, G., I. Grattagliano, and E. Altomare, *An update on the role of free radicals and antioxidant defense in human disease.* International Journal of Clinical and Laboratory Research, 1999. **29**(2): p. 49.
85. Poprac, P., et al., *Targeting free radicals in oxidative stress-related human diseases.* Trends in pharmacological sciences, 2017. **38**(7): p. 592-607.
86. Ganther, H.E., *Modification of methylmercury toxicity and metabolism by selenium and vitamin E: possible mechanisms.* Environmental health perspectives, 1978. **25**: p. 71.
87. Moniruzzaman, M., et al., *Synergistic effects of dietary vitamin E and selenomethionine on growth performance and tissue methylmercury accumulation on mercury-induced toxicity in juvenile olive flounder, Paralichthys olivaceus (Temminck et Schlegel).* Aquaculture Research, 2017. **48**(2): p. 570-580.
88. Hounkpatin, A.S., et al., *Protective effects of vitamin C on kidney, liver and brain: a study in wistar rats intoxicated with mercury.* 2017.
89. de Oliveira, M.R., et al., *Short-term vitamin A supplementation at therapeutic doses induces a pro-oxidative state in the hepatic environment and facilitates calcium-ion-induced oxidative stress in rat liver mitochondria independently from permeability transition pore formation.* Cell Biology and Toxicology, 2009. **25**(6): p. 545-560.
90. de Carvalho, R.M., et al., *Cytogenotoxicological defense of retinyl palmitate in the front damage of antineoplastics.* Experimental and Toxicologic Pathology, 2017. **69**(5): p. 293-297.
91. Murata, M. and S. Kawanishi, *Oxidative DNA damage by vitamin A and its derivative via superoxide generation.* Journal of Biological Chemistry, 2000. **275**(3): p. 2003-2008.
92. Klamt, F., et al., *Enhanced UV-mediated free radical generation; DNA and mitochondrial damage caused by retinol supplementation.* Photochemical & Photobiological Sciences, 2003. **2**(8): p. 856-860.
93. Klamt, F., et al., *Genotoxicity, recombinogenicity and cellular preneoplasic transformation induced by Vitamin a supplementation.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2003. **539**(1): p. 117-125.
94. Andersen, H.R. and O. Andersen, *Effects of dietary alpha-tocopherol and beta-carotene on lipid peroxidation induced by methyl mercuric chloride in mice.* Pharmacol Toxicol, 1993. **73**(4): p. 192-201.

95. Anderson, M.E. and A. Meister, *Transport and direct utilization of gamma-glutamylcyst(e)ine for glutathione synthesis.* Proc Natl Acad Sci U S A, 1983. **80**(3): p. 707-11.
96. Roos, D.H., et al., *Complex Methylmercury–Cysteine Alters Mercury Accumulation in Different Tissues of Mice.* Basic & Clinical Pharmacology & Toxicology, 2010. **107**(4): p. 789-792.
97. Hirayama, K., A. Yasutake, and T. Adachi, *Mechanism for Renal Handling of Methylmercury*, in *Advances in Mercury Toxicology*, T. Suzuki, N. Imura, and T.W. Clarkson, Editors. 1991, Springer US: Boston, MA. p. 121-134.
98. Yasutake, A. and K. Hirayama, *Acute effects of methylmercury on hepatic and renal glutathione metabolisms in mice.* Archives of Toxicology, 1994. **68**(8): p. 512-516.
99. Yasutake, A., et al., *Chronic effects of methylmercury in rats. I. Biochemical aspects.* Tohoku J Exp Med, 1997. **182**(3): p. 185-96.
100. Naganuma, A., et al., *Possible role of hepatic glutathione in transport of methylmercury into mouse kidney.* Biochem Pharmacol, 1988. **37**(2): p. 291-6.
101. Nielsen, J.B. and O. Andersen, *Elimination of recently absorbed methyl mercury depends on age and gender*. Pharmacol Toxicol, 1996. **79**(2): p. 60-4.
102. Suzuki, T., N. Imura, and T.W. Clarkson, *Advances in Mercury Toxicology*. 2013: Springer US.
103. Tanaka, T., et al., *Role of testosterone in γ-glutamyltranspeptidase-dependent renal methylmercury uptake in mice.* Toxicology and Applied Pharmacology, 1992. **112**(1): p. 58-63.
104. Hirayama, K. and A. Yasutake, *Sex and age differences in mercury distribution and excretion in methylmercury-administered mice*. Vol. 18. 1986. 49-60.
105. Yasutake, A. and K. Hirayama, *Sex and strain differences of susceptibility to methylmercury toxicity in mice*. Vol. 51. 1988. 47-55.
106. Vorojeikina, D., et al., *Glutathione S-Transferase Activity Moderates Methylmercury Toxicity During Development in Drosophila*. Vol. 157. 2017.
107. Sahu, S.C. and G.C. Gray, *Pro-oxidant activity of flavonoids: effects on glutathione and glutathione S-transferase in isolated rat liver nuclei.* Cancer Lett, 1996. **104**(2): p. 193-6.
108. Townsend, D.M., K.D. Tew, and H. Tapiero, *The importance of glutathione in human disease.* Biomed Pharmacother, 2003. **57**(3-4): p. 145-55.
109. Slezak, J., et al., *Hydrogen peroxide changes in ischemic and reperfused heart. Cytochemistry and biochemical and X-ray microanalysis.* The American Journal of Pathology, 1995. **147**(3): p. 772-781.
110. Janero, D.R., D. Hreniuk, and H.M. Sharif, *Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): nonperoxidative purine and pyrimidine nucleotide depletion.* J Cell Physiol, 1993. **155**(3): p. 494-504.
111. Yi, T. and B.M. Palmer, *Oxidative Stress by Hydrogen Peroxide Reduces Cardiac Contractile Protein Performance and Enhances Cardiomyocyte Calcium Release.* Biophysical Journal, 2013. **104**(2): p. 314a.
112. Fillion, M., et al., *A preliminary study of mercury exposure and blood pressure in the Brazilian Amazon.* Environ Health, 2006. **5**: p. 29.
113. Valera, B., E. Dewailly, and P. Poirier, *Association between methylmercury and cardiovascular risk factors in a native population of Quebec (Canada): a retrospective evaluation.* Environ Res, 2013. **120**: p. 102-8.
114. Thurston, S.W., et al., *Does prenatal methylmercury exposure from fish consumption affect blood pressure in childhood?* Neurotoxicology, 2007. **28**(5): p. 924-30.
115. Grandjean, P., et al., *Cardiac autonomic activity in methylmercury neurotoxicity: 14-year follow-up of a Faroese birth cohort.* J Pediatr, 2004. **144**(2): p. 169-76.
116. Yoshizawa, K., et al., *Mercury and the risk of coronary heart disease in men.* N Engl J Med, 2002. **347**(22): p. 1755-60.

117. Truong, J., R.J. Mailloux, and H.M. Chan, *Impact of methylmercury exposure on mitochondrial energetics in AC16 and H9C2 cardiomyocytes.* Toxicol In Vitro, 2015. **29**(5): p. 953-61.
118. Barp, J., et al., *Myocardial antioxidant and oxidative stress changes due to sex hormones.* Brazilian Journal of Medical and Biological Research, 2002. **35**: p. 1075-1081.
119. Razmara, A., et al., *Estrogen suppresses brain mitochondrial oxidative stress in female and male rats.* Brain Res, 2007. **1176**: p. 71-81.
120. Sengupta, P., *The Laboratory Rat: Relating Its Age With Human's.* International Journal of Preventive Medicine, 2013. **4**(6): p. 624-630.
121. Ogura, H., T. Takeuchi, and K. Morimoto, *A comparison of the 8-hydroxydeoxyguanosine, chromosome aberrations and micronucleus techniques for the assessment of the genotoxicity of mercury compounds in human blood lymphocytes.* Mutation research, 1996. **340**(2-3): p. 175-82.
122. Crespo-López, M.E., et al., *Methylmercury genotoxicity: A novel effect in human cell lines of the central nervous system.* Environment International, 2007. **33**(2): p. 141-146.
123. Betti, C., R. Barale, and B.L. Pool-Zobel, *Comparative studies on cytotoxic and genotoxic effects of two organic mercury compounds in lymphocytes and gastric mucosa cells of Sprague-Dawley rats.* Environmental and molecular mutagenesis, 1993. **22**(3): p. 172-80.
124. Amorim, M.I., et al., *Cytogenetic damage related to low levels of methyl mercury contamination in the Brazilian Amazon.* Anais da Academia Brasileira de Ciências, 2000. **72**(4): p. 497-507.
125. Pieper, I., et al., *Mechanisms of Hg species induced toxicity in cultured human astrocytes: genotoxicity and DNA-damage response.* Metallomics, 2014. **6**(3): p. 662-71.
126. Azqueta, A. and A.R. Collins, *Carotenoids and DNA damage.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2012. **733**(1): p. 4-13.
127. Yeum, K.-J., et al., *Antioxidant/Pro-oxidant Actions of Carotenoids*, in *Carotenoids: Volume 5: Nutrition and Health*, G. Britton, H. Pfander, and S. Liaaen-Jensen, Editors. 2009, Birkhäuser Basel: Basel. p. 235-268.

Table 1. Effects of MeHg, VitA and co-administration treatments on reproductive parameters of dams and their offspring.

Parameters	Treatments			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
N° of dams	8	7	8	7
Gestation weight gain (%)	3,5 ± 3,9	7,1 ± 5,8	4,9 ± 4,6	3,7 ± 3,8
Lactation weight gain (%)	14,6 ± 4,1	11,7 ± 3,5	10,7 ± 6,8	14,4 ± 1,6
Gestation length (days)	21,6 ± 0,5	21,4 ± 0,5	21,8 ± 0,5	21,9 ± 0,7
N° of implantations	8,7 ± 3,7	10,9 ± 3,3	10,3 ± 3,5	10,1 ± 3,0
Delivery index (%)	88 ± 20,6	88,9 ± 14,6	83,9 ± 24,3	75,1 ± 25,4
N° of pups delivered	9,8 ± 2,2	10,1 ± 2,2	9 ± 3,8	8,1 ± 3,0
Sex ratio of pups	0,500 ± 0,2	0,557 ± 0,1	0,425 ± 0,1	0,429 ± 0,2
Viability index (%)				
PND2	100	100	100	100
PND7	98,8 ± 3,5	98 ± 5,4	95,6 ± 9,0	98,7 ± 3,4
PND14	98,6 ± 3,9	100	98,2 ± 5,0	98,6 ± 3,8
PND21	100	100	100	100
Pup weight (g)				
PND2	7,3 ± 0,8	7,1 ± 0,9	7,1 ± 1,7	7,1 ± 1,4
PND7	13,7 ± 1,4	13,6 ± 2,3	13,5 ± 2,5	12,7 ± 2,7
PND14	25,1 ± 2,9	24,6 ± 4,6	25,2 ± 5,3	23,4 ± 2,3
PND21	39,3 ± 5,5	36,3 ± 9,4	40,2 ± 8,7	35,8 ± 2,9

Data are represented as mean ± SEM.
Gestation weight gain (%) = ((weight on PND0 – weight on GD0)/weight on GD0) x 100.
Lactation weight gain (%) = ((weight on PND21 – weight on PND0)/weight on PND0) x 100.
Delivery index (%) = (number of pups delivered/number of implantations) x 100.
Sex ratio of pups = number of male pups/total number of pups.
Viability index on PND2 (%) = (number of live pups on PND2/total number of pups delivered) x 100.
Viability index on PND7 (%) = (number of live pups on PND7/number of live pups on PND2) x 100.
Viability index on PND14 (%) = (number of live pups on PND14/number of live pups on PND7) x 100.
Viability index on PND21 (%) = (number of live pups on PND21/number of live pups on PND14) x 100.

Table 2. Total glycogen levels in both liver and heart from dams and their offspring.

Glycogen levels (% of control)	Treatments			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
Liver glycogen				
Dams	100 ± 10,05	126,1 ± 14,75	125,1 ± 15,57	118,5 ± 12,02
Offspring				
Males	100 ± 8,717	116,5 ± 7,92	108 ± 8,50	104,1 ± 10,03
Females	100 ± 13,62	95,6 ± 7,73	109,6 ± 9,57	95,0 ± 5,271
Heart glycogen				
Dams	87,5 ± 15,44	111,9 ± 7,96	105,1± 10,65	118,8 ± 14,62
Offspring				
Males	100 ± 2,91	106,2 ± 6,88	99,2 ± 2,09	100,3 ± 2,41
Females	100 ± 2,81	117 ± 12,4	102,7 ± 2,32	105,2 ± 3,43

Data are represented as mean ± SEM.

Table 3. Total retinoid content in liver and plasma from dams and their offspring.

Animals	Parameters	Treatments			
		Control	**MeHg**	**VitA**	**MeHg-VitA**
Dams	**Retinol**				
	Liver (µg/g wet weight)	170,7 ± 22,53	158,8 ± 20,07	**316,2 ± 45,57****	**285,5 ± 21,39***
	Plasma (µg/mL)	0,63 ± 0,09	0,46 ± 0,06	0,62 ± 0,08	0,64 ± 0,09
	Retinyl Acetate				
	Liver (µg/g wet weight)	1,74 ± 0,38	1,65 ± 0,30	5,22 ± 1,40	4,99 ± 1,49
Offspring					
Males	**Retinol**				
	Liver (µg/g wet weight)	8,86 ± 4,20	6,15 ± 2,10	7,96 ± 1,87	10,43 ± 4,42
	Plasma (µg/mL)	0,84 ± 0,14	1,06 ± 0,05	1,04 ± 0,04	1,08 ± 0,06
Females	**Retinol**				
	Liver (µg/g wet weight)	4,08 ± 0,65	4,23 ± 1,10	7,10 ± 1,10	6,31 ± 1,48
	Plasma (µg/mL)	0,95 ± 0,05	0,83 ± 0,03	0,91 ± 0,05	0,90 ± 0,05

Data are represented as mean ± SEM. **Bold for statistically significant difference**; *$p \leq 0,05$, **$p \leq 0,01$ compared to the control group.

Table 4. Total mercury content in liver samples from dams and their offspring.

Hg concentration (µg g^{-1})	Treatments		
	Control	**MeHg**	**MeHg-Vit A**
Dams	<LOD*	10,61 ± 1,9	8,214 ± 1,19
Offspring			
Males	<LOD*	0,21 ± 0,02	0,16 ± 0,01
Females	<LOD*	0,19 ± 0,03	0,22 ± 0,06

Data are represented as mean ± SEM; *Limit of detection (< 0.05 µg g-1)

Table 5. Effects of MeHg, VitA and co-administration treatments on redox parameters in maternal liver, kidney, and heart.

Redox parameters	Treatment			
	Control	MeHg	VitA	MeHg-VitA
No of dams examined	8	7	8	7
Liver				
TBARS content (nmol TBARS/mg protein)	0,1241 ± 0,02	0,2428 ± 0,05	0,2397 ± 0,04	**0,2788 ± 0,06***
Total reduced thiol content (nmol SH/mg protein)	42,21 ± 2,56	34,43 ± 1,95	33,71 ± 2,4	37,75 ± 3,68
Total non-protein thiol content (nmol SH/mg protein)	19,33 ± 6,73	25,27 ± 5,57	20,07 ± 8,73	17,27 ± 8,18
CAT activity (U CAT/mg protein)	133,8 ± 11,21	**79,13 ± 7,49****	99,32 ± 9,61	127,6 ± 13,6
SOD activity (U SOD/mg protein)	84,1 ± 7,56	70,87 ± 8,97	78,57 ± 5,98	68,86 ± 5,1
GST activity (U GST/mg protein)	0,2474 ± 0,01	0,2219 ± 0,004	**0,2054 ± 0,01***	0,2235 ± 0,01
GPx activity (U GPx/mg protein)	0,8453 ± 0,05	0,8213 ± 0,08	0,7775 ± 0,12	0,9886 ± 0,14
SOD/(GPx + CAT) ratio (arbitrary units)	1,58 ± 0,13	1,91 ± 0,17	2,49 ± 0,42	2,30 ± 0,55
Kidney				
TBARS content (nmol TBARS/mg protein)	0,4311 ± 0,03	0,498 ± 0,04	0,5098 ± 0,03	0,4043 ± 0,04
Total reduced thiol content (nmol SH/mg protein)	5,17 ± 0,53	4,34 ± 0,46	**3,57 ± 0,52***	4,45 ± 0,22
Total non-protein thiol content (nmol SH/mg protein)	14,18 ± 1,95	**28,48 ± 1,89****	16,58 ± 2,08	**35,15 ± 3,92********
CAT activity (U CAT/mg protein)	55,06 ± 10,95	48,81 ± 9,84	39,92 ± 7,65	52,93 ± 10,44
SOD activity (U SOD/mg protein)	59,5 ± 2,70	55,74 ± 4,84	51,47 ± 3,77	51,98 ± 3,96
GST activity (U GST/mg protein)	0,0141 ± 0,0008	**0,0217 ± 0,0009********	0,0124 ± 0,0009	**0,0222 ± 0,0012********
GPx activity (U GPx/mg protein)	0,0984 ± 0,04	0,1888 ± 0,05	0,2427 ± 0,08	0,0836 ± 0,03
SOD/(GPx + CAT) ratio (arbitrary units)	1,58 ± 0,32	1,51 ± 0,35	1,88 ± 0,41	1,68 ± 0,42
Heart				
TBARS content (nmol TBARS/mg protein)	0,2214 ± 0,02	0,2608 ± 0,03	0,2355 ± 0,02	0,2316 ± 0,03
Total reduced thiol content (nmol SH/mg protein)	13,35 ± 0,39	**11,73 ± 0,35***	13,24 ± 0,36	12,88 ± 0,46
Total non-protein thiol content (nmol SH/mg protein)	16,06 ± 4,34	17,72 ± 4,78	19,38 ± 4,11	22,0 ± 5,36
CAT activity (U CAT/mg protein)	26,72 ± 6,44	11,37 ± 6,26	30,16 ± 6,84	34,51 ± 6,89
SOD activity (U SOD/mg protein)	33,51 ± 3,68	26,3 ± 2,2	36,56 ± 3,11	33,27 ± 3,09
GST activity (U GST/mg protein)	0,0091 ± 0,0007	0,0083 ± 0,0005	0,0092 ± 0,0008	0,011 ± 0,0006
GPx activity (U GPx/mg protein)	0,178 ± 0,04	0,104 ± 0,03	0,086 ± 0,02	0,146 ± 0,03
SOD/(GPx + CAT) ratio (arbitrary units)	1,79 ± 0,42	1,87 ± 0,39	1,58 ± 0,35	1,13 ± 0,16

Data are represented as mean ± SEM. **Bold for statistically significant difference**; *p ≤ 0,05, **p ≤ 0,01, ****p ≤ 0,0001 compared to the control group.

Table 6. Effects of MeHg, VitA and co-administration treatments on redox parameters in the liver of the offspring.

Redox parameters	Treatment			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
No of litters examined	8	7	8	7
TBARS content (nmol TBARS/mg protein)				
Male	0,4138 ± 0,04	0,4464 ± 0,05	0,4636 ± 0,04	0,3436 ± 0,05
Female	0,49 ± 0,05	0,6631 ± 0,10	0,6529 ± 0,07	0,615 ± 0,09
Total reduced thiol content (nmol SH/mg protein)				
Male	19,09 ± 1,18	17,91 ± 1,5	19,83 ± 2,92	12,97 ± 1,92
Female	19,77 ± 1,76	17,47 ± 1,63	18,21 ± 1,93	18,85 ± 1,03
Total non-protein thiol content (nmol SH/mg protein)				
Male	21,96 ± 5,59	38,29 ± 7,69	18,32 ± 7,76	12,26 ± 4,98
Female	36,12 ± 10,61	29,27 ± 6,13	33,87 ± 7,19	39,34 ± 9,96
CAT activity (U CAT/mg protein)				
Male	85,03 ± 10,74	66,22 ± 10,9	90,96 ± 14,92	97,25 ± 16,08
Female	117,3 ± 16,44	**74,28 ± 6,78***	110,7 ± 12,15	108,3 ± 19,4
SOD activity (U SOD/mg protein)				
Male	65,54 ± 3,75	63,87 ± 8,94	65,39 ± 3,5	68,34 ± 6,73
Female	90,44 ± 9,32	96,84 ± 8,26	95,07 ± 4,54	99,03 ± 10,01
GST activity (U GST/mg protein)				
Male	0,1043 ± 0,005	0,1001 ± 0,006	**0,1238 ± 0,005***	0,113 ± 0,007
Female	0,1035 ± 0,006	0,09496 ± 0,004	0,09674 ± 0,005	0,09576 ± 0,003
GPx activity (U GPx/mg protein)				
Male	0,1212 ± 0,04	0,09256 ± 0,03	0,1865 ± 0,04	0,09598 ± 0,03
Female	0,1866 ± 0,06	0,1664 ± 0,05	0,147 ± 0,05	0,1152 ± 0,05
SOD/(GPx + CAT) ratio (arbitrary units)				
Male	2,17 ± 0,37	3,23 ± 0,50	2,09 ± 0,41	2,03 ± 0,47
Female	1,84 ± 0,25	**3,06 ± 0,57****	2,30 ± 0,26	1,95 ± 0,26

Data are represented as mean ± SEM; **Bold for statistically significant difference**; *$p \leq 0,05$, **$p \leq 0,01$ compared to the control group.

Table 7. Effects of MeHg, VitA and co-administration treatments on redox parameters in the kidney of the offspring.

Redox parameters	Treatment			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
No of litters examined	8	7	8	7
TBARS content (nmol TBARS/mg protein)				
Male	0,4793 ± 0,06	0,6055 ± 0,07	0,55 ± 0,06	0,4091 ± 0,08
Female	0,2531 ± 0,04	0,2679 ± 0,03	0,3053 ± 0,02	0,2757 ± 0,03
Total reduced thiol content (nmol SH/mg protein)				
Male	11,24 ± 0,75	**15,05 ± 0,82***	11,24 ± 1,09	10,43 ± 1,2
Female	7,9 ± 0,43	7,32 ± 0,63	7,25 ± 0,19	6,91 ± 0,36
Total non-protein thiol content (nmol SH/mg protein)				
Male	36,13 ± 3,19	**18,42 ± 4,16****	**18,59 ± 3,20****	**21,53 ± 2,20***
Female	31,12 ± 4,01	45,75 ± 7,40	36,75 ± 5,36	23,56 ± 5,52
CAT activity (U CAT/mg protein)				
Male	67,54 ± 12,87	**25,79 ± 4,92***	42,07 ± 9,24	68,94 ± 11,89
Female	21,67 ± 5,26	14,49 ± 7,76	10,00 ± 3,00	48,25 ± 18,71
SOD activity (U SOD/mg protein)				
Male	76,57 ± 8,86	73,58 ± 6,48	70,59 ± 5,93	63,61 ± 10,52
Female	59,03 ± 3,21	56,14 ± 5,65	58,42 ± 4,08	57,15 ± 7,91
GST activity (U GST/mg protein)				
Male	0,0170 ± 0,001	0,0188 ± 0,001	0,0153 ± 0,002	0,0152 ± 0,002
Female	0,0113 ± 0,0009	0,0098 ± 0,001	0,0111 ± 0,0008	0,0116 ± 0,0008
GPx activity (U GPx/mg protein)				
Male	0,1229 ± 0,05	0,1216 ± 0,06	0,1344 ± 0,03	0,1496 ± 0,04
Female	0,1426 ± 0,02	0,2384 ± 0,07	0,1368 ± 0,02	0,172 ± 0,05
SOD/(GPx + CAT) ratio (arbitrary units)				
Male	1,98 ± 0,47	3,25 ± 0,76	2,96 ± 0,99	1,36 ± 0,33
Female	2,58 ± 0,42	3,84 ± 0,76	4,43 ± 1,10	1,32 ± 0,44

Data are represented as mean ± SEM. **Bold for statistically significant difference**; *$p \leq 0,05$, **$p \leq 0,01$ compared to the control group.

Table 8. Effects of MeHg, VitA and co-administration treatments on redox parameters in the heart of the offspring.

Redox parameters	Treatment			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
No of litters examined	8	7	8	7
TBARS content (nmol TBARS/mg protein)				
Male	2,209 ± 0,2	1,927 ± 0,37	1,931 ± 0,31	1,507 ± 0,3
Female	0,79 ± 0,15	1,239 ± 0,44	1,29 ± 0,27	0,7476 ± 0,20
Total reduced thiol content (nmol SH/mg protein)				
Male	13,38 ± 0,33	14,92 ± 0,78	15,12 ± 0,63	14,8 ± 0,91
Female	13,15 ± 1,34	11,74 ± 2,65	**5,82 ± 1,69***	9,22 ± 1,47
Total non-protein thiol content (nmol SH/mg protein)				
Male	24,1 ± 3,75	24,71 ± 4,97	26,49 ± 3,48	19,47 ± 6,86
Female	22,62 ± 5,16	18,71 ± 3,42	21,05 ± 3,63	15,05 ± 4,21
CAT activity (U CAT/mg protein)				
Male	25,36 ± 6,81	23,12 ± 11,63	25,33 ± 4,18	11,3 ± 0,96
Female	22,22 ± 10,75	22,03 ± 8,67	18,45 ± 7,68	19,1 ± 1,65
SOD activity (U SOD/mg protein)				
Male	41,75 ± 4,08	41,36 ± 4,76	41,65 ± 3,77	39,15 ± 4,32
Female	49,39 ± 5,27	44,09 ± 6,75	51,74 ± 6,79	44,7 ± 2,44
GST activity (U GST/mg protein)				
Male	0,0013±0,0002	0,0011±0,0002	0,0016±0,0003	0,0016±0,0002
Female	0,0065±0,0005	0,0072±0,0004	0,0064±0,0004	0,0074±0,0007
GPx activity (U GPx/mg protein)				
Male	0,1604 ± 0,03	0,1616 ± 0,03	0,2456 ± 0,04	0,2096 ± 0,03
Female	0,2449 ± 0,03	0,3175 ± 0,07	0,2785 ± 0,03	0,3271 ± 0,06
SOD/(GPx + CAT) ratio (arbitrary units)				
Male	2,23 ± 0,74	1,50 ± 0,20	1,64 ± 0,17	**3,40 ± 0,37***
Female	2,95 ± 0,68	2,02 ± 0,89	3,68 ± 0,81	2,32 ± 0,06

Data are represented as mean ± SEM**. Bold for statistically significant difference**; *$p \leq 0,05$, **$p \leq 0,01$ compared to the control group.

Table 9. Effects of MeHg, VitA and co-administration treatments on maternal and offspring MNPCE frequencies.

	Treatments							
	Control		MeHg		VitA		MeHg-VitA	
N (dams/litters)	8		7		8		7	
MNPCE/2000 cells	**Mean ± SEM**	**Median 25th - 75th percentile**	**Mean ± SEM**	**Median 25th - 75th percentile**	**Mean ± SEM**	**Median 25th - 75th percentile**	**Mean ± SEM**	**Median 25th - 75th percentile**
Dams	0,38 ± 0,18	0,0 (0,0 - 1,0)	**2,0 ± 0,22***	2,0 (2,0 - 2,0)	**2,75 ± 0,25*****	3,0 (2,0 - 3,0)	**2,29 ± 0,36***	2,0 (2,0 - 3,0)
Offspring								
Male	0,31 ± 0,09	0,5 (0,0 - 0,5)	**2,4 ± 0,26*****	2,5 (2,0 - 3,5)	1,62 ± 0,23	2,0 (1,0 - 2,0)	**2,06 ± 0,26****	2,5 (2,0 - 3,0)
Female	0,09 ± 0,04	0,0 (0,0 - 0,3)	**1,9 ± 0,18******	2,0 (2,0 - 2,5)	1,0 ± 0,15	1,0 (0,5 - 1,5)	**1,56 ± 0,21****	1,5 (1,5 - 2,5)
Total	0,47 ± 0,11	0,3 (0,3 - 0,8)	**2,5 ± 0,19******	2,5 (2,0 - 2,8)	1,31 ± 0,13	1,5 (0,9 - 1,5)	**2,07 ± 0,22****	2,0 (1,8 - 2,5)

Data are represented as mean ± SEM. **Bold for statistically significant difference**; *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001, ****p ≤ 0,0001 compared to the control group.

CAPITULO II - METHYLMERCURY AND RETINYL PALMITATE CO-ADMINISTRATION IN RATS UNDER PREGNANCY AND BREASTFEEDING: BEHAVIORAL CHANGES AND OXIDATIVE STRESS UPON HIPPOCAMPUS OF DAMS AND THEIR OFFSPRING

Artigo a ser submetido no periódico: **Neurotoxicology**

“METHYLMERCURY AND RETINYL PALMITATE CO-ADMINISTRATION IN RATS UNDER PREGNANCY AND BREASTFEEDING: BEHAVIORAL CHANGES AND OXIDATIVE STRESS UPON HIPPOCAMPUS OF DAMS AND THEIR OFFSPRING”

Pedro Espitia-Pérez[1, *], Suelen Marin Albino[1], Lyda Espitia-Pérez[2], Hugo Brango[3], Helen da Rosa[1], Alexander Kleber Silveira[1], Diogo Pompéu Moraes[4], Camila Cerveira[4], Moara Mingori[1], Camila Tiefensee Ribeiro[1], Daniel Pens Gelain[1], Carlos Eduardo Schnorr[5] and José Cláudio Fonseca Moreira[1, **]

[1] Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600 Anexo Dpto. Bioquímica, Laboratório 32, CEP 90035-003, Porto Alegre, Rio Grande do Sul, Brazil.

[2] Facultad de Ciencias de la Salud, Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Calle 38 Carrera 1W, Barrio Juan XXIII, Montería, Córdoba, Colombia.

[3] Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil.

[4] Instituto de Química, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brazil.

[5] Departamento de Civil y Ambiental, Programa de Ingeniería Ambiental, Universidad de la Costa, Calle 58 #55– 66, Barranquilla, Atlántico, Colombia.

For correspondence with the authors:
E-mail adresses: * pjespitia@gmail.com (P. Espitia-Pérez); ** 00006866@ufrgs.br (J.C. Fonseca Moreira)

Abstract

Fish consumption and ubiquitous methylmercury (MeHg) exposure represents a public health problem globally. Additionally, micronutrients presented in fish affects MeHg uptake/distribution, but limited data is available. Vitamin A (VitA), another fish micronutrient is used in nutritional supplementation, especially during pregnancy. However there is no information about the health effects arising from their combined exposure. Therefore, the aim of the present study was to examine the effects of both MeHg and retinyl palmitate administered on pregnant and lactating rats on maternal and offspring hippocampus. Thirty *Wistar* female rats were orally supplemented with MeHg (0,5 mg/Kg/day) and retinyl palmitate (7500 µg RAE/Kg/day) via gavage, either individually or in combination from the gestational day 0 to weaning. Maternal behavior was scored in dams at postnatal day (PND) 5. In offspring, associative learning and neurodevelopment were evaluated through olfactory discrimination (PND 8) and eye opening (PNDs 12-16) tests respectively. Periadolescent male and female pups were assessed in open field (PND 28) and intersession habituation to open field tests (PND 30). Object recognition test using episodic-like memory paradigm (PND30) was used to test spatial and temporal components of memory in both male and female offspring. Redox parameters were evaluated at PND 31 for both. Our results showed no effects of MeHg-VitA co-administration in pregnant and lactating rats in terms of quality of maternal care, but showed subtle alterations in the pro-oxidant response of the hippocampus. In offspring, MeHg-VitA co-exposure affected early associative learning in neonatal offspring, with no further alterations in neurodevelopment, and no locomotor, exploratory or emotional alterations were evidenced in latter developmental stages. Habituation was altered in a sex-dependent manner, but no overall memory disturbances were encountered. Finally, MeHg-VitA co-administration reduced lipoperoxidation in male offspring hippocampus, possible attributed to the antioxidant properties of VitA *in vivo*. Further work is needed to confirm our findings and elucidate the molecular mechanisms of MeHg-VitA modulation and assess epidemiological implications. Pre-clinical assays are needed in order to demonstrate the potential therapeutical use of VitA in populations directly or indirectly exposed to MeHg.

