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EFEITO DO HIDROLISADO DE CLARA DE OVO COMO POSSÍVEL PROTETOR DAS ALTERAÇÕES NO SISTEMA CARDIOVASCULAR PROMOVIDAS PELA EXPOSIÇÃO AO CLORETO DE CÁDMIO EM RATOS.

DISSERTAÇÃO DE MESTRADO

Paola Zambelli Moraes

Uruguaiana, RS, Brasil

2019

PAOLA ZAMBELLI MORAES

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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 Mestra em Bioquímica.

Orientadora: Profª. Drª. Giulia A. Wiggers Peçanha

Co-orientadora: Drª. Caroline Silveira Martinez

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Uruguaiana, RS, Brasil
2019

"O que prevemos raramente ocorre; o que menos esperamos geralmente acontece."

Benjamin Disraeli

"Foi o tempo que dedicaste à tua rosa que a fez tão importante."

Antoine de Saint-Exupéry

Dedico este trabalho aos meus pais,
fonte de amor genuíno e inesgotável.
Tudo por vocês e para vocês.

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LISTA DE ABREVIATURAS

µg – micrograma

µg/kg – micrograma por quilograma

µg/kg/dia – micrograma por quilograma por dia

mg – miligrama

mg/kg – miligramas por quilograma

mg/kg/dia – miligramas por quilograma por dia

Kg – quilograma

ACE – Angiotensin-converting enzyme

ACh – Acetilcolina

APO – Apocinina

BAL – 2,3-dimercaptopropanol

Ca – Cálcio

Cd – Cádmio

$CdCl_2$ - Cloreto de cádmio

COX – enzima Ciclooxigenase

COX-2 – Isoforma 2 da Enzima Ciclooxigenase

Cu – Cobre

dAUC - Differences of area under the concentration-response curves

DHE - dihydroethidium

DPA – D-penicilamina

DMT1 – Transportador de metais divalentes 1

ECA - Enzima conversora da angiotensina

EDTA – Ácido Etilenodiaminotetracético

Emax – Resposta máxima

Fe – Ferro

KCl – Cloreto de potássio

L-NAME – N(ω)-Nitro-L-arginine methyl ester

LOS- Losartana

MDA – Malondialdehyde

MT – Metallothionein

NADPH oxidase – enzima Nicotinamida Adenina Dinucleotídeo Fosfato oxidase

NaOH – Hidróxido de Sódio

NO – Nitric Oxide

NOS – Oxido Nitrico Sintase

NPS – Nitroprussiato de Sódio

$O_2^{\cdot -}$ - Superoxide radical anion

Pb – Chumbo

Phe – Phenilephyne

RAS – Renin-angiotensin System

ROS – Reactive Oxygen Species

RNS - Reactive nitrogen species

SDS - Sodium dodecyl sulphate

SBP – Systolic Blood Pressure

SEM – Standard Error of the Mean

SHR – Spontaneous Hypertensive Rats

SRA – Sistema Renina Angiotensina

TBA - Ácido tiobarbitúrico

PARTE I

RESUMO

Dissertação de Mestrado

Programa de Pós Graduação em Bioquímica

Universidade Federal do Pampa

EFEITO DO HIDROLISADO DE CLARA DE OVO COMO POSSÍVEL PROTETOR DAS ALTERAÇÕES NO SISTEMA CARDIOVASCULAR PROMOVIDAS PELA EXPOSIÇÃO AO CLORETO DE CÁDMIO EM RATOS

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Local e Data da defesa: Uruguaiana, 27 de fevereiro de 2019

Introdução: O Cadmio (Cd) é um metal pesado ao qual estamos facilmente expostos através do consumo de água potável, alimentos e fumaça de cigarro. Seus efeitos deletérios atingem vários órgãos e sistemas, incluindo o sistema cardiovascular. Alimentos funcionais advindos de proteínas animal vem sendo investigados como alternativa ou complemento no tratamento de doenças que levam a danos oxidativos, inflamatórios e metabólicos ocasionados por metais pesados. **Objetivo:** Investigar se o hidrolisado de clara de ovo (EWH), um alimento funcional, pode ser eficaz no combate aos danos cardiovasculares causados pela exposição ao Cd. **Materiais e Métodos:** Ratos *Wistar* foram tratados por 14 dias com: A) Grupo não tratado – injeções intraperitoneais (i.p.) de água destilada e água potável por gavagem; B) Grupo Cd – injeções (i.p.) 1mg/kg de peso corporal/dia de cloreto de cádmio ($CdCl_2$) e água potável por gavagem; C) Grupo EWH – Injeções (i.p.) de água destilada e 1mg/kd/dia de EWH de pepsina por 8 horas diluído em água corrente por gavagem; D) Grupo CdEWH – ambos os tratamentos. Após o tratamento a pressão arterial sistólica (PAS) foi mensurada por pletismografia caudal; Os experimentos de reatividade vascular na aorta foram realizados em sistema de banho de órgãos, onde foram analisadas as respostas vasodilatadoras dependente e independente do endotélio e a resposta vasoconstritora à fenilefrina (Phe) na ausência e presença de endotélio, e de um inibidor da NOS (L-NAME), um inibidor da NADPH oxidase (Apocinina), um inibidor não selectivo da

COX (Indometacina), um inibidor selectivo da COX-2 (NS 398), e de um bloqueador dos receptores AT-1 (Losartan). A expressão da proteína COX-2 também foi realizada na aorta, enquanto a determinação da atividade da enzima conversora da angiotensina (ECA) foi medida em plasma. Níveis de peroxidação lipídica, espécies reativas de oxigênio (ROS) e capacidade antioxidante (FRAP) foram medidos em aorta. **Resultados:** O EWH preveniu o aumento da PAS e da reatividade vascular causada pelo Cd. Essas melhoras vasculares foram relacionadas à inibição dos prostanóides derivados da COX-2 pela redução da expressão dessa proteína, inibição da angiotensina II pela redução da atividade da ECA e a consequente redução do estresse oxidativo mediado pela NADPH oxidase. **Conclusão:** O EWH é um alimento funcional com propriedades benéficas e pode ser considerado como alternativa para o tratamento de danos cardiovasculares induzidos por Cd.

