UNIVERSIDADE FEDERAL DO PAMPA

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EFEITOS DO HIDROLISADO DA CLARA DE OVO SOBRE AS ARTÉRIAS MESENTÉRICAS DE RATOS EXPOSTO CRONICAMENTE AO CLORETO DE MERCURIO (HgCl$_2$)

DISSERTAÇÃO DE MESTRADO

ALYNE GOULART ESCOBAR

Uruguaiana

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

Orientadora: Giulia A. Wiggers Peçanha

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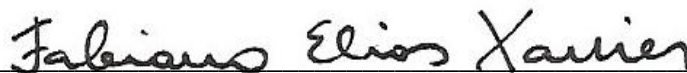
Área de concentração: Bioprospecção Molecular

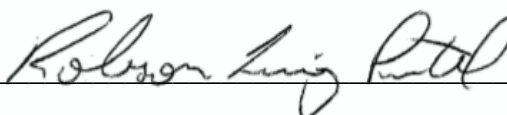**Dissertação defendida e aprovada em 07 de março de 2019.**

Banca examinadora:

Profª. Drª. Giulia Alessandra Wiggers Peçanha
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DEDICO

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

EFEITOS DO HIDROLISADO DA CLARA DO OVO SOBRE AS ARTÉRIAS MESENTÉRICAS DE RATOS EXPOSTOS CRONICAMENTE AO CLORETO DE MERCÚRIO (HgCl$_2$)

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Local e Data da defesa: 07 de março de 2019, Uruguaiana – RS

O hidrolisado de clara de ovo (HCO) possui propriedades antioxidantes, anti-inflamatórias e vasodilatadoras e pode ser uma alternativa eficiente para a prevenção ou tratamento de intoxicações por metais pesados. O objetivo foi investigar os potenciais efeitos benéficos da ingestão de HCO em distúrbios de reatividade vascular induzida por exposição crônica a baixas concentrações de Hg em artérias mesentérica de resistência (MRA), bem como esclarecer as possíveis vias envolvidas em seus efeitos. Ratos *Wistar* machos foram divididos em quatro grupos, tratados por 60 dias com: a) injeções intramusculares (i.m.) de solução salina a 0,9% e água por gavagem (Grupo Untreated); b) i.m. injecções de cloreto de mercúrio - HgCl$_2$, a primeira dose 4,6 µg / kg e doses subsequentes de 0,07 µg / kg / dia, para cobrir a perda diária, utilizando o modelo anteriormente descrito (Rizzetti *et al*., 2017a) e água por gavagem (Grupo HgCl$_2$); c) i.m. injeções de solução salina 0,9% e HCO (obtido por hidrólise por pepsina por 8 horas) diluído em água (1 g / kg / dia), por gavagem, segundo modelo previamente relatado (Rizzetti *et al*., 2016a) (Grupo HCO); d) ambos os tratamentos (HCO + HgCl$_2$). A pressão arterial sistólica (PAS) foi avaliada, a função vascular foi estudada na MRA em banho de órgãos isolado. Curvas concentração-resposta para acetilcolina (ACh) e nitroprussiato de sódio (NPS) foram realizadas. Resposta vasoconstritora à noradrenalina (NE) na presença e ausência de endotélio e na presença de um inibidor da enzima óxido nítrico sintase (NOS) (L-NAME), um inibidor não seletivo da ciclooxigenase (COX) (INDO), um co-fator essencial para síntese de NO (BH4), um inibidor da NADPH oxidase (VAS2870)

e um mimético da superóxido dismutase (TEMPOL) foram analisados. Medimos a produção in situ de ânion superóxido, liberação de NO, espécies reativas de oxigênio vascular (ROS), peroxidação lipídica, capacidade antioxidante e níveis de NPSH na MRA. O estudo foi aprovado pelo Comitê de Ética em Uso Animal (052014 - Unipampa). Os resultados foram expressos como média ± SEM, comparados por análise one-way ou two-way ANOVA seguida pelo teste post hoc de Fisher. ($P < 0,05$). O co-tratamento com HCO: a) preveniu o aumento da PAS, a disfunção endotelial e o aumento da resposta vasoconstritora ao NE promovidas pela exposição prolongada a $HgCl_2$ em MRA; b) restaurou a modulação endotelial mediada por NO; c) inibiu o estresse oxidativo derivado da NAD(P)H oxidase e as vias inflamatórias induzidas pelo metal nesses vasos, normalizando o estado antioxidante. Concluimos que o HCO parece ser capaz de neutralizar os efeitos tóxicos vasculares da exposição a longo prazo ao $HgCl_2$, o que aponta para possíveis efeitos terapêuticos baseados em alimento funcional contra um contaminante ambiental altamente difundido.

ABSTRACT

Dissertation of Master´s Degree
Post-Graduate Program in Biochemistry
Federal University of Pampa

EFFECT OF EGG WHITE HYDROLYSATE ON MESENTERIC ARTERIES IN RATS ON THE CHRONIC MERCURY CHLORIDE EXPOSURE (HgCl$_2$)

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Place and date of defense: March 7, 2019, Uruguaiana – RS.

Egg white hydrolysate (EWH) has antioxidant, anti-inflammatory and vasodilator properties and could be an efficient alternative for prevention or treatment in heavy metal poisoning. The aim was to investigate the potential beneficial effects of EWH intake on vascular reactivity disorders induced by chronic exposure to low concentrations of Hg in mesenteric resistance arteries (MRA) as well as to clarify the possible pathways involved in its effects. Male *Wistar* rats were divided into four groups, treated for 60 days with: a) intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage (Untreated); b) *i.m.* injections of mercury chloride − HgCl$_2$, the 1st dose 4.6 μg/kg, and subsequent doses of 0.07 μg/kg/day, to cover daily loss, using the model previously described (Rizzetti *et al.*, 2017a) and tap water by gavage (HgCl$_2$); c) *i.m.* injections of saline solution 0.9% and EWH from pepsin for 8 h diluted in tap water (1 g/kg/day), by gavage, according to model prior reported (Rizzetti *et al.*, 2016a) (EWH); d) both treatments (EWH+HgCl$_2$). Systolic blood pressure (SBP) it was evaluated, vascular function was studied in MRA in isolated organ bath. Concentration-response curves to acetylcholine (ACh) and sodium nitroprusside (NPS) were performed. Vasoconstrictor response to noradrenaline (NE) in presence and absence of endothelium and in presence of a nitric oxide synthase (NOS) inhibitor (L-NAME), a non-selective cyclooxygenase (COX) inhibitor (INDO), a essential cofactor for NO synthesis (BH4), a NADPH oxidase inhibitor (VAS2870) and a superoxide dismutase mimic (TEMPOL) were analyzed. We measured i*n situ* production of superoxide anion, NO release,

vascular reactive oxygen species (ROS), lipid peroxidation, antioxidant capacity and NPSH levels in MRA. The study was approved by the Ethics Committee on Animal Use (052014 – Unipampa). Results were expressed as mean and SEM, compared by one- or two-way ANOVA analysis followed by the Fisher post hoc test. ($P<0.05$). EWH: a) prevented the increased SBP, the endothelial dysfunction and the increased vasoconstrictor response to NE observed in MRA after prolonged $HgCl_2$ exposure; b) restored the NO-mediated endothelial modulation; c) inhibited the NAD(P)H oxidase-derived oxidative stress and the inflammatory pathways induced by the metal in these vessels, normalizing the antioxidant status. As a conclusion, the EWH seems to be able to counteract the vascular toxic effects of long-term exposure to $HgCl_2$, which points to possible therapeutic effects based on functional food against a highly widespread environmental contaminant.

Keywords: Egg White Hydrolyzate; Bioactive Peptides; Mercury; Toxicology; Oxidative stress; Vascular Reactivity.

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LISTA DE ABREVIATURAS E SIGLAS

µg – microgramas

µg/dia – microgramas por dia

µg/g – microgramas por grama

µg/kg – microgramas por quilograma

µg/kg/dia – microgramas por quilograma por dia

µM – micromolar

ACh – acetylcholine

Ala – Alanina

Arg - Arginina

BAL - 2,3-dimercaptopropanol

BH4 - (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride

CAT – Catalase

COX - Ciclooxigenase

Cys – cisteína

DAF - 4,5-diaminofluorescein

dAUC – difference Area Under the Curve

DCF – Fluorescent Dichlorofluorescein

DCHF-DA - 2′, 7′-dichlorofluorescein diacetate

DHE – dihydroethidium

DL50 – Dose Letal Mediana

DMPS – sulfonato dimercaptopropanol

DMSA – ácido dimercaptosuccínico

DNA – Deoxyribonucleic Acid

DPA – D-penicilamina (DPA)

DTNB – 5,5'-dithio-bis (2-nitrobenzoic acid)

ECA – enzima Conversora de Angiotensina

EDTA – ácido etilenodiaminotetracético

EPA – Environmental Protection Agency

EROs – Espécies Reativas de Oxigênio

EtHg – Timerosal EUA – Estados Unidos da América

EUA – Estados Unidos da América

EWH – Egg White Hydrolysate

FRAP – Ferric Reducing Antioxidant Power

FU – Fluorescence

Glu - Glutamato

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – Glutationa reduzida

HCO – Hidrolisado de Clara de Ovo

Hg – mercúrio

Hg^0 – mercúrio elementar

Hg^{1+} – íon mercuroso

Hg^{2+} – íon mercúrio

$HgCl_2$ – cloreto de mercúrio

HgS – sulfeto de mercúrio

HO-1 - expressão do gene heme oxigenase-1

Ile - Isoleucina

im – intramuscular injections

ip – intraperitoneal injections

KCl - potassium chloride

KHS - Krebs–Henseleit solution

Leu – leucina

L-NAME – Nω-Nitro-L-arginine methyl ester

Lys – lisina/lysine

M – molar

MDA – malondialdehyde

MeHg – metilmercúrio

Met – metionina

mg/kg – miligramas por quilograma

min – minute

ml – milliliter

MRA - mesenteric artery of resistance

mRNA – Messenger Ribonucleic Acid

NADPH oxidase – enzima Nicotinamida Adenina Dinucleotídeo Fosfato oxidase

NE – Noradrenalina

NE - Noradrenaline

NF-kB - fator nuclear kappa β

ng/l – nanogramas por litro

nM – nanomolar

NO – Nitric Oxide

NPSH – Non-proteic Thiol Groups

NRC – National Research Council

OMS – Organização Mundial da Saúde

PAS – Pressão Arterial Sistólica

PBS – phosphate buffered saline

pH – potencial hidrogeniônico

Phe – phenylephrine

Pro – prolina

RAS – Renin-angiotensin System

Rmax – Resposta máxima

ROS – Reactive Oxygen Species

SBP – Systolic Blood Pressure

SDS – sodium dodecil sulphate

SEM – Standard Error of the Mean

SH – grupamentos tióis/ thiol groups

SHR – Ratos Espontaneamente Hipertensos

SRA – Sistema Renina Angiotensina

SNC – Sistema Nervoso Central

SNP – sodium nitroprusside

SOD – Superóxido Dismutase

TBA – thiobarbituric acid

TBARS – Thiobarbituric Acid Reactive Substances

TCA – trichloroacetic acid

TEMPOL - 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl

TNF-α – Tumor Necrosis Factor Alpha

TPTZ – 2,4,6-Tripyridyl-s-Triazine

Trp – triptofano

Tyr – tirosina

VAS2870 - 1,3-Benzoxazol-2-yl-3-benzyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7-yl sulfide

vol – volume

WHO – World Health Organization

SUMÁRIO

PARTE I

INTRODUÇÃO

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PARTE I

INTRODUÇÃO

1 Mercúrio

1.1 O metal mercúrio

O mercúrio (Hg) é um metal pesado que ocupa o terceiro lugar na lista de substâncias toxicas segundo a Agência para Substâncias Tóxicas e Registro de Doenças (CLARKSON, 2002; ANDRADE et al., 2017) e seu potencial de toxicidade está relacionado com grande risco de contaminação ambiental (BUDNIK & CASTELEYN, 2018).

O Hg disponível no ambiente está dividido em três formas químicas que constituem seu ciclo biogeoquímico, sendo elas, a forma elementar (Hg0), o Hg inorgânico (Hg2+) e suas formas orgânicas, principalmente representadas pelo metilmercúrio (MeHg) (LI *et al*., 2015). Este metal pode formar compostos orgânicos e inorgânicos, variando sua conformação química e por consequência sua probabilidade de interagir com as barreiras biológicas, o que determina seus niveis de toxidade (KIM *et al*., 2015).