Keywords: Methylmercury, Retinyl palmitate, Pregnancy, Co-exposure, Behavior, Hippocampus, Oxidative stress

1. Introduction

Currently MeHg is considered a global ubiquitous contaminant [1]. Bioaccumulation, biomagnification and high persistence of MeHg can be observed in both freshwater and seawater ecosystems, especially in fish and shellfish, which possess 75 to 90% of organic mercury content in the form of MeHg [2]. However, 84% of total Hg burden enters our organism through fish consumption [3], which comprises the mayor route of chronic exposure to MeHg in humans [4, 5]. However, nutrients also present in fish like selenium and n-3 long chain polyunsaturated fatty acids are known modulators of MeHg-induced toxicity. Complementary factors like protein intake and other micronutrients presented in fish such as choline, iodine and iron might affect mercury uptake/distribution in the body [6]. Despite evidence, studies regarding MeHg interaction with micronutrients during development are scarce, and possible modulations effects during co-exposure events remains unknown [7].

Recently, increasing number of studies supports the hypothesis that humans and animals can be exposed to a mixture of chemicals from different sources [8-11]. Food consumption allows human exposure to intentional/unintentional/coincidental type of mixtures, comprising multiple chemicals from different sources and routes [12]. Studies underlying mechanisms of combined effects of mixtures are currently limited due to lack of data [13]. However, recent experimental evidence showed possible mixture effects with chemicals even combined at low concentrations [14]. Considering the latter, another compound that can be associated with MeHg via food consumption is vitamin A (VitA). This VitA may be obtained from both vegetal (provitamin A) and animal (preformed) diet [15, 16]. High contents of preformed VitA can be encountered in liver and fish, whereas green, orange and yellow vegetables are the most common dietary sources of provitamin A [17]. Recent reports demonstrated that global per capita fish consumption has risen to above 20 kg a year with estimates of further growth [18]. Concomitantly, strong evidences suggests that over 75% of people in developed nations may routinely ingest VitA above the recommended dietary allowance [19]. Additionally, another source of VitA are dietary supplements which contains variable portions of both preformed and provitamin A, mainly in the forms of retinol and β-carotene respectively [17]. Only in United States (US) 28 to 37% of adults ingest supplements containing VitA [20] and approximately 30 to 38% supplement consumption correspond to women in reproductive age (15 to 49 years old), more prominent when compared to men of the same age interval (25 to 32%) [20], suggesting that VitA may be obtained from both diet and as a supplementation, either for clinical use or even inadvertently [16].

Women are probably most influenced by the possible co-exposure effects of MeHg and VitA. The nutritional requirements in women increases during pregnancy, and fish and shellfish are the primary source of long-chain polyunsaturated fatty acids, which are essential nutrients for optimal neurodevelopment of the fetus [21]. Additionally, in pregnant women, VitA contributes to early fetal development, especially in the central nervous system (CNS) contributing to cellular differentiation, apoptosis and homeostasis during adulthood [22-25]. Regarding fish consumption, an estimated 400 million women of reproductive age in the world rely on seafood for at least 20% of their intake of animal protein [1], and only in the US, fish consumption has increased among pregnant women in recent years [21, 26]. Moreover, ingestion of VitA is higher among women rather than men, mostly in the form of provitamin A [27], whereas during pregnancy, VitA supplementation is commonly recommended as safe doses at 3000 or 7500 μg RAE/Kg/day, independently of previous VitA consumption [28]. Nutritional demand during pregnancy may increase the bioavailability of MeHg and VitA and therefore exposing the fetal environment to unknown effects.

In terms of MeHg, epidemiological investigations demonstrated that pregnant women with high fish intake are at greater risk of giving birth to children with brain affectations [29, 30]. However, increasing experimental evidence demonstrated the relation between chronic low dose MeHg exposure and neurodevelopment disorders [31, 32]. Also, epidemiological studies shows the possible association between prenatal exposure to low doses of MeHg and disturbances in memory, attention and language, encountered in children and adolescents [33-35]. Supporting the latter, a recent study demonstrated disrupted exploratory behavior, recognition memory, spatial learning, and acquisition of aversive memories in rats with prenatal MeHg exposure at low doses [36]. On the other hand, the detrimental effects of excessive VitA intake on behavior (i. e. confusion, irritability, anxiety, depression) were observed after chronic vitamin A exposition [16]. Additionally, evidence in animal models demonstrated that recommended VitA supplementation at safe doses induced anxiety-like behavior and decreased both locomotory and exploratory activities in adult male Wistar rats under a 28-day treatment. Furthermore, prenatal exposure to VitA supplementation induced behavioral disturbances in dams and their offspring [37].

Despite these well documented behavioral disturbances induced by either MeHg or VitA exposure alone, currently there is a lack of information about the health effects arising from their combined exposure. Therefore, the aim of the present study was to examine the effects of MeHg and VitA co-exposure on pregnant and lactating Wistar rats in order to evaluate behavioral and biochemical changes in the brain of the dams and their offspring.

2. Experimental procedures

Experimental procedures were conducted in accordance with the Principles of Laboratory Animal Care (US National Research Council Committee, 2011) [38], and were in compliance with recommendations of the Brazilian Society for Science in Laboratory Animals (SBCAL-COBEA). The Ethical Committee for Animal Experimentation (CONCEA) ascribed to Federal University of Rio Grande do Sul (UFRGS) approved the research protocol under authorization number 31672.

2.1. Animal and housing conditions

Male and female Wistar rats (*Rattus novergicus*) were obtained from our breeding colony and housed in groups of four animals under a 12 h light-dark cycle (7:00 – 19:00h) at constant temperature (21 ± 1°C). Standard commercial food (CR1 Lab Chow, Nuvilab, Curitiba, Brazil; 3600 µg RAE/Kg) and filtered water were provided *ad libitum*. Female nulliparous rats (200-250g) with 120 days were checked daily for their estrous cycle using direct vaginal smear evaluation under light microscopy [39]. Estrous cycle monitoring was performed for two weeks. Sexually receptive females (confirmed proestrous) were caged overnight with a single mature male (1F:1M). The next morning the presence of viable sperm in the vaginal smear was considered as successful mating and designated as gestation day 0 (GD0). Pregnant dams were caged individually and allowed to litter naturally. Animals were supplied daily with standard food and filtered water *ad libitum*. The date of delivery was defined as postnatal day 0 (PND0).

Restrain stress in late pregnancy (GD17-21) affects pup performance in pre-frontal cognitive tasks [40]. In order to avoid stress-related effects on offspring, dams were minimally handled during a 4-day period (comprising GD20 and PND2) and pups were only manipulated and registered on PND2. Full sized litters were maintained with the dam until weaning (PND21). Pups were separated from the dam and immediately sexed on PND21. Separated litters were co-housed in groups of the same sex in order to eliminate interferences on behavior related with sexual hormones [41]. No further treatment was applied to male and female pups, which continued in the same room conditions as their parents with standard food and filtered water *ad libitum*.

2.2. Treatment

Methylmercury (II) chloride was obtained from Spectrum (Spectrum, New Brunswick, NJ, United States). A stock solution of 0,39 $\mu g/\mu L$ of MeHg was prepared in mineral oil. Retinyl palmitate (a commercial form of vitamin A dissolved in mineral oil) was purchased from Embrafarma, (Embrafarma, Sao Paulo, SP, Brazil) as a stock solution of 45000 µg RAE.

Thirty pregnant dams were randomly divided into four experimental groups, as described: Group 1 (control), where rats where treated with mineral oil; Group 2 (MeHg): where rats received methylmercury (II) chloride at a dose of 0,5 mg/Kg/day; Group 3 (VitA): where rats received retinyl palmitate at a dose of 7500 µg RAE/Kg/day; Group 4 (MeHg-VitA): where rats received methylmercury (II) chloride (0,5 mg/Kg/day) and retinyl palmitate (7500 µg RAE/Kg/day) both mixed. All solutions were prepared in mineral oil.

All treatments were prepared daily and administrated orally with a metallic gastric tube (via gavage) in a maximum volume of 0,5 mL. Pregnant rats were treated once a day from GD0 until weaning at PND21. In dams, no evidence of motor alterations, or general health disturbances were observed during gestation and lactation in either of the experimental groups.

2.3. Behavioral tests and procedures

Behavioral tasks were always performed during the light phase between 9:00 and 15:00h in specifically designed rooms with bright controlled-light (overhead fluorescents lamps of 40W, light intensity 90 Lux), with the exception of the maternal observational test which was performed in the colony housing room. Experimental design of behavioral test is depicted in Figure 1.

Handling of dams and pups were performed accordingly in order to ensure minimal distress in animals during testing period. Behavioral recording and analysis was performed using ANY-maze Video Tracking System Version 5.14 (Stoelting Co., Wood Dale, IL, USA).

2.4. Maternal behavior

In animal models, maternal behavior test evaluate the latency, frequency and duration of stereotypic behaviors during the perinatal period in dams [42]. The test can be used to assess the effects of early stressors and contaminants which can affect the quality of maternal care and, therefore, influence stress response, anxiety and aversion reactivity in offspring through epigenetic regulation [42-44]. Observation of dam behavior in home cage and pup retrieval behavior were assessed in a two-set battery of test on PND5 for each of the dams in the experiment.

2.4.1. Observation of dam behavior in home cage

The test was performed in a single session between 12:00 and 12:30h using the methodology described by Rocha *et al*. [45]. Observations were registered over a 30-min limit using 10 spot checks at 3 min. Information collected in each spot check was immediately recorded,

including whether dams were in or out from the nest. For dams located in the nest, pup retrieval, pup licking, or breastfeeding behaviors were considered and registered as nursing. Results were expressed as % of time spent on nursing.

2.4.2. Pup retrieval behavior

Dams were tested for pup retrieval behavior between 14:00 and 16:00h. Retrieval behavior was assessed using a modification of the single day test protocol described in Rocha *et al*. [45]. Briefly, the home cage was transported from the colony room to a specifically designed soundproof room (testing room). Pups and dams were allowed to acclimate at least 1 hour prior to testing. The dam was then separated from the litter and maintained inside a holding cage for 3 min. The pups were kept inside a plastic container with wood shaving. After separation period, the home cage was placed in the marked site for testing and behavior recording. Four randomly selected pups from the litter were scattered over the floor of the home cage opposite to the nest. The dam was returned to the nest and the number of retrieved pups were recorded. The test continued until 7 min had elapsed. When litters were outnumbered (the number of pups were less than four) all pups were used for testing.

2.5. Maternal odor preference test

Maternal odor preference test measures the associative learning in neonatal pups regarding its ability to recognize the mother´s odor and other olfactory cues in their home environment [46]. This type of rapid classic conditioning allows the pup to approach to its mother for suckling [46, 47]. Maternal odor preference test was performed on PND8 using the protocol described elsewhere [48, 49]. Briefly, the test area consisted of a polypropylene cage (40x32x16 cm) divided in half by a 2-cm wide neutral zone. In both sides of the cage, two-odor choice areas were determined, one with nest material and another with fresh bedding. Approximately 300 mL of fresh or nest bedding was placed in adjacent corners. The dams were separated from the litter and transferred to another holding cage. One male pup was selected randomly from each litter for evaluation. Each pup was individually placed in the 2-cm neutral zone facing the cage wall and were evaluated in five trials of 1 min each and an inter-test interval of 2 min, during which the pup was placed in the home cage with their littermates. During the 1-min trial the amount of time the pup (the head or the whole body) spent in each of the two areas was recorded. During the inter-test interval of 2 min, location of the beddings were switched between both sides of the cage. After testing, the bedding material was collected, and the cage was cleaned with 70% ethanol in order to remove trace odors. Experiments were recorded for posterior analysis. The total exploration time on each area was

obtained by the sum of all five trials. Data analysis was performed using a modification described in Polan and Hofer [50] and results were expressed as % of preference for maternal bedding material for each pup, calculated as $\%_{Preference} = (T_M/T_{C+M}) \times 100\%$, where T_M was the total time exploring the maternal area, and T_{C+M} was the total exploration time for both control and maternal areas.

2.6. Eye opening

Eye opening was assessed once daily, from PND12 until 16, for all pups in each litter as previously described [51]. The dam was briefly separated from the pups in a holding cage, and each pup was gently examined and rated as follows: 0 – both eyes closed; 1 – one eye open and 2 – both eyes open.

2.7. Open field test

Open field test (OFT) is a relatively simple technique, traditional and commonly used for the assessment of the locomotive and explorative behavior as well as the emotional state in rodents [52, 53]. Pup testing was performed on PND28 (novel OFT) once a day using two male and two female pups from each litter selected randomly. After selection, pups were tail-marked for posterior identification in subsequent tests. They were transferred from the colony room to a specifically designed soundproof room (testing room) and allowed to habituate for 30 min. The OFT apparatus consists of a square open field (57x57x60cm) with black-painted walls. All the animals were gently placed in the periphery of the arena from a fixed position, and were allowed to freely explore it for 10 min. When test time ended, they were returned to their home cages. Following each test, the cage was cleaned with 70% ethanol to remove trace odors. Locomotion and exploration of animals were assessed by measuring total distance, average speed and center entries. Vertical activity was evaluated using total rearings and groomings as parameters [54]. However, due to technical difficulties during image registration, only on-wall rearings were measured for both male and female pups. Finally, anxiety was assessed as thigmotaxis index (i.e. ratio between distance covered alongside the walls to the total distance covered, expressed as percentage) [55].

2.8. Open field habituation test

Subjects were re-tested in the same open field on PND29 (familiar OFT) in order to assess intra and intersession habituation to the test apparatus and whether their behavior was reflective of exploratory activity specific to the novel environment [56]. Pups entered from the same fixed

position as the novel OFT. Animal behavior was monitored using a high-quality video camera, and exploration was analyzed off-line with ANY-maze tracking system. The same parameters measured in the novel OFT were analyzed in the habituation test. The total time spent in the periphery was used as a parameter in intersession habituation, as previously described [57].

2.9. Object recognition test

Episodic memory is defined as the ability to consistently evoke previous information obtained in a temporary basis and through spatiotemporal relations [58, 59]. Object recognition test (ORT) using the *what-when-where* paradigm was used to assess episodic-like memory in both male and female pups. Testing was performed at PND30 using two male and two female pups from each litter. We used the protocol described by Inostroza *et al.*, [60] and recently applied for Lopez-Pigozzi *et al.*, [61] with some modifications. Briefly, the test apparatus consisted of the same square open field previously used during OFT testing and the same conditions of illumination (90 lux) were maintained. Animal behavior was recorded and exploration was analyzed off-line via ANY-maze tracking system. Object recognition tasks were performed in a single three-phase session in the same testing room as OFT testing. Before the task, animals were previously habituated in two-day OFT sessions, meaning that only tail-marked males and females from OFT testing were chosen for object recognition testing.

Objects were chosen in order to avoid animals for climbing or dropping during testing. The object A consisted of a soda glass bottle (15 cm tall, 10 cm base) and the object B consisted on a soda can (10 cm tall, 10 cm base). During the task, animals were confronted with four copies of the same object during a first sample phase (objects A, old familiar) and were allowed to freely explore the objects for 5 min. Object exploration was considered as the rat being close to an object and directing its nose performing either sniffing or placing two paws as sign of interest. After a 50 min inter-test interval, animals encountered a second sample phase consisting of four copies of another object (objects B, recent familiar) and were permitted to explore for 5 min. Second objects were settled in either new or the same locations as object from the first sample phase. The test phase followed a 50 min inter-trial period after the second sample phase and consisted of exposing animals during 5 min to two old and two recently familiar objects seen in the previous sampling phases. Pups entered the arena from a previously fixed position, maintained during the two-day OFT period. Along the inter-trial periods, both objects and square area were cleaned with 70% ethanol to remove trace odors, and pups were maintained in their home cages with food and water *ad libitum*.

For assessment of episodic-like memory, a "when" discrimination index was defined in order to quantify the exploration of the old stationary object (Figure 7A, A1 black) compared to a recent stationary object (Figure 7A, B1 light blue). Additionally, a "where" discrimination index was calculated as the proportion of time exploring a recently displaced object (Figure 7A, B2 magenta) versus a recent object that remained stationary (B1). Calculations of the "when" and "where" indexes are fully detailed in previous work of Inostroza *et al.* [60]. For these ratios a value of zero indicated chance level performance and therefore no preference for the object.

Finally, in order to evaluate against chance exploration and assessing object preference, discrimination indexes were also defined for all tested objects during the test phase by dividing the total time exploring an object by the total exploratory time of all objects. For these ratios a value of 0,25 indicated chance level performance and therefore no preference for the object.

2.10. Tissues extraction and sample preparation

All animals were killed by decapitation. Dams were killed 24h after the last treatment at PND21, whereas male and female pups were killed on PND31. Maternal and offspring samples of hippocampus, prefrontal cortex and olfactory bulb were immediately identified, dissected in ice and stored in -80°C for posterior analysis. For sample preparation, tissues were homogenized in 50 mM phosphate buffer (pH 7.0) and centrifuged (3000 *x g*, 5 min) to remove cellular debris and obtained supernatants were employed for determination. Results were normalized by protein content according to Lowry technique using bovine albumin as standard [62]. For mercury determination, a small portion of the occipital cortex (130-180 mg of wet tissue) was isolated and stored in -80°C for posterior analysis. For sample preparation, maternal and offspring samples of cortex were weighted in an analytical balance (XS105 Excellence XS, Mettler Toledo, Switzerland) and pretreated using wet ash digestion procedure. Sample aliquots (0.1 to 0.2 g) were treated with concentrated HNO_3 using an initial microwave-assisted heating (4 cycles of 5 s duration) and posterior overnight (12 h) step was applied. A final cycle of heating assisted by microwave was performed and sequentially H_2O_2 was added before sonication step using ultrasonic bath (30 min). Finally, total volume was completed to 10 mL with water and centrifugation was performed to remove solid particles from wet ash procedure.

2.11. Mercury determination in cortex samples by chemical vapor generation coupled to atomic absorption spectrometry (CVG-AAS)

Mercury measurements were carried out with an atomic absorption spectrometer (AAnalyst 200, Perkin Elmer, USA) equipped with a quartz T-cell (160 mm length, 7 mm i.d.) at 100 ºC. A mercury electrodeless discharge lamp (EDL) at 220 mA was used as radiation source with deuterium lamp as continuum source for background correction. Wavelength was set to 253.7 nm and spectral band pass to 2.7/1.05 nm (width and height, respectively). All measurements were made in integrated absorbance (peak area). Sample injection was performed via flow injection analysis (FIAS 100, Perkin-Elmer, USA) using a sampling loop of 500 µL. The solution of 1 mol L^{-1} hydrochloric acid at flow rate of 4.8 mL min^{-1} and 0.75% (m/v) sodium tetrahydroborate at flow rate of 1.8 mL min^{-1} were used as carrier and reductant, respectively. Argon was used as carrier gas at flow rate of 50 ml min^{-1}. The arrangement of FIAS system was carried out using a polytetrafluorethylene (PTFE) tubes 1.0 mm i.d. and 200 mm (reaction coil) to transport the solutions to gas-liquid separator. Milli-Q water (18.2 MΩ cm, Millipore, USA) and analytical-grade reagents were used throughout the analysis. Standards solutions were prepared on the day of use by serial dilution of 1000 mg L-1 stock solution of Hg (Fluka Analytical, Sigma-Aldrich, USA). These stock standard solutions were also used for the analytes recoveries tests. The calibration solutions were prepared in 10% (v/v) HNO_3 in the range of 2.5 - 20 µg L^{-1} for Hg determination by CVG-AAS. All laboratory materials such as glasses and polypropylene vials were cleaned before use by soaked in 10% (v/v) HNO_3 bath for seven days.

2.12. Redox profile in isolated CNS structures

2.12.1. Cellular oxidative damage

Quantification of thiobarbituric acid reactive species (TBARS) was perfomed according to the protocol described by Draper and Hadley [63]. The procedure is widely accepted for measure oxidative damage in lipids. Briefly, protein from homogenized samples was extracted using 15% TCA and supernatants were treated with 0,67% thiobarbituric acid and heated at 100°C on a microplate dry bath for 25 min. TBARS content was determined spectrophotometrically at 532 nm. Results were expressed as nmol TBARS/mg protein.

Total reduced thiol content is a measure of intracellular redox status, mainly attributed to proteins as well as glutathione molecules. Quantification was performed using the protocol described by Ellman [64]. Briefly, homogenized samples were treated with 5,5-dithiobis-2-nitrobenzoic acid 0,01 M in ethanol. The developed yellow color was measured spectrophotometrically at 412 nm after 60 min. Results were expressed as nmol SH/mg protein.

2.12.2. Enzymatic antioxidant activities

Superoxide dismutase (SOD) activity was determined using the method described by Misra and Fridovich [65], using the quantification of adrenaline-dependent inhibition of superoxide auto oxidation in spectrophotometer at 480 nm. Results were expressed as units SOD/mg protein.

Catalase (CAT) activity was measured by the method of Aebi [66]. Using hydrogen peroxide as substrate, the decrease in absorbance due to H_2O_2 degradation was monitored spectrophotometrically at 240 nm for 1 min. Results were expressed as units CAT/mg protein.

Glutathione-S-transferase (GST) activity was determined using the method described by Habig and Jakoby [67] by the increase in conjugation rate of glutathione with 1-cloro-2,4-dinitrobenzene measured spectrophotometrically as absorbance for 3 minutes at 340 nm. Results were expressed as nmol GST/mg protein.

Glutathione peroxidase (GPx) activity was estimated using the method described by Wendel [68], by monitoring the oxidation of NADPH in spectrophotometer at 340 nm. Results were expressed as nmol GPx/mg protein.

2.13. Statistical analysis

Data analysis and graphics were performed using the software GraphPad Prism version 7.01 (GraphPad Software Inc., San Diego, CA, USA). Specific statistical testing was carried out using software R version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria) and the Statistical Package for the Social Sciences software version 18.0 (SPSS Inc., Chicago, IL, USA — SPSS version 18.0) software. All results are expressed as mean ± SEM and differences were considered significant when $p \leq 0,05$.

For behavioral testing, normality was confirmed by D´Agostino-Pearson omnibus test. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) and *post hoc* analysis by Holm-Sidak test or Fisher´s LSD test to detect differences between experimental groups and their respective controls. Non-parametric analyses were performed when needed (Kruskal-Wallis test with Dunn´s multiple comparison). Two-way ANOVA using treatment and sex as factors was used in order to assess differences in OFT testing and redox profile parameters from hippocampus, prefrontal cortex and olfactory bulb. When needed, *post hoc* comparisons were performed using Bonferroni and Sidak tests, and one-way ANOVA with Tukey's comparisons, especially to evaluate differences among oxidative stress parameters between experimental groups and their controls, and equally in object exploration in animals. For eye opening behavior a two-way ANOVA and *post*

hoc Chi^2 test was used to evaluate differences among groups at different days of testing. For preference assessment during olfactory testing and object exploration, one sample *t* test was used for data inference. Comparisons of mercury contents were analyzed with unpaired *t* student´s test.

3. Results

3.1. Maternal behavior

Nursing and pup retrieval behavior were evaluated in both control and treated dams and results are summarized in Figure 2. There was no differences on nursing (Figure 2A) and pup retrieval behaviors (Figure 2B) when treated dams were compared to controls, indicating no interference of MeHg and VitA treatments, either alone or administered in combination, with ongoing maternal behavior.

3.2. Pup maternal odor preference

Results are depicted in the Figure 3. Pups from Control (t = 3,086, df = 7, p = 0,018) and VitA (t = 5,306, df = 7, p = 0,001) exposed groups presented a significant preference for maternal bedding material. Prenatal/lactation treatment with MeHg impaired maternal odor preference (t = 0,051, df = 6, p = 0,961) and MeHg-VitA co-administration also disrupted olfactory learning response in offspring, since preference from MeHg-VitA treated pups was in the chance level (t = 0,742, df = 6, p = 0,486). No differences were encountered in any of the experimental groups regarding total exploration times, discriminated either by trials or as a total (data not shown) indicating that pup preference was not influenced by differential exploration between groups.

3.3. Eye opening behavior

Two-way ANOVA showed a highly significant effect of treatments ($F_{[3,\ 1285]}$ = 3,984, p = 0,0077), day of testing ($F_{[4,\ 1285]}$ = 734,4, p < 0,0001), and treatments x day of testing interaction ($F_{[12,\ 1285]}$ = 1,805, p = 0,0427). *Post hoc* testing revealed that eye opening was significantly affected by the treatments, principally at PNDs 14 ($\chi^2_{[5,\ 1285]}$ = 14,114, p = 0,028) and 15 ($\chi^2_{[5,\ 1285]}$ = 16,443, p = 0,012) showing a significant delay in eye opening in particular for MeHg treated pups (Figure

4). Furthermore, VitA and MeHg-VitA treatments showed no differences in eye opening behavior when compared with control group.

3.4. Open field test

Results for OFT are depicted in Figure 5. A general multivariate linear model was used for assessment of OFT parameters. *Post hoc* testing indicated no differences in total distance (Figure 5A), average speed (Figure 5B) or center entries (Figure 5E) among treatments for both male and female pups, indicating no evidence of motor or exploration dysfunctions among animals. Multiple comparison analysis also revealed no differences in rearing or grooming behaviors, with exception of MeHg female pups (Figure 5C), which presented a significant decrease in total rearings (non-parametric Kruskal-Wallis test with Dunn´s multiple comparison, K = 16,14, p = 0,001). The thigmotaxis index showed no significant differences between experimental groups, for both males and females rats, as revealed by posterior *post hoc* testing (data not shown). Conjunctively, our results may indicate no affectations in locomotor, exploratory and/or emotional activity in tested animals.

3.5. Habituation to novelty and minute-by-minute locomotor activity analysis

A minute-by-minute analysis of locomotor activity in both OFT sessions was performed and results are depicted for both male (Figures 6A and 6B) and female pups (Figure 7A and 7B). During the first 3 min of OFT Session 1, male pups from control and MeHg-VitA groups presented a significant increase in the difference in distance traveled between the 1st and the 3rd minutes of testing when compared with MeHg and VitA treated groups ($F_{[3,\ 52]}$ = 6,062, p = 0,0013), demonstrating that control and MeHg-VitA pups presented a strong habituation to novelty in OFT Session 1 (Figure 6C). However, during the first 3 min of OFT Session 2, male pups from MeHg, VitA and MeHg-VitA showed a significant increase in the difference in distance traveled between the 1st and the 3rd minutes of testing ($F_{[3,\ 52]}$ = 5,642, p = 0,0020), which indicates the beginning of habituation response during OFT Session 2. In general, a new exposure of OFT shows control male pups begins habituation the first 3 minutes of the OFT Session 1 (t = 2,206, df = 104, p = 0,0296), with normal habituation locomotive response at the final 4 min of further testing, differently of experimental groups shows ($F_{[1,\ 104]}$ = 23,76, p < 0,0001), which displayed a significant difference in distance traveled the last 4 min of testing, indicating a new habituation response when re-exposed to OFT Session 2 (Figure 6D).

Female pups showed no differences in distance traveled during the first 3 min of both OFT sessions when comparing experimental with it respective control groups (Figure 7C). However, during the first 3 min of OFT Session 2, all female pups from control and experimental groups showed a significant increase in the difference in distance traveled between the 1st and the 3rd minutes of testing ($F_{[3, 106]}$ = 2,744, p = 0,0467), indicating the beginning of habituation response. Control and VitA treated females displayed a significant decrease in distance traveled only in the last 4 min of testing when comparing both OFT sessions ($F_{[1, 106]}$ = 19,32, p < 0,0001), however, female rats from MeHg and MeHg-VitA groups showed no habituation response (Figure 7D).

3.6. Intersession habituation to open field

Results for intersession habituation to open field are summarized on Figure 8. Total distance traveled was used to compare locomotion in both OFT sessions. In terms of total distance, two-way ANOVA showed a significant effect of novelty for both males ($F_{[1, 101]}$ = 18,63, p < 0,0001) and females ($F_{[1, 106]}$ = 11,28, p = 0,0011). *Post hoc* Fisher´s LSD test for comparison between OFT sessions revealed significant differences for male pups from MeHg-VitA treated group (novel OFT vs familiar OFT, *t* = 2,645, df = 101, p = 0,0095) (Figure 8A). However, despite no differences in total distance traveled were encountered for female rats from MeHg-VitA group when comparing both OFT sessions, differential locomotion response was encountered for control (novel OFT vs familiar OFT, *t* = 2,228, df = 106, p = 0,028) and VitA treated females (novel OFT vs familiar OFT, *t* = 2,404, df = 106, p = 0,018) (Figure 8B).

On the other hand, intersession rearing was used to compare exploration in both OFT sessions. Rearing was significantly affected by the novelty factor in both male ($F_{[1, 104]}$ = 6,655, p = 0,011) and female offspring ($F_{[1, 106]}$ = 6,105, p = 0,015). However, treatment ($F_{[3, 106]}$ = 3,497, p = 0,018) and novelty x treatment interaction ($F_{[3, 106]}$ = 2,727, p = 0,048) influenced rearing only in female pups. Two-session comparison using *post hoc* Fisher´s LSD test revealed no differences in intersession rearing for both male (Figure 8C) and female pups (Figure 8D) from MeHg-VitA group. Differences in intersession rearing were encountered specifically for male pups from control (novel OFT vs familiar OFT, *t* = 2,272, df = 104, p = 0,025) and VitA group (novel OFT vs familiar OFT, *t* = 2,094, df = 104, p = 0,039) (Figure 8C). The same behavior was encountered in females rats from control (novel OFT vs familiar OFT, *t* = 2,954, df = 106, p = 0,004) and VitA groups (novel OFT vs familiar OFT, *t* = 2,390, df = 106, p = 0,019) (Figure 8D).

Finally, time spent in the periphery was used as a measure of intersession thigmotaxis. This parameter was significantly affected by the novelty factor in both males ($F_{[1, 104]}$ = 27,15, p <

0,0001) and females ($F_{[1, 106]}$ = 12,58, p = 0,0006). *Post hoc* Fisher´s LSD test revealed differences when comparing intersession parameters in male pups from MeHg-VitA groups (novel OFT vs familiar OFT, *t* = 2,518, df = 104, p = 0,013) (Figure 8E). However, no differences in intersession thigmotaxis were encountered for female rats from MeHg-VitA group (Figure 8F). Contrarily, control (novel OFT vs familiar OFT, *t* = 2,282, df = 106, p = 0,025) and VitA treated females (novel OFT vs familiar OFT, *t* = 2,572, df = 106, p = 0,012) showed differences in intersession thigmotaxis (Figure 8F).

Together, our results show that male from MeHg-VitA group displayed a high degree of intersession habituation when compared to female rats from the same group. Additionally, male and female rats from control and VitA groups presented better habituation response, indicating that changes in other tested groups were possibly treatment-related to MeHg exposure.