Palavras-chave: Hipertensão; Reatividade Vascular; Disfunção Vascular; Estresse oxidativo; Hidrolisado de clara de ovo; Peptídeos bioativos; Cádmio.

ABSTRACT

Masters Dissertation

Program of Post-Graduation in Biochemistry

Federal University of Pampa

EFFECT OF EGG WHITE HYDROLYZATE AS POSSIBLE PROTECTOR OF CARDIOVASCULAR DAMAGE PROMOTED BY EXPOSURE TO CADMIUM CHLORIDE ($CdCl_2$) IN RATS

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Co-advisor: Caroline Silveira Martinez

Site and Date of defence: Uruguaiana, February 27st, 2019

Introduction: Cadmium (Cd) is a heavy metal that we are easily exposed through the consumption of drinking water, food and cigarette smoke. Its deleterious effects affect several organs and systems, and the cardiovascular system is one of the affected. In contrast, functional foods derived from animal proteins have been investigated as an alternative or complementary treatment for innumerable pathologies, including oxidative, inflammatory and metabolic damages caused by heavy metals, bypassing these effects through bioactive peptides. **Objective:** To investigate whether egg white hydrolyzate (EWH), a functional food, may be effective in combating cardiovascular damage caused by exposure to Cd. **Materials and Methods:** Male *Wistar* rats were treated for 14 days with: A) Untreated group - intraperitoneal (ip) injections of distilled water and potable water by gavage; B) Group Cd - injections (i.p.) 1mg / kg of body weight / day of cadmium chloride ($CdCl_2$) and potable water by gavage; C) EWH Group - Injections (i.p.) of distilled water and 1 mg / kd / day of pepsin EWH for 8 hours diluted in running water by gavage; D) CdEWH Group - both treatments. After treatment, systolic blood pressure (SBP) was measured by tail plethysmography; Vascular reactivity experiments in the aorta were performed in an organ bath, where the vasopressin dependent and endothelial independent responses were analyzed and the

vasoconstricting response to phenylephrine (Phe) in the absence and presence of endothelium, and an inhibitor of NOS - (L-NAME), an inhibitor of NADPH oxidase (apocynin), a non-selective COX inhibitor (indomethacin), a selective COX-2 inhibitor (NS 398), an AT-1 (losartan) receptor blocker. In situ expression of COX-2 protein was also performed in the aorta, while the determination of angiotensin converting enzyme (ACE) activity was measured in plasma. Levels of lipid peroxidation, reactive oxygen species (ROS) and antioxidant capacity (FRAP) were measured in pots. **Results:** EWH prevented the increase in SBP and vascular reactivity caused by Cd. These vascular improvements were related to the inhibition of COX-2 derived prostanoids by the reduction of expression of this protein, inhibition of angiotensin II by the reduction of ACE activity and consequent reduction of oxidative stress mediated by NADPH oxidase. **Conclusion:** EWH could be considered as an alternative for the treatment of cardiovascular damage induced by Cd.

Keywords: Hypertension; Vascular Reactivity; Vascular Dysfunction; Oxidative stress; Egg white hydrolyzate; Bioactive peptides; Cadmium;

INTRODUÇÃO

1 CÁDMIO

1.1 Características físico-químicas do cádmio

Cádmio (Cd) é um metal pesado, tóxico, não essencial de coloração cinza clara, dúctil e maleável que, na presença de oxigênio pode ser facilmente oxidado a óxido de cádmio (WHO, 2010). Trata-se de um metal de transição, pertencente ao grupo IIb da tabela periódica, o mesmo grupo ocupado pelo zinco (Zn), e pelo mercúrio (Hg), possui massa atômica de 112.4u, estado sólido em temperatura ambiente, e o seu estado de oxidação mais comum é o +2. Este metal foi descoberto em 1817, pelo professor da Universidade de Gottinger, na Alemanha, Friendrich Stromeyer (NORDBERG, G., 2009). É um metal atualmente muito utilizado na indústria, mineração e refinamento de Cd, Zn, sendo que o Cd é o principal sub-produto da mineração, fundição e refinamento de outros metais, tais como chumbo (Pb), cobre (Cu) e Zn. Também é encontrado na eletrodeposição metálica, baterias de Cd-Ni, plásticos, pigmentos, eletrônicos, entre outros. Seus efeitos tóxicos já comprovados no ser humano devem-se a exposição ocupacional, ingestão de água, alimentos contaminados ou inalação ativa ou passiva de fumaça de cigarro. Seus efeitos podem ser perpetuados devido à longa meia vida (10 a 30 anos) deste metal e dos baixos níveis de excreção, (WHO, 1992; ATSDR, 2005; WHO, 2010).