Quando se combina com elementos como cloro, enxofre ou oxigênio, o Hg forma compostos inorgânicos, também designados como sais de Hg (sais mercurosos e mercúricos). Essa forma pode ser encontrada em certos cremes de clareamento da pele, medicamentos homeopáticos, desinfetantes e pesticidas (CLARKSON, 1997; AZEVEDO, 2003; WHO, 2003; LI et al., 2015). Aproximadamente 80% do Hg elementar inalado e 0,01% do ingerido é absorvido pelo organismo. Para o Hg inorgânico, absorção por via inalatória e gastrointestinal são iguais e em torno de 10%, enquanto que 2-3% do Hg inorgânico é absorvido através da pele. O Hg orgânico, se ingerido ou inalado, é quase completamente (95-100%) absorvido e representa a forma mais tóxica do metal (SOLENKOVA et al., 2014). A eliminação do Hg do organismo ocorre pelos rins, fígado (via bile), mucosa intestinal, glândulas sudoríparas e salivares, pele e leite materno, sendo as vias urinária e fecal as mais importantes (SWIFT, 1997). Embora a maior parte do Hg absorvido seja eliminada em cerca de 60 a 70 dias, traços deste metal podem ser detectados no organismo por meses ou anos, pois se deposita nos tecidos, demonstrando que o contato humano com este metal constitui uma ameaça à saúde.

As fontes de liberação de Hg são as naturais ou as antropogênicas. De forma natural é liberado a partir de depósitos de mercúrio, volatilização do oceano, atividades vulcânicas e outros processos geotérmicos (LIANG *et al*., 2017). Através das fontes antropogênicas, os

principais eventos são as queimas de combustíveis fósseis, que forma o vapor de mercúrio na atmosfera, a progressiva utilização do Hg para fins industriais, o uso de pesticidas contendo mercúrio na agricultura, são as principais ações humanas que fazer o Hg ser acumulado no solo ou na água (DONG *et al*., 2018).

Embora praticamente todas as formas de Hg sejam tóxicas a contaminação ambiental e contaminação humanas são variáveis e os limites seguros de exposição devem ser sempre revistos pelas agências responsáveis de saúde pública (REHMAN *et al*., 2018).

1.2   **Populações de Risco e Limites de Exposição**

Estamos expostos a diferentes formas de Hg, e a fundição de metais não ferrosos e combustão de combustíveis fósseis são atualmente considerados as principais fontes antropogênicas de emissão de Hg (DI MARZIO *et al*., 2018). Uma vez introduzido para o meio ambiente, o Hg pode ser transformado em sua forma orgânica altamente tóxica e ser e bioacumulado em organismos vivos pela biomagnificação na cadeia alimentar até atingir o homem que está no topo dela (LEE et al., 2017; KIMAKOVA et al., 2018). Outras formas mais simplificadas como vapor de mercúrio de amálgamas dentárias e a partir de exposições ocupacionais são fontes de contaminação humana relevantes, assim como o consumo de alimentos contaminados (MERGLER *et al*., 2007).

A população está freqüentemente exposta ao MeHg através do consumo de peixe, especialmente em populações ribeirinhas onde há grande atividade de garimpo (GIBB *et al*., 2014). Esta forma de Hg apresenta os maiores níveis de toxicidade (LI *et al*., 2015; DOREA & MARQUES, 2016). A distribuição de metais pesados em peixes depende do tipo de sua alimentação ou a composição da água onde habitam, uma das possíveis razões dos peixes terem um alto teor de Hg é através do alto teor de proteínas funcionais no músculo do peixe, que tem uma alta afinidade com o Hg (BRAZOVA *et al*., 2012) . No entanto outras formas de Hg podem também ser tóxicas ao organismo, uma vez que o Hg orgânico entra no sistema, se liga ao agrupamentos -SH, conseguindo atravessar facilmente as barreiras biologicas, como por exemplo a barreira hematoencéfalica nesses compartimentos pode então ser convertido em Hg inorgânico (CARICCIO *et al*., 2019).

A dose letal média (DL50) se diferencia de acordo com a forma química do Hg. Em sua forma elementar a DL50 é de 63 mg/kg via intraperitoneal (*ip*) em ratos (OVERJORDET *et al*., 2014). Na forma inorgânica (HgCl$_2$) e orgânica (MeHg) estes valores são de 14 a 57

mg/kg e 20 a 60 mg/kg respectivamente (USEPA, 1998; SALGADO *et al.*, 2003). Esta variabilidade também ocorre em relação à meia-vida das formas de Hg no corpo humano, o Hg metálico possui meia-vida de 35 a 90 dias, o Hg inorgânico de 29 a 50 dias, enquanto o Hg orgânico pode apresentar meia-vida de 50 a 70 dias. No entanto, sua capacidade de bioacumular, o tempo de permanência no organismo gerando depositos de Hg que são fonte de auto-contaminação são preocupantes (PAMPHLETT *et al.*, 2018; RITGER *et al.*, 2018).

No Brasil o limite seguro para consumo de pescado foi fixado em 0,5 mg/kg de Hg para peixes não predadores e 1,0 mg/kg para peixes predadores (LEI 685, 1998). De acordo com a OMS, o consumo máximo de Hg por meio da dieta recomendado por semana é de 1,6 µg/kg para as mulheres em idade fértil e 3,3 µg/kg para a população em geral (LI et al., 2015). Para as vacinas, as concentrações de timerosal (EtHg) variam de 12,5 a 25 µg de Hg para cada dose de 0,5 ml de vacina. Porém, segundo a EPA, o ideal seria que não ultrapassasse a concentração de 0,1 µg/kg/dia. O Ministério do Trabalho e a Associação Brasileira de Normas Técnicas (NBR 10004, 2004) também normatizaram os valores de tolerância do metal para o ambiente de trabalho, fixando o mesmo em até 0,04 mg de Hg/m^3.

Para pessoas não expostas ao Hg, a OMS considera seguros a concentração sanguínea média de 5 a 10 µg/l (WHO, 1990). Já o NRC (National Research Council) identifica 2 µg/l como a concentração sanguínea média para populações com pouco ou nenhum consumo de peixe nos EUA (National Academy os Sciences, 2000). A EPA considera segura concentração sanguínea de Hg de até 5,8 ng/ml, valor significativamente menor que o estipulado pela OMS (USEPA, 1998).

Muitos são os valores de referencia e estudos relativos a consumo de peixes e vacinas (DOREA., 2017). O consumo de peixe pela população é preocupante porque os metais, principalmente o Hg é capaz de se acumular em animais aquaticos e tem leva muito tempo para se decompor na natureza, logo a população que se alimenta desses pescados tem um forte risco de serem afetados pela toxidade do Hg (DE PAIVA *et al.*, 2017; PESTANA *et al.*, 2019), por outro lado o consumo de peixe demonstra ter vários beneficios nutricionais como por exemplo os ácidos graxos poliinsaturados de cadeia longa ômega-3, -6 e -9 que protege a saúde cardiovascular e cerebral e é encontrados em peixes (ABUAJAH *et al.*, 2015).

Embora muitas sejam as as evidências quanto ao risco de toxicidade para a saúde humana relativo as diversas fontes as quais estamos expostos, os esforços das agências de publicas de saúde são para estabelecer de uma forma mais concreta e segura para os níveis

aceitáveis de exposição, em uma situação global de aumento de emissão antropogênica deste metal e com o passar dos anos surgem mais relatos de altas concentrações deste metal no organismo associado à prevalência de doenças (ROY *et al.,* 2017; BUDNIK *et al*., 2018; MELELEO *et al*., 2018).

1.3 Efeitos tóxicos do Hg no organismo

Uma vez absorvido pelo organismo o mercúrio é distribuído primariamente para o sistema nervoso central e rins. A eliminação do metal geralmente se dá pela urina e fezes (BRUDKIN *et al*., 2018). Assim, os níveis máximos recomendados diariamente e seu valor tóxico correspondente também variam de acordo com forma de apresentação deste metal. O fato é que as doses de referência não servem para estimativa de risco, mas somente como guia para a população (MAGOS & CLARKSON, 2006).

Os íons de Hg não existem em um estado iônico livre nos sistemas biológicos, em vez disso eles formam fortes ligações com grupos sulfidrilos livres (grupos tióis) (PEI *et al*., 2015), presentes em biomoléculas como cisteína, glutationa e / ou albumina (ZALUPS, 2000) assim consegue este metal bioacumula-se no organismo (MILLS *et al*., 2019).

Todas as formas de Hg são reconhecidas como poderosos oxidantes capazes de gerar rapidamente um fenômeno denominado estresse oxidativo. Embora os tióis conjugados ao Hg pareçam causar menor dano oxidativo nas células do que íons de mercúrio livres, ainda assim são absorvidos nos compartimentos intracelulares, e os íons mercúricos podem dissociar-se e liberar-se nos tecidos.. Durante o processo de troca, os íons de Hg não consilidados podem causar dano oxidativo intracelular significativo (FARINA *et al*., 2013).

Vários estudos relatam danos resultantes de exposições ao Hg em diferentes tempos de exposição, bem como dose, forma do metal e via de administração. Está bem documentado na literatura danos ao sistema renal (ZALUPS, 2000), hepático (AANSAR *et al*., 2016), respiratório (SENER *et al*., 2007), nervoso (MELLO-CARPES *et al*., 2013), reprodutor (MARTINEZ *et al*., 2014) e cardiovascular (WIGGERS *et al*., 2008; PEÇANHA *et al*., 2010; FURIERI *et al*., 2011; WIGGERS *et al*., 2016; RIZZETTI *et al*., 2017b VASSALLO *et al*., 2019).

O Hg inorgânico se acumula rapidamente no tecido renal, após a administração de Hg cerca de 50% se deposita nas celulas renais apenas algumas horas após a exposição, vários estudos relataram evidências de lesão renal após exposição aguda e crônica a várias formas de

Hg, associando a exposição ao metal com disfunção e necrose nas celulas renais (AGRAWAL *et al*., 2014; HA *et al*., 2017; BRIDGES *et al*., 2018).

A acumulação de Hg no fígado pode causar hepatotoxicidade, isso se deve pela captação dos íons de Hg pela membrama sinusoidal do hepatócito, sendo assim um mecanismo especificos de transporte ativo na membrana plasmática do hepatócito, através desse mecanismo já foi descrito diferentes graus de nefrotoxicidade induzida po $HgCl_2$ em ratos *Wistar* machos e fêmeas (BRIDGES & ZALUPS, 2017; HAZELHOFF *et al*., 2018).

Com relação ao sistema respiratório, a intoxicação aguda ao Hg causa bronquiolite e pneumonite, em longo prazo, acarretaram fibrose pulmonar em humanos. A exposição a baixas concentrações de Hg presente no solo foi relacionada ao câncer de pulmão em Taiwan, porém os mecanismos ainda necessitam ser esclarecidos (HUANG *et al*., 2018; TELAHIGUE *et al*., 2018).

Diferentes formas de Hg são capazes de causar disordens neurologicas, por exemplo o metilmercúrio pode ligar-se a grupos tiol e permear facilmente a barreira hematoencefálica, chegando à células glias e neurônios, onde é convertido em mercúrio inorgânico. Além disso, o mercúrio inorgânico inibe a absorção de glutamato, promovendo sua liberação no espaço extracelular e causando efeitos neurotóxicos no sistemas nervoso central (MELLO-CARPES *et al*., 2013; IWAI-SHIMADA *et al*., 2016; CARRICIO *et al*., 2019).

Quanto ao sistema reprodutor já foram descritas alterações morfológicas testiculares, danos à espermatogênese e apoptose de células germinativas foram observadas em ratos expostos ao metal na forma orgânica, a administração do Hg inorgânico altera a performance reprodutiva, tanto em 30 quanto em 60 dias de exposição ao $HgCl_2$ em baixas concentrações, semelhante ao contato ocupacional humano, houve o desenvolvimento de disfunção reprodutiva em machos associada ao desequilíbrio hormonal e ao aumento do estresse oxidativo (JACKSON et al., 2011; MARTINEZ et al., 2014; SCHREIER et al., 2015).