3.7. Object recognition test

Since object exploration is essential for testing, we compare total exploration time for both male and female pups evaluated in ORT. One-way ANOVA showed no significant effect of treatments either for male (Figure 9B) or female pups (Figure 10B). Additionally, we confirmed our results using multivariate analysis. Two-way ANOVA with exploration times of each explored object (A1, A2, B1, B2) as the within-subject factor and treatment as the between-subject factor revealed a significant effect of exploration for both males ($F_{[3, 156]}$ = 7,123, p = 0,0002) and females ($F_{[3, 148]}$ = 13,73, p < 0,0001), but no for treatment factor. Exploration patterns were analyzed for males and females separately, for both stationary and displaced objects used during the test phase. Male control pups (n = 13) presented differential exploration, spending more time exploring an old stationary object (A1) than the recent stationary object (B1) (Figure 9C, A1 black vs B1 light blue, p ≤ 0,0001, Tukey´s multiple comparison test) and the recent displaced object (B2) (Figure 9C, A1 black vs B2 magenta, p ≤ 0,0001, Tukey´s multiple comparison test). For VitA (n = 10) and MeHg-VitA (n = 10) male pups, preferential exploration was focused in the old displaced object (A2) rather than the recent stationary object (B2) (Figure 9C; VitA, A2 purple vs B1 light blue, p ≤ 0,05, Tukey´s multiple comparison test; MeHg-VitA, A2 purple vs B1 light blue, p ≤ 0,05, Tukey´s multiple comparison test). Similar to male controls, MeHg-VitA male pups presented also preference for the old stationary object (A1) rather than the recent stationary object (B1) (Figure 9C, A1 black vs B1 light blue, p ≤ 0,05, Tukey´s multiple comparison test).

On the other hand, female control pups (n = 8) showed increased exploration time towards old stationary object (A1) against a recent displaced object (B2) (Figure 10C, A1 black vs B2

magenta, $p \leq 0,05$, Tukey´s multiple comparison test). The same exploration pattern was encountered in VitA treated females (n = 10) (Figure 10C, A1 black vs B2 magenta, $p \leq 0,05$, Tukey´s multiple comparison test), and preference was significantly increased towards the old stationary object (A1) in MeHg-VitA female rats (n = 11) (Figure 10C, A1 black vs B2 magenta, $p \leq 0,0001$, Tukey´s multiple comparison test).

Overall, our results indicate that male and female rats from control, VitA and MeHg-VitA groups preferred to explore mostly the old stationary object (A1) and the old displaced object (A2) rather than both recent objects (B1 and B2). However, MeHg treated animals showed no discrimination either for stationary or displaced objects for both male (n = 10) and female (n = 12) pups (Figures 9C and 10C).

Analysis of "when" discrimination index was performed for assessment of temporal component of testing for male (Figure 9D) and female pups (Figure 10D). In terms of performance, one sample *t* test revealed that all pups presented better-than-chance performance, with exception of male and female pups from VitA and MeHg treatments respectively. In terms of "where" discrimination index, results are displayed for male and female pups in Figures 9E and 10E respectively. Offspring from MeHg-VitA group presented a mean ratio greater than zero, indicating better performance in spatial component of testing among groups, for both male ($t = 2,956$, df = 9, $p = 0,016$) and female rats ($t = 2,396$, df = 10, $p = 0,038$).

In order to evaluate the effect of treatments in both components of the test, "when" and "where" discrimination indexes from experimental groups were compared to its respective controls. One-way ANOVA with *post hoc* Fisher´s LSD test showed that temporal component of testing was significantly affected by MeHg treatment in male pups ($t = 2,192$, df = 39, $p = 0,034$), whereas MeHg-VitA co-administration in male pups significantly improved performance in the spatial component of testing ($t = 2,088$, df = 39, $p = 0,043$). In contrast, female pups showed no significant differences in "when" and "where" discrimination indexes among treatments.

Next, we wanted to evaluate object preference in order to assess whether chance exploration was presented during test phase. We used the discrimination index for both stationary (DI A1) and displaced (DI B2) objects and analyzed with one sample *t* test. Exploration preferences were accounted for control male ($t = 3,854$, df = 12, $p = 0,002$) and female rats ($t = 5,010$, df = 7, $p = 0,002$) which preferred to explore mostly the old stationary object (A1), similar to VitA ($t = 5,860$, df = 9, $p = 0,0002$) and MeHg-VitA treated female rats ($t = 2,669$, df = 10, $p = 0,024$) (Figures 9F and 10F respectively). For the recent displaced object (B2), no differential exploration was encountered among tested animals (Figures 9G and 10G). Corroborating our previous results, the preference accounted for stationary object A1 was significantly treatment-dependent for male pups

(Figure 9F), but not for female rats (Figure 10F), as revealed by one-way ANOVA with *post hoc* Fisher´s LSD test ($F_{[3, 39]}$ = 5,678, p = 0,003). Additionally, the non-preferential exploration of the recent displayed object was unrelated to treatments, as revealed when comparing DI B2 for male and female rats, therefore confirming our previous results (Figures 9G and 10G respectively).

Finally, to discard biased exploration in tested animals due to certain object features or treatment effects, we compare exploration times during both sample phases of testing. No significant effects of treatments were encountered for total object exploration in either sample phases of testing, or differences in exploration among objects A or B (data not shown). Taken together, our results confirms that differential exploration in tested animals towards the old stationary object (A1) rather than the recent displaced object (B2) was driven by specific temporal component of memory.

3.8. Mercury determination in cortex samples

Total Hg contents from cortex samples isolated from both dams and their offspring were expressed as mean ± SEM. MeHg treated dams showed a mean total Hg content of 2,31 ± 0,19 µg.g of wet tissue^{-1}, whereas dams from the MeHg-VitA group presented a total of 2,55 ± 0,19 µg.g of wet tissue^{-1}. Unpaired *t* student´s test showed no differences between Hg contents in both MeHg and MeHg-VitA treated dams (*t* = 0,703, df = 12, p = 0,496). Hg contents from control and VitA treated dams were below limit of detection (LOD) of the analysis (< 0,05 µg.g^{-1}). For the offspring, all cortex samples showed Hg contents below LOD.

3.9. Redox profile in isolated CNS structures

3.9.1. Maternal hippocampus

Results from redox profile of hippocampus (HC) samples isolated from treated dams are displayed in Table 1. In general, no changes on lipoperoxidation or total thiol content were evidenced when treatment groups were compared with control group. CAT activity decreased in MeHg and VitA treated dams ($F_{[3, 33]}$ = 3,152, p = 0,038).

Additionally, there was a decrease in GPx activity in MeHg and MeHg-VitA groups ($F_{[3, 23]}$ = 3,564, p = 0,030). No changes in SOD or GST activity were encountered through treatments. SOD to GPx plus CAT ratio (SOD/(GPx + CAT)) showed no differences among experimental groups.

3.9.2. Offspring hippocampus

Results from redox profile of HC samples isolated from treated male and female pups are summarized in Table 2. Lipoperoxidation was decreased in male pups from MeHg-VitA group, whereas in MeHg treated females increased ($F_{[3,\ 25]}$ = 3,285, p = 0,037) when compared to its respective controls. Total thiol content decreased in male pups from the MeHg-VitA group ($F_{[3,\ 47]}$ = 3,816, p = 0,016).

Moreover, GPx activity decreased only in MeHg treated male pups ($F_{[3,\ 47]}$ = 3,439, p = 0,034). No changes on CAT, SOD and GST activities were encountered in any of experimental groups when compared to control group. Finally, a significant increase in the SOD/(GPx + CAT) ratio was encountered for MeHg male rats (*K* = 8,132, p = 0,043).

3.9.3. Maternal and offspring prefrontal cortex

Results from redox profile of prefrontal cortex (PFC) for both dams and their offspring are presented in Table 3. Total thiol content presented a significant increase in dams from the MeHg-VitA group ($F_{[3,\ 56]}$ = 4,168, p = 0,009), whereas the same tendency was encountered in male pups from the VitA group ($F_{[3,\ 54]}$ = 4,719, p = 0,005) when compared to control groups. In MeHg-VitA female pups, despite statistical significance was found by one way ANOVA ($F_{[3,\ 54]}$ = 3,523, p = 0,021), *post hoc* Bonferroni test showed no differences in total thiol content among the groups when compared to control group. Lipoperoxidation showed no differences among experimental groups when compared to control group.

In terms of enzymatic activity, GST decreased only in MeHg-VitA male pups ($F_{[3,\ 56]}$ = 3,828, p = 0,015) and GPx activity decreased merely in MeHg treated male pups ($F_{[3,\ 20]}$ = 3,907, p = 0,024), when compared to control groups.

3.9.4. Maternal and offspring olfactory bulb

Results from redox profile of olfactory bulb (OB) for both dams and their offspring are presented in Table 3. MeHg-VitA female pups showed an increase in total thiol content ($F_{[3,\ 48]}$ = 4,556, p = 0,007), but no differences were encountered in either dams or their male offspring for any of the experimental groups when compared to control groups. In terms of lipoperoxidation, no differences were found between experimental and control groups. No differences in GST or GPx activities were encountered between experimental and control groups.

4. Discussion

In the present study, we used a gestational/lactational animal exposure model in order to evaluate neurodevelopmental endpoints and biochemical parameters in rats following MeHg and VitA administration, single or in combination. Since no evidence in literature has been reported regarding MeHg-VitA interaction, we considered the hypothesis that pregnant women is a sensitive group of population where MeHg indirect exposure through fish consumption confronts the nutritional benefits from VitA supplementation and therefore exposing the fetus to possible unknown additive/antagonic/synergistic effects from their combined exposure, either intentional or inadvertently. In terms of MeHg we use 0,5 mg/Kg/day, a highly used dose which has been previously reported in animal models and considered an environmentally relevant low MeHg dose [69-73]. On the other hand, we used 7500 μg RAE/Kg/day, a safe/therapeutically used dose in VitA supplementation [74-77].

We first investigated the quality of the dam-offspring interaction through the evaluation of maternal care behavior in the dams following exposure to both chemicals. An increased maternal behavior can influence offspring behavior through epigenomic regulation and hypothalamic-pituitary-adrenal (HPA) response modulation to stress [78]. In fact, increased nursing and pup retrieval behaviors suggests a possible more "overprotective" mothering style, driven by a maternal stress response [79]. Our results showed no differences between dams from experimental groups when compared to control dams (Figure 2A and 2B). We can therefore hypothesize that any response in the offspring was not related to increased maternal behavior or an affected stress response.

We next decided to focus on neurodevelopmental behavioral responses in the offspring prior to weaning. Our results showed that both MeHg and MeHg-VitA treatment impaired olfactory preference in neonatal rats (PND8), with no affectations in their locomotor activity, indicating that evidenced nest-seeking behavior is completely olfactory-guided and any preference response towards olfactory cues in the test may be from the pup´s own degree of associative learning (pups have their eyes closed on PND8). On the other hand, MeHg infant rats showed a marked delay in eyelid opening on PNDs 14 and 15 (Figure 4). However, when comparing MeHg-VitA pups, surprisingly we observed a normal behavior, similar to control pups (Figure 4). This result showed that MeHg-VitA co-administration produced no additional effects in eye opening behavior. Disruption of olfactory learning in the co-exposure group may be attributed solely to MeHg, although there is no sufficient evidence regarding direct MeHg-induced olfactory disruption in

neonatal rats, increasing literature supports that MeHg affects cell proliferation during embryonic neurogenesis, affecting several areas of the CNS [80, 81]. In fact, MeHg affects transduction mediated by tyrosine kinase receptors (*trk´s*) *in vivo*, an important process in neuronal differentiation during early stages of development, which may be related to possible affectations in brain areas with high *trk´s* immunoreactivity, like the olfactory bulb [82]. Additionally, previous work supports that during early postnatal stages, MeHg disrupts mitochondrial-mediated apoptosis through caspase-3 in hippocampus, which is responsible for learning and memory processes. Impaired neurogenesis in the olfactory bulb and increased apoptosis in the hippocampal region could explain the early onset of disrupted neurobehavioral response in MeHg-VitA neonatal pups. In contrast, the unaffected eye opening behavior showed in MeHg-VitA infant rats possibly indicates no affectations in brain development at later postnatal periods or compensatory mechanisms triggered by VitA co-administration, since delayed eyelid opening was evidenced solely in MeHg treated pups, indicating a delay or alteration in the development of the brain [83]. Eye opening is both a reflex and automatic response from pups, and involves the GABAergic and glutamatergic neuronal circuits. Any affectation evidenced in eyelid opening may reflect an alteration of at least one of these synaptic systems [84]. Extensive evidence supports that GABAergic synaptic transmission is affected during low-level prenatal and postnatal exposure to MeHg, with long-lasting effects in offspring [85-88]. However, retinoic acid (RA), an active metabolite of retinyl palmitate, regulates gene expression through postnatal and neuronal activity-dependent phase of olfactory sensory map formation [89] and sub ventricular zone-olfactory bulb neurogenesis [90]. RA also regulates hippocampal neurogenesis during early stages of development [91] and promotes GABAergic synaptic plasticity [92-94], which controls the automatic response of eye opening [84]. Collectively, from our analysis of olfactory discrimination and eye opening behaviors we can hypothesize that under our exposure protocol, MeHg dose administered single or in combination with VitA was able to induce a disrupted olfactory response in early stages of neonatal life, which were less evident in MeHg-VitA infant rats, as continuous VitA co-administration proceeds at later stages of neurodevelopment, possibly via RA regulation.

Results of OFT (Figure 5) in male and female post-weanling rats revealed no detectable locomotor deficits in offspring for any of the treatments. Our results are consistent with literature for both MeHg [95, 96] and VitA treatments [74] using our same exposure paradigm and doses. Our next evaluated factor in OFT was exploration, assessed as number of rearings and total center entries performed by the animal inside the OFT area. Generally, we encountered no differences in exploration among tested animals; however, a significant decrease in total rearings in MeHg female rats was encountered, in concordance with literature [96-98]. Remarkably, the index of thigmotaxis

data assessed in OFT revealed no differences for both male and female rats from the MeHg-VitA group, indicating no anxiety-like behavior among tested animals. Previous work showed no influence of low-dose MeHg on anxiety behavior in offspring [99]. However, regarding VitA exposure our data conflicts with previous work from our laboratory, which encountered anxiety-like behavior after sub-acute VitA supplementation [77] and after gestational/lactational exposure to safe VitA doses in offspring [74]. We can assume that observed differences in behavior are possibly related to the pharmacological formulation used, since previous work from our laboratory used water-soluble form of retinyl palmitate, whereas the used in this study was oil-soluble. In fact, oil-based formulations of retinyl esters possess slower absorption rate [100, 101], and lower toxicity than water-based formulations [102], possibly affecting the toxicokinetic of retinyl esters in evaluated offspring. In addition to thigmotaxis analysis, we focused on grooming behavior. An increased frequency of stereotypical grooming in rats may indicate anxiety as an arousal response due to immersion into a novel environment, and this immersion can be considerate in OFT as a mild-stressor [103]. Confirming our results, we demonstrated using multivariate analysis that grooming behavior showed no differences between MeHg-VitA treated offspring when compared to controls. Conjunctively, our results may suggest that MeHg-VitA co-administration produced no effects on the emotional response of the offspring.

In terms of intersession habituation, animals exposed to MeHg and MeHg-VitA co-administration showed no difference in intersession OFT parameters, with the sole exception of MeHg-VitA male pups, which showed a reduced total distance traveled (Figure 8A) and decreased thigmotaxis (Figure 8E), between both OFT sessions, which is the classic behavioral phenotype of habituated animals [104]. Perinatal low-dose MeHg exposure impairs habituation response and learning/memory [105]. However, the locomotor activity in MeHg-VitA male pups was not affected. In fact, surprisingly showed a similar decrease pattern as controls during the first 3 min of novel OFT testing (Figure 6C) indicating a better reaction towards novelty. Locomotor activity is an important parameter in OFT habituation, since allows differentiating animals according to their information-processing ability, regarding how rapidly the animals adjust their behavior when evaluated inside an open field [106]. Additionally, the complex phenotype of habituation memory seems to be related to cholinergic stimulation of the hippocampus after sensory stimulation [107, 108]. We may assume that MeHg-VitA male pups seems more sensitive towards information gathering rather than female pups from the same group and also MeHg treated pups. Furthermore, VitA co-administration would affect this response. Based in our results, we can therefore hypothesize that a possible component of hippocampal memory or synaptic circuitry was affected during MeHg and MeHg-VitA co-administration, with the sole exception of male pups from the co-

exposure group which showed a better overall habituation response compared with female pups from the same group. However, analysis of the first 3 min of familiar OFT (Figure 6C) revealed a new habituation response in MeHg-VitA male pups when compared to habituated controls, which may indicate the existence of possible disturbances in long-term memory.

Our results of the assessment of episodic-like memory in offspring, showed that MeHg-VitA post-weanling pups presented no alterations in both temporal and spatial components of memory showing an overall better performance than other experimental groups (Figures 9D and 10D – "When" Index; Figures 9E and 10E – "Where" Index). Evidence supports that neuronal inputs from cortical layers transport information to the hippocampus, allowing contextual processing, and therefore episodic-like memory depends on hippocampal ability to bind this information into a spatiotemporal representation [59, 109-111]. Recent evidence supports that low-dose MeHg exposure can be associated with a decrease in neural stem cell proliferation in the hippocampal dentate gyrus, which progresses at weaning, affecting spatial learning performance in adolescent rats [112]. In fact, exposure during PND7, a period of hippocampal-vulnerability to MeHg, is responsible for irreversibly affecting late onset neurogenesis in older rats [113]. We can therefore hypothesize that, VitA co-administration until weaning, under our exposure protocol, can counteract the deleterious effects evidenced in both male and female rats exposed to MeHg, which showed low performance in spatial and temporal components of testing. Our results seems to corroborate previous findings during eye opening assessment, corroborating the protective effect of VitA co-administration at posterior developmental stages.

Neurochemical basis of behavioral alterations can be related to disruption in neurotransmitter systems, which in turn may be mediated by several molecular mechanisms, such as oxidative stress. Evidence in literature supports that MeHg behavioral disturbances and neurotoxicity can be attributed to a disruption in glutathione/oxidized glutathione ratio (GSH/GSSG) in neural cells, alongside with GPx activity inhibition [114-116]. Regarding VitA, disruption in locomotor activity and pro-oxidant effects in HC isolated from dams and their offspring following perinatal supplementation of retinyl palmitate were demonstrated in our laboratory [74].

In order to further understand our results on memory acquisition, we analyzed HC redox status for both dams and their offspring. In MeHg-VitA exposed dams a decrease in the hippocampal GPx activity was evidenced, which is particular during MeHg exposure [115, 117]. Curiously, despite MeHg and VitA administration alone impaired CAT activity as evidenced in literature [118, 119], no effects were encountered in hippocampal CAT activity for MeHg-VitA

dams. We can hypothesize that GPx inhibition in the co-exposure group may lead to free peroxide hydrogen (H_2O_2) increase, indicating a potential hippocampal pro-oxidative state, affecting signaling processes in neural cells due to oxidative stress. This deleterious process inhibits neuron formation, and alters the functionality of dendritic network in the HC [120, 121].

In terms of the hippocampal redox status in offspring, a decrease in TBARS content was found in MeHg-VitA male pups, suggesting that the presence of VitA co-administration potentizes the decrease in lipid peroxidation in HC, a type of oxidative damage extensively related with MeHg [122-125]. Although the decrease in total thiol content in the HC of MeHg-VitA male pups was significant, possibly indicating small-scale free radical generation in the tissue, our results showed no other alterations in enzyme plasticity. Curiously, these pups showed an improved learning response than other groups when we evaluated their performance in ORT. In addition, these pups displayed a better habituation learning response in terms of locomotion inside the OFT area. These results conjunctively may suggest a protective role of VitA co-administration in the HC, possibly sex-dependent. We may hypothesize that the antioxidant properties of VitA could possibly counteract *in vivo* the oxidative stress induced by MeHg. Extensive evidence in the past shows the protective effect of antioxidants like vitamin E against MeHg toxicity [126, 127]. However, a recent work demonstrated the protective effect of the carotenoid-rich extract of the Amazonian fruit *Mauritia flexuosa L.* against cognitive deficits and increased lipid peroxidation in the HC produced by MeHg [128]. Finally, despite OB showed no alterations in redox balance for both dams and their offspring, we can only highlight the sole exception of MeHg-VitA male pups, which showed a decrease in GST activity in the PFC. Modification in GST activity may be related to a redox disruption in the tissue, therefore inducing a signaling cascade and dysregulation of kinase pathways [129, 130]. We may hypothesize that PFC impairment in male pups possibly affected working memory, since PFC is involved in long-term storage memory [131] and impaired signaling process in PFC is related to dysfunction in working memory [132, 133]. Curiously, MeHg-VitA male pups presented a new habituation response during OFT re-exposure 24 hours later which, as described earlier, may indicate possible disturbances in long-term memory (Figure 6C). Further studies are needed in order to corroborate our hypothesis.

In summary, our results showed that MeHg-VitA co-administration in pregnant and lactating rats have no effects in terms of emotional response, but showed subtle alterations in the pro-oxidant response of the hippocampus. Additionally, MeHg-VitA co-exposure affected early associative learning in neonatal offspring, with no alterations in neurodevelopment in later infant stages of development. Periadolescent male and female rats showed no locomotor, exploratory or

emotional alterations. However, despite the habituation was altered in a sex-dependent manner, no overall memory disturbances were encountered. Finally, MeHg-VitA co-administration reduced lipoperoxidation in male offspring HC, possible attributed to the antioxidant properties of VitA *in vivo*, and decrease GST activity in PFC. Our hypothesis considers that VitA co-administration, under our exposure protocol, can counteract the deleterious neurodevelopmental effects solely attributed to low-dose MeHg in a tissue-specific mechanism, possibly via promoting active RA signaling. However, the exact protection mechanism remains to be elucidated in future studies.

5. Conclusions

Based on our data, we can suggest that low-dose MeHg in co-administration with a supplementation dose of VitA can induce a subtle, yet pro-oxidant state in maternal hippocampus. In offspring the antioxidant properties of this retinoid prevented oxidative stress and neurodevelopmental affectations produced by MeHg, preventing late-onset memory disruptions. However, co-exposure affects learning in a sex-dependent manner. Further work is needed to confirm our findings and elucidate the molecular mechanisms of MeHg-VitA modulation. Epidemiological implications of our results remains to be determined in future studies. To our knowledge, our results represents the first insights of the possible protective effects of VitA against MeHg toxicity, and further pre-clinical assays are needed in order to demonstrate the potential therapeutical use of VitA in populations directly or indirectly exposed to MeHg.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS), Brazil.

REFERENCES

1. Sheehan, M.C., et al., *Global methylmercury exposure from seafood consumption and risk of developmental neurotoxicity: a systematic review.* Bulletin of the World Health Organization, 2014. **92**(4): p. 254-269F.
2. Hong, Y.-S., Y.-M. Kim, and K.-E. Lee, *Methylmercury Exposure and Health Effects.* Journal of Preventive Medicine and Public Health, 2012. **45**(6): p. 353-363.

3. WHO, *Trace Elements in Human Nutrition and Health*. 1996, Geneva: World Health Organization.
4. Crespo-Lopez, M.E., et al., *[Mercury and neurotoxicity]*. Rev Neurol, 2005. **40**(7): p. 441-7.
5. Berzas Nevado, J.J., et al., *Mercury in the Tapajos River basin, Brazilian Amazon: a review.* Environ Int, 2010. **36**(6): p. 593-608.
6. Li, P., X. Feng, and G. Qiu, *Methylmercury Exposure and Health Effects from Rice and Fish Consumption: A Review.* International Journal of Environmental Research and Public Health, 2010. **7**(6): p. 2666-2691.
7. Black, P., et al., *Modulation of the effects of methylmercury on rat neurodevelopment by co-exposure with Labrador Tea (Rhododendron tomentosum ssp. subarcticum).* Food and Chemical Toxicology, 2011. **49**(9): p. 2336-2342.
8. CDC, *Fourth National Report on Human Exposure To Environmental Chemicals. Center for Disease Control and Prevention. Prevention. U.S. Department of Health and Human Services, Atlanta, USA.* 2009.
9. Zeliger, H.I., *Exposure to lipophilic chemicals as a cause of neurological impairments, neurodevelopmental disorders and neurodegenerative diseases.* Interdiscip Toxicol, 2013. **6**(3): p. 103-10.
10. Zeliger, H.I., *Lipophilic chemical exposure as a cause of cardiovascular disease.* Interdisciplinary Toxicology, 2013. **6**(2): p. 55-62.
11. Zeliger, H.I., *Lipophilic chemical exposure as a cause of type 2 diabetes (T2D).* Rev Environ Health, 2013. **28**(1): p. 9-20.
12. Kienzler, A., et al., *Regulatory assessment of chemical mixtures: Requirements, current approaches and future perspectives.* Regulatory Toxicology and Pharmacology, 2016. **80**: p. 321-334.
13. Evans, R.M., et al., *Should the scope of human mixture risk assessment span legislative/regulatory silos for chemicals?* Science of The Total Environment, 2016. **543**: p. 757-764.
14. Kortenkamp, A., *Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment.* Current Opinion in Pharmacology, 2014. **19**: p. 105-111.
15. Napoli, J.L., *Physiological insights into all-trans-retinoic acid biosynthesis.* Biochim Biophys Acta, 2012. **1821**(1): p. 152-67.
16. Oliveira, M.R., *The neurotoxic effects of vitamin A and retinoids.* An Acad Bras Cienc, 2015. **87**(2 Suppl): p. 1361-73.
17. Ross, C.A., ed. *Vitamin A.* 2 ed. Encyclopedia of Dietary Supplements 2nd Edition, ed. P.M. Coates, et al. 2010, Informa Healthcare: New York. 778-91.
18. FAO, *The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all*. 2016, Rome: Food and Agriculture Organization of the United Nations. 200.
19. Penniston, K.L. and S.A. Tanumihardjo, *The acute and chronic toxic effects of vitamin A.* Am J Clin Nutr, 2006. **83**(2): p. 191-201.
20. Bailey, R.L., et al., *Dietary supplement use in the United States, 2003-2006.* J Nutr, 2011. **141**(2): p. 261-6.
21. Razzaghi, H. and S.C. Tinker, *Seafood consumption among pregnant and non-pregnant women of childbearing age in the United States, NHANES 1999–2006.* Food & Nutrition Research, 2014. **58**: p. 10.3402/fnr.v58.23287.
22. (WHO), W.H.O., *WHO Guidelines Approved by the Guidelines Review Committee*, in *Guideline: Use of Multiple Micronutrient Powders for Point-of-Use Fortification of Foods Consumed by Pregnant Women*. 2016, World Health Organization Copyright (c) World Health Organization 2016.: Geneva.

23. Lane, M.A. and S.J. Bailey, *Role of retinoid signalling in the adult brain.* Prog Neurobiol, 2005. **75**(4): p. 275-93.
24. McCaffery, P., J. Zhang, and J.E. Crandall, *Retinoic acid signaling and function in the adult hippocampus.* J Neurobiol, 2006. **66**(7): p. 780-91.
25. McCaffery, P., et al., *Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen.* Eur J Neurosci, 2003. **18**(3): p. 457-72.
26. Cusack, L.K., et al., *Regional and temporal trends in blood mercury concentrations and fish consumption in women of child bearing Age in the united states using NHANES data from 1999–2010.* Environmental Health, 2017. **16**: p. 10.
27. IOM, *Front Matter*, in *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. 2001, The National Academies Press: Washington.
28. (WHO), W.H.O., *Global Prevalence of Vitamin A deficiency in Populations at Risk 1995-2005. WHO Global Database on Vitamin A Deficiency. Geneva. World Health Organization.* 2009.
29. Grandjean, P., et al., *Impact of maternal seafood diet on fetal exposure to mercury, selenium, and lead.* Arch Environ Health, 1992. **47**(3): p. 185-95.
30. Grandjean, P., et al., *Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury.* Neurotoxicol Teratol, 1997. **19**(6): p. 417-28.
31. Fujimura, M., J. Cheng, and W. Zhao, *Perinatal exposure to low-dose methylmercury induces dysfunction of motor coordination with decreases in synaptophysin expression in the cerebellar granule cells of rats.* Brain Res, 2012. **1464**: p. 1-7.
32. Karagas, M.R., et al., *Evidence on the Human Health Effects of Low-Level Methylmercury Exposure.* Environmental Health Perspectives, 2012. **120**(6): p. 799-806.
33. Al-Saleh, I., et al., *Mercury (Hg) exposure and its effects on Saudi breastfed infant's neurodevelopment.* Int J Hyg Environ Health, 2016. **219**(1): p. 129-41.
34. Debes, F., et al., *Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years.* Neurotoxicology and teratology, 2006. **28**(3): p. 363-375.
35. Hsi, H.C., et al., *Methylmercury Concentration in Fish and Risk-Benefit Assessment of Fish Intake among Pregnant versus Infertile Women in Taiwan.* PLoS One, 2016. **11**(5): p. e0155704.
36. Albores-Garcia, D., et al., *Early Developmental Low-Dose Methylmercury Exposure Alters Learning and Memory in Periadolescent but Not Young Adult Rats.* 2016. **2016**: p. 6532108.
37. Schnorr, C.E., et al., *The effects of vitamin A supplementation to rats during gestation and lactation upon redox parameters: increased oxidative stress and redox modulation in mothers and their offspring.* Food Chem Toxicol, 2011. **49**(10): p. 2645-54.
38. NIH, *Guide for the Care and Use of Laboratory Animals: Eighth Edition. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals*. 2011: National Academies Press.
39. Marcondes, F.K., F.J. Bianchi, and A.P. Tanno, *Determination of the estrous cycle phases of rats: some helpful considerations.* Braz J Biol, 2002. **62**(4a): p. 609-14.
40. Paris, J.J. and C.A. Frye, *Juvenile offspring of rats exposed to restraint stress in late gestation have impaired cognitive performance and dysregulated progestogen formation.* Stress (Amsterdam, Netherlands), 2011. **14**(1): p. 23-32.
41. Dridi, I., et al., *Dietary early-life exposure to contaminated eels does not impair spatial cognitive performances in adult offspring mice as assessed in the Y-maze and the Morris water maze.* Nutrition Research, 2014. **34**(12): p. 1075-1084.
42. Pires, G.N., et al., *Maternal behavior in basic science: translational research and clinical applicability.* Einstein (São Paulo), 2013. **11**: p. 256-260.