1.2 Contaminação Ambiental e Exposição Humana

A exposição ao Cd se dá por fontes naturais e antropogênicas, este é um metal que pode ser encontrado em baixas concentrações na natureza, sendo que sua principal fonte natural de emissão para o ambiente são os vulcões e o intemperismo das rochas. Cerca de 140-1500 toneladas de cádmio foram lançadas para a atmosfera através de erupções vulcânicas em 1983 (NRIAGU, 1989). Ainda sobre este mesmo ano, Nriagu e Pacyna (1988) estimaram que a contaminação atmosférica por fontes antropogênicas foi de 7600 toneladas de Cd, dessa forma superando as fontes naturais e intensificando a emissão do metal para o meio ambiente. Esse dado persiste atualmente, onde as fontes antropogênicas seguem sendo as principal via de contaminação ambiental e por consequência contaminação humana pelo consumo de água e alimentos contaminados (WHO, 1992; ATSDR, 2005; WHO, 2010). Essas fontes antropogênicas derivam da

queima de combustíveis fósseis, mineração e fundição, deposição atmosférica de indústrias metalúrgicas eliminação de resíduos como plástico, baterias e lodo de esgoto pelo despejo ou de incineração (PACYNA e PACYNA, 2001; KHAN *et al.*, 2017; SAH *et al.*, 2018).

As indústrias, além de ocasionar a contaminação ocupacional, acabam por ser responsáveis pela contaminação de populações de áreas vizinhas podendo afetar um maior número de indivíduos, com ou sem acidentes ambientais justamente por aumentar os níveis de Cd na atmosfera (WHO, 1992; HOUTMAN, 1993; ATSDR, 2005). Dentre os acidentes mais conhecidos envolvendo Cd já descritos, o acidente que deu origem as principais evidências dos seus efeitos deletérios, ocorreu por volta de 1950, na região de Funchu Machi, no Japão, onde o despejo de resíduos da jazida de chumbo e zinco - Kamioka e da sua usina de processamento, localizada 50 km de distância do rio Jintsu contaminou uma vasta região e afetou centenas de pessoas que desenvolveram a doença, posteriormente conhecida como a doença de *Itai-Itai*, caracterizada pela disfunção tubular renal, osteomalácea severa, pseudofraturas e anemia. Existem evidências de que moradores dessa região chegavam a consumir através de alimentos cerca de 1000 µg/dia de Cd (SHUTO, 2005).

No Brasil no ano de 1996, em Itaguaí- RJ, uma fábrica de zinco eletrolítico foi denunciada por despejar mais de 50 milhões de litros de água e lama contendo Zn e Cd, contaminando manguezais que abrigavam caranguejos, siris, ostras e mexilhões, por sua vez consumidos pela população da região (GONÇALVES, *et al.*, 1996). Análises referentes ao ambiente aquático mostram que moluscos filtradores são os principais acumuladores de metais, independente da poluição ambiental, porém a concentração de metal nesses seres pode aumentar consideravelmente quando as águas são contaminadas (WHYTE, *et al.*, 2009), exemplo disso é observado nas ostras da costa da Nova Zelândia, onde devido a contaminação desta área, chegam a ter duas vezes maior quantidade de cádmio do que as do resto do oceano pacífico, onde a concentração de cádmio nas ostras já foi quantificada em 13,5 mg/kg de peso seco (COPES, *et al.*, 2008).

O acidente ambiental mais recente envolvendo Cd ocorreu em uma região autônoma da China, chamada Guangxi Zhuang, em 2012, nesta região encontra-se a maior área de produção de grãos e estão concentradas indústrias de metais não ferrosos. Com a descarga irregular de esgoto industrial da mineração de metais não-ferrosos

poluiu diretamente o rio Longjiang, e em 2012 as concentrações de metal na água excedeu 80 vezes o padrão ambiental. Essa poluição extrema afetou de várias formas a agricultura, a aquicultura e meios de subsistência dos moradores. Como medida para reverter a situação o governo local utilizou a tecnologia de precipitação química alcalina, que se mostrou efetiva na precipitação de Cd no rio (ZHANG *et al*., 2017).

No Brasil, recentemente ocorreram dois grandes desastres ambientais, em Mariana/MG em 2015 e outro há poucos dias em Brumadinho/MG. Ambos os desastres derivaram do rompimento de barragens contendo dejetos de extração de ferro com grandes quantidades de Cd e outros metais. Os efeitos ao meio ambiente (SEGURA et al., 2016; GOMES et al., 2018) e para saúde humana estão sendo registrados e serão vistos e sentidos durante décadas.

Normalmente este metal está presente em baixas concentrações no ambiente, mas a sua larga utilização, na produção de baterias, pigmentos e até mesmo de plástico, ocasionou seu aumento na atmosfera, tornando-o um contaminante comum de água e alimentos (ATSDR, 2008; WHO, 2010). Uma das formas de exposição a este metal ocorre através da utilização cada vez mais intensa de fertilizantes fosfatados que apresentam Cd em sua constituição, aumentando a incidência de intoxicação de pessoas que não são expostas ocupacionalmente. Estes fertilizantes promovem o acúmulo deste metal em alimentos, principalmente em vegetais folhosos, legumes, oleaginosas e folhas de tabaco consumido pela população (WHO, 1992). No entanto, a principal forma de exposição não-ocupacional ao cádmio deriva do tabagismo. As folhas de tabaco por acumularem altos níveis de cádmio do solo, tornam-se um potente agente de exposição humana a este metal. Fumantes apresentam aproximadamente o triplo da concentração de cádmio no sangue (1.58µg/L) do que a encontrada na população não fumante (0.47µg/L) (ATSDR, 2008).