Grande maioria dos efeitos deletérios ocasionados pelo Hg são gerados a partir de uma situação de estresse oxidativo, os mecanismos pelos quais as espécies de Hg causam este fenômeno ainda não foram totalmente elucidados, porém estudos sugerem que a exposição leva a formação do complexo Cys-S-Hg-S-Cys causando modicações na função cellula (ORR *et al*., 2019)

Sabe-se que o metal pode ativar à peroxidação de lipídeos, aumento do números de especies reativas do oxigênio (EROS) promover o aumento da produção de EROS, a exposição ao Hg também pode ocasionar alterações no sistema de defesa do organismo ao

reduzir os níveis enzimáticos das enzimas superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR) e glutationa peroxidase (GPx), como também da glutationa (GSH), contribuindo para o estresse oxidativo e como conseqüência gerar o estresse oxidativo (KHAN & WANG, 2018; ORR *et al*., 2019). Além disso já foi descrito a ação do metal de ativar a cascata de inflamação, já foi demostrado a sua atuação sobre a via da COX, promovendo o aumento dos prostanóides vasoconstritores derivados da COX-2 tanto na exposição aguda quanto crônica ao metal (WIGGERS *et al*., 2008; PEÇANHA *et al.,* 2011).

Atualmente muito se sabe sobre a ação do Hg no organismo com um todo, no entanto os estudos que abordam especificamente seus efeitos sobre o sistema cardiovascular nós relatam importantes ações desse metal.

1.4   Toxicidade do Hg no sistema cardiovascular

Existe forte associação entre exposição ao mercúrio e doenças cardiovasculares, tais como hipertensão, aterosclerose carotídea, infarto do miocárdio e doença arterial coronariana (SALONEN *et al*., 2000; HOUSTON *et al*., 2007; GENCHI et al., 2017; CHOWDHURY *et al*., 2018).

A exemplo do que ocorre em outros sistemas o estresse oxidativo parece ser um dos principais mecanismos implicados que neste caso contribui para a geração de lipoproteína de baixa densidade oxidada, e subsequentemente aterosclerose (MITRA *et al*. 2011). A geração de produtos finais da glicação avançada e a participação de células inflamatórias também promovem a manutenção da injúria vascular em ratos expostos a Hg (HARJA *et al*., 2015). O endotélio vascular é outro alvo do metal que tanto em baixas como em altas concentrações promove dano vascular, demonstrando a importância e a necessidade de elucidar os mecanismos pelos quais o metal promove o desenvolvimento de doenças cardiovasculares (VASSALLO *et al.*, 2011; GENCHI et al., 2017; GHIZONI *et al*., 2017).

A exposição crônica ao mercúrio inorgânico, uma forma dita menos tóxica, produz concentrações sanguíneas compatíveis àquelas encontradas em indivíduos expostos, e também representa um fator de risco cardiovascular. A exposição a este tipo de metal influencia a atividade da ECA, aumenta o estresse oxidativo e promove alterações nos niveis pressoricos (VASSALLO *et al*., 2019). Estudo em animais experimentais de nosso grupo de pesquisa relatam que exposição crônica, durante 30 dias, a baixas doses de HgCl$_2$, mimetizando a concentração sanguínea na exposição humana, produziu aumento na reatividade vascular em artérias aorta, mesentéricas, coronárias e basilares sem provocar alterações na pressão arterial

dos ratos evidenciando que as alterações vasculares precedem o aumento da pressão arterial encontrada em exposições mais prolongadas (PEÇANHA *et al*., 2010; FURIERI *et al*., 2011; WIGGERS *et al*., 2008; WIGGERS *et al*., 2016).

Recentemente desenvolvemos um modelo de exposição crônica a baixas concentrações por 60 dias e foi observado o aumento da pressão arterial a partir da sexta semana de exposição acompanhado de incremento da resposta contratil a fenilefrina e disfunção endotelial (RIZZETTI *et al*., 2017a). Estes efeitos estão associados e foram observados em outros estudos com diferentes tempos e doses de exposição também evidenciaram aumento do estresse oxidativo sistêmico e do tecido vascular, aumento da peroxidação lipídica do plasma, redução das enzimas antioxidantes superóxido dismutase (SOD) e glutationa peroxidase, aumento da expressão protéica vascular de enzimas pró-oxidantes, como a NADPH oxidase (WIGGERS *et al*., 2008; PEÇANHA *et al*., 2010; FURIERI *et al*., 2011; RIZZETTI *et al.,* 2013), redução da biodisponibilidade de óxido nítrico (NO), disfunção endotelial (ROSSONI *et al*., 1999; DA CUNHA *et al.,* 2000; WIGGERS *et al*., 2008a) e aumento na liberação de prostanóides vasoconstritores derivados da COX-2 (PEÇANHA *et al*., 2010; VASSALO 2019).

Diferentemente ao que ocorre em outros modelos animais de hipertensão, para as disfunções vasculares observadas durante a exposição ao Hg, a ativação da via da COX-2 não parece ser secundária ao estresse oxidativo gerado pela via da NADPH oxidase (AGUADO *et al*., 2013; RIZZETTI *et al*., 2013). As alterações observadas em nosso modelo experimental, são importantes para demonstrarmos que, mesmo em concentrações próximas aos limites considerados seguros pelas organizações de saúde e de meio ambiente, a exposição prolongada ao Hg constitui um sério problema de saúde pública, e alternativas visando sua remoção do ambiente e a prevenção de seus danos aos sistemas devem ser continuamente estudadas.

Nesse modelo o Hg promoveu importantes alterações nos parâmetros cardiovasculares, os níveis pressóricos aumentados nos remetem uma série de novas perguntas, ainda não esclarecidas como por exemplo a participação as artérias resistência no aumento de pressão promovido por este modelo. Sabemos que essas artérias são determinantes na regulação daresistência vascular periférica, e por conseguinte na manutenção da pressão arterial. A resistência vascular varia inversamente ao raio do vaso a quarta potência (Lei de Poiseuille), dessa maneira, pequenas alterações do diâmetro luminar

podem influenciar grandemente na resistência vascular periférica (GUYTON & HALL, 2016).

No cenário atual podemos observar diferentes formas de exposição ao Hg que podem desempregar vários efeitos maleficos em todo organismo, com isso se faz cada vez mais necesário o estudo de recursos terapêuticos que possam neutralizar as ações desse metal, como compostos sinteticos e naurais como por exemplo os alimentos funcionais.

1.5   Alternativas terapêuticas contra os danos promovidos pelo Hg

A terapia quelante é uma das principais alternativas e constitui o tratamento central de várias intoxicações por metais como o Hg devido à exposição ambiental, alimentar ou ocupacional. Há vários relatos evidenciando de maneira clara a eficáz o seu uso para intoxicações agudas e crônicas a metais pesados, principalmente o Hg, e os efeitos colaterais dos diferentes agentes quelantes em seres humanos (GUHA MAZUMDER *et al*., 2001; ANDERSEN *et al*., 2016).

Antigamente as intoxicações por Hg eram tratadas com os quelantes como 2,3-dimercaptopropanol (BAL) e d-penicilamina (DPA), no entanto, o BAL passou a ser considerado contraindicada no processo de desintoxicação devido ao aumento da deposição cerebral de Hg inorgânico e orgânico observada (COHEN *et al*., 2013; BJORKLUND *et al*., 2017). Atualmente esses agentes são considerados ultrapassados, pois suas contra-indicações podem superar seus benefícios (AASERT *et al*., 2015). Utiliza-se o dimercaptosuccínico (DMSA) que age como um antídoto eficiente na intoxicação aguda por Hg e seu análogo o sulfonato dimercaptopropanol (DMPS) facilita a excreção de Hg na urina, reduzindo a quantidade de Hg na massa corpórea (AASERT *et al*., 2015), no entanto ainda há relatos de casos clínicos de toxicidade após seu uso (ANDERSEN & AASETH, 2016).

Em estudo prévio demonstramos que o co-tratamento de ratos expostos ao mercúrio com apocinina, um inibidor da NADPH oxidase, preveniu parcialmente o aumento de reatividade vascular induzido em aorta, normalizou a disfunção endotelial e preveniu o estresse oxidativo resultando em melhora na biodisponibilidade do NO (RIZZETTI et al., 2013). Este estudo foi importante para evidenciar que compostos antioxidantes sintéticos são eficazes para melhorar a disfunção vascular induzida pelo metal.

Sabendo a dificuldade de inserção de compostos antioxidante para a adesão ao tratamento humano alguns estudos também relataram produtos alimentares como potentes quelantes de metais pesados, e associaram seu consumo com a redução da concentração de Hg

no cérebro e sangue (WANG *et al*., 2018). Compostos naturais, especialmente alimentos de origem protéica podem reduzir a absorção ou a reabsorção de metais tóxicos e apoiar vias de desintoxicação naturais, a sua eficiência como um composto quelante deve-se a presença de enxofre na composição química das proteínas, que possui grande afinidade por metais pesados aumentando e melhorando sua excreção (SEARS 2013).

O consumo de antioxidantes presentes em determinados alimentos como derivados de frutas, vegetais e proteinas já foram descritos com potente ação contra os efeitos tóxicos causados pela exposição a metais, uma vez que protege a célula de danos no DNA e muda o seu estado redox (ABARIKWU *et al.*, 2016). Levando em consideração os elevados custos para os sistemas de saúde para o tratamento das diversas doenças induzidas pela exposição crônica ao Hg, outros compostos naturais provenientes da dieta de ação específica sobre sua toxicidade devem ser investigados e inseridos como estratégia terapêutica nessas condições (SOLENKOVA *et al*., 2014). Com isso, a OMS recomenda que os nutrientes que alterem a toxicidade induzida pelos contaminantes ambientais, tais como Hg, sejam melhores investigados e elucidados para serem utilizados como possíveis tratamentos ou complementarem alternativas terapêuticas existentes (OMS, 1990).

Neste contexto, o consumo de alimentos funcionais representam uma boa estratégia para prevenção e tratamento de inúmeros distúrbios de saúde, e os estudos acerca do tema vem ganhando espaço e importância na comunidade científica, principalmente por serem uma alternativa baixo custo e segura, praticamente isenta de efeitos adversos (BOONLA *et al*., 2015; GARCÉS-RAMÓN *et al*., 2016).

2 Alimentos Funcionais

Nos últimos anos, devido ao elevado número de doenças relacionadas a um estilo de vida inadequado, preocupações e busca por alternativas saudáveis de alimentação tornaram-se crescentes. Nesse sentido, o consumo de alimentos naturais representa uma boa estratégia para prevenção e tratamento de inúmeros distúrbios de saúde, e os estudos acerca do tema vem ganhando espaço e importância na comunidade científica, principalmente por serem uma alternativa baixo custo e segura, praticamente isenta de efeitos adversos (BOONLA et al., 2015; GARCÉS-RAMÓN *et al*., 2016).

2.1 Peptídeos bioativos

Os péptidos bioativos são definidos como moléculas inativas na proteína de origem, sua ação e função biológica é facilitada a partir de uma hidrolise *in vitro* ou *in vivo*. Fragmentos específicos de proteínas de origem animal como ovo e leite exercem um impacto positivo nas funções ou condições corpóreas, como melhora nos parametros lipidicos, normalização dos níveis pressóricos, entre outros, podendo influenciar positivamente o estado de saúde. Atualmente, sabe-se que os peptídeos bioativos são seqüências específicas de aminoácidos com atividade similar a um fármaco ou hormônio, que eventualmente modulam a função fisiológica ao se ligarem a receptores específicos da célula alvo (LIAO *et al.,* 2018).

Além de suas propiedades nutricionais, algumas proteínas alimentares podem exercer também atividade biológia mediante a liberação dos peptideos bioativos durante o processo de digestão, atraves da hidrolise de enzimas do trato grastro intestinal (MAJUMDER *et al.,* 2013). Estes peptídeos separam-se de sua proteína precursora pelo processo de hidrólise enzimática parcial ou total. Além da atividade enzimática as condições de pH, temperatura e tempo de hidrólise modificam o resultado e propriedades do produto final (ECKERT *et al.*, 2013).