43. Champagne, F.A., et al., *Variations in maternal care in the rat as a mediating influence for the effects of environment on development.* Physiol Behav, 2003. **79**(3): p. 359-71.
44. Cromwell, H.C., et al., *Effects of polychlorinated biphenyls on maternal odor conditioning in rat pups.* Physiol Behav, 2007. **91**(5): p. 658-66.
45. Rocha, J.B., F.A. Soares, and C.F. De Mello, *Influence of the test situation on pup retrieval behavior of normal and undernourished lactating rats.* Braz J Med Biol Res, 2002. **35**(1): p. 91-7.
46. Gregory, E.H. and D.W. Pfaff, *Development of olfactory-guided behavior in infant rats.* Physiology & Behavior, 1971. **6**(5): p. 573-576.
47. Sullivan, R.M. and D.A. Wilson, *Molecular biology of early olfactory memory.* Learn Mem, 2003. **10**(1): p. 1-4.
48. Kirsten, T.B., et al., *Prenatal LPS exposure reduces olfactory perception in neonatal and adult rats.* Physiol Behav, 2011. **104**(3): p. 417-22.
49. Raineki, C., et al., *Neonatal handling and the maternal odor preference in rat pups: involvement of monoamines and cyclic AMP response element-binding protein pathway in the olfactory bulb.* Neuroscience, 2009. **159**(1): p. 31-8.
50. Polan, H.J. and M.A. Hofer, *Olfactory preference for mother over home nest shavings by newborn rats.* Developmental Psychobiology, 1998. **33**(1): p. 5-20.
51. Schneider, T. and R. Przewlocki, *Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism.* Neuropsychopharmacology, 2005. **30**(1): p. 80-9.
52. Tobach, E., *Experimental approaches to the study of emotional behavior.* The Sciences, 1968. **8**(3): p. 20-38.
53. Prut, L. and C. Belzung, *The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review.* Eur J Pharmacol, 2003. **463**(1-3): p. 3-33.
54. Sestakova, N., et al., *Determination of motor activity and anxiety-related behaviour in rodents: methodological aspects and role of nitric oxide.* Interdiscip Toxicol, 2013. **6**(3): p. 126-35.
55. Simon, P., R. Dupuis, and J. Costentin, *Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions.* Behav Brain Res, 1994. **61**(1): p. 59-64.
56. Bolivar, V.J., *Intrasession and Intersession Habituation in Mice: From Inbred Strain Variability to Linkage Analysis.* Neurobiology of learning and memory, 2009. **92**(2): p. 206-214.
57. Thiel, C.M., et al., *High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments.* Neuroscience, 1999. **93**(1): p. 243-251.
58. Tulving, E. and H.J. Markowitsch, *Episodic and declarative memory: role of the hippocampus.* Hippocampus, 1998. **8**(3): p. 198-204.
59. Davachi, L., *Item, context and relational episodic encoding in humans.* Current Opinion in Neurobiology, 2006. **16**(6): p. 693-700.
60. Inostroza, M., et al., *Specific impairment of "what-where-when" episodic-like memory in experimental models of temporal lobe epilepsy.* J Neurosci, 2013. **33**(45): p. 17749-62.
61. Lopez-Pigozzi, D., et al., *Altered Oscillatory Dynamics of CA1 Parvalbumin Basket Cells during Theta-Gamma Rhythmopathies of Temporal Lobe Epilepsy.* eNeuro, 2016. **3**(6).
62. Lowry, O.H., et al., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 265-75.
63. Draper, H.H. and M. Hadley, *Malondialdehyde determination as index of lipid peroxidation.* Methods Enzymol, 1990. **186**: p. 421-31.
64. Ellman, G.L., *Tissue sulfhydryl groups.* Archives of Biochemistry and Biophysics, 1959. **82**(1): p. 70-77.
65. Misra, H.P. and I. Fridovich, *The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase.* J Biol Chem, 1972. **247**(10): p. 3170-5.

66. Aebi, H., *Catalase in vitro.* Methods Enzymol, 1984. **105**: p. 121-6.
67. Habig, W.H. and W.B. Jakoby, *Assays for differentiation of glutathione S-transferases.* Methods Enzymol, 1981. **77**: p. 398-405.
68. Wendel, A., *Glutathione peroxidase.* Methods Enzymol, 1981. **77**: p. 325-33.
69. Gandhi, D. and D. K Rajput, *Effects of maternal exposure to methylmercury (MeHg) on F1 generation behavior in the rat*. Vol. 1. 2016. 6-16.
70. Gandhi, D.N., G.M. Panchal, and D.K. Dhull, *Influence of gestational exposure on the effects of prenatal exposure to methyl mercury on postnatal development in rats.* Cent Eur J Public Health, 2013. **21**(1): p. 30-5.
71. Gandhi, D.N., G.M. Panchal, and D.K. Dhull, *Neurobehavioral toxicity in progeny of rat mothers exposed to methylmercury during gestation.* Annali dell'Istituto Superiore di Sanità, 2014. **50**: p. 28-37.
72. Newland, M.C., et al., *Response inhibition is impaired by developmental methylmercury exposure: acquisition of low-rate lever-pressing.* Behav Brain Res, 2013. **253**: p. 196-205.
73. Paletz, E.M., M.C. Craig-Schmidt, and M.C. Newland, *Gestational exposure to methylmercury and n-3 fatty acids: effects on high- and low-rate operant behavior in adulthood.* Neurotoxicol Teratol, 2006. **28**(1): p. 59-73.
74. Schnorr, C.E., et al., *Vitamin A supplementation in rats under pregnancy and nursing induces behavioral changes and oxidative stress upon striatum and hippocampus of dams and their offspring.* Brain Res, 2011. **1369**: p. 60-73.
75. Behr, G.A., et al., *Increased cerebral oxidative damage and decreased antioxidant defenses in ovariectomized and sham-operated rats supplemented with vitamin A.* Cell Biol Toxicol, 2012. **28**(5): p. 317-30.
76. de Oliveira, M.R., et al., *Vitamin A supplementation induces a prooxidative state in the striatum and impairs locomotory and exploratory activity of adult rats.* Brain Res, 2007. **1169**: p. 112-9.
77. de Oliveira, M.R., et al., *Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: effects of sub acute vitamin A supplementation at therapeutic doses.* Neurotoxicology, 2007. **28**(6): p. 1191-9.
78. Weaver, I.C.G., et al., *Epigenetic programming by maternal behavior.* Nature Neuroscience, 2004. **7**: p. 847.
79. Kan, J.M. and R. Richardson, *Ghosts of mother's past: Previous maternal stress leads to altered maternal behavior following a subsequent pregnancy in rats.* Dev Psychobiol, 2018.
80. Ceccatelli, S. and M. Aschner, *Methylmercury and Neurotoxicity*. 2012: Springer New York.
81. Ceccatelli, S., et al., *Long-lasting neurotoxic effects of exposure to methylmercury during development.* J Intern Med, 2013. **273**(5): p. 490-7.
82. Barone, S., et al., *Gestational exposure to methylmercury alters the developmental pattern of trk-like immunoreactivity in the rat brain and results in cortical dysmorphology.* Developmental Brain Research, 1998. **109**(1): p. 13-31.
83. Govindaraj, S., A. Shanmuganathan, and R. Rajan, *Maternal psychological stress-induced developmental disability, neonatal mortality and stillbirth in the offspring of Wistar albino rats.* PLoS ONE, 2017. **12**(2): p. e0171089.
84. Heinen, K., et al., *GABAA receptor maturation in relation to eye opening in the rat visual cortex.* Neuroscience, 2004. **124**(1): p. 161-71.
85. Yuan, Y. and W.D. Atchison, *Methylmercury differentially affects GABA(A) receptor-mediated spontaneous IPSCs in Purkinje and granule cells of rat cerebellar slices.* The Journal of Physiology, 2003. **550**(Pt 1): p. 191-204.
86. Dasari, S. and Y. Yuan, *Low level postnatal methylmercury exposure in vivo alters developmental forms of short-term synaptic plasticity in the visual cortex of rat.* Toxicology and applied pharmacology, 2009. **240**(3): p. 412-422.

87. Yuan, Y. and W.D. Atchison, *Action of methylmercury on GABA(A) receptor-mediated inhibitory synaptic transmission is primarily responsible for its early stimulatory effects on hippocampal CA1 excitatory synaptic transmission.* J Pharmacol Exp Ther, 1997. **282**(1): p. 64-73.
88. Yuan, Y. and W.D. Atchison, *Comparative effects of methylmercury on parallel-fiber and climbing-fiber responses of rat cerebellar slices.* J Pharmacol Exp Ther, 1999. **288**(3): p. 1015-25.
89. Öztokatli, H., et al., *Retinoic acid receptor and CNGA2 channel signaling are part of a regulatory feedback loop controlling axonal convergence and survival of olfactory sensory neurons.* The FASEB Journal, 2012. **26**(2): p. 617-627.
90. Wang, T.-W., H. Zhang, and J.M. Parent, *Retinoic acid regulates postnatal neurogenesis in the murine subventricular zone-olfactory bulb pathway.* Development, 2005. **132**(12): p. 2721-2732.
91. Jacobs, S., et al., *Retinoic acid is required early during adult neurogenesis in the dentate gyrus.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(10): p. 3902-3907.
92. Sarti, F., et al., *Rapid suppression of inhibitory synaptic transmission by retinoic acid.* J Neurosci, 2013. **33**(28): p. 11440-50.
93. Chatzi, C., T. Brade, and G. Duester, *Retinoic acid functions as a key GABAergic differentiation signal in the basal ganglia.* PLoS Biol, 2011. **9**(4): p. e1000609.
94. Crandall, J.E., et al., *Retinoic acid influences neuronal migration from the ganglionic eminence to the cerebral cortex.* J Neurochem, 2011. **119**(4): p. 723-35.
95. Onishchenko, N., et al., *Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice.* Toxicol Sci, 2007. **97**(2): p. 428-37.
96. Albores-Garcia, D., et al., *Early Developmental Low-Dose Methylmercury Exposure Alters Learning and Memory in Periadolescent but Not Young Adult Rats.* Biomed Res Int, 2016. **2016**: p. 6532108.
97. Carratu, M.R., et al., *Acute exposure to methylmercury at two developmental windows: focus on neurobehavioral and neurochemical effects in rat offspring.* Neuroscience, 2006. **141**(3): p. 1619-29.
98. Baraldi, M., et al., *Cognitive deficits and changes in gene expression of NMDA receptors after prenatal methylmercury exposure.* Environmental Health Perspectives, 2002. **110**(Suppl 5): p. 855-858.
99. Vitalone, A., et al., *Neurobehavioral assessment of rats exposed to low doses of PCB126 and methyl mercury during development.* Environ Toxicol Pharmacol, 2008. **25**(1): p. 103-13.
100. Karpe, F., et al., *Quantification of postprandial triglyceride-rich lipoproteins in healthy men by retinyl ester labeling and simultaneous measurement of apolipoproteins B-48 and B-100.* Arterioscler Thromb Vasc Biol, 1995. **15**(2): p. 199-207.
101. Renuka, K.D., et al., *Use of water-miscible retinyl palmitate as markers of chylomicrons gives earlier peak response of plasma retinyl esters compared with oil-soluble retinyl palmitate.* British Journal of Nutrition, 2007. **86**(4): p. 427-432.
102. Myhre, A.M., et al., *Water-miscible, emulsified, and solid forms of retinol supplements are more toxic than oil-based preparations.* Am J Clin Nutr, 2003. **78**(6): p. 1152-9.
103. Moody, T.W., Z. Merali, and J.N. Crawley, *The Effects of Anxiolytics and Other Agents on Rat Grooming Behaviora.* Annals of the New York Academy of Sciences, 1988. **525**(1): p. 281-290.
104. Giovannini, M.G., et al., *Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats*. Neuroscience, 2001. **106**(1): p. 43-53.

105. Fischer, C., A. Fredriksson, and P. Eriksson, *Coexposure of neonatal mice to a flame retardant PBDE 99 (2,2 ',4,4 ',5-pentabromodiphenyl ether) and methyl mercury enhances developmental neurotoxic defects.* Toxicological Sciences, 2008. **101**(2): p. 275-285.
106. Brenes, J.C., M. Padilla, and J. Fornaguera, *A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats.* Behavioural Brain Research, 2009. **197**(1): p. 125-137.
107. Nilsson, O.G., et al., *Acetylcholine release in the rat hippocampus as studied by microdialysis is dependent on axonal impulse flow and increases during behavioural activation.* Neuroscience, 1990. **36**(2): p. 325-338.
108. Gray, J.A. and N. McNaughton, *Comparison between the behavioural effects of septal and hippocampal lesions: A review.* Neuroscience & Biobehavioral Reviews, 1983. **7**(2): p. 119-188.
109. Eichenbaum, H., A.P. Yonelinas, and C. Ranganath, *The medial temporal lobe and recognition memory.* Annu Rev Neurosci, 2007. **30**: p. 123-52.
110. MacDonald, C.J., et al., *Hippocampal "time cells" bridge the gap in memory for discontiguous events.* Neuron, 2011. **71**(4): p. 737-49.
111. Naya, Y. and W.A. Suzuki, *Integrating what and when across the primate medial temporal lobe.* Science, 2011. **333**(6043): p. 773-6.
112. Sokolowski, K., et al., *Neural stem cell apoptosis after low methylmercury (MeHg) exposures in postnatal hippocampus produce persistent cell loss and adolescent memory deficits.* Developmental neurobiology, 2013. **73**(12): p. 10.1002/dneu.22119.
113. Obiorah, M., et al., *Hippocampal developmental vulnerability to methylmercury extends into prepubescence.* Frontiers in Neuroscience, 2015. **9**: p. 150.
114. Farina, M., J.B.T. Rocha, and M. Aschner, *Mechanisms of methylmercury-induced neurotoxicity: Evidence from experimental studies.* Life Sciences, 2011. **89**(15–16): p. 555-563.
115. Franco, J.L., et al., *Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase.* Free Radic Biol Med, 2009. **47**(4): p. 449-57.
116. Henry, K.A., et al., *Oxidative stress in songbirds exposed to dietary methylmercury.* Ecotoxicology, 2015. **24**(3): p. 520-6.
117. Zemolin, A.P.P., et al., *Evidences for a role of glutathione peroxidase 4 (GPx4) in methylmercury induced neurotoxicity in vivo.* Toxicology, 2012. **302**(1): p. 60-67.
118. Abdel-Hamid, H.A., F.C. Fahmy, and I.A. Sharaf, *Influence of free radicals on cardiovascular risk due to occupational exposure to mercury.* J Egypt Public Health Assoc, 2001. **76**(1-2): p. 53-69.
119. Shimizu, N., K. Kobayashi, and K. Hayashi, *The reaction of superoxide radical with catalase. Mechanism of the inhibition of catalase by superoxide radical.* J Biol Chem, 1984. **259**(7): p. 4414-8.
120. Cui, Y., et al., *Deficits in Water Maze Performance and Oxidative Stress in the Hippocampus and Striatum Induced by Extremely Low Frequency Magnetic Field Exposure.* PLoS ONE, 2012. **7**(5): p. e32196.
121. Huang, T.T., D. Leu, and Y. Zou, *Oxidative stress and redox regulation on hippocampal-dependent cognitive functions.* Arch Biochem Biophys, 2015. **576**: p. 2-7.
122. Ercal, N., H. Gurer-Orhan, and N. Aykin-Burns, *Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage.* Curr Top Med Chem, 2001. **1**(6): p. 529-39.
123. Franco, J.L., et al., *Mercurial-induced hydrogen peroxide generation in mouse brain mitochondria: protective effects of quercetin.* Chem Res Toxicol, 2007. **20**(12): p. 1919-26.
124. Ali, S.F., C.P. LeBel, and S.C. Bondy, *Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity.* Neurotoxicology, 1992. **13**(3): p. 637-48.

125. Yee, S. and B.H. Choi, *Oxidative stress in neurotoxic effects of methylmercury poisoning.* Neurotoxicology, 1996. **17**(1): p. 17-26.
126. Chang, L.W., M. Gilbert, and J. Sprecher, *Modification of methylmercury neurotoxicity by vitamin E.* Environ Res, 1978. **17**(3): p. 356-66.
127. Welsh, S.O., *The protective effect of vitamin E and N,N'-diphenyl-p-phenylenediamine (DPPD) against methyl mercury toxicity in the rat.* J Nutr, 1979. **109**(10): p. 1673-81.
128. Leao, L.K.R., et al., *Mauritia flexuosa L. protects against deficits in memory acquisition and oxidative stress in rat hippocampus induced by methylmercury exposure.* Nutr Neurosci, 2017. **20**(5): p. 297-304.
129. Sahu, S.C. and G.C. Gray, *Pro-oxidant activity of flavonoids: effects on glutathione and glutathione S-transferase in isolated rat liver nuclei.* Cancer Lett, 1996. **104**(2): p. 193-6.
130. Townsend, D.M., K.D. Tew, and H. Tapiero, *The importance of glutathione in human disease.* Biomed Pharmacother, 2003. **57**(3-4): p. 145-55.
131. Blumenfeld, R.S. and C. Ranganath, *Prefrontal cortex and long-term memory encoding: an integrative review of findings from neuropsychology and neuroimaging.* Neuroscientist, 2007. **13**(3): p. 280-91.
132. Arnsten, A.F.T., *Stress signalling pathways that impair prefrontal cortex structure and function.* Nature reviews. Neuroscience, 2009. **10**(6): p. 410-422.
133. Bañuelos, C., et al., *Prefrontal Cortical GABAergic Dysfunction Contributes to Age-Related Working Memory Impairment.* The Journal of Neuroscience, 2014. **34**(10): p. 3457-3466.

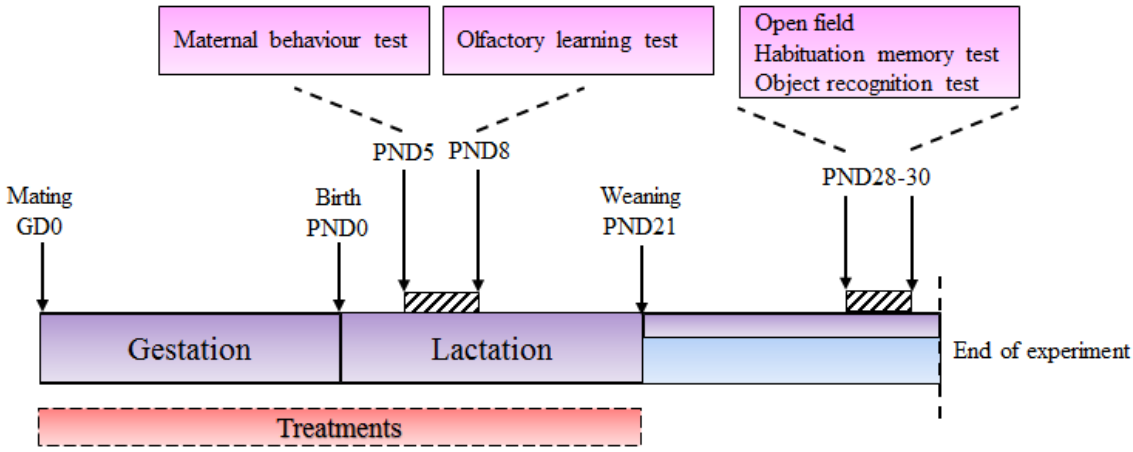

Figure 1. Behavioral testing

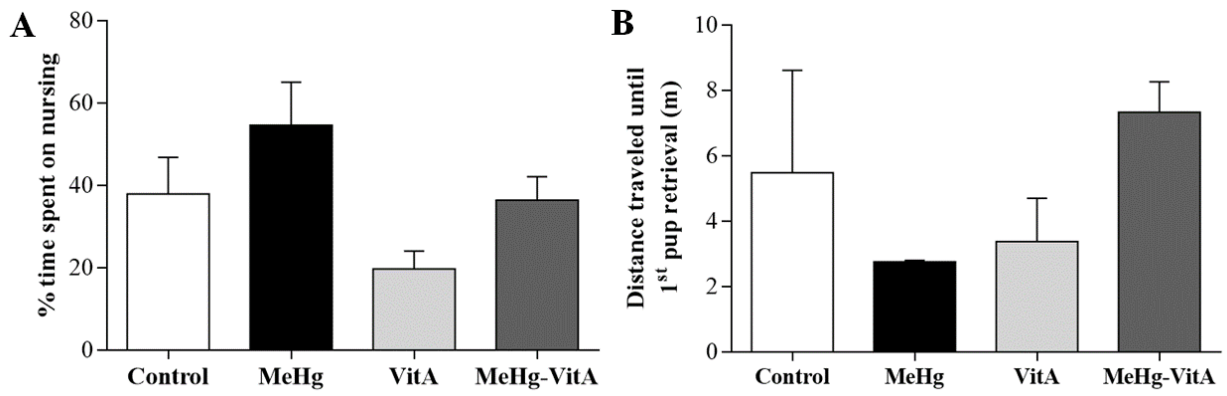

Figure 2. Maternal behavior analysis.
The quality of dam-offspring interaction was evaluated by nursing behavior frequency (A) and total distance traveled until 1st pup retrieval (B). All tests were performed at PND 5. Data are represented as mean ± SEM.

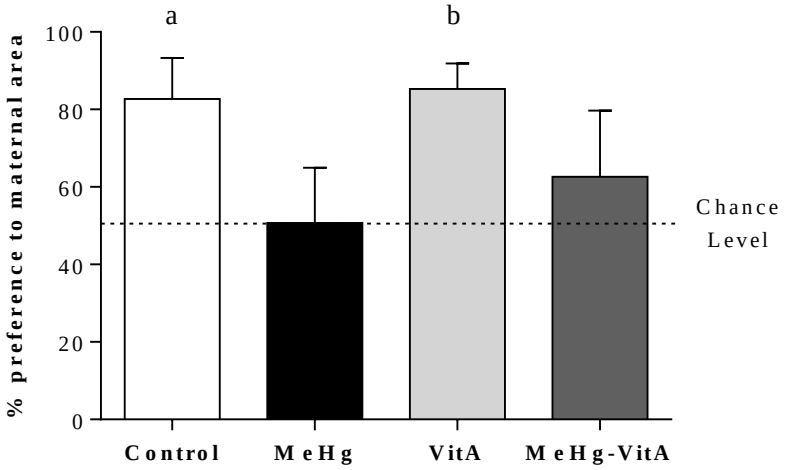

Figure 3. Olfactory Learning Test.
Pup maternal odor preference was evaluated at PND8 and is represented as % of time on maternal bedding material in randomly selected male pups from litters of the four experimental groups. Data are represented as mean ± SEM. (a) $p \leq 0{,}01$; (b) $p \leq 0{,}001$ statistically significant from reference value (50% preference to maternal area) with one sample *t* test.

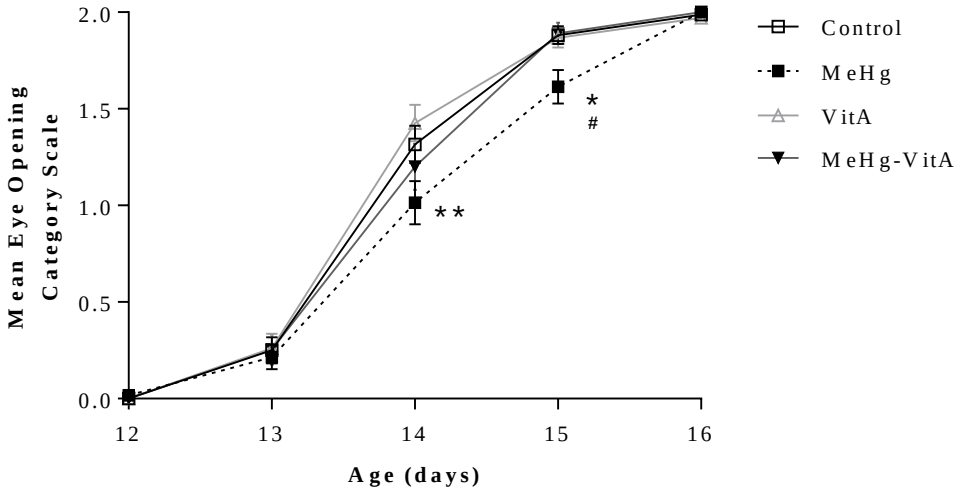

Figure 4. Eye opening day.
Observation of eyelid opening behavior was performed for all pups from the litter during PND 12, 13, 14 15 and 16. Data are represented as mean ± SEM. *$p \leq 0,05$ when different from control group; **$p \leq 0,01$ when different from control group; $^{\#}p \leq 0,05$ when different from MeHg-VitA group.

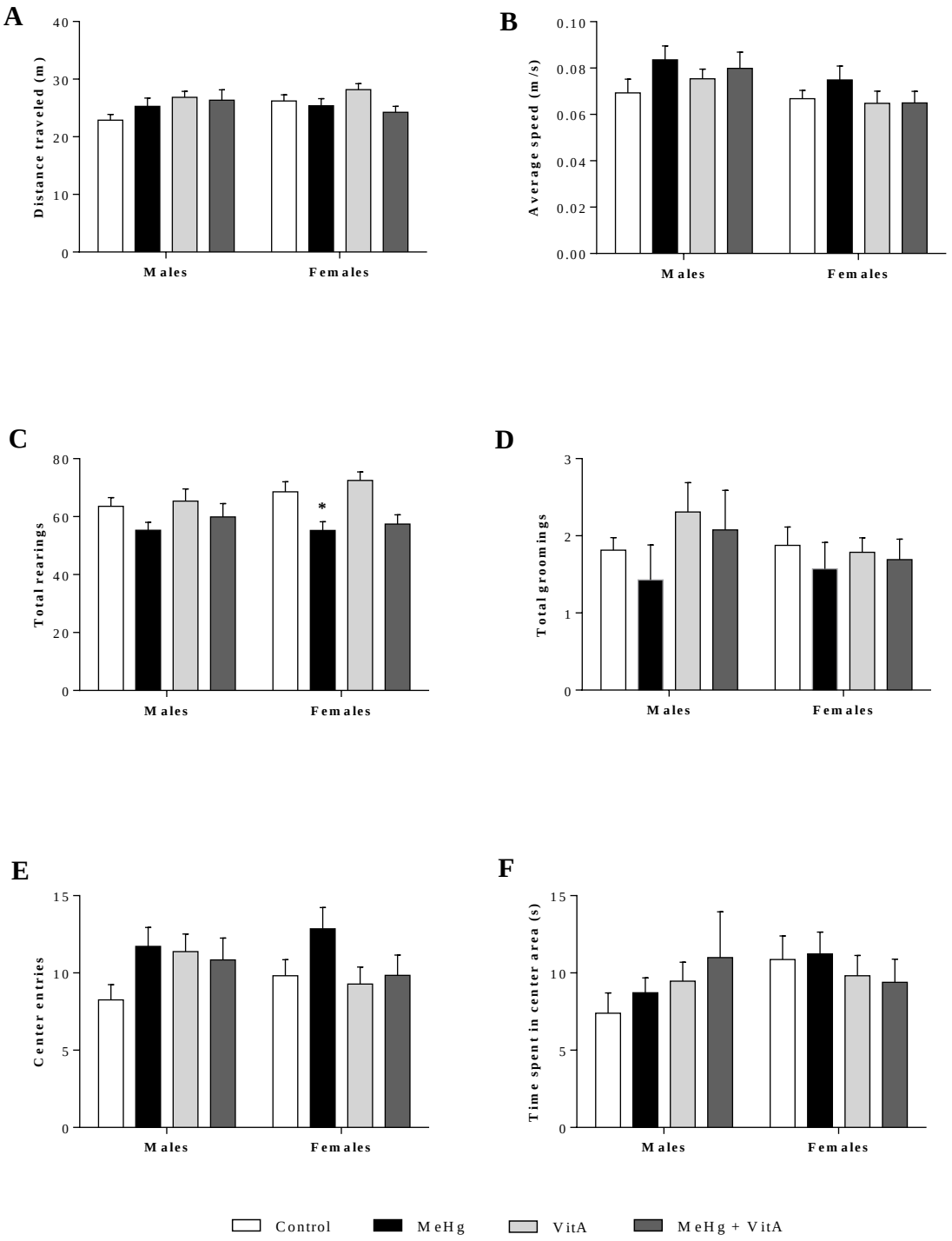

Figure 5. Open Field Test.
The total distance traveled (A), the average speed (B), the number of rearings (C), the number of groomings (D), the number of center entries (E), and the total time spent in center area (F) were analyzed in control and exposed pups for both male and female offspring. Open field testing was performed at PND 28 for a total 10 min exploration. Data are represented as mean ± SEM. *p ≤ 0,05 when different from control group

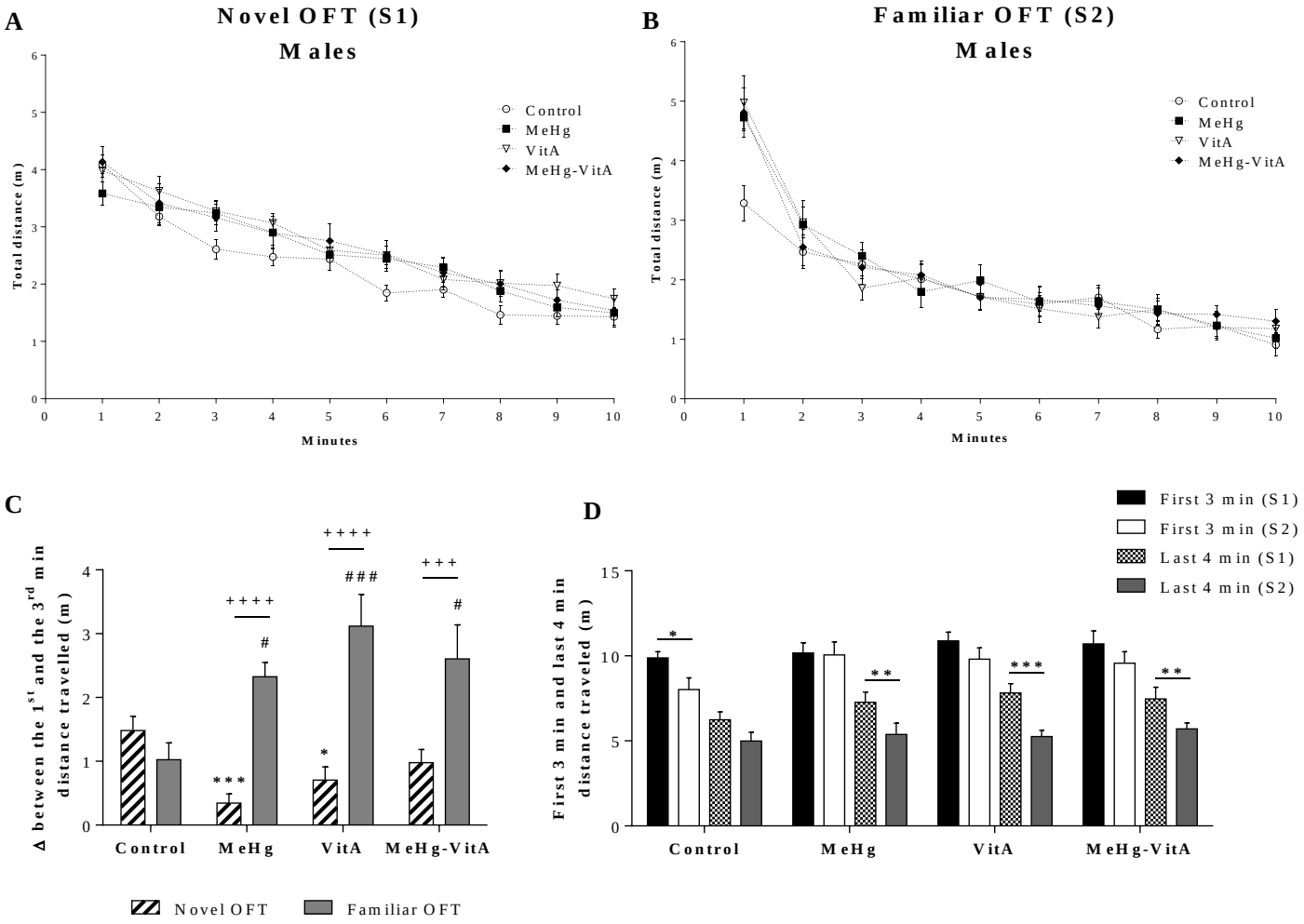

Figure 6. Effects of treatments on habituation to novelty and locomotor activity in the OFT for male pups.
Minute-by-minute analysis of locomotor activity of male pups during Session 1 OFT (A) and Session 2 OFT (B). Each point represent the mean ± SEM of each group. The change in the distance traveled between the 1st and 3rd minute of testing for both OFT sessions (C) and the distance traveled during the first 3 min and last 4 min of testing for both OFT sessions (D). Each column represents the mean ± SEM. Data were analyzed using a two-way ANOVA followed by Dunnett´s post-hoc tests. Session 1 OFT (Novel OFT – PND28); Session 2 OFT (Familiar OFT – PND29). Each OFT session was assessed for a total 10 min exploration. **Figure 6C** (* $p \leq 0,05$, *** $p \leq 0,001$ compared to the respective control at Novel OFT (first exposure); # $p \leq 0,05$, ### $p \leq 0,001$ compared to the respective control at Familiar OFT (second exposure); +++ $p \leq 0,001$, ++++ $p \leq 0,0001$ when comparing both OFT sessions); **Figure 6D** (* $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$ when comparing both OFT sessions).