É importante ressaltar que enquanto a população em geral ingere em média 1µg/dia de Cd via alimentação, consumo de água contaminada e pela inalação da fumaça de cigarro, os fumantes absorvem cerca de 1-3µg/dia para cada maço de cigarro consumido (ATSDR, 2008; ATSDR, 2012). Deve-se levar em conta também que a absorção pulmonar chega a ser dez vezes maior que a gastrointestinal (GOERING, *et al.*, 1994), o que significa que uma pessoa fumante ativa apresenta aproximadamente o dobro de carga corporal de cádmio quando comparadas a pessoas não fumantes (LEWIS, *et al.*, 1972). Atualmente mais de um bilhão de pessoas no mundo são fumantes e estima-se

que esse total poderá chegar a dois bilhões em 2030 (FILHO, *et al.*, 2010), sendo este um dos fatores que nos leva a acreditar que o índice de exposição humana ao cádmio irá aumentar gradativamente nos próximos anos, gerando aumento no número de pessoas com doenças provocadas por este metal.

A Organização Mundial de Saúde estabeleceu um limite de segurança de 7µg/semana/kg de peso corporal para ingestão de cádmio. Esse valor foi obtido através da concentração renal crítica de cádmio, que está entre 100 e 200 µg/g de peso seco o que corresponde a concentração urinária de cádmio de 5 a 10 µg/g de creatinina (WHO, 1993). Apesar desse valor de ingestão ser considerado seguro, já foram observados efeitos renais prejudiciais, em concentrações abaixo destes valores (SATARUG e MOORE, 2004), o que é um indicativo de que a exposição a este metal pode ser bastante perigosa, mesmo em baixas concentrações.

1.3 Cinética do Cádmio

O cádmio não apresenta função essencial conhecida para o organismo, consequentemente não tem mecanismos específicos para sua absorção, transporte e entrada nas células, desta forma acaba aproveitando o mesmo mecanismo utilizado por metais essenciais como Zn, magnésio (Mg), cálcio (Ca) e ferro (Fe), o transportador de metais divalentes 1 (DMT 1). A sua absorção por via intestinal está relacionada ao estado nutricional do indivíduo e o cádmio compete diretamente com os metais essenciais por esse carreador de metais divalentes. Aumento nos níveis de DMT 1 intestinal, estão associados a deficiência de ferro e esse aumento eleva a capacidade de absorver Fe e consequentemente Cd. Estudos envolvendo mulheres tailandesas (SATARUG *et al.,* 2004), mulheres e indivíduos ex fumantes do sul da Suécia (OLSSON *et al.,* 2002) com deficiência de Fe, mostram que estes possuem uma carga corporal de Cd de 3 a 4 vezes maior do que indivíduo sem essa deficiência (SATARUG & MOORE, 2004).

O Cd tem meia vida longa, cerca de 10 - 30 anos e uma baixa excreção (1-2 µg/dia), podendo ocorrer variações entre indivíduos (WHO, 1992; GOERING *et al.*, 1995). Este longo tempo de permanência no organismo é atribuído em grande parte, a sua alta afinidade e forte ligação com a metalotioneína (MT), principalmente em órgãos com altos níveis desta proteína, como rins e fígado por exemplo. Esta proteína age como

protetora do organismo, propiciando uma desintoxicação do metal de forma temporária (KLAASSEN *et al.,* 1999). A MT é uma proteína rica em cisteína, de baixo peso molecular que possui alta afinidade por metais essenciais e não essenciais (CAI *et al.*, 2010; NORBERG *et al.,* 2004), a união da MT com o Cd ocorre através do grupo sulfidrila presentes na estrutura dessa proteína e pela afinidade que o Cd possui por estes grupos (KLAASSEN *et al.,* 2009). No entanto, mesmo que o Cd se ligue a MT, formando o complexo MT-Cd^{2+} e dessa forma impedindo que esse metal se ligue a outras macromoléculas, a formação desse complexo dificulta a sua excreção renal (KLAASSEN *et al.,* 2009). Quando o complexo MT-Cd^{2+} chega nos rins, ele é filtrado e reabsorvido pelas células dos túbulos proximais, onde a ligação desse complexo é rompida e ocorre a liberação do cádmio bivalente na circulação. Uma vez que a quantidade de cádmio excede a produção e a ligação dessa proteína, a concentração de Cd livre aumenta e causa seus efeitos danosos sobre diferentes sistemas (FRIBERG *et al.,* 1986).

1.4 Efeito do Cádmio nos diferentes sistemas

As evidências dos efeitos tóxicos do Cd começaram a surgir no final do século XIX e começo do século XX (NORDBERG, 2009), com maior evidência a partir do desastre no Japão e o desenvolvimento da doença de *Itai-Itai.* Atualmente este metal ocupa o sétimo lugar na lista de substâncias perigosas da Agência de Substâncias Tóxicas e Registro de Doenças, que classifica compostos conforme a sua ameaça à saúde humana devido a sua toxicidade conhecida ou até mesmo suspeita, levando em consideração a potencial exposição humana (ATSDR, 2011). Além disso, a Agência Internacional para Pesquisa Sobre o Câncer classifica o cádmio como um agente carcinogênico do grupo 1, podendo causar diversos tipos de câncer (IARC, 1993; WAALKES, 2003).

Estas colocações estão associadas ao fato do metal acumular-se nos órgãos, possuir meia vida longa e da sua ligação com a metalotioneína, já citados anteriormente. Alguns órgão e sistemas bastante atingidos por esse metal são pulmões (KLIMISCH, 1993; LUCHESE *et al.*, 2007), fígado (JOHANSEN, *et al.,* 2006), rins (JIHEN, *et al.*, 2008), testículo (HAOUEM, *et al.,* 2008), cérebro, ossos e sistema sanguíneo (MESSAOUDI *et al.,* 2010).