Muitos estudos investigam as seqüências de peptídeos específicos isoladas que são liberados após o processo de hidrólise (MIGUEL *et al*., 2004). No entanto, recentemente está sendo verificado que a administração de hidrolisados completos poderia ter maior relevância em nível fisiológico do que a administração de um único peptídeo isolado, bem como um efeito biológico muito mais complexo, além de serem considerados produtos mais interessantes e elaborados para o desenvolvimento de alimentos funcionais do ponto de vista de custo beneficio, organoléptico e nutricional (LIU *et al*., 2014). Dentre os componentes biologicamente ativos de alimentos funcionais incluem-se os compostos fitoquímicos derivados de plantas, verduras, legumes, frutas e cereais, os ácidos graxos poliinsaturados de cadeia longa ômega-3, -6 e -9 encontrados em peixes, os compostos probióticos de produtos lácteos fermentados e os peptídeos bioativos encontrados em proteínas de origem vegetal e animal (ABUAJAH *et al*., 2015; SRIVIDYA *et al*., 2017).

2.2 Atividades biológicas dos peptídeos bioativos

Desde seu descobrimento por volta de 1979, esses peptídeos tem se destacado por suas diversas atividades biológicas como atividade antioxidante, antihipertensiva,

inmunomoduladora, antimicrobiana, antinflamatória, opioide, etc (MOUGHAN *et al*., 2014). Uma vez liberado de sua proteína precursora, os peptídeos bioativos podem exercer diferentes atividades biológicas no organismo, as quais estão relacionadas à composição e seqüência de aminoácidos, assim como o tamanho dos peptídeos (RAO *et al*., 2012).

Com relação às propriedades antioxidantes, vários estudos demonstraram a presença acentuada dessa atividade biológica em hidrolisados ou peptídeos bioativos derivados de fontes protéicas vegetais ou animais como amendoim (WANG *et al*., 2014), farelo de arroz (REVILLA *et al*., 2008), folha de alfafa (XIE *et al*., 2008), milho (LI et al., 2008), pele de rã (QIAN *et al*., 2008), ovo (MIGUEL et al., 2004), leite (LIU *et al*., 2005), caseína (MIGUEL *et al*., 2010) e animais marinhos (FENG *et al*., 2015). O exato mecanismo por meio do qual os peptídeos bioativos promovem atividade antioxidante ainda não foi completamente compreendido, porém vários estudos têm demonstrado que esses compostos podem ser inibidores da peroxidação lipídica (MOURE *et al.,* 2006; QIAN *et al*., 2008; SARMANDI & ISMAIL, 2010), scavenger de radicais livres (RAJAPAKSE *et al*., 2005; MOURE *et al.,* 2006) e quelantes de íons metálicos (RAJAPAKSE *et al*., 2005; WANG *et al.,* 2014). Além disso, tem sido relatado que os peptídeos antioxidantes protegem as células de danos causados por EROS através da capacidade de indução de genes específicos. Foi demonstrado que o dipeptídeo Met-Tyr derivado de proteína muscular da sardinha previne o estresse oxidativo por estimular a expressão do gene heme oxigenase-1 (HO-1) e da ferritina em células endoteliais (ERDANN *et al*., 2006). Outros estudos também mostraram que alguns hidrolisados protéicos vegetais são capazes de aumentar a atividade das enzimas antioxidantes glutationa peroxidase (GPx) e superóxido dismutase (SOD) *in vivo* (FU, 2003).

Nos últimos anos, esses componentes alimentares com atividade biológica foram utilizados para desenvolver novos alimentos chamados alimentos funcionais. Estes são definidos como alimentos que, além de suas propriedades nutricionais, elas podem exercer uma atividade biológica benéfica no organismo depois de ingerido. Nesse sentido, até hoje foram extraídos e obtidos um grande número de componentes ou ingredientes funcionais de alimentos de origem animal e vegetal, que demonstraram exercer atividade na melhora da síndrome metabólica ou em alguns dos suas complicações (BROWN *et al*., 2015). Um exemplo desses compostos são os flavonóides, componentes bioativos dos alimentos de origem vegetal.

Os alimentos funcionais foram propostos como uma alternativa relacionada com a modificação dos hábitos alimentares, que consistiria em incluir na dieta de pacientes

componentes de alimentos com atividade biológica que contribuir com melhorias substâncias nas alterações funcionais e/ou metabólicas para desenvolver em pacientes obesos ou com síndrome metabólica, e assim limitar e/ou evitar a implantação da utilização de várias alternativas medicamentosas nestes sujeitos (MIGUEL & ALEIXANDRE, 2006; ANDERSEN & FERNANDEZ, 2013; BROWN *et al*., 2015; ROCHLAIN *et al*., 2017). Adicionalmente, estes componentes de origem alimentar possuem a vantagem de não causar efeitos adversos ou dependência (LIU *et al*., 2010).

2.3   Peptídeos bioativos derivados do ovo

O ovo de galinha é hoje um dos alimentos mais consumidos, devido ao seu fácil acesso, baixo preço, alta versatilidade nas aplicações culinárias e também porque supõe uma fonte muito equilibrada de nutrientes de grande qualidade e alta biodisponibilidade (Instituto de Estudos do Ovo, 2009; CAMPOS ZANI *et al*., 2018; LIAO et al., 2018). Recentemente, o ovo tornou-se um alimento muito interessante para a obtenção de peptídeos bioativos (MAJUMDER *et al*., 2015; NIMALARATNE *et al*. 2015; SUN *et al*, 2016; LIU *et al*., 2017).

Do isolamento e identificação do primeiro peptídeo bioativo derivado de clara de ovo (FUJITA *et al*., 1995), pesquisa e desenvolvimento de peptídeos bioativos em torno desta fonte a proteína teve um crescimento exponencial. É especificamente de ano de 2010, quando estudos e pesquisas sobre a produção de peptídeos dos derivados bioativos de ovos começam a ser mais relevantes. Nesse sentido, existem hidrolisados obtidos e peptídeos bioativos de todas as estruturas do ovos que incluem proteínas em sua composição, como clara de ovo (albumina), gema e a membrana interna da casca (membrana do testacea) (JAIN & ANAL, 2017).

As proteínas do ovo, que estão principalmente presentes na clara, são consideradas de alto valor biológico e importantes fontes de nitrogênio na dieta. Este alimento também desempenha um papel fundamental na nutrição humana. Entretanto, apesar de o ovo ser uma fonte muito valiosa de proteínas para a alimentação humana, devido a sua variedade e capacidade funcional, poucos peptídeos bioativos são descritos como provenientes de suas proteínas. Os hidrolisados de ovo mais abundantes são aqueles que vêm da clara do ovo, provavelmente devido à facilidade de obter esta parte do ovo e também devido ao alto teor de proteína que possui (MIGUEL e ALEIXENDRE, 2006; GARCÉS-RIMÓN *et al*., 2016; JOVANOVIC *et al*., 2016).

2.4   Ação biologica do hidrolisado da clara de ovo (HCO)

A clara do ovo branco representa cerca de 58% do peso total do ovo de galinha e é quase exclusivamente composto por água (88-90%) e proteínas (10-12%). A riqueza e o equilíbrio nos aminoácidos essenciais da proteína da clara do ovo fazem dela um alimento de valor biológico muito alto e é usado como uma proteína de referência para avaliar a qualidade de outras proteínas alimentares (Instituto de Estudos do Ovo, 2009, YU *et al.,* 2011, GARCÉS-RIMÓN *et al*., 2016, LIAO *et al*.,2018). Além disso, graças à sua alta capacidade de saciedade, é uma fonte de proteína de grande interesse na produção de dietas hipocalóricas para pacientes com excesso de peso (DU *et al*., 2017).

As proteínas derivadas da clara de ovo, principalmente a ovoalbumina, demonstraram possuir ação antioxidante e funções benéficas para a saúde humana (SUN *et al*., 2016). Previamente, estudo desenvolvido por Miguel *et al*. (2004), mostrou que a hidrólise de proteínas de clara de ovo com diferentes enzimas digestivas como pepsina por 3 horas geram hidrolisados com capacidades biológicas anti-hipertensivas e antioxidantes *in vitro*. Deste HCO foram isolados e identificados vários peptídeos que mostraram atividade sobre a inibição da ECA (MIGUEL *et al*., 2004) e/ou uma potente atividade antioxidante (DÁVALOS *et al*., 2004). É importante notar que vários fatores podem condicionar a atividade desses peptideos quando administrado oralmente, entre esses fatores é importante destacar os fatores fisiológicos, incluindo resistência a enzimas gastrointestinal, pH, e biodisponibilidade (absorção, transporte e capacidade para alcançar seus locais de ação) (LIAO *et al*., 2018, SANTOS-HERNANDEZ *et al*., 2018).

Alguns estudos relatam a ação protetora do HCO de forma diferenciada de acordo com o tratamento de hidrólise utilizado. O HCO com potencial atividade biológica obtidos e testados, o primeiro com pepsina por 8 horas e o segundo com 433P peptidase por 24 horas demonstraram atividade antioxidante e de inibição da inibidor da ECA *in vitro e* atividade antioxidante e hipocolesterolêmico *in vitro* respectivamente (MIGUEL *et al*., 2004; DÁVALOS *et al*., 2004). Posteriormente, foi demonstrado que o HCO com pepsina 8 horas mostrou-se ser mais promissor, devido sua capacidade de reduzir o estresse oxidativo e inflamação produzidas em ratos obesos *Zucker*, além de reduzir a esteatose hepática deste modelo animal (GARCÉS-RIMÓN *et al*., 2016). Este HCO também mostrou atividade moduladora da microbiota intestinal, por reversão da disbiose produzida no intestino dos ratos obesos *Zucker* (REQUENA *et al*., 2017).

Em estudos recentes do nosso grupo de pesquisa foi realizado um co-tratamento deste mesmo HCO em ratos *Wistar* expostos cronicamente a baixas doses de Hg por 60 dias e esse HCO foi capaz de reverter as alterações cardiovasculares, reprodutivas e neurológicas associado a um aumento no estresse oxidativo causado pela exposição a metal (RIZZETTI *et al*., 2016a; RIZZETTI *et al*., 2016b; RIZZETTI *et al*., 2017b; RIZZETTI *et al*., 2017c). Inicialmente, pensamos que as atividades descritas anteriormente sobre o HCO como a de ser inibidor da ECA, vasodilatador e antioxidante, poderia ser útil para reverter as alterações produzidas neste modelo de toxicidade por Hg. Porém, é sugerido que HCO com pepsina por 8 horas pode estar exercendo uma atividade de quelantes de metais relacionados com seu mecanismo de ação antioxidante, o que impediria o depósito de Hg no organismo (RIZZETTI *et al*., 2016a).

3 JUSTIFICATIVA

O hidrolisado de clara do ovo um composto da classe dos alimentos funcionais, carate-rizado por peptídeos bioativos com importantes características de cardioproteção como des-crito por Miguel *et al.*, (2004), apresentou tanto in vitro como in vivo capacidade inibição da ECA, capacidade anti-inflamatória e antioxidante.

O mercúrio é um metal tóxico para o ser humano e provoca inúmeras alterações e ris-cos à saúde. Já está bem descrito na literatura que baixas concentrações sanguíneas deste me-tal, como as adquiridas no consumo de peixes contaminados ou no contato ou uso de amál-gamas dentárias, são suficientes para alterar parâmetros fisiológicos importantes, inclusive no sistema cardiovascular.

Considerando os efeitos já conhecidos, principalmente através do estudo de efeitos crônicos com técnicas de administração controlada de mercúrio tendo como base o modelo desenvolvido por Wiggers *et al.,* (2008) e evidenciados ainda mais quando o modelo de expo-sição por maior tempo e observou-se o aumento do pressão arterial, o aumento da reatividade vascular em artéria de condutância, com maior participação da via do óxido nítrico e das es-pécies reativas de oxigênio descrito por Rizzetti *et al.*, (2017a).

Em estudos recentes do nosso grupo de pesquisa foi realizado um co-tratamento deste mesmo HCO em ratos *Wistar* expostos cronicamente a baixas doses de Hg por 60 dias e esse HCO foi capaz de reverter as alterações cardiovasculares, reprodutivas e neurológicas associado a um aumento no estresse oxidativo causado pela exposição a metal, principalmente no sistemas cardiovascular preveniu o aumento de pressão arterial e disfunções de vasoconstrição e vasorelaxamento em arterias de condutância Rizzetti *et al.*, (2017b).

No entanto, no presente estudo procuramos aprofundar os estudos nas artéria de resitência que são as principais envolvidas nas alterações de pressão, também investigar novos mecanismos de ação que possam estar envolvidos com a ação do Hg e HCO e por consequência determinar uma nova alternativa terapêutica para intoxição por Hg através do consumo de um alimento funcional.