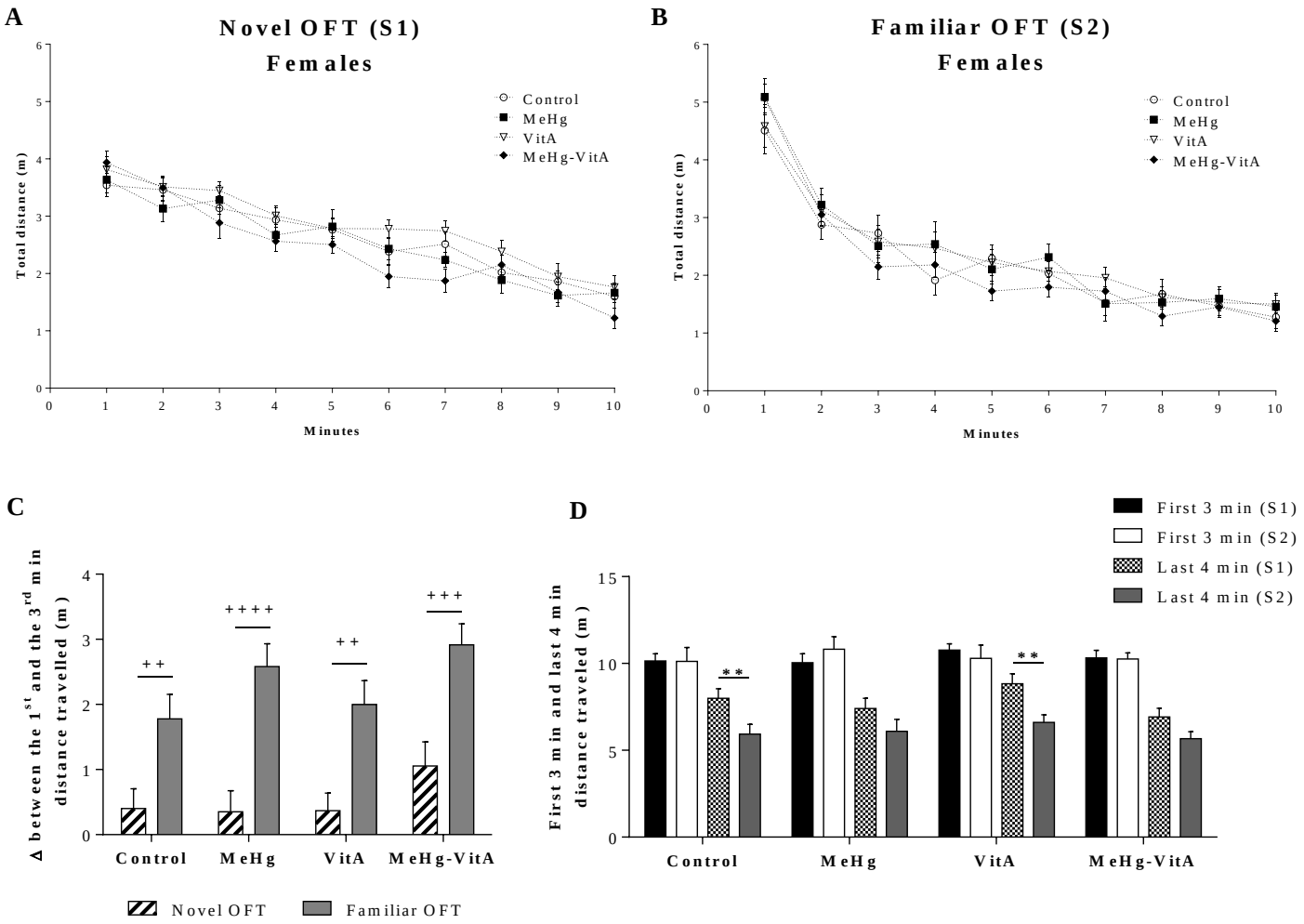

Figure 7. Effects of treatments on habituation to novelty and locomotor activity in the OFT for female pups.
Minute-by-minute analysis of locomotor activity of female pups during Session 1 OFT (A) and Session 2 OFT (B). Each point represent the mean ± SEM of each group. The change in the distance traveled between the 1st and 3rd minute of testing for both OFT sessions (C) and the distance traveled during the first 3 min and last 4 min of testing for both OFT sessions (D). Each column represents the mean ± SEM. Data were analyzed using a two-way ANOVA followed by Dunnett´s post-hoc tests. Session 1 OFT (Novel OFT – PND28); Session 2 OFT (Familiar OFT – PND29). Each OFT session was assessed for a total 10 min exploration. **Figure 7C** (++ $p \leq 0,01$, +++ $p \leq 0,001$, ++++ $p \leq 0,0001$ when comparing both OFT sessions); **Figure 7D** (** $p \leq 0,01$ when comparing both OFT sessions).

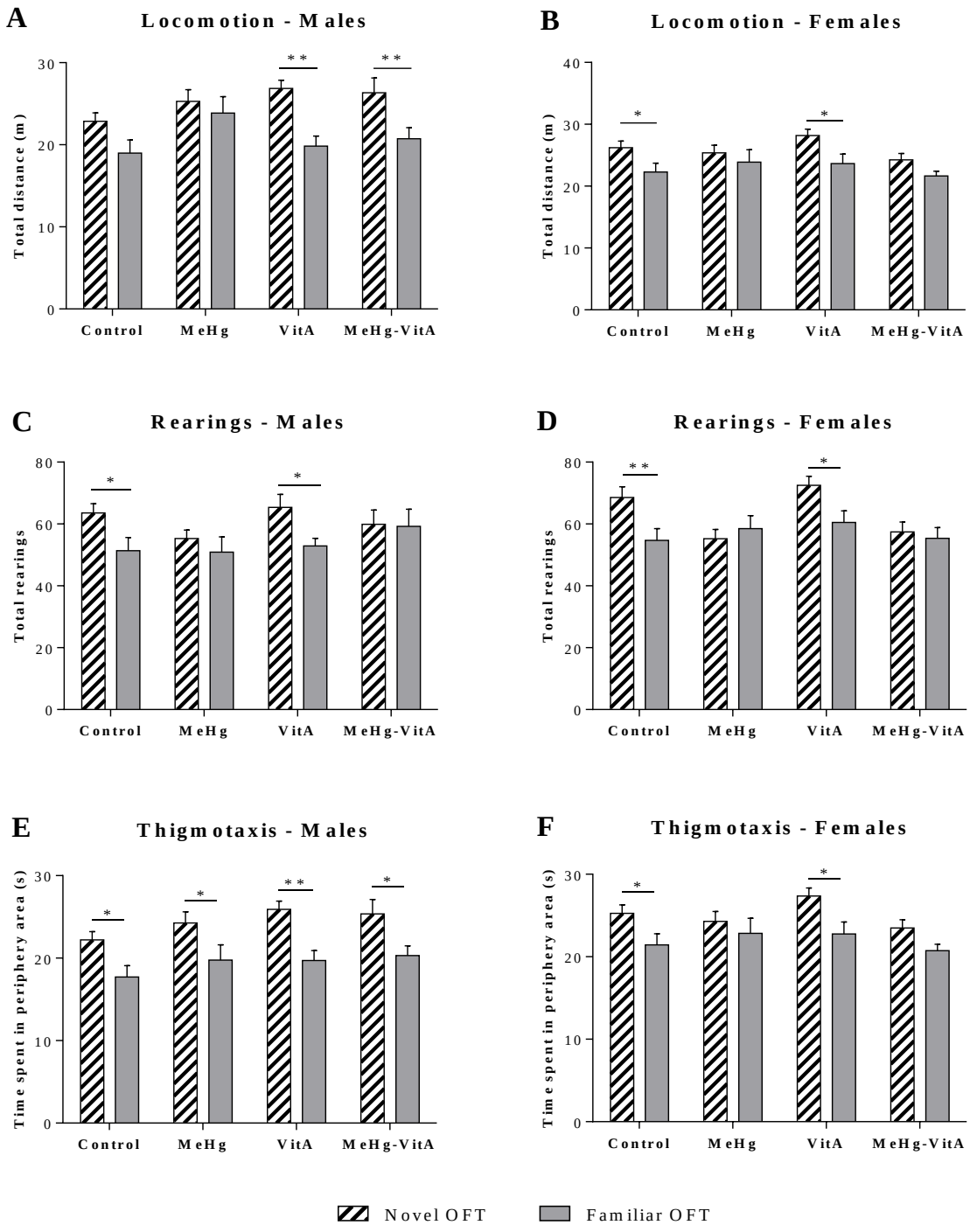

Figure 8. Intersession habituation.
Locomotion, measured as total distance traveled (A – male pups, B – female pups), total rearings (C – male pups, D – female pups) and thigmotaxis, measured as time spent in the periphery (E – male pups, F – female pups) over the two-day OF testing. Each bar represents parameters on day 1 (Novel OFT) and day 2 (Familiar OFT). Intersession habituation was assessed at PND 29 for a total 10 min exploration. Data are represented as mean ± SEM. *p ≤ 0,05 when different from the same parameter at novel OFT, **p ≤ 0,01 when different from the same parameter at novel OFT.

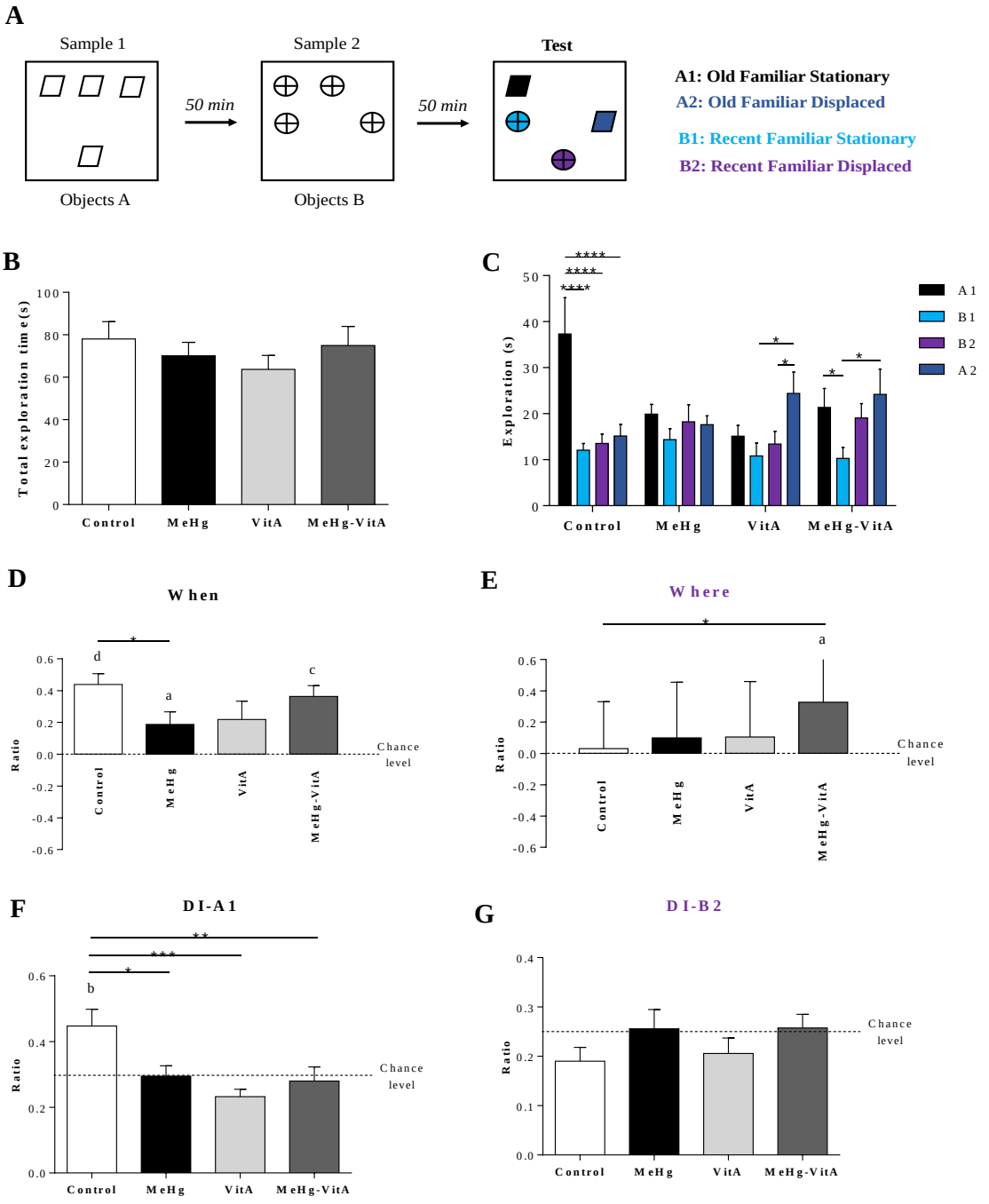

Figure 9. Episodic-like memory assessment for male pups performed at PND 30.
(A) Testing consisted in exposing animals to two set of object samples and the final test phase, each of 5 min duration, with an inter-test interval of 50 min; (B) total exploration times by treatment; (C) exploration times for each of the objects tested; (D) "when" discrimination index; (E) "where" discrimination index; (F) discrimination index for the old stationary object (A1, black) in the test phase; and (G) discrimination index for the recent displaced object (B2, magenta) in the test phase. Data are represented as mean ± SEM. *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001, ****p ≤ 0,0001; (a) p ≤ 0,05; (b) p ≤ 0,01; (c) p ≤ 0,001; (d) p ≤ 0,0001 statistically significant from reference value with one sample *t* test (0 for "when" or "where" discrimination indexes; 0,25 or 25% preference for single object discrimination indexes).

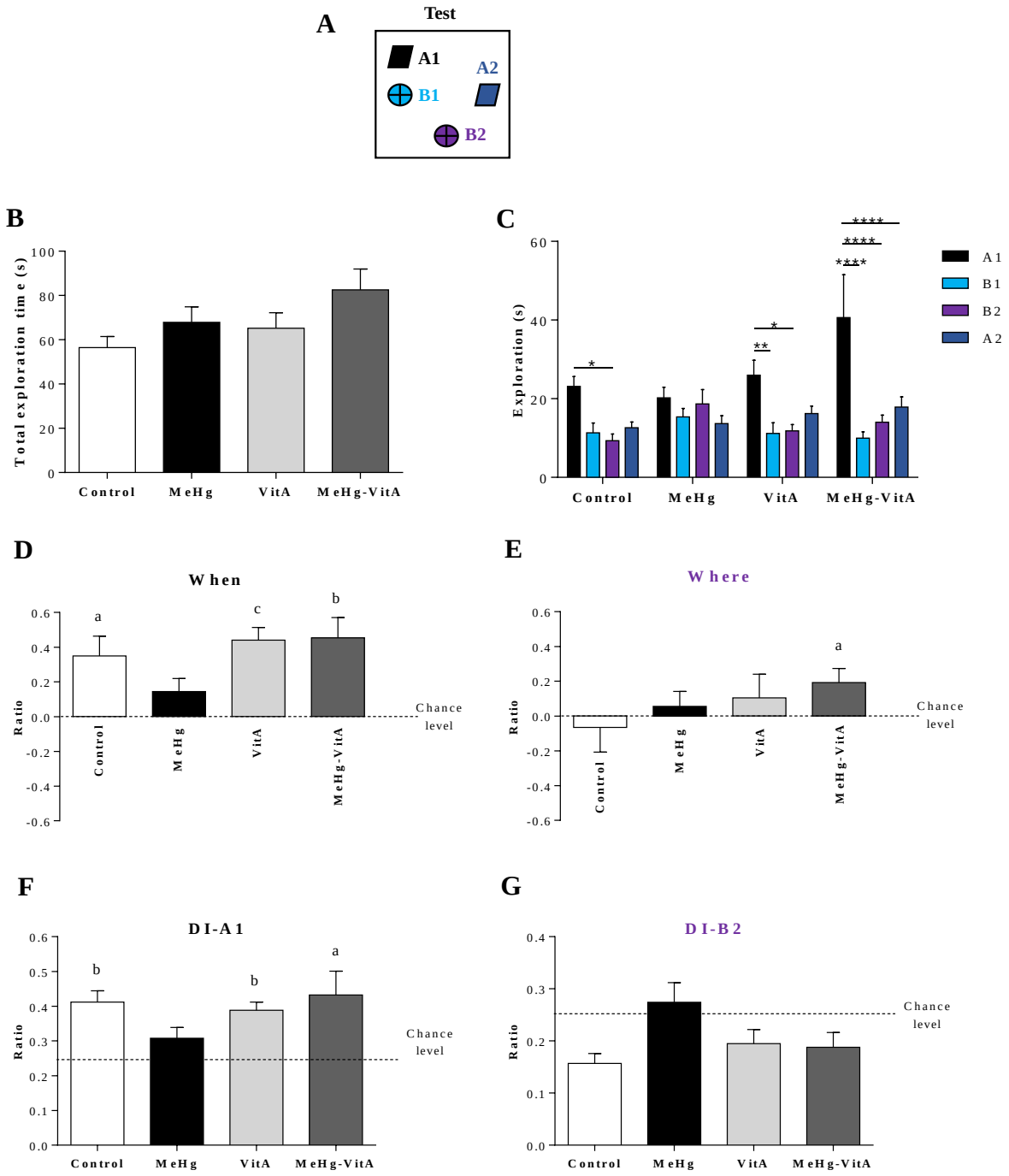

Figure 10. Episodic-like memory assessment for female pups performed at PND 30.
(A) Objects location during final test phase (5 min) duration, posterior to sample phase (as described on Figure 7); (B) exploration times for each of the objects tested; (C) total exploration times by treatment; (D) "when" discrimination index; (E) "where" discrimination index; (F) discrimination index for the old stationary object (A1, black) in the test phase; and (G) discrimination index for the recent displaced object (B2, magenta) in the test phase. Data are represented as mean ± SEM. *p ≤ 0,05, **p ≤ 0,01, ****p ≤ 0,0001; (a) p ≤ 0,05; (b) p ≤ 0,01; (c) p ≤ 0,001; (d) p ≤ 0,0001 statistically significant from reference value with one sample *t* test (0 for "when" or "where" discrimination indexes; 0,25 or 25% preference for single object discrimination indexes).

Table 10. Redox profile in maternal hippocampus (HC).
Effects of treatments administered during gestation and lactation upon maternal HC redox parameters. Data are represented as mean ± SEM. *p ≤ 0,05 when different from control group.

	Treatment			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
No of dams examined	8	7	8	7
TBARS content (nmol TBARS/mg protein)	0,40 ± 0,04	0,48 ± 0,05	0,47 ± 0,04	0,45 ± 0,05
Total thiol content (nmol SH/mg protein)	14,55 ± 0,89	14,94 ± 0,76	13,34 ± 0,79	13,88 ± 0,45
CAT activity (U CAT/mg protein)	1,273 ± 0,34	**0,3625 ± 0,13***	**0,4221 ± 0,06***	0,8392 ± 0,35
SOD activity (U SOD/mg protein)	43,32 ± 5,00	38,77 ± 2,72	37,47 ± 3,62	37,15 ± 5,85
GST activity (nmol/min/mg protein)	62,08 ± 3,13	54,57 ± 3,00	54,57 ± 4,31	62,07 ± 2,06
GPx activity (nmol/min/mg protein)	42,63 ± 2,82	**31,11 ± 1,10***	39,41 ± 3,89	**31,9 ± 2,19***
SOD/(GPx + CAT) ratio (arbitrary units)	0,99 ± 0,07	1,24 ± 0,07	1,04 ± 0,17	1,15 ± 0,17

Table 11. Redox profile in offspring hippocampus (HC).
Effects of treatments administered during gestation and lactation upon HC redox parameters for both male and female offspring. Data are represented as mean ± SEM. *p ≤ 0,05, **p ≤ 0,01 when different from control group; # p ≤ 0,05 when different from MeHg group; + p ≤ 0,05 when different from VitA group.

	Treatment			
	Control	**MeHg**	**VitA**	**MeHg-VitA**
No of litters examined	8	7	8	7
TBARS content (nmol TBARS/mg protein)				
Male	0,25 ± 0,02	0,21 ± 0,01	0,25 ± 0,03	**0,18 ± 0,01*** **#** **+**
Female	0,23 ± 0,02	**0,46 ± 0,15***	0,24 ± 0,03	0,2 ± 0,03
Total thiol content (nmol SH/mg protein)				
Male	11,19 ± 0,83	9,03 ± 0,34	9,88 ± 0,76	**8,25 ± 0,52****
Female	12,37 ± 0,47	12,01 ± 0,60	13,74 ± 0,56	11,48 ± 1,10
CAT activity (U CAT/mg protein)				
Male	0,87 ± 0,21	0,86 ± 0,41	0,65 ± 0,20	0,7 ± 0,26
Female	0,70 ± 0,17	0,81 ± 0,34	0,42 ± 0,23	1,27 ± 0,62
SOD activity (U SOD/mg protein)				
Male	45,76 ± 4,69	40,48 ± 2,30	44,36 ± 4,03	43,41 ± 4,60
Female	32,78 ± 3,96	33,11 ± 4,45	36,19 ± 3,12	40,15 ± 4,67
GST activity (U GST/mg protein)				
Male	27,5 ± 2,00	22,38 ± 1,10	26,57 ± 1,84	26,85 ± 1,90
Female	23,88 ± 1,32	22,8 ± 0,64	26,61 ± 1,61	22,93 ± 2,13
GPx activity (U GPx/mg protein)				
Male	36,13 ± 0,98	**22,28 ± 2,48***	31,87 ± 3,56	30,35 ± 3,23
Female	29,42 ± 1,67	26,79 ± 1,87	31,37 ± 2,70	25,88 ± 2,44
SOD/(GPx + CAT) ratio (arbitrary units)				
Male	1,24 ± 0,09	**1,83 ± 0,16***	1,43 ± 0,12	1,5 ± 0,22
Female	1,1 ± 0,10	1,21 ± 0,11	1,22 ± 0,16	1,51 ± 0,15

Table 12. Redox profile in prefrontal cortex (PFC) and olfactory bulb (OB) isolated from dams and their offspring.
Effects of treatments administered during gestation and lactation upon maternal and offspring redox parameters in dissected PFC and OB. Data are represented as mean ± SEM (n = dams/litters). *p ≤ 0,05, **p ≤ 0,01 when different from control group.

	Prefrontal Cortex (PFC)				**Olfactory Bulb (OB)**			
Treatment/parameter	**Control**	**MeHg**	**VitA**	**MeHg-VitA**	**Control**	**MeHg**	**VitA**	**MeHg-VitA**
	(n = 8)	(n = 7)	(n = 8)	(n = 8)	(n = 8)	(n = 7)	(n = 8)	(n = 8)
TBARS content (nmol TBARS/mg protein)								
Dams	0,42 ± 0,04	0,41 ± 0,04	0,39 ± 0,04	0,35 ± 0,02	0,37 ± 0,06	0,41 ± 0,03	0,51 ± 0,08	0,46 ± 0,06
Offspring								
Male	0,92 ± 0,05	0,92 ± 0,10	0,89 ± 0,10	0,84 ± 0,05	0,80 ± 0,13	0,93 ± 0,11	0,87 ± 0,10	0,69 ± 0,07
Female	0,36 ± 0,03	0,42 ± 0,04	0,36 ± 0,04	0,4 ± 0,02	0,62 ± 0,11	0,90 ± 0,06	0,85 ± 0,11	0,62 ± 0,09
Total thiol content (nmol SH/mg protein)								
Dams	1,52 ± 0,32	1,53 ± 0,33	2,55 ± 0,31	**2,87 ± 0,40***	3,85 ± 0,40	3,40 ± 0,24	3,68 ± 0,42	3,50 ± 0,57
Offspring								
Male	1,71 ± 0,24	1,14 ± 0,40	**2,36 ± 0,23***	2,21 ± 0,32	1,85 ± 0,26	2,06 ± 0,38	2,23 ± 0,39	1,55 ± 0,33
Female	1,80 ± 0,24	1,40 ± 0,22	1,82 ± 0,38	2,83 ± 0,36	2,13 ± 0,49	1,52 ± 0,55	2,32 ± 0,31	**4,45 ± 0,86***
GST activity (U GST/mg protein)								
Dams	65,99 ± 1,70	66,94 ± 2,29	66,95 ± 1,76	68,85 ± 2,60	56,17 ± 4,68	63,16 ± 3,05	61,62 ± 2,16	63,84 ± 4,06
Offspring								
Male	43,51 ± 1,65	41,95 ± 1,59	41,13 ± 1,89	**33,92 ± 3,19****	43,93 ± 3,87	52,03 ± 4,14	48,23 ± 4,04	44,7 ± 3,49
Female	33,23 ± 1,66	32,72 ± 2,06	36,8 ± 1,91	32,03 ± 2,10	35,05 ± 4,09	36,07 ± 2,67	38,95 ± 3,86	33,93 ± 3,34
GPx activity (U GPx/mg protein)								
Dams	44,18 ± 3,95	36,7 ± 3,16	41,77 ± 6,78	33,76 ± 5,01	54,15 ± 5,80	58 ± 5,19	62,64 ± 6,04	53,88 ± 5,19
Offspring								
Male	25,89 ± 4,09	**9,12 ± 2,55***	21,24 ± 2,95	26,55 ± 2,66	53,12 ± 10,04	57,42 ± 8,14	65,41 ± 10,1	59,23 ± 8,29
Female	19,08 ± 2,46	13,74 ± 1,92	26,17 ± 5,75	17,61 ± 3,95	58,21 ± 11,07	64,12 ± 11,38	68,14 ± 10,21	55,43 ± 8,30

PARTE 3

4. DISCUSSÃO

Atualmente, existe um fenômeno de contaminação ubíqua pelo mercúrio a nível global (Oken et al., 2012, Sheehan et al., 2014). Estudos recentes demostram uma alta biodisponibilidade de mercúrio na população geral. Nos Estados Unidos e Coréia, o consumo de peixe e mariscos aporta entre 60 a 80% respectivamente da carga de mercúrio disponível na população (Dong et al., 2015, Kim et al., 2016). Além disso, cerca de 47% do MeHg consumido pelos norte-americanos vem de duas fontes comuns: atum enlatado e o camarão (Sunderland et al., 2018). O aumento no consumo de peixe pela população global, de maneira geral, é um problema preocupante, pois se consome em média 20 Kg a mais de peixe a cada ano (FAO, 2016). Em países sub-desenvolvidos, o aumento do consumo de peixe é de até 15 vezes maior em relação a populações de países desenvolvidos. (Cisneros-Montemayor et al., 2016). Apesar do alto consumo de peixes, mariscos e derivados, a exposição ao MeHg ocorre ainda em doses baixas. Estudos epidemiológicos reforçam o risco da exposição crônica materna ao MeHg em doses baixas, sobre o desenvolvimento de alterações em funções motoras, linguagem, atenção e memória de crianças e adolescentes (Debes et al., 2006, Al-Saleh et al., 2016, Hsi et al., 2016).

Por outro lado, através de modelos animais, nosso laboratório já demonstrou os efeitos deletérios da suplementação em doses recomendadas e terapêuticas de VitA (de Oliveira et al., 2007a, de Oliveira et al., 2007b, de Oliveira et al., 2008, Oliveira, 2015, Schnorr et al., 2015), e a teratogenicidade induzida por doses de suplementação de VitA, aparentemente sem risco como 3000 e 7500 µg RAE/Kg/dia, usadas dentro do período gestacional e lactacional (Schnorr et al., 2011a, Schnorr et al., 2011b). Estudos transversais mostram que o consumo de suplementos de VitA tem aumentado (del Balzo et al., 2014) ou

mantendo-se constante nos últimos anos na população mundial (Beltran-de-Miguel et al., 2015, Kantor et al., 2016). É importante ressaltar, que além do crescimento do consumo de peixes, há também aumento do consumo de alimentos enriquecidos com VitA em alimentos fortalecidos, medicamentos e suplementos (NIH, 2016). Concomitantemente, o panorama de consumo de peixe no mundo entre mulheres gravidas é variável. Na União Europeia, devido ao consumo de mariscos, apenas 5% das gestantes possui níveis de Hg no cabelo acima da concentração de 1 µg/g preconizado pela USEPA (Castaño et al., 2015). Já nos EUA (Razzaghi e Tinker, 2014, Cusack et al., 2017) e alguns países da Ásia (Kim et al., 2006, Tsuchiya et al., 2008, Jo et al., 2010) a biodisponibilidade de Hg aumentou entre mulheres grávidas nos últimos anos. Na literatura não há evidências sobre os possíveis efeitos moduladores do MeHg em combinação com a VitA, e devido a sua particular demanda nutricional, as mulheres grávidas podem apresentar uma incrementada susceptibilidade aos efeitos deletérios dos dois compostos. O objetivo deste trabalho foi avaliar os efeitos de uma dose baixa ambientalmente relevante de MeHg e uma dose de suplementação de palmitato de retinol (na forma de provitamina A) e seus efeitos tóxicos em modelo animal de exposição gestacional e lactacional nas mães e seus filhotes

No caso do MeHg, a dose usada neste trabalho é considerada ambientalmente relevante, o seja, que constitui uma dose aproximada a casos de exposição humana (Obiorah et al., 2015). A dose escolhida foi 0,5 mg/Kg/dia, referenciada em trabalhos prévios e usada em vários paradigmas de exposição usando modelos animais (Reed et al., 2008, Vitalone et al., 2008, Gandhi et al., 2013, Newland et al., 2013, Gandhi et al., 2014, Gandhi e K Rajput, 2016). Sabe-se que o tratamento com MeHg na água de consumo (exposição crônica) ou através de dose única (exposição aguda) por injeção sob cutânea,

ambas produzem efeitos inibitórios sobre a neurogênese hipocampal (Fujimura et al., 2012, Sokolowski et al., 2013). 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 (Newland et al., 2015). Portanto, nossa dose de MeHg começo a ser administrada durante o primer dia gestacional (GD0) e foi continua até o desmame dos filhotes (DPN21), sendo um modelo de exposição crônica. Segundo a literatura, modelos animais com exposição nesse período pós-natal levam a um pico da atividade de neurogênese (DPN7), o qual é equivalente em humanos ao terceiro mês de gestação, onde ocorre o desenvolvimento hipocampal (Obiorah et al., 2015).

Em relação a dose de VitA, 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 melhor descrita por Schnorr e colaboradores (Schnorr et al., 2011a, Schnorr et al., 2011b). A dose é baseada em um fator de incerteza de 10 vezes para diferentes espécies e 10 vezes entre as espécies, e é considerada uma dose segura para suplementação de VitA em mulheres grávidas sem pré consumo deste micronutriente (Schnorr et al., 2011b). 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 aquisição estável de VitA do feto ao longo da suplementação (Schnorr et al., 2011b).