É sabido que o Cd tanto em altas quanto em baixas doses afeta principalmente as mulheres, devido a sua maior capacidade de absorver Cd no trato gastrointestinal do que homens (ATSDR, 2012). Pesquisas populacionais realizadas no Japão, Tailândia, Austrália, Polônia, Bélgica e Suécia que avaliaram os efeitos da exposição humana ao Cd e os seus mecanismos, identificaram seis fatores de riscos do Cd nos efeitos à saúde presentes em mulheres que pode agravar a sua exposição, são eles: A) Tipo mais grave de disfunção tubular renal; B) Diferença no metabolismo do cálcio e seus hormônios reguladores; C) Maior sensibilidade renal; diferenças no fenótipo P450; D) Gravidez; E) Níveis reduzidos de armazenamento de Fe corporal e F) Fatores genéticos (NISHIJO *et al.*, 2004).

Outros estudos levando em consideração a associação da exposição ao Cd à redução ou impedimento da função tubular renal e glomerular, mostrou perda da capacidade reabsortiva de nutrientes, minerais e vitaminas. Este efeito, por sua vez, influencia outros órgãos e sistemas, como por exemplo, os ossos que com a redução do cálcio sanguíneo, sofre desmineralização, isto justifica o fato da exposição a esse metal estar relacionada a osteomalácia e osteoporose (WHO, 1992; INABA *et al.* 2005).

Os rins são órgãos alvo da toxicidade do Cd, sendo que a disfunção renal ocasionada pela exposição ao metal se dá pelo dano as células tubulares glomerulares que reabsorvem ativamente o Cd juntamente ao Zn, glicose e aminoácidos no filtrado glomerular. As disfunções englobam proteinúria, aminocidúria, glicosúria e necrose de células tubulares proximais renais (MADDEN & FOWLER, 2000; SATARUG *et al.*, 2017).

A Diabetes Mellitus tipo II também vem sendo associada com a exposição a este metal que acumula no pâncreas e exerce efeito diabetogênico. Estudo transversal populacional com 8.722 norte americanos revelou uma associação entre os altos níveis de Cd urinário e o aumento dos níveis de glicemia em jejum e diabetes (SCHWARTZ *et al.*, 2003). Já foi descrito que a ligação entre diabetes, doença renal terminal que necessita de diálise ou transplante renal, e hipertensão (SATARUG *et al.*, 2017; MADRIGAL *et al.*, 2018).

Como já mencionado anteriormente, o Cd está bastante associado a vários tipos de câncer (IARC, 1993), principalmente ao câncer de pulmão onde há um risco aumentado de 20% em trabalhadores expostos ocupacionalmente em comparação

aqueles não expostos (SORAHAN e LANCASHIRE, 1997). Além disso, muitos estudos *in vitro* com exposição ao Cd mostram aumento do estresse oxidativo (CUYPERS *et al.,* 2010), atividade modificada de fatores de transcrição de células epiteliais alveolares (WATKIN *et al.,* 2003), e posterior inibição de reparação do DNA (JIN *et al.,* 2003). Esses fatores ocasionam aumento da mutagênese, propiciando o desenvolvimento de células cancerígenas. Em fumantes que estão expostos a esse metal através da principal via de exposição a este metal, já foi comprovado uma associação da redução do volume expiratório forçado em um segundo e o aumento do cádmio urinário (LAMPE *et al.,* 2008), gerando assim uma possível associação onde a doença pulmonar de fumantes pode ser mediada em parte pela exposição ao Cd.

Outro sistema acometido por este metal é o sistema reprodutor, onde o Cd age afetando a reprodução e o desenvolvimento de formas diversificadas, englobando todos os estágios do processo reprodutivo (THOMPSON e BANNIGAN, 2008; UJAH *et al*, 2018). Os danos testiculares envolvem desde lesões diretas até redução da qualidade do sêmen e consequente infertilidade (WIJESEKARA, F.; WIJERATHNA; BANDARA, 2015). Tanto a intoxicação aguda quanto subcrônica no sistema reprodutor de animais e humanos (DE ANGELIS *et al*, 2017) promovem aumento do estresse oxidativo, seja por aumento de radicais livres ou pela inibição de defesas celulares enzimáticas ou não enzimáticas, morte celular e inflamação (SANTOS *et al.*, 2004a; ACHARYA *et al.*, 2008; OGNJANOVIC´ *et al.*, 2010; RINALDI et al., 2017; UJAH *et al.*, 2018).

A neurotoxicidade também é presente na intoxicação por Cd, estudos mostram que a captação de Cd no cérebro é dependente da idade, sendo que roedores neonatos apresentam uma captação maior do que roedores adultos, e isso parece estar associado à ontogenia do sistema vascular cerebral (CHOUDHURI, S. *et al.,* 1996; VALOIS, A.A. e WEBSTER, W.S., 1989). O Cd acumula-se no parênquima cerebral, corpo estriado e nos neurônios (NISHMURA T. *et al.,* 2006; KWAKYE *et al*, 2018), e os efeitos observados tanto em humanos como em modelos experimentais são déficit de atenção, memória e disfunção olfatória e estão relacionado com o desenvolvimento de doenças neurodegenerativas como Alzheimer, Parkinson, esclerose lateral amiotrófica e esclerose múltipla. (ROSE, C.S. *et al*., 1992; LUKAWSKI, K. *et al.,* 2005; BRANCA *et al.,* 2018).