4 OBJETIVO

4.1 Geral

Investigar se o co-tratamento com o HCO é capaz de promover efeitos benéficos sobre as disfunções hemodinâmicas e vasculares causadas pela intoxicação crônica a baixas concentrações de Hg em ratos.

4.2 Específicos

- Avaliar a reatividade vascular em vasos de resistência (artérias mesentéricas) de ratos expostos a 60 dias de $HgCl_2$ e co-tratados com HCO.

- Estudar as possíveis vias envolvidas, especificamente a via do óxido nítrico, das espécies reativas de oxigênio e da via ciclooxigenase nos efeitos vasculares promovidos pela exposição ao Hg e os possíveis efeitos protetores do co-tratamento com HCO

- Analisar o efeito dos tratamentos sobre os biomarcadores de estresse oxidativo em artérias mesentéricas.

PARTE II

Este artigo será submetido ao *FOOD AND CHEMICAL TOXICOLOGY*

Benefits of Egg White Hydrolysate intake on mesenteric resistance arteries after mercury induced damage in rats.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Abstract

Egg white hydrolysate (EWH) has antioxidant, anti-inflammatory and vasodilator properties and could be an efficient alternative for prevention or treatment in heavy metal poisoning. The aim was to investigate the potential beneficial effects of EWH intake on vascular reactivity disorders induced by chronic exposure to low concentrations of Hg in mesenteric resistance arteries (MRA) as well as to clarify the possible pathways involved in its effects. Male *Wistar* rats were divided into four groups, treated for 60 days with: a) intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage (Untreated); b) *i.m.* injections of mercury chloride − $HgCl_2$, the 1st dose 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day, to cover daily loss, using the model previously described (Rizzetti *et al.*, 2017a) and tap water by gavage ($HgCl_2$); c) *i.m.* injections of saline solution 0.9% and EWH from pepsin for 8 h diluted in tap water (1 g/kg/day), by gavage, according to model prior reported (Rizzetti *et al.*, 2016a) (EWH); d) both treatments (EWH+$HgCl_2$).Vascular function was studied in MRA in isolated organ bath. Concentration-response curves to acetylcholine (ACh) and sodium nitroprusside (NPS) were performed. Vasoconstrictor response to noradrenaline (NE) in presence and absence of endothelium and in presence of a nitric oxide synthase (NOS) inhibitor (L-NAME), a non-selective cyclooxygenase (COX) inhibitor (INDO), a essential cofactor for NO synthesis (BH4), a NADPH oxidase inhibitor (VAS2870) and a superoxide dismutase mimic (TEMPOL) were analyzed. We measured i*n situ* production of superoxide anion, NO release, vascular reactive oxygen species (ROS), lipid peroxidation, antioxidant capacity and NPSH levels in MRA. The study was approved by the Ethics Committee on Animal Use (052014 – Unipampa). Results were expressed as mean and SEM, compared by one- or two-way ANOVA analysis followed by the Fisher post hoc test. (P<0.05). EWH: a) prevented the increased systolic blood pressure (SBP), the endothelial dysfunction and the increased vasoconstrictor response to NE observed in MRA after prolonged $HgCl_2$ exposure; b) restored the NO-mediated endothelial modulation; c) inhibited the NAD(P)H oxidase-derived oxidative stress and the inflammatory pathways induced by the metal in these vessels, normalizing the antioxidant status. As a conclusion, the EWH seems to be able to counteract the vascular toxic effects of long-term exposure to $HgCl_2$, which points to possible therapeutic effects based on functional food against a highly widespread environmental contaminant.

Introduction

Mercury (Hg) is one of the most dangerous pollutants in the ecosystem, it is found in different physical and chemical forms and several toxicity levels as well as serious deleterious biological effects have already been demonstrated (Clarkson and Magos, 2006). These effects are dose and time-dependent and studies have shown damage to kidneys (Zalups, 2000), liver

(Huang et al., 2019), nervous system (Mello-Carpes et al., 2013), reproductive system (Martinez et al., 2014) and cardiovascular system (Rizzetti et al., 2017c) induced by small doses of this metal.

It is known that Hg is able to promote oxidative stress and it seems that this is the main pathway of tissue damage (Mahboob et al., 2001). In the cardiovascular system, Hg exposure at low doses for 30 days was able to induce oxidative stress, activation of cyclooxygenase (COX) pathway and stimulation of angiotensin II participation in contractile responses of conductance and resistance arteries from rats, leading to endothelial dysfunction and increased vascular reactivity in these vessels. However, no changes in blood pressure were observed (Pecanha et al., 2010; Wiggers et al., 2008). Recently, we have demonstrated that an increase in the exposure time at low doses of this metal (60 days) promotes worsening of previously observed vascular damage, leading to an increase in systolic blood pressure (SBP) (Rizzetti et al., 2017c).

As a preventive or therapeutic alternative against the deleterious consequences generated by the Hg, different compounds were studied and showed beneficial effects, such as chelants (Andersen and Aaseth, 2016), plant compounds (Abarikwu et al., 2017), synthetic antioxidants (Rizzetti et al., 2013) as well as bioactive peptides derived from egg white hydrolysate (EWH) (Rizzetti et al., 2017a). Bioactive peptides are defined as inactive molecules in the parent protein that, upon their release by hydrolysis *in vitro* or *in vivo*, may begin to develop biological activities (Liao et al., 2018). The EWH obtained by albumin hydrolysis with pepsin for 8 hours proposed in this study demonstrated to be a potent antioxidant, angiotensin-converting enzyme (ACE) inhibitor (Miguel et al., 2004), increase mitochondrial proliferation and bound gene expression (Moreno-Fernandez et al., 2018b) in addition to improving inflammatory biomarkers in animal models (Moreno-Fernandez et al., 2018a). It is known that EWH improves the Hg-induced damage in parameters related to memory deficits (Rizzetti et al., 2016a), peripheral nervous disorders (Rizzetti et al., 2016b), male reproductive dysfunction (Rizzetti et al., 2017b), as well as conductance arteries injury (Rizzetti et al., 2017a). In conductance arteries of long-term Hg-exposed rats, EWH prevented the increased vascular reactivity and endothelial dysfunction promoted by the metal. Moreover, normalized SBP values were observed in these rats. Taking into account that resistance arteries contribute significantly to the creation of the resistance to flow and regulation of blood pressure, the aim of this study was to investigate the potential beneficial effects of EWH intake on vascular disorders induced by chronic exposure to low

concentrations of Hg in mesenteric resistance arteries (MRA) as well as to clarify the possible pathways involved in its effects.

Materials and Methods

Animals and reagents

Three-month-old male *Wistar* rats (250g-350g) were obtained from Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were maintained at a constant room temperature, humidity, and light/dark cycle, and they had access to water and feed *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethic Committee on Animal Use at Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (institutional review board 052014). The experiments were designed to minimize the number of animals used and their suffering during the execution of the protocols. All reagents were high purity and purchased from Sigma-Aldrich (St. Louis, MO, USA), and all of the solutions were prepared with purified water by a Milli-Q high purity water device (Millipore, Bedford, MA, USA).

Experimental groups

Rats were divided into four groups, treated for 60 days with: a) intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage (Untreated); b) *i.m.* injections of mercury chloride − $HgCl_2$, the 1st dose 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day, to cover daily loss, using the model described previously (Rizzetti et al., 2017c) and tap water by gavage ($HgCl_2$); c) *i.m.* injections of saline solution 0.9% and EWH from pepsin for 8 h diluted in tap water (1 g/kg/day), by gavage, according to model prior reported (Rizzetti et al., 2016a) (EWH); d) both treatments (EWH+$HgCl_2$). During the treatment, the manipulation of the animals was performed following the appropriate safety measures and general health, body weight, food and water intakes were recorded once a week.

Blood pressure measurements

We weekly measured the SBP of the rats by noninvasive tail-cuff plethysmography (Bunag, 1973). For this, rats were kept before the measurement at 37 °C for 10 min to make the pulsations of the tail artery detectable. To determine the value of SBP 10 measurements were taken by the equipment (AD Instruments Pty Ltd, Bella Vista, NSW, Australia) and the

average of all of them was obtained. All measurements were taken by the same person in the same peaceful environment to minimize stress-induced variations in blood pressure. Moreover, we established a training period of 2 weeks before the actual trial time to guarantee the reliability of the measurements and during this period the rats were accustomed to the procedure.

Vascular reactivity experiments of the mesenteric arteries

Following 60 days of treatment, rats were anesthetized (ketamine − 87 mg/kg plus xylazine − 13 mg/kg, *i.p.*). The third-order MRA were dissected and segments of 2 mm in length were mounted in a small vessel chamber myograph (Multi Wire Myograph System, DMT620, ADInstruments, Australia) for isometric tension measurements (Mulvany and Halpern, 1977). Subsequently they were placed in oxygenated Krebs–Henseleit solution (KHS, in mM: 115 NaCl, 25 NaHCO$_3$, 4.7 KCl, 1.2 MgSO$_4$ 7H$_2$O, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 11.1 glucose, and 0.01 Na$_2$EDTA) the buffer was equilibrated with a gas mixture of 95% O2 and 5% CO2 to pH 7.4 at 37°C. The vessel segments were stretched to their optimal luminal diameter for the active tension measurements, washed with KHS and left to equilibrate for 30 minutes to check their functional integrity. After, concentration–response curves to acetylcholine (ACh, 0.01 nM–30 mM) were performed in mesenteric resistance previously contracted with noradrenaline (NE), at a concentration that produced approximately 50% of the contraction induced by KCl. Relaxation equal to or greater than 80% represented a positive demonstration of the functional integrity of the endothelium. Then, after a washout period of the 30 minutes, increasing concentrations of NE (10 nM–30 μM) were applied. A concentration-response curve was generated, and tension was measured once a plateau was reached. The influence of the endothelium on the response to NE was investigated after its mechanical removal, which was accomplished by rubbing the vessel lumen. The absence of the endothelium was confirmed by the inability of 10 μM ACh to induce relaxation greater than 10% of the previous contraction to NE. A parallel study was performed to determine the effects of the nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 mM, an essential cofactor for NO synthesis, tetrahydrobiopterin (BH4, 100 μM), a non-selective cyclooxygenase inhibitor (Indomethacin, 10 μM), a NAD(P)H oxidase inhibitor (VAS2870, 10 μM), a superoxide dismutase mimetic (TEMPOL, 10 μM). These drugs were added 30 min prior to the generation of the NE concentration-response curves. To evaluate the relaxation dependent and independent of the endothelium, concentration-

response curves to ACh (0.1 nM −3.5 mM) and sodium nitroprusside (SNP) (0.1 nM − 3.5 mM) were respectively performed.

Nitric oxide release

NO release was measured as previously described (Avendano et al., 2014). After an equilibration period of 60 min in Krebs-HEPES buffer at 37°C, mesenteric segments were incubated with 4,5-diaminofluorescein (DAF-2, 2 μM) for 45min. Thereafter, the medium was collected to measure basal NO release. Subsequently, the induced NO release was measured after segments were incubated with NE 0.1 nM and relaxed with ACh 10 μM. The fluorescence was measured using a spectrofluorometer (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA) with excitation at 492 nm and emission at 515 nm. The induced NO release was obtained by subtracting basal NO release from that evoked by ACh. Blank samples were measured in the medium without segments to subtract background emission. The amount of NO released was expressed as arbitrary units/g tissue. Data were expressed as percentage of results obtained for Untreated rats.

Superoxide anion production in MRA

To evaluate *in situ* superoxide anion production in mesenteric arteries, the oxidative fluorescent dye dihydroethidium (DHE) was used as previously described (Martin et al., 2012). Hydroethidine freely permeates cells and is oxidized in the presence of superoxide anion to ethidium bromide, which is trapped by intercalation with DNA. Frozen mesenteric segments were cut into 14-μm-thick sections and placed on a glass slide. Serial mesenteric sections were equilibrated in a Krebs-HEPES buffer containing (in mM) 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, and 11 glucose, pH 7.4. Fresh buffer containing DHE (2 μM, 30 min, 37°C) was applied topically onto each tissue section, cover slipped, incubated for 30 min in a light-protected humidified chamber at 37°C, and then viewed by a fluorescence microscope (Eclipse 50i55i Epi-fluorescence Nikon, Tokyo, Japan, magnification: ×40). To quantify the fluorescence emitted by ethidium bromide, five artery cuts per animal were used. The total ring area for each experimental condition were analyzed using Image J version V1.56 (National Institutes of Health, Bethesda, Maryland, USA) software, for color images an external camera was used.