4.1. Avaliação de alterações fisiológicas, metabólicas e deposição de mercúrio em tecidos das mães e seus filhotes

Nossas primeiras observações durante o período gestacional das mães, e ao longo do período pós-natal nos filhotes, não mostraram alterações nas condições de saúde nos animais. Como era esperado, as doses utilizadas de MeHg e VitA não apresentaram, de

maneira geral, sintomas de intoxicação como sinais de dano neurológico (tremores e ataxia) perda de peso, redução da capacidade reprodutiva ou alterações fenotípicas (anormalidades em estruturas do corpo). Além disso, não foram encontradas alterações fisiológicas nas mães ou filhotes durante as fases experimentais, como vômito ou diarreia. Por último, durante o sacrifício dos animais não observamos alterações morfológicas evidentes nos tecidos de órgãos internos.

Apesar que o MeHg ser um e inibidor da enzima glicose-6-fosfatase, responsável pelo processo de gliconeogênese (Snell et al., 1977), não foram encontradas alterações metabólicas gerais sobre a deposição de glicogênio no tecido hepático ou cardíaco entre os grupos experimentais, incluindo mães e filhotes do grupo MeHg-VitA, como vimos no Capítulo I. Além disso, a quantificação de retinóides no fígado das mães mostra um aumento do conteúdo de retinol, indicando disfunção na homeostase de VitA. Apesar de ser uma dose baixa de acordo com a LOAEL, o tratamento crônico com VitA incrementa a frequência de micronúcleos em eritrócitos policromáticos (MNPCE) nas mães, indicando a presença de dano mutagênico pela ação pró-oxidante da VitA, que induz quebras no DNA e posterior ruptura cromossômica (Yeum et al., 2009, Veloso et al., 2013). No entanto, ao contrário do tratamento com apenas VitA, o tratamento MeHg-VitA consegue aumentar a frequência de MNPCE nos filhotes, provavelmente pelo dano clastogênico *in vivo* produzido pelo MeHg (Crespo-Lopez et al., 2016). Sendo um parâmetro de avaliação do estado regulatório do fígado (Shirakami et al., 2012), e dado que não encontramos diferença no acúmulo de retinóides no fígado e plasma, nós podemos concluir que a co-exposição MeHg-VitA não gerou nos filhotes alterações metabólicas no tecido hepático, mas mostram um dano elevado ao DNA.

Apesar da baixa dose de MeHg, o acúmulo de Hg nos tecidos das mães e dos filhotes foi considerável. Nos Capítulos I e II desta dissertação o conteúdo de Hg total em fígado e córtex occipital foi quantificado para todos os grupos experimentais. Só nos grupos MeHg e MeHg-VitA foi encontrado um padrão de desmetilacão de MeHg, o qual ocorre no fígado da mãe para logo ser transferido como Hg inorgânico ao feto. No entanto, nós não conseguimos demostrar acúmulo de Hg nas amostras de córtex dos filhotes machos e fêmeas. Vários autores reforçam que a exposição pré-natal/lactacional ao MeHg, mesmo considerando sua toxicocinética, se encontra relacionada com sua eliminação no organismo, a qual persiste mesmo após a primeira exposição (Sakamoto et al., 2002, Hu et al., 2010). Trabalhos prévios no modelo de exposição pré-natal demostraram que a concentração de Hg alcança um pico duas semanas depois da última exposição ao MeHg, diminuindo na semana posterior, e finalmente permanecendo indetectável 40 dias depois da exposição (Hu et al., 2010). Sendo esse um modelo que inclui tratamento durante a lactação, a exposição pós-natal ao Hg nos filhotes e completamente através do leite materno até o desmame, o que promove transporte limitado de Hg (Sakamoto et al., 2002, Albores-Garcia et al., 2016). Adicionalmente, depois do desmame, os filhotes permaneceram sem tratamento e possivelmente aumentando a eliminação do Hg. Sherin e Sumathi (Sherin e Sumathi, 2016), usando a mesma dose de MeHg (0,5 mg/Kg/dia), obtiveram conteúdos baixos de Hg no cérebro dos filhotes, os quais foram menores do limite de detecção de nossa análise.

4.2. Avaliação de parâmetros de estresse oxidativo em fígado, rim e coração isolado das mães e seus filhotes

Sendo o fígado, rins e o coração, órgãos metabolicamente ativos durante o estresse oxidativo, além de desempenhar um papel importante nos mecanismos de certas patologias,

nós decidimos avaliar também o perfil redox nesses órgãos, das mães e seus filhotes (Romero et al., 1998, Korge et al., 2008). Os resultados obtidos dentro deste trabalho foram descritos no Capítulo I desta dissertação e são resumidos na Figura 2.

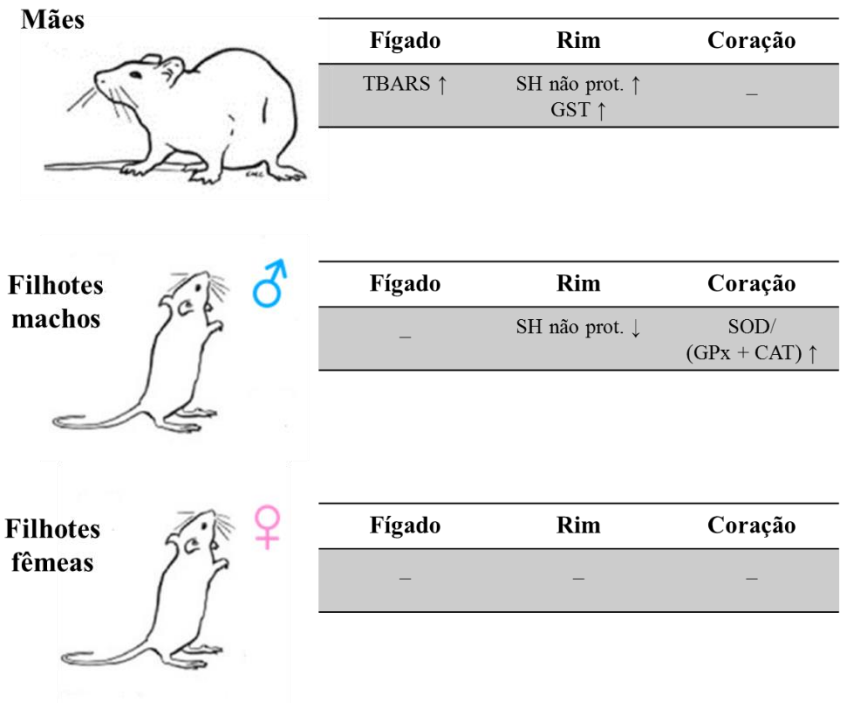

Figura 2. Resultados do perfil redox em fígado, rim e coração isolados de mães e filhotes *Wistar* expostos a metilmercúrio coadministrado com palmitato de retinol (MeHg-VitA) durante o período de gestação e lactação.

Um dos principais parâmetros de dano oxidativo é TBARS, sendo uma das principais espécies químicas deste tipo o malondialdeído (MDA), que é o principal produto da peroxidação de ácidos graxos poli-insaturados (PUFA), principais componentes da membrana celular, sendo a elevação nos níveis de TBARS relacionada ao incremento na peroxidação lipídica e um indicativo de dano sobre as membranas celulares (Freeman e Crapo, 1981). Autores como Uchendu e colaboradores (Uchendu et al., 2014) estudando

pesticidas, atribuem o incremento de TBARS durante episódios de co-exposição a capacidade de compostos de natureza lipofílica de afetar a integridade da membrana celular de maneira potenciada. O palmitato de retinol possui natureza lipofílica, além alta concentração no fígado. Além disso, o MeHg possui um grupo metil (CH_3) que confere sua lipofilicidade (Halbach, 1990). Evidências em modelos animais demostram o aumento da peroxidação lipídica no tecido hepático induzidas tanto pelo MeHg (Andersen e Andersen, 1993), quanto pela VitA (Murata e Kawanishi, 2000). Nós acreditamos que o incremento na peroxidação lipídica encontrado no grupo MeHg-VitA pode ser devido ao efeito combinado pró-oxidante ou de potenciação a nível celular, devido ao aumento no estresse oxidativo produzido pela ação combinada do MeHg e a VitA, interagindo com a membrana celular dos hepatócitos.

Não foram evidenciadas alterações visíveis no rim, provavelmente atribuída a pouca ação fisiológica deste tecido (Smith et al., 1983). No entanto, existe uma resposta de detoxificação visto através do aumento de tióis não proteicos, que constituem 90% da glutationa livre (GSH) no rim materno, impulsada pela administração crônica de MeHg (Woods et al., 1992). O mecanismo de geração de GSH tem a função de complexar as formas mercúricas circulantes no organismo materno. De fato, os conjugados de GSH com Hg e MeHg têm sido encontrados na bile e na urina de animais expostos, além de sua absorção e processamento pelo rim (Zalups, 2000, Rubino, 2015). Por outro lado, o incremento na atividade da GST nas ratas tratadas com MeHg-VitA pode ser devido ao desbalanço do equilíbrio pró-oxidante/antioxidante em razão das reações oxidativas, também encontradas em certas patologias renais (Stępniewska et al., 2014, Sureshbabu et al., 2015). Apesar de seu caráter antioxidante, a VitA subministrada não atua de maneira

compensatória no rim durante a co-exposição com MeHg. Em consequência, nossa hipótese planteia que durante a ativação oxidativa da GST (Aniya et al., 1993) e a eliminação ulterior do complexo GSH-mercúrio, se produz uma condição de estresse oxidativo leve não compensada pela VitA co-administrada, o que pode desencadear uma resposta ativadora de cascada de sinalização e respostas de autofagia como resposta adaptativa ao nível celular (Townsend et al., 2003, Sureshbabu et al., 2015).

O tratamento MeHg-VitA no fígado não alterou os parâmetros redox enzimáticos e não enzimáticos nos filhotes, o que pode ser explicado pelo mecanismo protetor da gestação e a lactação (Yoshida et al., 2002). Por outro lado, a análise do perfil redox do rim nos filhotes mostrou resultados interessantes. Apesar de não terem sido feitas quantificações da deposição de Hg no rim, nós conseguimos evidenciar pequeno aumento nos conteúdos de Hg no fígado, e níveis indetectáveis de Hg no córtex occipital, que é esperado em modelos murinos, onde animais machos na pré-adolescência recentemente expostos ao MeHg começam a resposta hormonal de ativação da via GSH/γ-glutamiltranspeptidase (γ-GTP) e eliminação de Hg circulante, o que diminui as quantidades de GSH (Nielsen e Andersen, 1996). Os resultados obtidos para as fêmeas do grupo MeHg-VitA não só no rim, mas em fígado e coração, de maneira geral, não mostram alterações nos parâmetros redox (Figura 2). Isso pode ser explicado pela ação protetora do estrógeno nas fêmeas. Estudos em modelos de deficiência dietética de cobre em ratas fêmeas ovariectomizadas (OVX) demostram o caráter protetor do estrógeno sobre o estresse oxidativo (Bureau et al., 2003). Nas fêmeas a produção de estrógeno começa na infância (14 dias após nascimento) (Weniger et al., 1993).

Por outro lado, neste trabalho não encontramos alterações no balanço redox do tecido cardíaco das mães submetidas ao tratamento de co-exposição (Figura 2). No entanto, o análise do tecido cardíaco dos filhotes machos mostrou um incremento na relação SOD/(GPx + CAT). O aumento desta razão está relacionado com a maior formação de peróxido de hidrogênio (H_2O_2) livre dentro do tecido, isso porque a SOD catalisa a reação de dismutação do radical $O_2^{\bullet-}$ a H_2O_2 e oxigênio, entretanto a CAT e a GPx catalisam a conversão de H_2O_2 em taxas menores (Schnorr et al., 2011b). Tanto a CAT como a GPx reduzem peróxido de hidrogênio (H_2O_2) à água, com a única diferença que a GPx também reduz uma grande quantidade de peróxidos orgânicos utilizando GSH (Arthur, 2000). O peróxido livre no tecido é convertido via reação de Fenton em o radical hidroxil ($OH^{\bullet}$), uma poderosa molécula pró-oxidante, capaz de interagir com biomoléculas e produzir dano oxidativo (Halliwell, 2006, Veal et al., 2007, Halliwell e Gutteridge, 2015).

4.3. Alterações comportamentais nas mães e seus filhotes

No Capítulo II da presente dissertação, demostramos que os tratamentos, em particular a co-exposição MeHg-VitA nas doses utilizadas durante uma exposição crônica sobre as mães durante a gestação e lactação, foram capazes de induzir alterações comportamentais nos filhotes. No caso do MeHg, inúmeras evidências demonstram os déficits motores e sensoriais produzidos por este poluente em modelos animais (Bisen-Hersh et al., 2014), além da sua capacidade de induzir comportamento depressivo (Onishchenko et al., 2007) e alterações na memória e aprendizado (Bisen-Hersh et al., 2014). Por outro lado, trabalhos prévios do nosso laboratório têm demostrado os efeitos da suplementação com VitA na indução de ansiedade e diminuição da atividade exploratória

em modelos animais (de Oliveira et al., 2007b). Os resultados obtidos dentro deste trabalho foram descritos no Capítulo II desta dissertação e são resumidos na Figura 3.

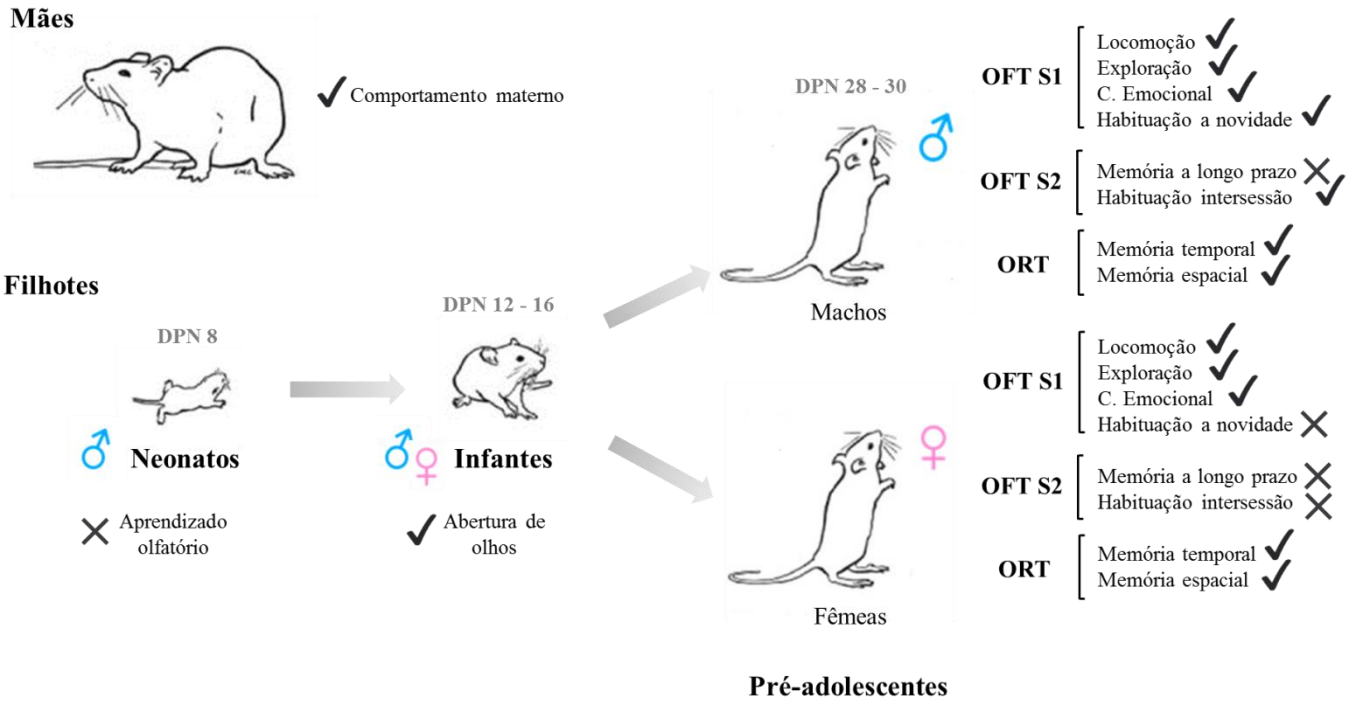

Figura 3. Resultados dos analises de comportamento de mães e filhotes *Wistar* expostos a metilmercúrio coadministrado com palmitato de retinol (MeHg-VitA) durante o período de gestação e lactação. ✓ Sem diferença com o controle; ✕ Diferente do controle; DPN (dia pós-natal).

A avaliação dos parâmetros de comportamento materno não mostrou diferenças entre as mães tratadas com MeHg-VitA, sendo um indicativo da ausência de alterações comportamentais nos filhotes que podam ser induzidas por excessiva proteção materna. No entanto, nossos resultados de avaliação comportamental nos filhotes neonatos (DPN 8) e posteriormente infantes (DPN 12 – 16) mostraram resultados interessantes.

Entre os neonatos machos (DPN 8) do grupo MeHg-VitA não foi encontrada preferência pelo odor materno através do teste de aprendizado olfatório. A resposta deletéria dentro do grupo co-exposto pode ser atribuída diretamente ao MeHg, devido a que este composto foi relacionado com afetações de aprendizado em modelo animal (Lucena et al., 2013). O mecanismo mais provável para explicar essa diminuição do aprendizado gerado pelo MeHg seja devido a sua capacidade para promover a perturbação na capacidade de progressão normal das células-tronco neurais (NSCs), além de induzir sua morte celular por apoptose pela ativação de ambas caspases e calpaína proteases (Tamm et al., 2006, Ceccatelli et al., 2013). A resposta deletéria do MeHg na dose de co-exposição não alterou a exploração dos filhotes (Anexo II desta dissertação), portanto não foram vistas alterações nas primeiras respostas locomotoras dos filhotes. Porém, afetou diretamente o mecanismo de aprendizado, provavelmente via inibição na proliferação das NSCs. No entanto, em etapas posteriores de desenvolvimento dos filhotes (DPN 12 – 16), machos e fêmeas mostraram atraso na aparição do reflexo de abertura de olhos quando expostos ao MeHg, mas não apresentaram alterações no grupo co-exposto. A abertura de olhos constitui uma resposta reflexa automática do sistema nervoso periférico (SNP) comum em todos os mamíferos. O desempenho desta resposta, junto com outros reflexos do neurodesenvolvimento (reflexos primitivos) é um indicativo usado na pratica clínica para avaliar a maturação do sistema nervoso (Schneider e Przewlocki, 2005, Nguyen et al., 2017). Neste trabalho, utilizamos o procedimento descrito por Schneider e Przewlocki (Schneider e Przewlocki, 2005) que consiste em fazer o registro da aparição do reflexo de apertura de olhos do dia pós-natal 12 até o 16 (dia aproximado da aparição dos reflexos em todos os filhotes). Este modelo é usado para avaliar neurodesenvolvimento em ratas com exposição pré-natal ao ácido valproico (VPA), um modelo animal de indução de autismo.

No caso do MeHg, estudos com modelos de exposição perinatal não mostram alterações na abertura de olhos (Paletz et al., 2006, Gandhi et al., 2014, Gandhi e K Rajput, 2016), mas para o nosso conhecimento, nenhum estudo até agora utilizou nosso procedimento. Nossa hipótese sobre os resultados de aprendizado olfatório e apertura de olhos, é que durante o período gestacional e ao longo do período infantil, a co-administração de VitA surgiu como um mecanismo compensatório progressivo ante os efeitos deletérios produzidos pelo MeHg, o qual foi intensificado nos filhotes. Existe evidência sobre o papel do ácido retinóico (RA), o metabólito ativo do palmitato de retinol, no desenvolvimento no sistema olfatório e de sua reparação depois de lesões, além da capacidade deste retinoide em aumentar a memória olfatória e de combate aos efeitos do envelhecimento em camundongos (Sell, 2014).

É interessante reconhecer que, os efeitos hereditários do MeHg em modelo experimental de NSC especificadas anteriormente respondem a modificações epigenéticas. Existem evidências crescentes na literatura sobre o MeHg e seu envolvimento na hipometilação do DNA (Anway et al., 2005, Baccarelli e Bollati, 2009, Pilsner et al., 2010). Além disso, NSC expostas a MeHg apresentam um diminuição da 5-metilcitosina, indicando uma baixa metilação do DNA (Bose et al., 2012), acompanhada de uma expressão diminuída no mRNA da DNA (citosina-5-)-metiltransferase (DNMT) 3b (Ceccatelli et al., 2013). A mesma inibição da DNMT está envolvida em um decrescimento na proliferação celular, indução da senescência e perda da diferenciação neural, o qual foi evidenciado usando um modelo experimental de células tronco multipotentes derivadas de sangue de cordão umbilical humano (So et al., 2011, Ceccatelli et al., 2013). Adicionalmente ao descrito, as modificações epigenéticas poderiam explicar os efeitos a

longo prazo evidenciados depois da exposição pré-natal ao MeHg, especialmente no comportamento. Onishchenko e colaboradores (Onishchenko et al., 2008) demostraram, usando camundongos, que o comportamento do tipo depressivo produzido por uma dose baixa de MeHg teve relação com baixa expressão gênica do Fator Neurotrófico Derivado do Cérebro (BDNF) no hipocampo. Além disso, na região promotora do BDNF o tratamento com MeHg induz hipermetilação do DNA, incremento na trimetilação da histona H3-K27 (H3-K27me3) e um decrescimento na acetilação H3 no promotor IV do BDNF. Curiosamente, trabalhos recentes mostram que o RA, atuando sobre os receptores de ácido retinóico (RARs), promove o decrescimento da marca repressiva epigenética provocada pela H3-K27me3, permitindo a ativação transcricional das células tronco (Urvalek e Gudas, 2014). O anterior poderia explicar como a coadministração de VitA poderia modular progressivamente os efeitos do MeHg durante a infância dos filhotes. No entanto, mais estudos são requeridos para confirmar nossa hipótese.

Após o desmame e após o desenvolvimento dos filhotes pré-adolescentes (PND 28 – 30), o tratamento MeHg-VitA não induziu alterações locomotoras, exploratórias ou de comportamento emocional (ansiedade) no teste de campo aberto (OFT). Em relação a este último parâmetro, nós não encontramos diferenças nos índices de tigmotaxía para nenhum dos tratamentos durante o OFT (Anexo III desta dissertação). Por outro lado, a avaliação dos parâmetros intersessão no OFT sobre os filhotes expostos a MeHg, a suplementação de VitA e ao tratamento de co-exposição apresentados neste trabalho, mostrou diferenças sexo-dependentes. Os animais machos do grupo co-exposição, mostraram maior resposta de habituação em comparação com as fêmeas, significando uma diminuição na locomoção e na tigmotaxia entre as duas sessões de OFT efetuadas (dias pós-natais 28 e 29). Os

parâmetros da locomoção e do *rearing* (exploração vertical) são usados convencionalmente como uma medida do aprendizado de habituação no OFT (Platel e Porsolt, 1982, Dai et al., 1995, Thiel et al., 1999). No entanto, o comportamento de *varredura tigmotáxica*, isso é, o tempo de locomoção (provavelmente exploratório) que o animal mante ao longo das paredes do campo aberto, também decresce com exposição repetida ao teste, sendo uma alternativa ou ainda melhor parâmetro para a medição da habituação (Thiel et al., 1999).

No caso dos filhotes das mães suplementadas com VitA incluindo o grupo co-exposição, a resposta de habituação pode ter sido favorecida pelo RA, que nós estabelecemos previamente durante nossa discussão do comportamento de abertura de olhos. O RA é um promotor da neurogênese, pois contribui para o desenvolvimento e a plasticidade hipocampal durante o neurodesenvolvimento (Bonnet et al., 2008). A neurogênese hipocampal, um processo que ocorre em roedores (Cameron et al., 1993) e humanos (Knoth et al., 2010) leva ao aumento do número de células neuronais do giro denteado (DG), após tarefas de aprendizado mediadas pelo hipocampo, ou seja, tarefas que incluam navegação espacial (ex: Em ratos, *Morris Water Maze*) (Leussis e Bolivar, 2006, Yau et al., 2015). Por tanto, o DG é crucial em funções de aprendizado e memória (curto e longo prazo, além da memória espacial) podendo estar envolvida na resposta de habituação (Leussis e Bolivar, 2006, Lieberwirth et al., 2016). Trabalhos prévios têm demostrado a vulnerabilidade hipocampal ao MeHg, e o fato que a exposição a doses baixas de este contaminante afeta a neurogênese hipocampal (Sokolowski et al., 2013). Por outro lado, várias linhas de evidencia mostram que a VitA previne déficits na memória, devido a que o RA pode contrapor-se aos efeitos do déficit na memória espacial produzidos pelo dano na neurogênese hipocampal (Bonnet et al., 2008, Touyarot et al., 2013).

Alterações provocadas durante a neurogênese hipocampal são acompanhadas de modificações epigenéticas. Recentemente, inúmeras evidências nos apontam que os processos de memória e os efeitos deletérios dos processos cognitivos pelo processo de envelhecimento, estão relacionados com a metilação do DNA (Liu et al., 2009, Yu et al., 2011, Heyward e Sweatt, 2015, Oliveira, 2016). Além disso, o impacto de estressores sobre a neurogênese desenvolvida em grandes taxas no DG hipocampal, e a metilação do DNA, estão relacionadas com afetações cognitivas (Chen et al., 2013, Penner et al., 2016). Nossa hipótese sobre a disfunção da neurogênesis hipocampal poderia explicar as diferenças especificadas previamente na resposta diferenciada de habituação dos filhotes machos e das fêmeas. Estudos recentes demostraram em modelo de rato *Wistar* que, sob condições de estresse crónico a proliferação e supervivência de neurônios maduros é menor nas fêmeas do que os machos (Hillerer et al., 2013). Além disso, no mesmo modelo animal o estresse no período perinatal aumenta a redução na neurogênesis hipocampal nos períodos prévios a puberdade no caso das fêmeas, melhorando em etapas perto da idade adulta (Loi et al., 2014). Nossa hipótese sobre o efeito do desenvolvimento e respostas diferenciadas de habituação pode ser explicado pela presencia de hormônios sexuais. De fato, os filhotes se encontravam na peri-adolescência, e para as ratas fêmeas, depois do desmame e perto do dia pós-natal 32 começa o processo de maduração sexual e abertura vaginal (Sengupta, 2013), não sendo descartada a possibilidade que as fêmeas tinhas começado seu ciclo estral.

Por último, as alterações sexo-dependentes na habituação não foram acompanhadas de um decrescimento na memória episódica, as quais se encontram acentuados durante o envelhecimento e inúmeras condições neurológicas como a doença de Alzheimer e certas formas de epilepsia (Inostroza et al., 2013). Nossa única limitação foi demostrar a tendência discriminatória no componente espacial do teste, o seja, a preferência

pelos objetos deslocados foi completamente aleatória. O anterior pode significar que para nossos ratos, o objeto antigamente mostrado seja novamente novidade em relação aos objetos mostrados recentemente, e as diferencias no desempenho nos componentes de memória sejam produto da mesma exploração diferencial dos filhotes. No caso dos filhotes do grupo da co-exposição (machos e fêmeas), esses ratos possuem um tempo maior de exploração pelo objeto deslocado em comparação com os outros ratos, mas sem mostrar preferência.

4.4. Avaliação de parâmetros de estresse oxidativo em hipocampo, córtex pré-frontal e bulbo olfatório isolados das mães e seus filhotes

Adicional a nossos resultados de comportamento, no Capítulo II decidimos aumentar o escopo de nosso trabalho avaliando parâmetros de estresse oxidativo em tecidos do SNC. A ideia sobre a relação entre o estresse oxidativo e comportamento tem sido demostrada ao longo dos anos, e atualmente ainda é reforçada por vários modelos experimentais. Estudo recente demostrou a atividade anti-epileptogênica e neuroprotetora ante déficits cognitivos do antioxidante ácido ferulico (Hassanzadeh et al., 2017). No outro estudo, usando um modelo animal de autismo (ácido valpróico), os déficits comportamentais e o estresse oxidativo induzido pelo composto foram atenuados pelo tratamento com o carotenoide astaxantina (Al-Amin et al., 2015). Os resultados obtidos dentro deste trabalho foram descritos no Capítulo II desta dissertação e são resumidos na Figura 4.

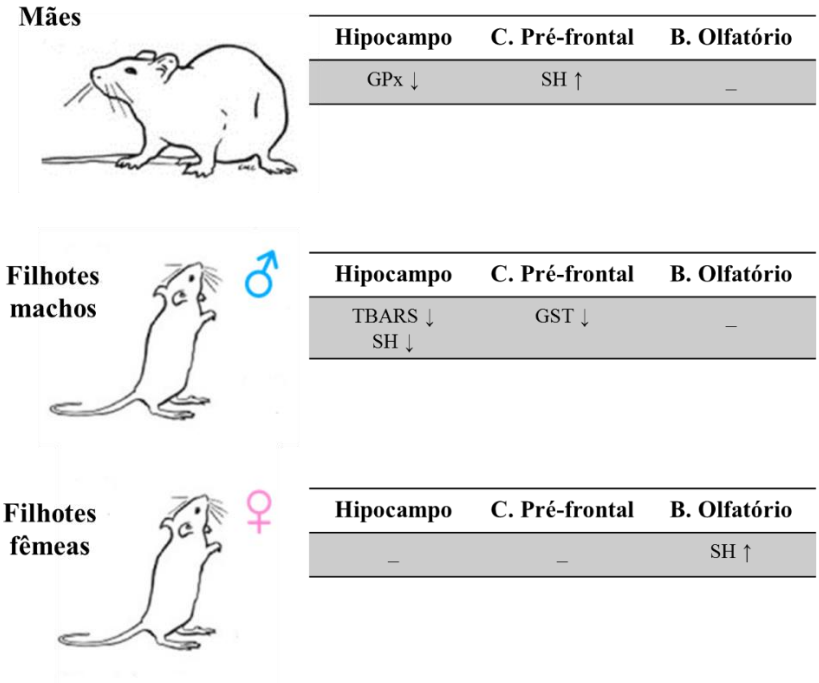

Figura 4. Resultados do perfil redox em hipocampo, córtex pré-frontal e bulbo olfatório isolados de mães e filhotes *Wistar* expostos a metilmercúrio coadministrado com palmitato de retinol (MeHg-VitA) durante o período de gestação e lactação.