1.5 Efeitos do Cádmio no Sistema Cardiovascular

Os relatos de efeitos da exposição ao Cd ocorrem desde o começo do sec. XX, desde então vários efeitos cardiovasculares foram relatados, tais como a redução de produção de NO pelas células endoteliais associada à inibição direta da sua migração (KOLLURO *et al.,* 2006), maior chance de desenvolver doença arterial periférica, relacionado à níveis aumentados de Cd no sangue e na urina (NAVAS-ACIEN *et al.,* 2004; NAVAS-ACIEN *et al.,* 2005), aumento da incidência de acidente vascular isquêmico (BORNÉ *et al.,* 2017), aterosclerose ocasionada pelo influxo de componentes séricos pró-aterogênicos e o recrutamento de leucócitos que é facilitado pela alteração na integridade das camadas de células endoteliais (KNOFLACH *et al.,* 2011; SANTOS-CALLEGO & JIALAL, 2016; BORNÉ *et al.,* 2017; TINKOV *et al.,* 2018), e maior incidência de hipertensão e aterosclerose associados aos maiores níveis de Cd na urina e à populações que habitam áreas contaminadas (HOUTMAN, 1993; TINKOV *et al.,* 2018).

Um dos efeitos mais conhecidos da exposição ao cádmio é a hipertensão, acredita-se que essa doença venha acompanhada de alterações na função das células endoteliais, e este seria um dos caminhos para sua origem, visto que a disfunção endotelial contribui para a manutenção do aumento da resistência vascular, favorecendo assim o processo hipertensivo (CANNON III, 1998; TRIGGLE *et al.,* 2003; KOLLURU *et al.,* 2010). A concentração sanguínea de Cd esta associada com a hipertensão, onde indivíduos com maiores níveis de Cd no sangue, apresentam essa patologia (TELLEZ-PLAZA *et al.,* 2008; MADRIGAL *et al.,* 2018). Outros mecanismos que também podem estar envolvidos no desenvolvimento de hipertensão após a exposição ao Cd, estão relacionados com a indução de dano no túbulo renal proximal, retenção de sal e aumento do volume sanguíneo (SATARUG *et al,* 2005; VALKO *et al,* 2005), além de desequilíbrio dos sistemas oxidante e antioxidante gerando estresse oxidativo como a depleção da glutationa, e peroxidação lipídica (YIIN *et al,* 1999; VALKO et al., 2005 OLIVEIRA *et al.,* 2019). Outra possibilidade é a interação do Cd nos canais de cálcio, estimulação do sistema nervoso simpático e reduzindo a liberação de agentes vasodilatadores o que contribuiria para o desencadeamento de hipertensão arterial sistêmica (FADLOUN e LEACH, 1980; BALARAMAN *et al,* 1989; SKOZYNSKA e MARTYNOWICZ, 2005; DONPUNHA et al., 2011; MADRIGAL *et al.,* 2018).

Inúmeros estudos indicam que o endotélio é o principal alvo da intoxicação pelo Cd no sistema cardiovascular, podendo ocasionar, desta forma, o desequilíbrio na biodisponibilidade de substâncias vasodilatadoras e vasoconstritoras, resultando no aumento do tônus vascular (MARTINOWICS *et al.*, 2004; KOLLURU *et al.*, 2006; DONPUNHA *et al.*, 2011). Isso é ocasionado principalmente porque o Cd é capaz de promover uma redução da isoforma da eNOS e na produção de NO (YOOPAN *et al.*, 2008), através do aumento de ânion superóxido, gerado pela NADPH oxidase (ALMENARA *et al.*, 2013) que acaba reagindo rapidamente com o NO, reduzindo a sua biodisponibilidade e levando à produção de peróxido de nitrito, considerado um potente agente oxidante (STROES *et al*, 1998; KOLLURO *et al.*, 2006; FROSTERMANN e MUNZEL, 2006; TAKAYA *et al*, 2007; OLIVEIRA et al., 2019).

A redução na biodisponibilidade de NO é considerada um dos mais importantes fatores associados a doenças cardiovasculares (STROES *et al*, 1998; KERR *et al*, 1999; FROSTERMANN e MUNZEL, 2006), pois este é um dos principais agentes vasodilatadores, advindos do endotélio que atua por diversos mecanismos no músculo liso vascular (PALMER *et al*, 1987; MONCADA *et al*, 1991; CANNON III, 1998; FERNANDES *et al.*, 2017).

O aumento de EROs afeta muitas das funções do endotélio e do músculo liso vascular, no endotélio por exemplo, essas alterações englobam a redução da biodisponibilidade de NO, a apoptose, aumento na adesão de monócitos e angiogênese (TANIYAMA & GRIENDLIG, 2003). Já no músculo liso vascular as alterações causadas pelo aumento de espécies reativas, pode promover crescimento celular, migração de mediadores inflamatórios, desorganização da matriz extracelular e aumento no tônus muscular (TANIYAMA e GRIENDLING, 2003). A soma desses efeitos levam ao dano vascular e consequentemente hipertensão arterial (GRIENDLING e USHIO-FUKAI, 1994; KERR *et al.*, 1999; CRUZADO *et al.*, 2005; TOUYZ e SCHINFFRIN, 2004; TOUYZ & SCHINFFRIN, 2008; ALMENARA *et al.*, 2013).

Todos os achados envolvendo a toxicidade do Cd, mesmo em doses próximas aos limites considerados seguros pela OMS, nos diversos sistemas, constitui um sério problema de saúde pública, visto que a principal fonte de intoxicação deste metal se dá pelo consumo de água e alimentos e a exposição à fumaça de cigarro, fontes difíceis de serem controladas, o que impulsiona o interesse em pesquisas que busquem alternativas para sua remoção do ambiente e prevenção dos seus danos.