Biochemical Assays

For biochemical assays, segments of MRA were homogenized in 50 mM Tris-HCl, pH 7.4, (1/10, w/v), centrifuged at 2400g for 10 min at 4°C and the resulting supernatant fraction

was frozen at -80 °C for further assay. Reactive Oxygen Species (ROS) levels in MRA were determined by spectrofluorimetric method (Loetchutinat et al, 2005). For this, samples were diluted (1:5) in 50 mM Tris-HCl (pH 7.4) and 2′, 7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCHF, which is then rapidly oxidized to form highly fluorescent 2′, 7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 488 nm excitation) for 60 min at 15 min intervals (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA). The amount of ROS formed was expressed as fluorescence units; data were expressed as percentage of those obtained for Untreated rats.

We also measured lipid peroxidation levels were measured in MRA by colorimetric method (Ohkawa et al., 1979), with modifications. An aliquot of tissue was incubated with thiobarbituric acid (TBA) 0.8%, acetic acid buffer (pH 3.2) and sodium dodecil sulphate (SDS) 8% at 95°C for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA). The results were expressed as nmol of malondialdehyde (MDA) per gram of tissue.

The total antioxidant capacity was measured in samples mesenteric arteries by Ferric Reducing/Antioxidant Power (FRAP) assay (Benzie and Strain, 1996). This method is based on the ability of samples to decrease ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s-triazina (TPTZ) the chelate complex Fe^{+2}-TPTZ. Briefly, 10 μL of MRA was added to 1 mL of freshly prepared and pre warmed (37°C) FRAP reagent (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1) in a test tube and incubated at 37°C for 10 min. The absorbance of the blue colored complex was read against reagent blank (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). Standard dose-response curve of Trolox (50–1000 μM – water soluble analog of vitamin E) was performed and results are presented with particular reference to Trolox equivalents.

Non-protein thiols (NPSH) in mesenteric arteries were determined by the method of Ellman (1959). To do this, an aliquot of tissue was added to potassium phosphate buffer 1 M, pH 7.4, and 5,5′- dithio-bis (2-nitrobenzoic acid) (DTNB) 10 mM. The color reaction was spectrophotometrically measured at 412 nm (Spectrophotometer Femto600S, São Paulo, Brazil). The results were expressed as nmol of NPSH per gram of tissue.

Statistical analyses

Vasoconstrictor responses induced by NE were expressed as the % of the tone generated by 120 mM KCl. Vasodilator responses induced by ACh were expressed as the % of the previous tone in each case. The maximum response (Emax) and pD2 values were calculated by nonlinear regression analysis of each individual concentration-response curve using a computer program (GraphPad Prism 6 Software, San Diego, CA, USA). To compare the effect of endothelium removal and the drugs on the response to NE in segments from the four groups, some results are expressed as the differences of areas under the concentration-response curves (dAUC) in the control and experimental situations. AUCs were calculated from the individual concentration-response curve plots using the same software; the differences were expressed as the % of the AUC of the corresponding control situation. The results are expressed as the mean $\pm$ SEM (standard error of the mean) of the number of animals used in each experiment; differences were analyzed using one- or two-way analyses of variance (ANOVA), followed by the Fisher post hoc test. Differences were considered statistically significant at $P < 0.05$.

Results

The water and food intake did not differ between the groups during the treatment (data not shown). Moreover, no differences in body weight gain were observed after the treatment (body weight gain, in g: Untreated: 58.5 ± 4.1; $HgCl_2$: 60.3 ± 4.9; EWH: 59.7 ± 3.8; EWH+$HgCl_2$: 61.1 ± 5.3; n = 8; P > 0.05). As previously shown (Rizzetti et al., 2017a), increased SBP values were verified in Hg-treated rats. However, co-treatment with EWH was able to prevent this increment in SBP (SBP values, in mmHg: Untreated: 120.1 ± 1.9; $HgCl_2$: $135.2 \pm 2.8^*$; EWH: 124.5 ± 1.5; EWH+$HgCl_2$: $122.0 \pm 2.2^{\#}$; n = 8; P < 0.05). In the vascular reactivity experiments, the response to KCl was not modified by different treatments (in mN/mm, Untreated: 14.1 ± 0.3; $HgCl_2$: 14.2 ± 0.5; EWH: 14.3 ± 0.1; EWH+$HgCl_2$: 14.1 ± 0.2; n=8, one-way ANOVA, P > 0.05), demonstrating that neither Hg nor EWH alter vascular integrity of MRA.

Hg treatment exposure for 60 days increased the contractile responses induced by NE while it reduced the endothelium-dependent vasodilator response to ACh without affecting the endothelium-independent vasodilator response to NPS in MRA. Interestingly, EWH-treatment was able to prevent the increase in the vasoconstrictor response to NE and the decrease in

vasodilator response to ACh, suggesting that EWH intake prevented the endothelial dysfunction promoted by chronic exposure to low doses of Hg in rat mesenteric arteries (Table 1; Fig 1 A-C)

Endothelium removal or the incubation with the NOS inhibitor L-NAME caused a significant increase in the contractile response to NE in MRA of Untreated, EWH and EWH+HgCl$_2$ groups. However, NE responses remained unchanged in HgCl$_2$ group, as demonstrated by the dAUC values (Fig 2A-E, a-e). These findings evidence absence of endothelial participation in the vasoconstrictor response to NE in this group. Interestingly, EWH treatment did not modify the effect of both endothelium removal or L-NAME; thus, mesenteric segments from animals that received treatment with EWH in combination with HgCl$_2$ showed similar effects of endothelium removal or L-NAME to the Untreated group (Fig 2A-E, a-e), suggesting that EWH prevented these reduced endothelial modulation by NO in MRA of rats exposed to chronic Hg treatment, as shown in conductance arteries (Rizzetti et al., 2017a). In agreement, ACh-induced NO release was decreased in MRA from Hg-treated rats. On the other hand, rats receiving EWH alone and those receiving the combination of HgCl$_2$ and EWH increased the percentage of NO release when compared to the untreated group (Fig 2a′). This finding suggests that EWH was able to induce NO production by NOS.

Previously we demonstrated that the oxidative stress is an important factor responsible for the reduction in the NO bioavailability produced by Hg (Rizzetti et al., 2017a; Rizzetti et al., 2017c; Wiggers et al., 2008). ROS derived from the enzyme NAD(P)H oxidase are involved in reducing NO bioavailability in the vasculature and may induce endothelial dysfunction in aortic arteries of Hg-exposed rats (Rizzetti et al., 2017a; Rizzetti et al., 2017c; Wiggers et al., 2008). In addition, NOS isoforms may also promote the formation of ROS when they are uncoupled, reducing NO bioavailability and causing vascular damage. In the eNOS, uncoupling is caused by the absence of the co-factors L-arginine and tetrahydrobiopterin (BH4) (Andrews and Wang, 2002; Vasquez-Vivar et al., 1998). In order to investigate whether ROS arising from NAD(P)H oxidase and eNOS depletion would be involved in the vascular alterations of MRA from Hg-exposed rats, as well as the influence of EWH in the improvement of vascular oxidative stress, we performed *in vitro* experiments using an essential cofactor for NO synthesis, (BH4), a NAD(P)H oxidase inhibitor (VAS2870) and a superoxide dismutase mimetic (TEMPOL).

The cofactor for NO synthesis BH4 incubation reduced the vasoconstrictor response induced by NE in the MRA only from Hg-treated rats, demonstrating a reduction in BH4

bioavailability and possibly a uncoupled state of eNOS in this vessel. The co-treatment with EWH prevented the reduced BH4 bioavailability in MRA from Hg-treated rats, suggesting the improvement of eNOS and NO syntesis (Fig 3A-E).

The NAD(P)H oxidase inhibitor VAS2870 reduced the contractile response to NE in MRA only in Hg treated rats (Fig 3a-e). EWH prevented the increased ROS participation from NAD(P)H oxidase on contractile response to NE. The superoxide dismutase mimetic TEMPOL reduced the contractile response to NE in MRA only from Hg-exposed rats, thus the NE responses remained unchanged in the other groups (Fig 3a´-e´). This finding suggests that EWH prevented the increased ROS participation from NAD(P)H oxidase in MRA from Hg-treated rats. NAD(P)H oxidase is an important source of vascular ROS production. Thus, we investigated the production of reactive species *in situ*. We observed a significant increase in superoxide anion production in arteries of rats exposed to Hg, and EWH was able to prevent this increase (Fig 4). In accordance with our functional experiments, chronic Hg treatment for 60 days increased ROS levels and lipid peroxidation in MRA from exposed rats (Fig 5A, B). Moreover, the total antioxidant capacity and the NPHS levels were reduced in the Hg-exposed rats (Fig 5C, D). EWH was able to prevent the oxidative stress in MRA of Hg-treated rats, normalizing the pro-oxidant and antioxidant status (Fig 5A-D).

Our previous study also reported that low dose of $HgCl_2$ increased the participation of inflammatory pathways on vasoconstrictor responses to Phe in the aorta (Pecanha et al., 2010; Rizzetti et al., 2017c). Thus, to investigate the role of EWH on prostanoids involvement in vascular response to NE in MRA rings from Hg-exposed rats, the effects of indomethacin were analyzed. Indomethacin reduced the response to NE in MRA segments from only in Hg-treated rats (Fig 6 A-E), indicating increased participation of the COX pathway in these conditions. Interestingly, EWH co-treatment totally prevented the increase in the participation of this inflammatory pathway on vasoconstrictor responses in MRA from Hg-treated rats.

Discussion

Our results demonstrated, for the first time, that EWH is able to prevent the increased contractile responses and vascular dysfunction induced by chronic Hg exposure at low concentrations in MRA, which probably leads to a reduction in SBP values of Hg-exposed rats. These effects were associated, at least in part, with the capacity of the EWH in producing NO by eNOS and its antioxidant and anti-inflammatory properties. Its bioactive peptides

provide protection against the increased ROS from NAD(P)H oxidase and the activation of inflammatory COX pathways in MRA from Hg-treated rats, normalizing the NO modulation in this vasculature.

It is known that Hg is an environmental risk factor for cardiovascular diseases (Houston, 2007; Virtanen et al., 2007). There is evidence of the association between the exposure to this metal and the development of hypertension, atherosclerosis, coronary artery disease and myocardial infarction (Salonen et al., 2000; Vassallo et al., 2011). Acute Hg exposure promotes reduction of myocardial force development (Vassallo et al., 1999) and inhibition of myosin ATPase activity (Vassallo et al., 1999). Moreover, a subchronic exposure to this metal for 30 days, at doses similar to the human exposure, increases vascular reactivity in resistance and conductance vessels from rats (Pecanha et al., 2010; Wiggers et al., 2008). Recently we verified that a prolonged treatment at low doses of $HgCl_2$ for 60 days increased SBP and vascular dysfunction in conductance arteries, whose effects were related to stimulation of ACE activity, the NADPH oxidase-mediated oxidative stress and the activation of COX-2 inflammatory pathway in these vessels (Rizzetti et al., 2017a; Rizzetti et al., 2017c). In the current study we demonstrated that resistance arteries are also affected by prolonged exposure to Hg, which could explain the increased SBP values observed. Moreover, we confirmed that the oxidative stress, caused by the production of superoxide anion from NAD(P)H oxidase, and the participation of inflammatory mediators derived from COX are the main routes through which this metal exerts its toxic effects on the cardiovascular system. In addition, we verified for the first time the involvement of other pathways in Hg-induced damaging actions on vascular tissue, such as the possible eNOS uncoupling, which could be due to the increased vascular oxidative stress, possibly caused by the inflammatory stimuli from COX pathway. In this study we demonstrated the protective effect of EWH on resistance arteries, which are vessels responsible for controlling blood flow and controlling blood pressure affected by the metal (Magder, 2018; Nyvad et al., 2017). This EWH obtained from *in vitro* pepsin digestion after 8 h of hydrolysis has demonstrated different biological activities, such as antioxidant, free radical scavenger, ACE inhibitor, vascular-relaxing and anti-inflammatory activity (Miguel et al., 2004). Fourteen of its constituent peptides have already been identified and its actions have been reported when studied *in vitro* or *in vivo* (Miguel et al., 2006; Miguel et al., 2004).