Ainda que uma relação causa/efeito que possa explicar nossos resultados comportamentais está longe de ser obtida, o estresse oxidativo pode ser um fator determinante sobre os efeitos biológicos causados pela co-exposição ao MeHg-VitA, permitindo inferir sobre possíveis mecanismos de atuação a nível celular. Uma das consequências dos tratamentos com MeHg e VitA foi a modulação das defesas antioxidantes no hipocampo das mães e dos filhotes indicando a produção de espécies reativas de oxigênio neste tecido. No caso das mães tratadas com MeHg e VitA, se encontrou uma diminuição da atividade da CAT hipocampal, sem diferencia na atividade evidenciada na SOD. Evidencia na literatura tem mostrado que a atividade da CAT diminui, o que pode ser explicado pela inibição direta da enzima pelo MeHg (Abdel-Hamid

et al., 2001). No caso da VitA, tem evidência na literatura que demostra o incremento do radical $O_2^{\bullet -}$ após episódios de exposição a VitA (Klamt et al., 2005). Além disso, o mesmo radical $O_2^{\bullet -}$ tem um efeito de inibição alostérica sobre a CAT, porém diminuindo sua atividade (Shimizu et al., 1984). Por outro lado, nos grupos MeHg e MeHg-VitA, a atividade da enzima que ficou mais alterada foi a GPx, apresentando uma diminuição significativa. De fato, a GPx constitui uma enzima essencial para contra-atacar os efeitos pró-oxidantes do MeHg (Franco et al., 2009). O principal mecanismo de ação do MeHg e inibir a síntese de GPx, devido a que constitui uma selênio-enzima. No SNC, o MeHg forma um complexo insolúvel com o selênio livre (Se-Hg) o que impede a síntese *de novo* de proteínas selênio-dependentes (Zemolin et al., 2012). Os níveis de CAT nas mães do grupo MeHg-VitA não apresentam diferenças. Tanto a GPx como a CAT competem pelo mesmo substrato (peroxido), então devido a inibição do MeHg o mecanismo compensatório esperado seria um crescimento na atividade da GPx (Machi et al., 2016). Devido ao anterior nós podemos hipotetizar que existe um incremento na produção de H_2O_2 gerando uma situação pró-oxidante no hipocampo

Nossos resultados sugerem um possível efeito protetor *in vivo* da VitA sobre os efeitos da toxicidade do MeHg evidenciado no hipocampo dos filhotes. De fato, nos filhotes submetidos ao tratamento MeHg-VitA sob coadministração, mostraram uma melhor resposta antioxidante. Existe evidencia na literatura sobre os efeitos dos antioxidantes durante condições de estresse oxidativo no SNC. É amplamente conhecido o efeito protetor da vitamina E, além dos seus análogos (tocoferóis e tocotrienóis) prevenindo a peroxidação lipídica e, portanto, a neurotoxicidade do MeHg (Osakada et al., 2004, Khanna et al., 2006, Nabi, 2014d). Outro composto com provável atividade antioxidante e o

trolox (Ácido-6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico), outro derivado da vitamina E, que possui efeito protetor *in vivo* reduzindo a apoptose na camada granular cerebelar de ratas expostas ao MeHg (Usuki et al., 2001). Entre as hipóteses sobre efeito protetor dos compostos antioxidantes sobre o SNC se encontram o *scavenging* e remoção de radicais livres, reversão ao dano da absorção de glutamato, inibição da liberação do citocromo c e ativação da via das caspases (Aschner et al., 2007, Nabi, 2014d). Um dos mecanismos mais aceitos constitui a inibição do transporte de glutamato nos astrócitos por parte do MeHg, o que leva a uma sobre produção de ROS, mas os antioxidantes têm a habilidade de prevenir essa sobre produção, portanto atenuando a neurotoxicidade do MeHg (Aschner et al., 2007, Nabi, 2014d). No caso da VitA, até agora e para nosso conhecimento, não tem nenhum estúdio referente aos possíveis mecanismos de modulação deste micronutriente com o MeHg. Nossos resultados são os primeiros em mostrar os possíveis efeitos antioxidantes da VitA no hipocampo de ratos tratados com este poluente.

5. CONCLUSÕES

Baseados nos resultados apresentados na presente dissertacao, podemos concluir os seguintes pontos:

1) A co-exposição a uma dose baixa e ambientalmente relevante de MeHg junto com uma dose de suplementação com VitA em dose considerada como segura durante o período reprodutivo, não produz alterações toxicológicas visíveis ou metabólicas tanto nas mães como nos filhotes. O anterior não foi acompanhado de afetações nos parâmetros reprodutivos clássicos nas mães ou malformações congênitas nos filhotes;
2) O tratamento de co-exposição nestas mesmas doses não alterou o comportamento materno, mas foi capaz de induzir alterações comportamentais nos filhotes como preferência aleatória no teste de aprendizado olfatório (PND 8), mas sem produzir afetações no teste de apertura de olhos (PND 12 -16), Os resultados podem indicar que a co-exposição a MeHg junto com uma suplementação de VitA interfere nos processos de aprendizado em etapas iniciais da vida neonatal, mas não afeta de maneira geral o neurodesenvolvimento em etapas posteriores da etapa infantil;
3) A coadministração em mães e seus filhotes de MeHg em doses baixas e ambientalmente relevantes junto com a suplementação com VitA foi capaz de modular de forma tecido-especifica a atividade das enzimas glutationa dependentes; induzir ou alterar a lipoperoxidação; e provocar modificações na relação SOD/(GPx + CAT) que podem indicar a dismutação de $O_2^{\bullet -}$ pela SOD e não metabolização do ulterior H_2O_2 a taxas normais;

4) O tratamento de co-exposição a uma dose ambientalmente relevante de MeHg e suplementação segura de VitA não induz de maneira geral alterações metabólicas, toxicológicas ou sobre a atividade locomotor-exploratória-emocional, em filhotes pré-adolescentes (DPN 31). Apesar de que o tratamento não alterou as funções cognitivas de memória episódica (PND 30), afetou a resposta de habituação ao Campo Aberto de maneira sexo-dependente (PND 29), apresentando os machos um melhor desempenho em comparação com as fêmeas. As mesmas diferencias sexuais produziram diferentes respostas no balanço redox de maneira tecido-especifica, favorecendo as fêmeas provavelmente pela ação de hormônios sexuais com natureza protetora como o estrógeno.

6. PERSPECTIVAS

Baseados nos resultados mostrados na presente dissertação de Mestrado podemos estabelecer uma serie de perspectivas em torno a refinamento e utilização de outras técnicas ou modelos biológicos para estudar os mecanismos moleculares da modulação MeHg-VitA, um aprofundamento sobre efeitos biológicos ulteriores ao estresse oxidativo e parâmetros adicionais a nível comportamental e protocolos específicos que envolvam outras áreas do SNC. Entre algumas propostas para continuação do trabalho podemos especificar as seguintes:

1) Efetuar uma transposição em modelo *in vitro* focando em linhagem celulares neuronais como neuroblastoma derivado de humano (SH-SY5Y) ou célula-tronco neural (NSC) as quais são amplamente utilizadas para avaliação dos efeitos neurotóxicos do MeHg, sendo interessante corroborar efeitos de metabolitos ativos do palmitato de retinol como o ácido retinóico ou retinol;
2) Estudar e aprofundar outros parâmetros em modelos *in vitro* ou *in vivo* baseados no neurodesenvolvimento que permitam avaliar a neurogênesis durante episódios de co-exposição a MeHg-VitA em áreas como o hipocampo (especificamente o giro denteado);
3) Avaliar os efeitos de outros parâmetros redox específicos como a 3-nitrotirosina usada em modelos in vivo usando como foco a função cardíaca afetada pelo MeHg. Além disso, focar na avaliação da cadeia transportadora de elétrons, sendo a mitocôndria um conhecido alvo da toxicidade do MeHg;

4) Investigar outros parâmetros comportamentais ou tarefas de memória espacial hipocampo-dependentes como o Morris Water Maze, ou de aprendizado simples como o teste de Aversão ao Sabor;
5) Estudar os efeitos da combinação de outros compostos antioxidantes além da VitA, como a vitamina E (um conhecido neuroprotetor) ou vitamina C em termos de modular os efeitos tóxicos do MeHg. Nossos resultados mostraram que nos tecidos hepáticos e cardíaco foram apresentados efeitos pró-oxidantes que podem ser combatidos por outros antioxidantes mais seletivos;
6) Investigar o panorama de metabolitos do palmitato de retinol em outras estruturas do SNC que possam existir durante episódios de co-exposição com MeHg, em forma de estudos qualitativos e quantitativos usando metodologias de analise químico;
7) Investigar as modificações epigenéticas (metilação do DNA, metilação e acetilação de histonas) que podem acontecer durante episódios de co-exposição a MeHg e VitA a nível do SNC como de outros órgãos em modelos *in vitro* e *in vivo*.

REFERENCIAS

Abdel-Hamid, H. A., Fahmy, F. C. Sharaf, I. A. (2001). Influence of free radicals on cardiovascular risk due to occupational exposure to mercury. J Egypt Public Health Assoc 76(1-2): 53-69.

Al-Amin, M. M., Rahman, M. M., Khan, F. R., Zaman, F. Mahmud Reza, H. (2015). Astaxanthin improves behavioral disorder and oxidative stress in prenatal valproic acid-induced mice model of autism. Behavioural Brain Research 286: 112-121.

Al-Saleh, I., Nester, M., Abduljabbar, M., Al-Rouqi, R., Eltabache, C., Al-Rajudi, T. Elkhatib, R. (2016). Mercury (Hg) exposure and its effects on Saudi breastfed infant's neurodevelopment. Int J Hyg Environ Health 219(1): 129-141.

Albores-Garcia, D., Acosta-Saavedra, L. C., Hernandez, A. J., Loera, M. J. Calderon-Aranda, E. S. (2016). Early Developmental Low-Dose Methylmercury Exposure Alters Learning and Memory in Periadolescent but Not Young Adult Rats. Biomed Res Int 2016: 6532108.

Andersen, H. R. Andersen, O. (1993). Effects of dietary alpha-tocopherol and beta-carotene on lipid peroxidation induced by methyl mercuric chloride in mice. Pharmacol Toxicol 73(4): 192-201.

Aniya, Y., Shimoji, M. Naito, A. (1993). Increase in liver microsomal glutathione S-transferase activity by phenobarbital treatment of rats. Possible involvement of oxidative activation via cytochrome P450. Biochem Pharmacol 46(10): 1741-1747.

Anway, M. D., Cupp, A. S., Uzumcu, M. Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308(5727): 1466-1469.

Arthur, J. R. (2000). The glutathione peroxidases. Cell Mol Life Sci 57(13-14): 1825-1835.

Aschner, M. Aschner, J. L. (1990). Mercury neurotoxicity: mechanisms of blood-brain barrier transport. Neurosci Biobehav Rev 14(2): 169-176.

Aschner, M. Clarkson, T. W. (1988). Uptake of methylmercury in the rat brain: effects of amino acids. Brain Research 462(1): 31-39.

Aschner, M., Syversen, T., Souza, D. O., Rocha, J. B. Farina, M. (2007). Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. Braz J Med Biol Res 40(3): 285-291.

Azaïs-Braesco, V. Pascal, G. (2000). Vitamin A in pregnancy: requirements and safety limits. The American Journal of Clinical Nutrition 71(5): 1325s-1333s.

Baccarelli, A. Bollati, V. (2009). Epigenetics and environmental chemicals. Curr Opin Pediatr 21(2): 243-251.

Behr, G. A., Schnorr, C. E., Simoes-Pires, A., da Motta, L. L., Frey, B. N. Moreira, J. C. (2012). Increased cerebral oxidative damage and decreased antioxidant defenses in ovariectomized and sham-operated rats supplemented with vitamin A. Cell Biol Toxicol 28(5): 317-330.

Beltran-de-Miguel, B., Estevez-Santiago, R. Olmedilla-Alonso, B. (2015). Assessment of dietary vitamin A intake (retinol, alpha-carotene, beta-carotene, beta-cryptoxanthin) and its sources in the National Survey of Dietary Intake in Spain (2009-2010). Int J Food Sci Nutr 66(6): 706-712.

Belle, L. P., De Bona, K. S., Abdalla, F. H., Pimentel, V. C., Pigatto, A. S. Moretto, M. B. (2009). Comparative evaluation of adenosine deaminase activity in cerebral cortex and

hippocampus of young and adult rats: effect of garlic extract (Allium sativum L.) on their susceptibility to heavy metal exposure. Basic Clin Pharmacol Toxicol 104(5): 408-413.
Bellovino, D., Apreda, M., Gragnoli, S., Massimi, M. Gaetani, S. (2003). Vitamin A transport: in vitro models for the study of RBP secretion. Molecular Aspects of Medicine 24(6): 411-420.
Bellum, S., Bawa, B., Thuett, K. A., Stoica, G. Abbott, L. C. (2007). Changes in biochemical processes in cerebellar granule cells of mice exposed to methylmercury. Int J Toxicol 26(3): 261-269.
Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S. Kalayci, O. (2012). Oxidative Stress and Antioxidant Defense. The World Allergy Organization journal 5(1): 9-19.
Bisen-Hersh, E. B., Farina, M., Barbosa, F., Rocha, J. B. T. Aschner, M. (2014). Behavioral Effects of Developmental Methylmercury Drinking Water Exposure in Rodents. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS) 28(2): 117-124.
Bjornberg, K. A., Vahter, M., Berglund, B., Niklasson, B., Blennow, M. Sandborgh-Englund, G. (2005). Transport of methylmercury and inorganic mercury to the fetus and breast-fed infant. Environ Health Perspect 113(10): 1381-1385.
Blaner, W. S., Li, Y., Brun, P.-J., Yuen, J. J., Lee, S.-A. Clugston, R. D. (2016). Vitamin A Absorption, Storage and Mobilization. The Biochemistry of Retinoid Signaling II: The Physiology of Vitamin A - Uptake, Transport, Metabolism and Signaling. M. A. Asson-Batres and C. Rochette-Egly. Dordrecht, Springer Netherlands: 95-125.
Bonnet, E., Touyarot, K., Alfos, S., Pallet, V., Higueret, P. Abrous, D. N. (2008). Retinoic acid restores adult hippocampal neurogenesis and reverses spatial memory deficit in vitamin A deprived rats. PLoS One 3(10): e3487.
Bose, R., Onishchenko, N., Edoff, K., Janson Lang, A. M. Ceccatelli, S. (2012). Inherited Effects of Low-Dose Exposure to Methylmercury in Neural Stem Cells. Toxicological Sciences 130(2): 383-390.
Bridges, C. C. Zalups, R. K. (2005). Molecular and ionic mimicry and the transport of toxic metals. Toxicology and applied pharmacology 204(3): 274-308.
Bureau, I., Gueux, E., Mazur, A., Rock, E., Roussel, A. M. Rayssiguier, Y. (2003). Female rats are protected against oxidative stress during copper deficiency. J Am Coll Nutr 22(3): 239-246.
Cameron, H. A., Woolley, C. S., McEwen, B. S. Gould, E. (1993). Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56(2): 337-344.
Castaño, A., Cutanda, F., Esteban, M., Pärt, P., Navarro, C., Gómez, S., Rosado, M., López, A., López, E., Exley, K., Schindler, B. K., Govarts, E., Casteleyn, L., Kolossa-Gehring, M., Fiddicke, U., Koch, H., Angerer, J., Hond, E. D., Schoeters, G., Sepai, O., Horvat, M., Knudsen, L. E., Aerts, D., Joas, A., Biot, P., Joas, R., Jiménez-Guerrero, J. A., Diaz, G., Pirard, C., Katsonouri, A., Cerna, M., Gutleb, A. C., Ligocka, D., Reis, F. M., Berglund, M., Lupsa, I.-R., Halzlová, K., Charlier, C., Cullen, E., Hadjipanayis, A., Krsková, A., Jensen, J. F., Nielsen, J. K., Schwedler, G., Wilhelm, M., Rudnai, P., Középesy, S., Davidson, F., Fischer, M. E., Janasik, B., Namorado, S., Gurzau, A. E., Jajcaj, M., Mazej, D., Tratnik, J. S., Larsson, K., Lehmann, A., Crettaz, P., Lavranos, G. Posada, M. (2015). Fish consumption patterns and hair mercury levels in children and their mothers in 17 EU countries. Environmental Research 141: 58-68.
Castoldi, A. F., Coccini, T., Ceccatelli, S. Manzo, L. (2001). Neurotoxicity and molecular effects of methylmercury. Brain Res Bull 55(2): 197-203.

CDC (2009). Fourth National Report on Human Exposure To Environmental Chemicals. Center for Disease Control and Prevention. Prevention. U.S. Department of Health and Human Services, Atlanta, USA.

Ceccatelli, S., Bose, R., Edoff, K., Onishchenko, N. Spulber, S. (2013). Long-lasting neurotoxic effects of exposure to methylmercury during development. J Intern Med 273(5): 490-497.

Cisneros-Montemayor, A. M., Pauly, D., Weatherdon, L. V. Ota, Y. (2016). A Global Estimate of Seafood Consumption by Coastal Indigenous Peoples. PLoS ONE 11(12): e0166681.

Clagett-Dame, M. Knutson, D. (2011). Vitamin A in Reproduction and Development. Nutrients 3(4): 385-428.

Cordier, S., Garel, M., Mandereau, L., Morcel, H., Doineau, P., Gosme-Seguret, S., Josse, D., White, R. Amiel-Tison, C. (2002). Neurodevelopmental investigations among methylmercury-exposed children in French Guiana. Environ Res 89(1): 1-11.

Crespo-Lopez, M. E., Costa-Malaquias, A., Oliveira, E. H. C., Miranda, M. S., Arrifano, G. P. F., Souza-Monteiro, J. R., Sagica, F. E.-S., Fontes-Junior, E. A., Maia, C. S. F., Macchi, B. M. do Nascimento, J. L. M. (2016). Is Low Non-Lethal Concentration of Methylmercury Really Safe? A Report on Genotoxicity with Delayed Cell Proliferation. PLoS ONE 11(9): e0162822.

Crespo-Lopez, M. E., Macedo, G. L., Arrifano, G. P., Pinheiro, M. C., Martins do Nascimento, J. L. Herculano, A. M. (2011). Genotoxicity of mercury: Contributing for the analysis of Amazonian populations. Environment International 37(1): 136-141.

Crespo-López, M. E., Macêdo, G. L., Pereira, S. I. D., Arrifano, G. P. F., Picanço-Diniz, D. L. W., do Nascimento, J. L. M. Herculano, A. M. (2009). Mercury and human genotoxicity: Critical considerations and possible molecular mechanisms. Pharmacological Research 60(4): 212-220.

Cusack, L. K., Smit, E., Kile, M. L. Harding, A. K. (2017). Regional and temporal trends in blood mercury concentrations and fish consumption in women of child bearing Age in the united states using NHANES data from 1999–2010. Environmental Health 16: 10.

Chapman, M. S. (2012). Vitamin a: history, current uses, and controversies. Semin Cutan Med Surg 31(1): 11-16.

Chen, Y., Ozturk, N. C. Zhou, F. C. (2013). DNA Methylation Program in Developing Hippocampus and Its Alteration by Alcohol. PLoS ONE 8(3): e60503.

Dai, H., Krost, M. Carey, R. J. (1995). A new methodological approach to the study of habituation: the use of positive and negative behavioral indices of habituation. Journal of Neuroscience Methods 62(1): 169-174.

de Oliveira, M. R., de Bittencourt Pasquali, M. A., Silvestrin, R. B., Mello, E. S. T. Moreira, J. C. (2007a). Vitamin A supplementation induces a prooxidative state in the striatum and impairs locomotory and exploratory activity of adult rats. Brain Res 1169: 112-119.

de Oliveira, M. R., Silvestrin, R. B., Mello e Souza, T. Moreira, J. C. (2008). Therapeutic vitamin A doses increase the levels of markers of oxidative insult in substantia nigra and decrease locomotory and exploratory activity in rats after acute and chronic supplementation. Neurochem Res 33(3): 378-383.

de Oliveira, M. R., Silvestrin, R. B., Mello, E. S. T. Moreira, J. C. (2007b). Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: effects of sub acute vitamin A supplementation at therapeutic doses. Neurotoxicology 28(6): 1191-1199.

Debes, F., Budtz-Jørgensen, E., Weihe, P., White, R. F. Grandjean, P. (2006). Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years. Neurotoxicology and teratology 28(3): 363-375.

del Balzo, V., Vitiello, V., Germani, A., Donini, L. M., Poggiogalle, E. Pinto, A. (2014). A Cross-Sectional Survey on Dietary Supplements Consumption among Italian Teen-Agers. PLoS ONE 9(7): e100508.

do Nascimento, J. L., Oliveira, K. R., Crespo-Lopez, M. E., Macchi, B. M., Maues, L. A., Pinheiro Mda, C., Silveira, L. C. Herculano, A. M. (2008). Methylmercury neurotoxicity & antioxidant defenses. Indian J Med Res 128(4): 373-382.

Dong, Z., Jim, R. C., Hatley, E. L., Backus, A. S. N., Shine, J. P., Spengler, J. D. Schaider, L. A. (2015). A Longitudinal Study of Mercury Exposure Associated with Consumption of Freshwater Fish from a Reservoir in Rural South Central USA. Environmental research 136: 155-162.

Dorea, J. G., Marques, R. C. Isejima, C. (2012). Neurodevelopment of Amazonian infants: antenatal and postnatal exposure to methyl- and ethylmercury. J Biomed Biotechnol 2012: 132876.

Dreiem, A., Gertz, C. C. Seegal, R. F. (2005). The effects of methylmercury on mitochondrial function and reactive oxygen species formation in rat striatal synaptosomes are age-dependent. Toxicol Sci 87(1): 156-162.

Eto, K. (2000). Minamata disease. Neuropathology 20 Suppl: S14-19.

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome, Food and Agriculture Organization of the United Nations.

Fergusson, J. E. (1990). The heavy elements: chemistry, environmental impact, and health effects, Pergamon Press.

Ferraro, L., Tomasini, M. C., Tanganelli, S., Mazza, R., Coluccia, A., Carratu, M. R., Gaetani, S., Cuomo, V. Antonelli, T. (2009). Developmental exposure to methylmercury elicits early cell death in the cerebral cortex and long-term memory deficits in the rat. Int J Dev Neurosci 27(2): 165-174.

Fox, D. A., Grandjean, P., de Groot, D. Paule, M. G. (2012). Developmental origins of adult diseases and neurotoxicity: epidemiological and experimental studies. Neurotoxicology 33(4): 810-816.

Franco, J. L., Posser, T., Dunkley, P. R., Dickson, P. W., Mattos, J. J., Martins, R., Bainy, A. C., Marques, M. R., Dafre, A. L. Farina, M. (2009). Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. Free Radic Biol Med 47(4): 449-457.

Franco, J. L., Teixeira, A., Meotti, F. C., Ribas, C. M., Stringari, J., Garcia Pomblum, S. C., Moro, A. M., Bohrer, D., Bairros, A. V., Dafre, A. L., Santos, A. R. Farina, M. (2006). Cerebellar thiol status and motor deficit after lactational exposure to methylmercury. Environ Res 102(1): 22-28.

Freeman, B. A. Crapo, J. D. (1981). Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J Biol Chem 256(21): 10986-10992.

Fujimura, M., Cheng, J. Zhao, W. (2012). Perinatal exposure to low-dose methylmercury induces dysfunction of motor coordination with decreases in synaptophysin expression in the cerebellar granule cells of rats. Brain Res 1464: 1-7.

Gale, C. R., O'Callaghan, F. J., Godfrey, K. M., Law, C. M. Martyn, C. N. (2004). Critical periods of brain growth and cognitive function in children. Brain 127(Pt 2): 321-329.

Gandhi, D. K Rajput, D. (2016). Effects of maternal exposure to methylmercury (MeHg) on F1 generation behavior in the rat. Int J Neural Sci Brain Res 1(1): 6-16.
Gandhi, D. N., Panchal, G. M. Dhull, D. K. (2013). Influence of gestational exposure on the effects of prenatal exposure to methyl mercury on postnatal development in rats. Cent Eur J Public Health 21(1): 30-35.
Gandhi, D. N., Panchal, G. M. Dhull, D. K. (2014). Neurobehavioral toxicity in progeny of rat mothers exposed to methylmercury during gestation. Ann Ist Super Sanita 50(1): 28-37.
Gasparotto, J., Petiz, L. L., Girardi, C. S., Bortolin, R. C., de Vargas, A. R., Henkin, B. S., Chaves, P. R., Roncato, S., Matte, C., Zanotto-Filho, A., Moreira, J. C. Gelain, D. P. (2015). Supplementation with vitamin A enhances oxidative stress in the lungs of rats submitted to aerobic exercise. Appl Physiol Nutr Metab 40(12): 1253-1261.
Glover, C. N., Zheng, D., Jayashankar, S., Sales, G. D., Hogstrand, C. Lundebye, A. K. (2009). Methylmercury speciation influences brain gene expression and behavior in gestationally-exposed mice pups. Toxicol Sci 110(2): 389-400.
Gracia, L., Marrugo, J. Alvis, E. (2010). Contaminación por mercurio en humanos y peces en el municipio de Ayapel, Córdoba, Colombia, 2009. Rev. Fac. Nac. Salud Pública 28(2): 118-124.
Gribble, M. O., Karimi, R., Feingold, B. J., Nyland, J. F., O'Hara, T. M., Gladyshev, M. I. Chen, C. Y. (2016). Mercury, selenium and fish oils in marine food webs and implications for human health. Journal of the Marine Biological Association of the United Kingdom. Marine Biological Association of the United Kingdom 96(1): 43-59.
Grotto, D., Valentini, J., Fillion, M., Passos, C. J. S., Garcia, S. C., Mergler, D. Barbosa Jr, F. (2010). Mercury exposure and oxidative stress in communities of the Brazilian Amazon. Science of The Total Environment 408(4): 806-811.
Halbach, S. (1990). Mercury compounds: lipophilicity and toxic effects on isolated myocardial tissue. Arch Toxicol 64(4): 315-319.
Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? J Neurochem 97(6): 1634-1658.
Halliwell, B. Gutteridge, J. M. C. (2015). Free Radicals in Biology and Medicine, Oxford University Press.
Hassanzadeh, P., Arbabi, E., Atyabi, F. Dinarvand, R. (2017). Ferulic acid exhibits antiepileptogenic effect and prevents oxidative stress and cognitive impairment in the kindling model of epilepsy. Life Sciences 179: 9-14.
Heyward, F. D. Sweatt, J. D. (2015). DNA Methylation in Memory Formation:Emerging Insights. The Neuroscientist 21(5): 475-489.
Hillerer, K. M., Neumann, I. D., Couillard-Despres, S., Aigner, L. Slattery, D. A. (2013). Sex-dependent regulation of hippocampal neurogenesis under basal and chronic stress conditions in rats. Hippocampus 23(6): 476-487.
Hsi, H. C., Hsu, Y. W., Chang, T. C. Chien, L. C. (2016). Methylmercury Concentration in Fish and Risk-Benefit Assessment of Fish Intake among Pregnant versus Infertile Women in Taiwan. PLoS One 11(5): e0155704.
Hu, G., Jin, M., Lin, X., Guo, C., Zhang, L. Sun, Z. (2010). Mercury distribution in neonatal rat brain after intrauterine methylmercury exposure. Environ Toxicol Pharmacol 29(1): 7-11.
Huang, C. F., Liu, S. H., Hsu, C. J. Lin-Shiau, S. Y. (2011). Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice. Toxicol Lett 201(3): 196-204.

Hughes, W. L. (1957). A physicochemical rationale for the biological activity of mercury and its compounds. Ann N Y Acad Sci 65(5): 454-460.
Inostroza, M., Brotons-Mas, J. R., Laurent, F., Cid, E. de la Prida, L. M. (2013). Specific impairment of "what-where-when" episodic-like memory in experimental models of temporal lobe epilepsy. J Neurosci 33(45): 17749-17762.
IOM (2001). Front Matter. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. Washington, The National Academies Press.
Jayashankar, S., Glover, C. N., Folven, K. I., Brattelid, T., Hogstrand, C. Lundebye, A. K. (2012). Cerebral gene expression and neurobehavioural responses in mice pups exposed to methylmercury and docosahexaenoic acid through the maternal diet. Environmental Toxicology and Pharmacology 33(1): 26-38.
Jensen, T. K., Grandjean, P., Jorgensen, E. B., White, R. F., Debes, F. Weihe, P. (2005). Effects of breast feeding on neuropsychological development in a community with methylmercury exposure from seafood. J Expo Anal Environ Epidemiol 15(5): 423-430.
Jo, E. M., Kim, B. G., Kim, Y. M., Yu, S. D., You, C. H., Kim, J. Y. Hong, Y. S. (2010). Blood mercury concentration and related factors in an urban coastal area in Korea. J Prev Med Public Health 43(5): 377-386.
Kanda, H., Shinkai, Y. Kumagai, Y. (2014). S-mercuration of cellular proteins by methylmercury and its toxicological implications. J Toxicol Sci 39(5): 687-700.
Kantor, E. D., Rehm, C. D., Du, M., White, E. Giovannucci, E. L. (2016). Trends in Dietary Supplement Use among US Adults From 1999–2012. JAMA 316(14): 1464-1474.
Karagas, M. R., Choi, A. L., Oken, E., Horvat, M., Schoeny, R., Kamai, E., Cowell, W., Grandjean, P. Korrick, S. (2012). Evidence on the human health effects of low-level methylmercury exposure. Environ Health Perspect 120(6): 799-806.
Kawaguchi, R., Zhong, M., Kassai, M., Ter-Stepanian, M. Sun, H. (2015). Vitamin A Transport Mechanism of the Multitransmembrane Cell-Surface Receptor STRA6. Membranes (Basel) 5(3): 425-453.
Khanna, S., Roy, S., Parinandi, N. L., Maurer, M. Sen, C. K. (2006). Characterization of the potent neuroprotective properties of the natural vitamin E alpha-tocotrienol. J Neurochem 98(5): 1474-1486.
Kienzler, A., Bopp, S. K., van der Linden, S., Berggren, E. Worth, A. (2016). Regulatory assessment of chemical mixtures: Requirements, current approaches and future perspectives. Regulatory Toxicology and Pharmacology 80: 321-334.
Kim, E. H., Kim, I. K., Kwon, J. Y., Kim, S. W. Park, Y. W. (2006). The Effect of Fish Consumption on Blood Mercury Levels of Pregnant Women. Yonsei Medical Journal 47(5): 626-633.
Kim, S.-A., Kwon, Y., Kim, S. Joung, H. (2016). Assessment of Dietary Mercury Intake and Blood Mercury Levels in the Korean Population: Results from the Korean National Environmental Health Survey 2012–2014. International Journal of Environmental Research and Public Health 13(9): 877.
Klamt, F., Roberto de Oliveira, M. Moreira, J. C. F. (2005). Retinol induces permeability transition and cytochrome c release from rat liver mitochondria. Biochimica et Biophysica Acta (BBA) - General Subjects 1726(1): 14-20.
Knoth, R., Singec, I., Ditter, M., Pantazis, G., Capetian, P., Meyer, R. P., Horvat, V., Volk, B. Kempermann, G. (2010). Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One 5(1): e8809.

Kondo, K. (1996). [Incidence of Minamata disease in communities along the Agano river, Niigata, Japan--patterns of the exposure and official diagnosis of patients]. Nihon Eiseigaku Zasshi 51(2): 599-611.

Kondo, K. (2000). Congenital Minamata disease: warnings from Japan's experience. J Child Neurol 15(7): 458-464.

Korge, P., Ping, P. Weiss, J. N. (2008). Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. Circ Res 103(8): 873-880.

Lane, M. A. Bailey, S. J. (2005). Role of retinoid signalling in the adult brain. Prog Neurobiol 75(4): 275-293.

Lepham, L., Cermichiari, E., Cox, C., Meyers, G., Baggs, R., Brewer, R., Shamlaye, C., Davidson, P. Clarkson , T. (1995). An analysis of autopsy of brain tissue from infants prematurely exposed to methylmercury. Neurotoxicology 16: 689–704.

Leussis, M. P. Bolivar, V. J. (2006). Habituation in rodents: A review of behavior, neurobiology, and genetics. Neuroscience & Biobehavioral Reviews 30(7): 1045-1064.

Liang, J., Inskip, M., Newhook, D. Messier, C. (2009). Neurobehavioral effect of chronic and bolus doses of methylmercury following prenatal exposure in C57BL/6 weanling mice. Neurotoxicol Teratol 31(6): 372-381.

Lieberwirth, C., Pan, Y., Liu, Y., Zhang, Z. Wang, Z. (2016). HIPPOCAMPAL ADULT NEUROGENESIS: ITS REGULATION AND POTENTIAL ROLE IN SPATIAL LEARNING AND MEMORY. Brain research 1644: 127-140.

Liu, L., van Groen, T., Kadish, I. Tollefsbol, T. O. (2009). DNA methylation impacts on learning and memory in aging. Neurobiol Aging 30(4): 549-560.

Loi, M., Koricka, S., Lucassen, P. J. Joëls, M. (2014). Age- and Sex-Dependent Effects of Early Life Stress on Hippocampal Neurogenesis. Frontiers in Endocrinology 5: 13.