2. ALTERNATIVAS TERAPÊUTICAS PARA OS DANOS CAUSADOS PELO CÁDMIO

Alternativas terapêuticas para minimizar os danos causados pela exposição a este metal já foram descritas, entre elas quelantes como Deferasirox e Deferiprone, que após duas doses de Cd de 20 e 40 mg/kg de peso corporal/dia durante 60 dias em ratos, em uma ação combinada consegue remover íons de Cd e regularizar os níveis de ferro (JAMILALDIN FATERMI, S. *et al.,* 2011). A glutationa administrada juntamente com EDTA também mostra efeitos quelantes positivos sendo uma alternativa eficaz em pacientes intoxicados por cádmio (GIL, H. *et al.,* 2010).

Os quelantes clássicos como a D-penicilamina (DPA), 2,3-dimercaptopropanol (BAL) e o ácido etilenodiaminotetracético (EDTA), que são utilizados há décadas na tentativa de combater os efeitos deletérios causados não só por Cd mas também por outros metais pesados como mercúrio e chumbo, atualmente estão sendo considerados ultrapassados ao passo que suas contraindicações, em diversas situações, superam seus efeitos benéficos (ANDERSEN e AASETH, 2002; AASETH *et al.,* 2015; RAFATI *et al.*, 2017).

Quando investigado o tratamento para disfunções cardiovasculares, encontramos na literatura a descrição do uso combinado de quercitina e alfa-tocoferol, onde o tratamento combinado possibilita uma proteção notável contra o estresse oxidativo e alterações no metabolismo lipídico, induzidos pela exposição ao Cd, reduzindo assim doenças cardiovasculares causadas por este metal (PRABU, S.M., *et al.,* 2010). A quercitina por sua vez, também é utilizada como alternativa no combate a neurotoxicidade induzida pelo estresse oxidativo (UNSAL C., *et al.*, 2013). A taurina, um aminoácido não essencial abundante no organismo, também se mostra benéfica contra os danos cardíacos, evitando deficiências oxidativas e a redução do poder antioxidante induzido pela exposição ao Cd (MANNA, P., *et al.,* 2008).

Cada vez mais ocorre o incentivo de pesquisas com compostos naturais, tanto pelo avanço nas descobertas de seus benefícios sem grandes contraindicações ou efeitos adversos e pelo seu baixo custo, O fato é que a ciência tem se voltado para a investigação desses componentes. Compostos naturais, especialmente de origem alimentar, que possam reduzir a absorção e a reabsorção de metais tóxicos e dessa

forma complementar vias de desintoxicação. Alguns agentes naturais que possuem efeitos positivos contra os malefícios da exposição ao Cd, já são descritos, como o suco de salsa (*Petroselium crispum Apiaceae*) que em camundongos melhorou alterações comportamentais associadas a este metal, além de reduzir o aumento da peroxidação lipídica e normalizar a atividade da glutationa peroxidase no cérebro (MAODAA, S.N. *et al.*, 2016). O extrato de mirtilo (*Vaccinium ashei Reade*) também demonstra potencial benéfico através de suas propriedades antioxidantes que por sua vez protegem pelo menos em parte o tecido ovariano da toxicidade do Cd em camundongos (IZAGUIRRY, A.P., *et al.*, 2015). A Mimosa (*Mimosa caesalpiniifolia*) uma planta nativa da América do Sul, que também possui propriedades antioxidantes, é capaz de prevenir a genotoxicidade induzida pela exposição ao Cd no fígado e nas células sanguíneas de ratos (SILVA, M.J.D. *et al.*, 2014). A Cúrcuma, por sua vez, mostra efeitos que atenuam a peroxidação lipídica, a depleção da glutationa e as alterações de enzimas antioxidantes (MOHAJERI et al., 2017).

Alimentos de origem proteica demonstram eficiência como quelantes, grande parte por possuírem enxofre na sua composição, o enxofre por sua vez, possui grande afinidade por metais pesados e é capaz de aumentar e melhorar a sua excreção (SEARS, 2013). Contrapondo-se aos altos custos que o tratamento para diversas doenças ocasionadas pela exposição ao Cd resultam para os sistemas de saúde, outros compostos naturais advindos da dieta devem ser investigados e inseridos como estratégias terapêuticas em situações com danos causados por metais pesados (SOLENKOVA *et al.*, 2014). A OMS recomenda que os nutrientes que tem capacidade de alterar a toxicidade induzida por contaminantes ambientais, sejam melhores investigados e detalhados para serem utilizados tanto como possíveis tratamentos quanto para complementar alternativas terapêuticas já existentes (OMS, 1990).

2.1. Alimentos Funcionais

Com o passar dos anos a sociedade se torna cada vez mais consciente e busca por estilos de vida mais saudáveis, isso inclui muitas vezes a modificação de alguns hábitos alimentares, pois existe uma importante relação entre a dieta e a saúde. Com isso, a linha de investigação nesta área cresce exponencialmente nos favorecendo em recursos para a prevenção e também para o tratamento de disfunções no nosso organismo (WILDMAN, 2001; ABUAJAH *et al.*, 2015).

Sabe-se que os componentes alimentares, além das suas já conhecidas propriedades nutricionais essenciais para a vida, também possuem capacidade de exercer diferentes atividades biológicas, podendo gerar benefícios para funções específicas ao organismo (HUANG *et al.,* 2010). Os componentes alimentares que apresentam atividade biológica são utilizados para desenvolver novos alimentos funcionais. A ANVISA (Resolução nº 18/99) estabelece que para ser considerado funcional, tanto alimentos quanto ingredientes devem além de possuir atividades nutricionais e efeitos metabólicos benéficos à saúde, devem ser cientificamente comprovados que o seu uso mesmo sem supervisão médica seja seguro (BRASIL, 1999).