The antihypertensive capacity of the EWH was previously reported in experimental models of spontaneously hypertensive rats (SHR) (Miguel et al., 2006) and this property was

attributed, at least in part, to the vasodilator peptides whose N-terminal position exhibits amino acids Arg or Tyr (Miguel et al., 2005; Miguel et al., 2004). This compound also demonstrated beneficial effects in metabolic syndrome parameters of high-fat/high-dextrose fed rats (Moreno-Fernandez et al., 2018a), improvement in glucose metabolism complications (Moreno-Fernandez et al., 2018b), decreased body weight and adipose tissue and ameliorated hepatic steatosis of Zucker fatty rats (Garces-Rimon et al., 2016).

Recently we reported beneficial effects of the EWH in a experimental model of long-term exposure to Hg at low doses, in which its compound was able to prevent neuropathic disorders (Rizzetti et al., 2016b); memory impairments (Rizzetti et al., 2016a); male reproductive injury (Rizzetti et al., 2017b) and cardiovascular dysfunction in conductance arteries (Rizzetti et al., 2017a) in these Hg-exposed rats. In the present study we demonstrated improvements in resistance arteries and, in consequence, in SBP levels of Hg-treated rats. The most part its effects on this system are probably due to its vascular-relaxing, antioxidant and anti-inflammatory properties.

Regarding the effects of EWH on the Hg-induced vasodilator dysfunction, it has been reported that alterations in eNOS gene increases the susceptibility to cardiovascular diseases in individuals after Hg exposure by modulating NO levels (de Marco et al., 2012). The deleterious vascular effects of Hg on the aorta, coronary and basilar arteries resulting from exposure to the metal at low concentrations are related to the increased vascular reactivity caused by the reduction of NO bioavailability in these vessels (Botelho et al., 2019; Furieri et al., 2011; Pecanha et al., 2010; Wiggers et al., 2008). Moreover, a study in mesenteric arteries from rats treated for 30 days with $HgCl_2$ showed the eNOS protein expression upregulation possible due to a compensatory mechanism against metal-induced endothelial dysfunction (Wiggers et al., 2008). Our results indicate that the reduction in NO bioavailability and endothelial dysfunction persists in MRA of this long-term exposure experimental model. Moreover, we showed that the reduced NO bioavailability occurs, at least in part, due to a decrease in NO release in resistance arteries. Our functional and biochemical findings suggest that this reduction in NO release could be associated with the uncoupling of eNOS promoted by the Hg. In this condition, eNOS shifts from NO production to overproducing superoxide anion, incrementing the oxidative stress in the vasculature (Antoniades et al., 2006). Here we observed a decrease in the BH4 bioavailability in MRA of Hg-treated rats, which represents an important cofactor for the production of NO by this enzyme, indicating that the metal induces eNOS uncoupling by reducing its cofactor.. This dysfunction has been described in

several cardiovascular disorders including diabetes, hypertension, and heart failure (Varadharaj et al., 2015).

Furthermore, at the vascular level, impaired NO bioavailability is also associated with overproduction of vascular ROS, generation of peroxynitrite and an inadequate antioxidant reserve (Faria et al., 2018). Interestingly, the oxidative stress has also been reported as an important mechanism responsible for the reduction in NO bioavailability induced by Hg (Rizzetti et al., 2017c; Wiggers et al., 2008) and it seems to be involved in Hg-induced vascular damage in our study. In addition, the oxidative stress can directly modify eNOS protein (Chen et al., 2010) or its cofactor BH4 and leading to an enzymatic dysfunction in vascular tissue (Dumitrescu et al., 2007; Kuzkaya et al., 2003). In this context, EWH is composed of several peptides that have vasodilator effects (Miguel et al., 2007). Other peptides derived from sources such as soybean (Yang et al., 2009), casein (Hirota et al., 2011), peptides isolated from fermented milk (Wakai et al., 2012) and fish protein (Kamoun et al., 2017) demonstrated similar activity, which was related to its capacity of increasing NO release by the rise in eNOS activity or the upregulation in protein expression of endothelial cells, L-NAME-induced hypertension, SHR and ethanol-induced oxidative stress animal models. Our study suggests that EWH was able to increase the NO release possibly enhancing the eNOS function, which is the major isoform involved in the control of vascular function and blood pressure (Shabeeh et al., 2017). The vasodilator power of EWH has already been described in SHR model and related to the fact of having amino acids like Arg or Tyr that are necessary in the N-terminal position for vasodilator activity (Garcia-Redondo et al., 2010).

Regard to the antioxidant effects of the EWH on oxidative damage in MRA of Hg-exposed rats, several amino acid sequences have been related to scavenging free radicals and antioxidant properties (Miguel et al., 2004). Amino acid sequences such as Tyr-Ala and Glu-Arg-Tyr-Pro-Ile-Leu were suggested as responsible for *in vitro* antioxidant properties (Davalos et al., 2004). However, it is known that antioxidant peptides and amino acids not only act individually, but also cooperatively and synergistically beyond the absorption and hydrolysis in the gastrointestinal system should also be considered (Davalos et al., 2004; Miner-Williams et al., 2014). In the present study EWH was able to prevent the oxidative stress in MRA of Hg-treated rats, avoiding the superoxide anion generation from NAD(P)H oxidase and normalizing the pro-oxidant and antioxidant status verified by the functional experiments and the oxidative stress biomarkers in vascular tissue. Previous study by our research group has showed reduced NOX-4 and p22phox mRNA levels in aorta from long-

term Hg exposed-rats after the co-treatment with EWH, which was related to the reduced vascular reactivity observed in this conductance vessels (Rizzetti et al., 2017a). Similar effect could be found in MRA in the present study to explain our findings. Moreover, recently, EWH demonstrated that its antioxidant properties decreased plasmatic MDA levels and increased the levels of reduced glutathione in the liver of Zucker obese animals (Garces-Rimon et al., 2016). Restored plasma antioxidant capacity was found in high-fat/high-dextrose fed rats with metabolic syndrome after EWH intake (Moreno-Fernandez et al., 2018a) In addition, normalized oxidative biomarkers were reported in neurological, reproductive and cardiovascular systems from Hg-exposed animals after EWH consumption (Rizzetti et al., 2016a; Rizzetti et al., 2016b; Rizzetti et al., 2017a; Rizzetti et al., 2017b).

Our results also evidence that Hg stimulates the COX inflammatory pathway in MRA of these metal-exposed rats. Different biochemical and physical stimuli can activate inflammatory leukocytes, resulting in the induction and synthesis of proinflammatory proteins and enzymes, in particular of the most important enzyme is COX-2 (Murakami and Ohigashi, 2007; Turini and DuBois, 2002). Molecular oxygen in tissues whereas COX-2 activity leads to the conversion of arachidonic acid to prostaglandin (PG)-H2, a precursor of PGE2, which contributes to the pathogenesis of inflammatory diseases (Udenigwe et al., 2013). It has been described that Hg induces NF-kB activation, resulting in the induced expression of COX-2 (Park and Youn, 2013). However, in the current study, the co-treatment with EWH avoided the activation of these inflammatory pathways in Hg-exposed rats. It has been described that the EHW consumption diminished the pro-inflammatory state and decreased the plasma levels of TNF-α in Zucker fatty rats (Garces-Rimon et al., 2016), increased expression of the anti-inflammatory hormone adiponectin and suppressed cytokine mediated inflammatory response in pre-adipocyte cell (Jahandideh et al., 2017). The EWH intake also contributes to the improvement of inflammation biomarkers in obese Zucker rats (Requena et al., 2017). Furthermore, this compound inhibited the release of inflammatory cells in testis from Hg-exposed rats (Rizzetti et al., 2017b), and totally prevented the increased participation of COX-2 pathway on vasoconstrictor responses in conductance arteries (Rizzetti et al., 2017a), corroborating our present findings. Furthermore, hydrolysates produced from other proteins have shown anti-inflammatory properties. Almond-derived peptides exhibited action on inflammatory pathways, reducing the mRNA expression levels of COX-2 enzymes and the activated macrophages (Udenigwe et al., 2013). Similarly our results also evidence EWH

totally prevented the increase in the participation inflammatory pathway COX participation, normalizing the NO production in MRA.

In summary, our results evidenced for the first time that a dietetic supplementation with EWH promotes protective effects against the endothelial dysfunction in MRA, and, in consequence, the increment in SBP of long-term Hg-exposed rats. Its benefits could be possibly related to its NO-induced vasodilatation capacity and its antioxidant and anti-inflammatory properties, reducing the increased ROS from NAD(P)H oxidase and the activation of inflammatory COX pathway in MRA from Hg-treated rats. The EWH could represent a good public health strategy against cardiotoxic pollutants.

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Table 1: Effect of EWH on sensitivity (pD2) and maximum response (Emax) to NE in mesenteric rings of rats exposed to low doses of HgCl2 for 60 days.

	pD$_2$				Emax(%)			
	Untreated	HgCl$_2$	EWH	EWH+HgCl$_2$	Untreated	HgCl$_2$	EWH	EWH+HgCl$_2$
Ct	5.3± 0.06	5.1 ± 0.03	4.9 ± 0.04	5.1 ± 0.05	98.3 ± 1.2	116.6 ±1.7	93.7 ± 2.6	92.7 ± 2.8
E-	5.4 ± 0.1	5.2 ± 0.05	5.0± 0.06	5.2 ± 0.06	109.6 ± 3.4	113.5 ± 2.5	106.6 ± 2.5	102.9 ±3.0
LNAME	5.0 ± 0.08	5.1 ± 0.08	5.0 ± 0.07	5,0 ± 0.1	109.6 ±2.3	113.9 ± 3.6	105.6 ± 3.6	108.1 ±4.0
BH4	4.9 ± 0.1	4.9 ± 0.06	4.8 ± 0.1	4.9 ± 0.08	89.7 ± 2.9	76.1 ± 12.0	87.7 ± 6.5	82.0 ± 3.4
VAS2870	5.0 ± 0.1	4.9 ± 0.08	4.8 ± 0.07	4.9 ± 0.05	80.4 ± 6.4	87.6 ±5.4	83.2 ±4.0	86.4 ± 4.2
TEMPOL	5.1 ± 0.1	4.8 ± 0.07	4.8 ± 0.07	5.1 ± 0.06	93.3 ± 2.6	87.7 ± 8.9	95.6 ± 4.9	101.1 ±4.6
INDO	4.8 ± 0.1	4.85 ± 0.08	4.8 ± 0.05	4.89 ± 0.04	89.9 ± 3.3	90.1± 4.1	83.3 ±3.4	90.6 ± 2.7

Parameters of sensitivity (pD2) and maximal response (Emax) of the concentration-response curves to NE in MRA from rats Untreated, treated with Mercury, Hydrolysate and Hydrolysate plus Mercury in intact (Ct) and endothelium removal (E-) segments and in the presence of L-NAME, BH4, VAS2870, TEMPOL and Indomethacin incubation. Results are expressed as mean ± SEM. Emax, maximal effect (expressed as a percentage of maximal response induced by 120mMKCl) and pD2 (expressed as −log one-half Emax) (8 of each group in parenthesis, one-way ANOVA, *P < 0.05 vs. compared to the corresponding control in each group; #P < 0.05 vs. Untreated group).

Figure legends

Figure 1. Effect of EWH co-treatment in MRA reactivity of rats exposure to Hg for 60 days. Concentration–response curves to (A) noradrenaline (NE), (B) acetylcholine and (C) sodium nitroprusside in MRA segments. The results (mean ± SEM) are expressed as a percentage of the response to 120mmol/l KCl. Two-Way ANOVA followed by Fisher test; n of each group in parenthesis; *$P<0,05$ vs Untreated and # vs $HgCl_2$.

Figure 2. Effects of EWH co-treatment in MRA of rats exposure to Hg for 60 days on the endothelium and NO mediated vascular response. Concentration-response curve to NE in the absence of endothelium (E−) (A-D) and L-NAME (100μM) (a-d) in mesenteric segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean ± SEM) are expressed as a percentage of the response to 120mmol/l KCl. Two-Way ANOVA followed by Fisher test; *$P<0,05$ vs Untreated and # vs $HgCl_2$. Differences in the area under the concentration-response curves (dAUC) in mesenteric segments are represented in E and e of the four experimental groups ; one-way ANOVA, *$P < 0.05$ vs. Untreated and # vs $HgCl_2$. In a´ represent the NO release in all experimental groups.