Lucena, G. M., Prediger, R. D., Silva, M. V., Santos, S. N., Silva, J. F. B., Santos, A. R. S., Azevedo, M. S. Ferreira, V. M. (2013). Ethanolic extract from bulbs of Cipura paludosa reduced long-lasting learning and memory deficits induced by prenatal methylmercury exposure in rats. Developmental Cognitive Neuroscience 3: 1-10.

Machi, J. F., Dias, D. d. S., Freitas, S. C., de Moraes, O. A., da Silva, M. B., Cruz, P. L., Mostarda, C., Salemi, V. M. C., Morris, M., De Angelis, K. Irigoyen, M.-C. (2016). Impact of aging on cardiac function in a female rat model of menopause: role of autonomic control, inflammation, and oxidative stress. Clinical Interventions in Aging 11: 341-350.

Manfroi, C. B., Schwalm, F. D., Cereser, V., Abreu, F., Oliveira, A., Bizarro, L., Rocha, J. B., Frizzo, M. E., Souza, D. O. Farina, M. (2004). Maternal milk as methylmercury source for suckling mice: neurotoxic effects involved with the cerebellar glutamatergic system. Toxicol Sci 81(1): 172-178.

Marques, R. C., Abreu, L., Bernardi, J. V. Dorea, J. G. (2016). Traditional living in the Amazon: Extended breastfeeding, fish consumption, mercury exposure, and neurodevelopment. Ann Hum Biol: 1-34.

Marques, R. C., Bernardi, J. V., Dorea, J. G., Leao, R. S. Malm, O. (2013). Mercury transfer during pregnancy and breastfeeding: hair mercury concentrations as biomarker. Biol Trace Elem Res 154(3): 326-332.

Marques, R. C., Dorea, J. G., Bernardi, J. V., Bastos, W. R. Malm, O. (2009). Prenatal and postnatal mercury exposure, breastfeeding and neurodevelopment during the first 5 years. Cogn Behav Neurol 22(2): 134-141.

Marrugo-Negrete, J., Benitez, L. Olivero-Verbel, J. (2008a). Distribution of Mercury in Several Environmental Compartments in an Aquatic Ecosystem Impacted by Gold Mining in Northern Colombia. Archives of environmental contamination and toxicology 55: 305–316.

Marrugo-Negrete, J., Olivero-Verbel, J., Ceballos, E. L. Benitez, L. N. (2008b). Total mercury and methylmercury concentrations in fish from the Mojana region of Colombia. Environmental geochemistry and health 30(1): 21-30.

Marrugo, J., Lans, E. Benítez, L. (2007). Hallazgo de mercurio en peces de la ciénaga de Ayapel, Córdoba, Colombia. Revista MVZ Córdoba 12(1): 878-886.

McCaffery, P., Adams, J., Maden, M. Rosa-Molinar, E. (2003). Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. Eur J Neurosci 18(3): 457-472.

McCaffery, P., Zhang, J. Crandall, J. E. (2006). Retinoic acid signaling and function in the adult hippocampus. J Neurobiol 66(7): 780-791.

McCauley, M. E., van den Broek, N., Dou, L. Othman, M. (2015). Vitamin A supplementation during pregnancy for maternal and newborn outcomes. Cochrane Database of Systematic Reviews(10).

Moretto, M. B., Funchal, C., Santos, A. Q., Gottfried, C., Boff, B., Zeni, G., Pureur, R. P., Souza, D. O., Wofchuk, S. Rocha, J. B. (2005). Ebselen protects glutamate uptake inhibition caused by methyl mercury but does not by Hg2+. Toxicology 214(1-2): 57-66.

Murata, M. Kawanishi, S. (2000). Oxidative DNA damage by vitamin A and its derivative via superoxide generation. Journal of Biological Chemistry 275(3): 2003-2008.

Nabi, S. (2014a). Exposure, Sources and Intoxication. Toxic Effects of Mercury, Springer: 59.

Nabi, S. (2014b). Mercury and Cognition. Toxic Effects of Mercury, Springer: 36-47.

Nabi, S. (2014c). Toxic Effects and Remedy. Toxic Effects of Mercury, Springer: 20.

Nabi, S. (2014d). Toxic Effects of Mercury, Springer India.

Napoli, J. L. (2012). Physiological insights into all-trans-retinoic acid biosynthesis. Biochim Biophys Acta 1821(1): 152-167.

Newland, M. C., Hoffman, D. J., Heath, J. C. Donlin, W. D. (2013). Response inhibition is impaired by developmental methylmercury exposure: acquisition of low-rate lever-pressing. Behav Brain Res 253: 196-205.

Newland, M. C., Reed, M. N. Rasmussen, E. (2015). A Hypothesis About How Early Developmental Methylmercury Exposure Disrupts Behavior in Adulthood. Behavioural processes 114: 41-51.

Nguyen, A. T., Armstrong, E. A. Yager, J. Y. (2017). Neurodevelopmental Reflex Testing in Neonatal Rat Pups. J Vis Exp(122).

Nielsen, J. B. Andersen, O. (1996). Elimination of recently absorbed methyl mercury depends on age and gender. Pharmacol Toxicol 79(2): 60-64.

NIH. (2016). Vitamin A. Fact Sheet for Health Professionals. Retrieved 15/08/2017, 2017, from https://ods.od.nih.gov/factsheets/VitaminA-HealthProfessional/#en1.

Obiorah, M., McCandlish, E., Buckley, B. DiCicco-Bloom, E. (2015). Hippocampal developmental vulnerability to methylmercury extends into prepubescence. Frontiers in Neuroscience 9: 150.

Ogura, H., Takeuchi, T. Morimoto, K. (1996). A comparison of the 8-hydroxydeoxyguanosine, chromosome aberrations and micronucleus techniques for the assessment of the genotoxicity of mercury compounds in human blood lymphocytes. Mutation Research/Reviews in Genetic Toxicology 340(2-3): 175-182.

Oken, E., Choi, A. L., Karagas, M. R., Marien, K., Rheinberger, C. M., Schoeny, R., Sunderland, E. Korrick, S. (2012). Which fish should I eat? Perspectives influencing fish consumption choices. Environ Health Perspect 120(6): 790-798.

Oliveira, A. M. M. (2016). DNA methylation: a permissive mark in memory formation and maintenance. Learning & Memory 23(10): 587-593.

Oliveira, M. R. (2015). The neurotoxic effects of vitamin A and retinoids. An Acad Bras Cienc 87(2 Suppl): 1361-1373.

Olivero-Verbel, J., Caballero-Gallardo, K. Marrugo Negrete, J. (2011). Relationship between localization of gold mining areas and hair mercury levels in people from Bolivar, north of Colombia. Biological trace element research 144(1-3): 118-132.

Olivero-Verbel, J. Johnson-Restrepo, B. (2002). El lado gris de la mineria del oro: La contaminación con mercurio en el norte de Colombia. Cartagena, Alpha Impresiones.

Olivero-Verbel, J., Johnson-Restrepo, B., Mendoza-Marín, C., Paz-Martínez, R. Olivero-Verbel, R. (2004). Mercury in the aquatic environment of the village of Caimito at the Mojana region, north of Colombia. Water, Air, and Soil Pollution 159: 409-420.

Olivero, J., Johnson, B. Arguello, E. (2002). Human exposure to mercury in San Jorge river basin, Colombia (South America). Science Of The Total Environment 289: 41–47.

Onishchenko, N., Karpova, N., Sabri, F., Castren, E. Ceccatelli, S. (2008). Long-lasting depression-like behavior and epigenetic changes of BDNF gene expression induced by perinatal exposure to methylmercury. J Neurochem 106(3): 1378-1387.

Onishchenko, N., Tamm, C., Vahter, M., Hokfelt, T., Johnson, J. A., Johnson, D. A. Ceccatelli, S. (2007). Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicol Sci 97(2): 428-437.

Osakada, F., Hashino, A., Kume, T., Katsuki, H., Kaneko, S. Akaike, A. (2004). Alpha-tocotrienol provides the most potent neuroprotection among vitamin E analogs on cultured striatal neurons. Neuropharmacology 47(6): 904-915.

Paletz, E. M., Craig-Schmidt, M. C. Newland, M. C. (2006). Gestational exposure to methylmercury and n-3 fatty acids: effects on high- and low-rate operant behavior in adulthood. Neurotoxicol Teratol 28(1): 59-73.

Penner, M. R., Parrish, R. R., Hoang, L. T., Roth, T. L., Lubin, F. D. Barnes, C. A. (2016). Age-related changes in Egr1 transcription and DNA methylation within the hippocampus. Hippocampus 26(8): 1008-1020.

Pilsner, J. R., Lazarus, A. L., Nam, D. H., Letcher, R. J., Sonne, C., Dietz, R. Basu, N. (2010). Mercury-associated DNA hypomethylation in polar bear brains via the LUminometric Methylation Assay: a sensitive method to study epigenetics in wildlife. Mol Ecol 19(2): 307-314.

Platel, A. Porsolt, R. D. (1982). Habituation of exploratory activity in mice: a screening test for memory enhancing drugs. Psychopharmacology (Berl) 78(4): 346-352.

Quadro, L., Hamberger, L., Gottesman, M. E., Wang, F., Colantuoni, V., Blaner, W. S. Mendelsohn, C. L. (2005). Pathways of vitamin A delivery to the embryo: insights from a new tunable model of embryonic vitamin A deficiency. Endocrinology 146(10): 4479-4490.

Ray, P. D., Huang, B.-W. Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling 24(5): 981-990.

Razzaghi, H. Tinker, S. C. (2014). Seafood consumption among pregnant and non-pregnant women of childbearing age in the United States, NHANES 1999–2006. Food & Nutrition Research 58: 10.3402/fnr.v3458.23287.

Reed, M. N., Banna, K. M., Donlin, W. D. Newland, M. C. (2008). Effects of gestational exposure to methylmercury and dietary selenium on reinforcement efficacy in adulthood. Neurotoxicol Teratol 30(1): 29-37.

Rice, G., Swartout, J., Mahaffey, K. Schoeny, R. (2000). Derivation of U.S. EPA's oral Reference Dose (RfD) for methylmercury. Drug Chem Toxicol 23(1): 41-54.

Romero, F. J., Bosch-Morell, F., Romero, M. J., Jareno, E. J., Romero, B., Marin, N. Roma, J. (1998). Lipid peroxidation products and antioxidants in human disease. Environ Health Perspect 106 Suppl 5: 1229-1234.

Ross, C. A., Ed. (2010). Vitamin A. Encyclopedia of Dietary Supplements 2nd Edition. New York, Informa Healthcare.

Rubino, F. M. (2015). Toxicity of Glutathione-Binding Metals: A Review of Targets and Mechanisms. Toxics 3(1): 20-62.

Sakamoto, M., Chan, H. M., Domingo, J. L., Kubota, M. Murata, K. (2012). Changes in body burden of mercury, lead, arsenic, cadmium and selenium in infants during early lactation in comparison with placental transfer. Ecotoxicol Environ Saf 84: 179-184.

Sakamoto, M., Kakita, A., Wakabayashi, K., Takahashi, H., Nakano, A. Akagi, H. (2002). Evaluation of changes in methylmercury accumulation in the developing rat brain and its effects: a study with consecutive and moderate dose exposure throughout gestation and lactation periods. Brain Res 949(1-2): 51-59.

Schneider, T. Przewlocki, R. (2005). Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30(1): 80-89.

Schnorr, C. E., Bittencourt Lda, S., Petiz, L. L., Gelain, D. P., Zeidan-Chulia, F. Moreira, J. C. (2015). Chronic retinyl palmitate supplementation to middle-aged Wistar rats disrupts the brain redox homeostasis and induces changes in emotional behavior. Mol Nutr Food Res 59(5): 979-990.

Schnorr, C. E., da Silva Morrone, M., Simoes-Pires, A., da Rocha, R. F., Behr, G. A. Moreira, J. C. (2011a). Vitamin A supplementation in rats under pregnancy and nursing induces behavioral changes and oxidative stress upon striatum and hippocampus of dams and their offspring. Brain Res 1369: 60-73.

Schnorr, C. E., Morrone Mda, S., Weber, M. H., Lorenzi, R., Behr, G. A. Moreira, J. C. (2011b). The effects of vitamin A supplementation to rats during gestation and lactation upon redox parameters: increased oxidative stress and redox modulation in mothers and their offspring. Food Chem Toxicol 49(10): 2645-2654.

Sell, C. S. (2014). Chemistry and the Sense of Smell, Wiley.

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. International Journal of Preventive Medicine 4(6): 624-630.

Shanker, G., Mutkus, L. A., Walker, S. J. Aschner, M. (2002). Methylmercury enhances arachidonic acid release and cytosolic phospholipase A2 expression in primary cultures of neonatal astrocytes. Brain Res Mol Brain Res 106(1-2): 1-11.

Sheehan, M. C., Burke, T. A., Navas-Acien, A., Breysse, P. N., McGready, J. Fox, M. A. (2014). Global methylmercury exposure from seafood consumption and risk of developmental neurotoxicity: a systematic review. Bulletin of the World Health Organization 92(4): 254-269F.

Sherin, J. Sumathi, T. (2016). Neurotoxic effects of gestational exposure of methyl mercury on different brain regions of F1 generation; neurobehavioural, biochemical and histological study during weaning period of rat.

Shimizu, N., Kobayashi, K. Hayashi, K. (1984). The reaction of superoxide radical with catalase. Mechanism of the inhibition of catalase by superoxide radical. J Biol Chem 259(7): 4414-4418.
Shirakami, Y., Lee, S.-A., Clugston, R. D. Blaner, W. S. (2012). HEPATIC METABOLISM OF RETINOIDS AND DISEASE ASSOCIATIONS. Biochimica et biophysica acta 1821(1): 124-136.
Smith, J. H., McCormack, K. M., Braselton, W. E., Jr. Hook, J. B. (1983). The effect of prenatal methylmercury administration on postnatal renal functional development. Environ Res 30(1): 63-71.
Snell, K., Ashby, S. L. Barton, S. J. (1977). Disturbances of perinatal carbohydrate metabolism in rats exposed to methylmercury in utero. Toxicology 8(3): 277-280.
Snodgrass, S. R. (1992). Vitamin neurotoxicity. Mol Neurobiol 6(1): 41-73.
So, A. Y., Jung, J. W., Lee, S., Kim, H. S. Kang, K. S. (2011). DNA methyltransferase controls stem cell aging by regulating BMI1 and EZH2 through microRNAs. PLoS One 6(5): e19503.
Sokolowski, K., Obiorah, M., Robinson, K., McCandlish, E., Buckley, B. DiCicco-Bloom, E. (2013). Neural stem cell apoptosis after low-methylmercury exposures in postnatal hippocampus produce persistent cell loss and adolescent memory deficits. Dev Neurobiol 73(12): 936-949.
Spiegler, E., Kim, Y.-K., Wassef, L., Shete, V. Quadro, L. (2012). Maternal-fetal transfer and metabolism of vitamin A and its precursor β-carotene in the developing tissues. Biochimica et biophysica acta 1821(1): 88-98.
Stępniewska, J., Dołęgowska, B., Popińska, M., Sałata, D., Budkowska, M., Gołembiewska, E., Myślak, M., Domański, M., Marchelek-Myśliwiec, M. Ciechanowski, K. (2014). Prooxidative-Antioxidative Balance of Cells in Different Types of Renal Replacement Therapy. Blood Purification 37(1): 4-11.
Steuerwald, U., Weihe, P., Jorgensen, P. J., Bjerve, K., Brock, J., Heinzow, B., Budtz-Jorgensen, E. Grandjean, P. (2000). Maternal seafood diet, methylmercury exposure, and neonatal neurologic function. J Pediatr 136(5): 599-605.
Stoltzfus, R. J. Underwood, B. A. (1995). Breast-milk vitamin A as an indicator of the vitamin A status of women and infants. Bulletin of the World Health Organization 73(5): 703-711.
Sunderland, E. M., Li, M. Bullard, K. (2018). Decadal Changes in the Edible Supply of Seafood and Methylmercury Exposure in the United States. Environ Health Perspect 126(1): 017006.
Sureshbabu, A., Ryter, S. W. Choi, M. E. (2015). Oxidative stress and autophagy: Crucial modulators of kidney injury. Redox Biology 4: 208-214.
Sutton, D., Tchounwou, P., Ninashvili, N. Shen, E. (2002). Mercury Induces Citotoxicity and Transcriptionally Activates Stress Genes in Human Liver Carcinoma ($HepG_2$) Cells. International Journal of Molecular Sciences 3: 965-984.
Takeuchi, T., Eto, N. Eto, K. (1979). Neuropathology of childhood cases of methylmercury poisoning (Minamata disease) with prolonged symptoms, with particular reference to the decortication syndrome. Neurotoxicology 1: 1–20.
Tamm, C., Duckworth, J., Hermanson, O. Ceccatelli, S. (2006). High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation. J Neurochem 97(1): 69-78.

Taylor, C. M., Golding, J. Emond, A. M. (2016). Blood mercury levels and fish consumption in pregnancy: Risks and benefits for birth outcomes in a prospective observational birth cohort. International Journal of Hygiene and Environmental Health 219(6): 513-520.

Thiel, C. M., Müller, C. P., Huston, J. P. Schwarting, R. K. W. (1999). High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments. Neuroscience 93(1): 243-251.

Thompson, L. P. Al-Hasan, Y. (2012). Impact of Oxidative Stress in Fetal Programming. Journal of Pregnancy 2012: 8.

Touyarot, K., Bonhomme, D., Roux, P., Alfos, S., Lafenetre, P., Richard, E., Higueret, P. Pallet, V. (2013). A mid-life vitamin A supplementation prevents age-related spatial memory deficits and hippocampal neurogenesis alterations through CRABP-I. PLoS One 8(8): e72101.

Townsend, D. M., Tew, K. D. Tapiero, H. (2003). The importance of glutathione in human disease. Biomed Pharmacother 57(3-4): 145-155.

Tsuchiya, A., Hinners, T. A., Burbacher, T. M., Faustman, E. M. Mariën, K. (2008). Mercury Exposure from Fish Consumption Within the Japanese and Korean Communities. Journal of Toxicology and Environmental Health, Part A 71(15): 1019-1031.

Uchendu, C., Ambali, S. F., Ayo, J. O., Esievo, K. A. N. Umosen, A. J. (2014). Erythrocyte osmotic fragility and lipid peroxidation following chronic co-exposure of rats to chlorpyrifos and deltamethrin, and the beneficial effect of alpha-lipoic acid. Toxicol Rep 1: 373-378.

UNEP. (2013). Global Mercury Assessment Report. New York:United Nations Environmental Programme. Retrieved 27 February 2017, from http://www.unep.org/PDF/PressReleases/GlobalMercuryAssessment2013.pdf.

Urvalek, A. M. Gudas, L. J. (2014). Retinoic acid and histone deacetylases regulate epigenetic changes in embryonic stem cells. J Biol Chem 289(28): 19519-19530.

USDA. (2015). Dietary Guidelines for Americans 2015-2020. Eight Edition. Retrieved 25/03/2018, 2018, from https://health.gov/dietaryguidelines/2015/guidelines/.

Usuki, F., Yasutake, A., Umehara, F., Tokunaga, H., Matsumoto, M., Eto, K., Ishiura, S. Higuchi, I. (2001). In vivo protection of a water-soluble derivative of vitamin E, Trolox, against methylmercury-intoxication in the rat. Neurosci Lett 304(3): 199-203.

Veal, E. A., Day, A. M. Morgan, B. A. (2007). Hydrogen peroxide sensing and signaling. Mol Cell 26(1): 1-14.

Veloso, C. A., Oliveira, B. F., Mariani, F. E., Fagundes-Neto, F. S., Volpe, C. M., Nogueira-Machado, J. A. Chaves, M. M. (2013). Vitamin complex (ascorbic acid, alpha-tocopherol and beta-carotene) induces micronucleus formation in PBMNC unrelated to ROS production. Redox Rep 18(6): 219-223.

Vieira, S. M., de Almeida, R., Holanda, I. B., Mussy, M. H., Galvao, R. C., Crispim, P. T., Dorea, J. G. Bastos, W. R. (2013). Total and methyl-mercury in hair and milk of mothers living in the city of Porto Velho and in villages along the Rio Madeira, Amazon, Brazil. Int J Hyg Environ Health 216(6): 682-689.

Vitalone, A., Catalani, A., Chiodi, V., Cinque, C., Fattori, V., Goldoni, M., Matteucci, P., Poli, D., Zuena, A. R. Costa, L. G. (2008). Neurobehavioral assessment of rats exposed to low doses of PCB126 and methyl mercury during development. Environ Toxicol Pharmacol 25(1): 103-113.

Wakabayashi, K., Kakita, A., Sakamoto, M., Su, M., Iwanaga, K. Ikuta, F. (1995). Variability of brain lesions in rats administered methylmercury at various postnatal development phases. Brain Res 705(1-2): 267-272.
Wassef, L. Quadro, L. (2011). Uptake of Dietary Retinoids at the Maternal-Fetal Barrier: IN VIVO EVIDENCE FOR THE ROLE OF LIPOPROTEIN LIPASE AND ALTERNATIVE PATHWAYS. The Journal of Biological Chemistry 286(37): 32198-32207.
Weniger, J. P., Zeis, A. Chouraqui, J. (1993). Estrogen production by fetal and infantile rat ovaries. Reprod Nutr Dev 33(2): 129-136.
WHO (1996). Trace Elements in Human Nutrition and Health. Geneva, World Health Organization.
WHO. (2003). Joint FAO/WHO Expert Committee on Food Additives, Sixty-first meeting 10-19 June 2003, Summary and Conclusions. Retrieved 12-06-2016, from ftp://ftp.fao.org/es/esn/jecfa/jecfa61sc.pdf.
WHO (2009). Global Prevalence of Vitamin A deficiency in Populations at Risk 1995-2005. WHO Global Database on Vitamin A Deficiency. Geneva. World Health Organization.
Woods, J. S., Davis, H. A. Baer, R. P. (1992). Enhancement of gamma-glutamylcysteine synthetase mRNA in rat kidney by methyl mercury. Arch Biochem Biophys 296(1): 350-353.
Yau, S.-y., Li, A. So, K.-F. (2015). Involvement of Adult Hippocampal Neurogenesis in Learning and Forgetting. Neural Plasticity 2015: 717958.
Yeum, K.-J., Aldini, G., Russell, R. M. Krinsky, N. I. (2009). Antioxidant/Pro-oxidant Actions of Carotenoids. Carotenoids: Volume 5: Nutrition and Health. G. Britton, H. Pfander and S. Liaaen-Jensen. Basel, Birkhäuser Basel: 235-268.
Yoshida, M., Satoh, M., Shimada, A., Yamamoto, E., Yasutake, A. Tohyama, C. (2002). Maternal-to-fetus transfer of mercury in metallothionein-null pregnant mice after exposure to mercury vapor. Toxicology 175(1-3): 215-222.
Yoshida, M., Suzuki, M., Satoh, M., Yasutake, A. Watanabe, C. (2011). Neurobehavioral effects of combined prenatal exposure to low-level mercury vapor and methylmercury. J Toxicol Sci 36(1): 73-80.
Yu, N.-K., Baek, S. H. Kaang, B.-K. (2011). DNA methylation-mediated control of learning and memory. Molecular Brain 4: 5-5.
Zalups, R. K. (2000). Molecular interactions with mercury in the kidney. Pharmacol Rev 52(1): 113-143.
Zareba, G., Cernichiari, E., Hojo, R., Nitt, S. M., Weiss, B., Mumtaz, M. M., Jones, D. E. Clarkson, T. W. (2007). Thimerosal distribution and metabolism in neonatal mice: comparison with methyl mercury. J Appl Toxicol 27(5): 511-518.
Zeliger, H. I. (2003). Toxic effects of chemical mixtures. Arch Environ Health 58(1): 23-29.
Zeliger, H. I. (2011). 2 - Health Effects of Exposures to Chemical Mixtures. Human Toxicology of Chemical Mixtures (Second Edition). Oxford, William Andrew Publishing: 7-13.
Zeliger, H. I. (2013a). Exposure to lipophilic chemicals as a cause of neurological impairments, neurodevelopmental disorders and neurodegenerative diseases. Interdiscip Toxicol 6(3): 103-110.
Zeliger, H. I. (2013b). Lipophilic chemical exposure as a cause of cardiovascular disease. Interdisciplinary Toxicology 6(2): 55-62.
Zeliger, H. I. (2013c). Lipophilic chemical exposure as a cause of type 2 diabetes (T2D). Rev Environ Health 28(1): 9-20.

Zemolin, A. P. P., Meinerz, D. F., de Paula, M. T., Mariano, D. O. C., Rocha, J. B. T., Pereira, A. B., Posser, T. Franco, J. L. (2012). Evidences for a role of glutathione peroxidase 4 (GPx4) in methylmercury induced neurotoxicity in vivo. Toxicology 302(1): 60-67.

ANEXOS

Anexo I. Parâmetros otimizados para as transições MRM do analise MS para retinóides.

Tabela S1. Parâmetros otimizados para as transições MRM do analise MS para retinóides. Potencial do Orifício (DP, Declustering Potential), Potencial de Entrada (EP, Entrance Potential), Energia de Colisão (CE, Collision Energy), e Potencial de Saída da Cela de Colisão (CXP, Collision Cell Exit Potential)

Q1 (m/z)	Q3 (m/z)	Tempo de permanencia	ID	DP (V)	EP (V)	CE (V)	CXP (V)
269,429	93	50	Retinyl Acetate S1	76	10	20	14
269,429	41	50	Retinyl Acetate S2	76	10	56	14
269,429	95	50	Retinyl Acetate S3	76	10	25	18
269,429	109,1	50	Retinyl Acetate S4	76	10	23	12
269,429	93	50	Retinol S1	76	10	20	14
269,429	41	50	Retinol S2	76	10	56	14
269,429	95	50	Retinol S3	76	10	25	18
269,429	109,1	50	Retinol S4	76	10	23	12

Anexo II. Tempos de exploração durante o teste de aprendizado olfatório (DPN 8)

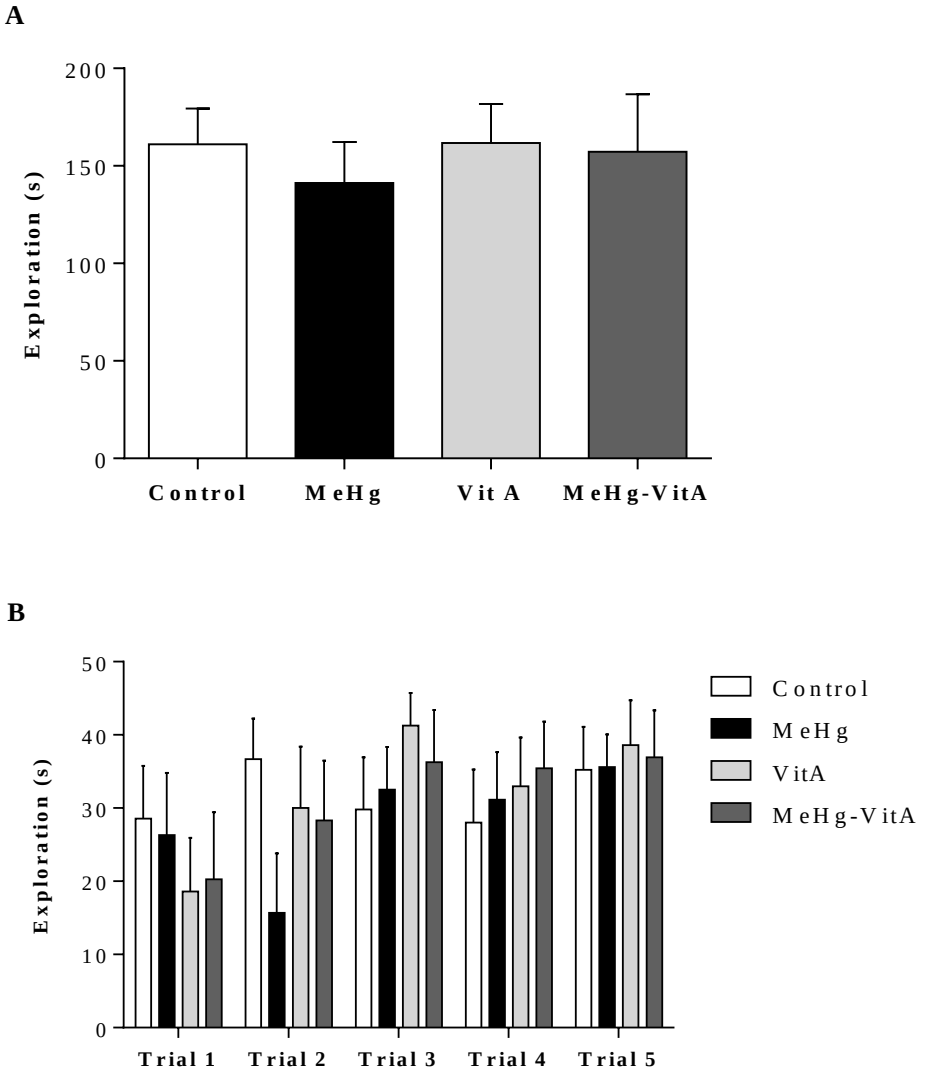

Figura S1. Tempos de exploração total (A) e discriminado por intentos (B) para o teste de aprendizado olfatório efetuado nos filhotes machos de cada ninhada no dia pós-natal 8. Os dados estão representados como a media ± SEM.

Anexo III. Índice de tigmotaxía calculado para os filhotes durante o novo open field (Novel OFT) (DPN 28)

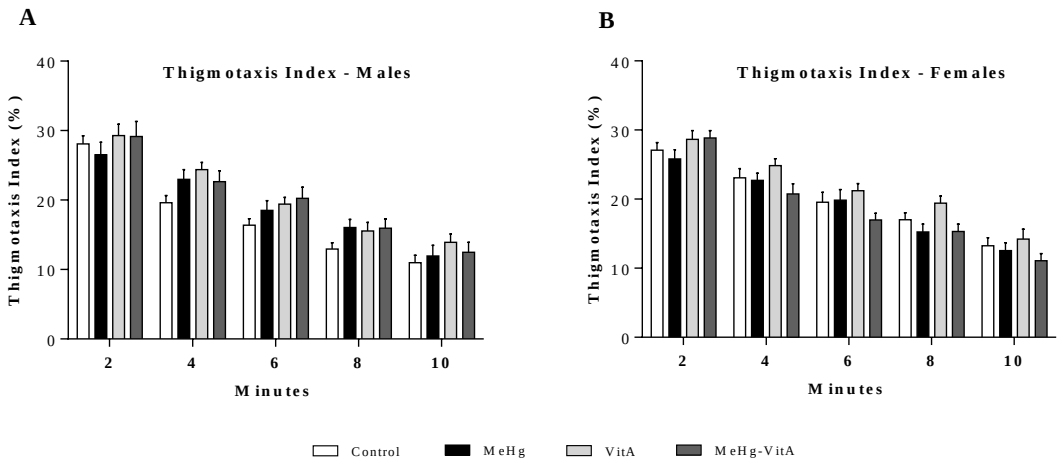

Figura S2. Índice de tigmotaxia para a primeira sessão de OFT (DPN 28). O índice de tigmotaxia foi calculado em intervalos de 2 minutos usando a distância percorrida pelo animal na periferia no intervalo de tempo estabelecido, dividido pela distância total percorrida total dentro do aparato de OFT, e expressado como porcentagem. (A) Índice de tigmotaxia para machos e (B) para fêmeas. Os dados estão representados como a media ± SEM.