Figure 3. Effects of EWH on NO cofactor modulation, participation of reactive oxygen species (ROS) from NAD(P)H oxidase and participation of superoxide anion in vasoconstrictor responses to NE in MRA from rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to NE in the absence (Ct), presence of the eNOS cofactor (BH4) (A-D), the presence of the NAD(P)H synthase inhibitor (VAS2870) (a-d) and the presence of the superoxide anion scavenger mimetic (TEMPOL) (a'-d') in mesenteric segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean ± SEM) are expressed as a percentage of the response to 120mmol/l KCl. Differences in the area under the concentration-response curves (dAUC) in mesenteric segments in the presence and the absence of BH4, VAS2870 and TEMPOL of the four experimental groups (E, e and e´); n of each group in parenthesis, one-way ANOVA, *$P < 0.05$ vs. Untreated and # vs HgCl2.

Figure 4: Effects of EWH on local anion superoxide production in mesenteric of rats exposed to low concentrations of $HgCl_2$ for 60 days. Superoxide Anion production in mesenteric from rats Untreated, treated with Mercury, with Hydrolysate or with Hydrolysate plus Mercury levels in mesenteric of all groups; n of each group in parenthesis, one-way ANOVA, *P < 0.05 vs. Untreated, # vs HgCl2.

Figure 5: Effects of EWH in the ROS (A), MDA (B), the total antioxidant capacity (C) and NPSH (D) in mesenteric of rats exposed to low concentrations of $HgCl_2$ for 60 days. Data are expressed as mean ± SEM. Number of animals is indicated in parentheses respectively, one-way ANOVA, *P < 0.05 vs. Untreated; # vs HgCl2.

Figure 6: Effects of EWH on contribution of COX pathway on vasoconstrictor responses to NE in mesenteric of rats exposed to low concentrations of $HgCl_2$ for 60 days. Concentration-response curve to NE in the absence (Ct), the presence of the non-selective COX inhibitor (Indomethacin) (A-D) in mesenteric segments from rats Untreated (A), treated with Mercury (B), with Hydrolysate (C) and with Hydrolysate plus Mercury (D). The results (mean ± SEM) are expressed as a percentage of the response to 120mmol/l KCl. Two-Way ANOVA followed by Fisher test; n of each group in parenthesis; *P<0,05 vs Untreated and # vs $HgCl_2$. . Differences in the area under the concentration-response curves (dAUC) in mesenteric segments are represented in E and e graphs (one-way ANOVA)
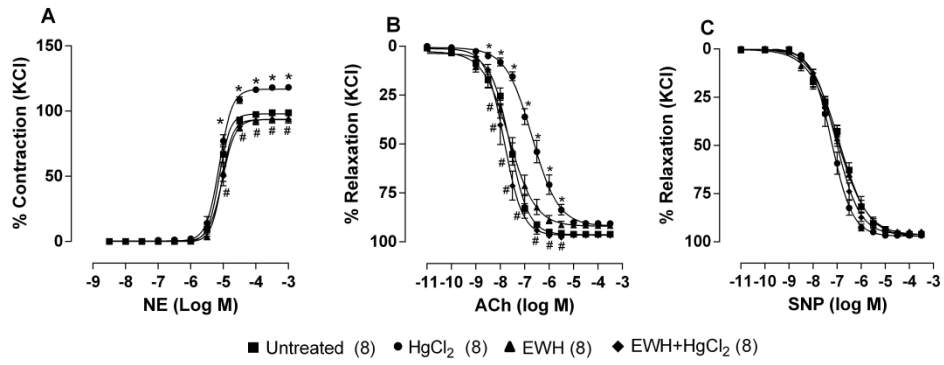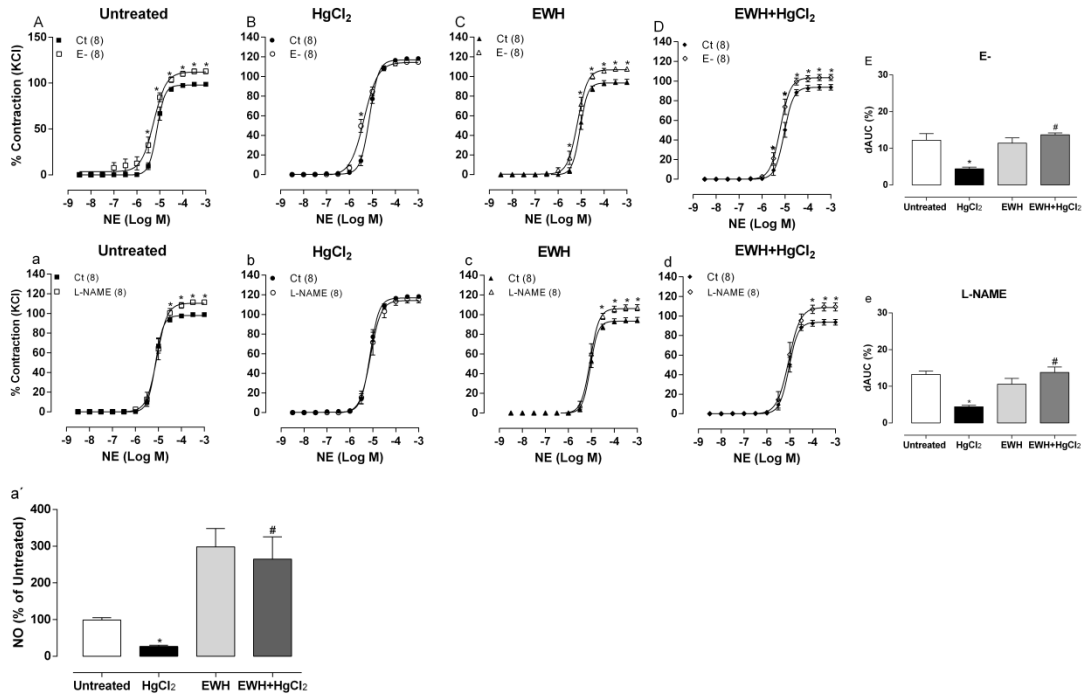
Figures

Figure 1

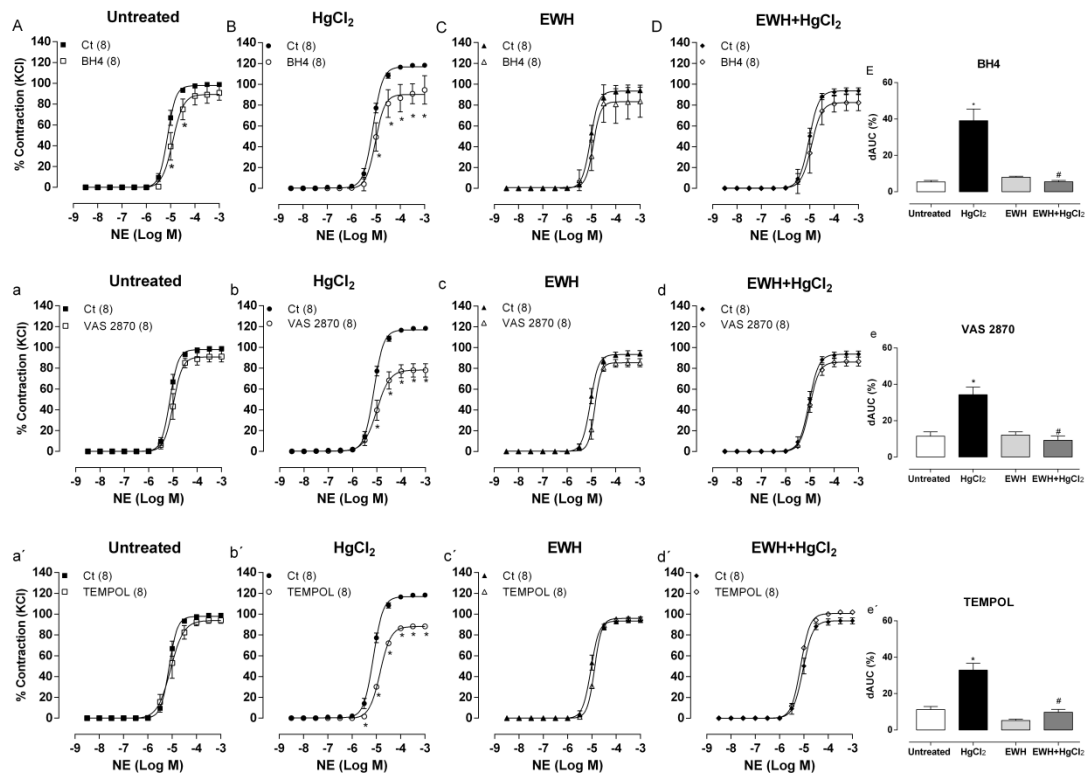■ Untreated (8)   ● HgCl$_2$ (8)   ▲ EWH (8)   ◆ EWH+HgCl$_2$ (8)

Figure 2

Figure 3

Figure 4

Figure 5

A. ROS levels

B. Lipid Peroxidation

C. Total Antioxidant Capacity

D. NPSH levels

Untreated (8) HgCl₂ (8) EWH (8) EWH+HgCl₂ (8)

Figure 6

A — Untreated

B — HgCl$_2$

C — EWH

D — EWH+HgCl$_2$
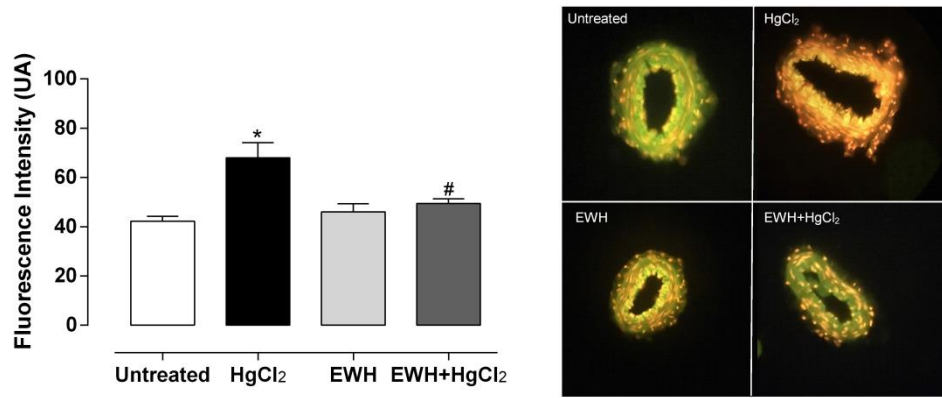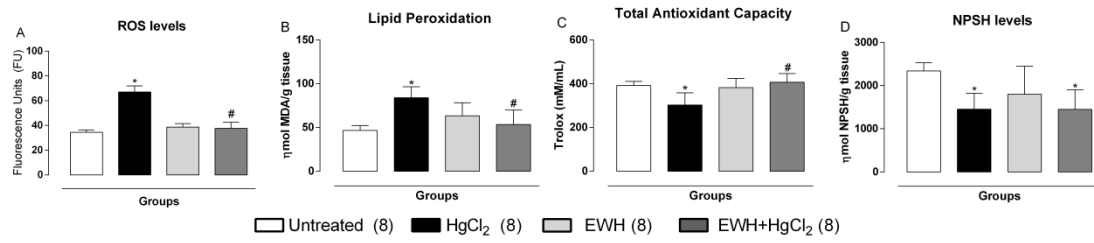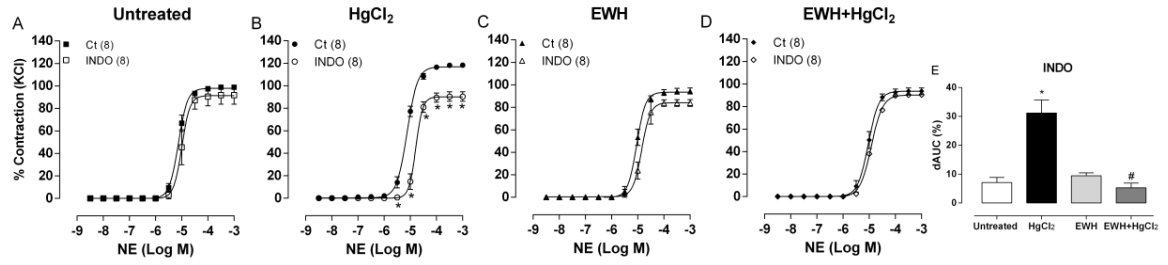
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