A linha que divide alimentos de medicamentos se torna cada vez mais tênue ao passo que indivíduos buscam cada vez mais por recursos naturais para prevenção e tratamento de determinadas doenças. Dentre os componentes biologicamente ativos de alimentos funcionais, podemos citar os compostos fitoquímicos advindos da natureza, através de plantas, verduras, legumes, frutas e cereais, de animais como o peixe, podemos ressaltar os ácidos graxos poliinsaturados de cadeia longa ômega-3, -6 e -9, os lácteos fermentados dão origem aos compostos probióticos e através proteínas de origem vegetal e animal encontramos peptídeos bioativos (SRIVIDYA *et al.,* 2010; ABUAJAH *et al.,* 2015).

2.2. O ovo como fonte de peptídeos bioativos

As proteínas da dieta, fonte de energia e aminoácidos essenciais para o adequado funcionamento fisiológico do organismo, exercem atividades biológicas potentes quando ingeridas e apresentam muitos benefícios comprovados *in vivo*, por esse motivo as proteínas estão sendo utilizadas como matéria prima para obtenção *in vitro* de peptídeos bioativos (KORHONEN e PIHLANTO-LEPPALA, 2002; SAMARANAYAKA e LI-CHAN, 2011). Os peptídeos bioativos são sequências específicas de aminoácidos com atividade que modula a função fisiológica ao se ligar em receptores específicos de células alvo (KORHONEN, 2009), eles contém de 3 a 20 resíduos de aminoácidos por molécula e normalmente são inativos dentro da sequência de proteína, mostrando sua atividade biológica apenas quando é liberado de sua proteína precursora (PIHLANTO-LEPPALA, 2000; GARCÉS-RAMÓN *et al.,* 2016), essa liberação ocorre através de hidrólise *in vitro* ou *in vivo* (LIAO *et al.,* 2018).

Desde que os peptídeos bioativos foram descobertos em 1979, são descritos na literatura diversos biopeptídeos com atividades biológicas distintas como antioxidante, anti-hipertensiva, imunomodulante, antimicrobiana, opióide, entre outras (MOUGHAN *et al.,* 2014), sendo que alguns desses peptídeos podem demonstrar atividades biológicas simultâneas (LIAO *et al.,* 2018). O processo de hidrólise para obtenção desses peptídeos pode ser total ou parcial e é dependente da utilização de enzimas. Para obtenção do peptídeo de interesse é necessário considerar alguns fatores além da proteína precursora como as condições de hidrólise (pH, temperatura e tempo) em que a proteína vai ser submetida (ECKERT *et al.,* 2013). Além disso, a grande maioria dos estudos das últimas décadas concentram-se em descrever as propriedades de biopeptídeos isolados, no entanto atualmente existem evidências de que a administração de hidrolisados completos tem maior relevância em nível fisiológico, bem como efeito biológico mais complexo (LIU *et al.,* 2017), podendo agir sobre aspectos diversos, beneficiando ainda mais quem faz o seu consumo.

O ovo de galinha é um alimento de fácil acesso, baixo preço e possui uma ampla gama de aplicações culinárias, além de ser uma fonte de nutrientes de alta qualidade, por isso é um dos alimentos mais consumidos pela sociedade e atualmente considerado um alimento muito importante na obtenção de peptídeos bioativos (INSTITUTO DE ESTUDIOS DEL HUEVO, 2009; ZANI *et al.,* 2018; MAJUMDER *et al.,* 2015; NIMALARATNE *et al*., 2015; SUN *et al.,* 2016; LIU *et al.,* 2017). A clara do ovo é responsável por 58% do peso total deste alimento e é composta exclusivamente por água (88-90%) e proteínas (10-12%). A riqueza de aminoácidos essenciais da proteina da clara do ovo faz com que ela seja uma rica fonte de valor biológico destes e também seja considerada fonte de proteínas de referencia para validar outras proteínas alimentares. Algumas proteínas que estão presentes na clara do ovo são metaloproteínas e contem enxofre na sua composição e isso é muito importante visto o mecanismo de ação do Cd (INSTITUTO DE ESTUDIOS DEL HUEVO, 2009; YU *et al.*, 2011; GARCÉS-RIMÓN *et al*., 2016b; LIAO *et al.*, 2018).

Nos últimos anos tornou-se possível obter hidrolisados e peptídeos bioativos procedentes de todas as estruturas do ovo que incluem proteínas na sua composição como a clara, a gema e a membrana interna da casca (JAIN e ANAL, 2017). Alguns peptídeos descritos derivados do ovo mostram atividade anti-hipertensiva bastante associada ao mecanismo vasodilatador derivado do endotélio (FUJITA *et al.,* 2001;

SCRUGGS *et al*., 2004) ou com sua capacidade de inibição da ECA tanto *in vitro* quanto *in vivo* (YU *et al.,* 2011; AHMAD *et al.,* 2012). Outros peptídeos derivados de proteínas do ovo apresentaram efeitos de redução de estresse oxidativo associado à inflamação (HUANG *et al.,* 2010) ou propriedades antiinflamatórias diretas através da via de NF-kB (CHAKRABARTI *et al*., 2014). Além de atividade hipoglicêmica (YU *et al.*, 2012), anti-apoptótica (LIU *et al.,* 2014) e antioxidantes (JUNG *et al*., 2001), que por sua vez está associada às suas propriedades redox e capacidade de atuar como agente quelante de metais (DING *et al.,* 2015).

2.3. Peptídeos bioativos derivados da clara do ovo

Os hidrolisados mais abundantes em peptídeos bioativos do ovo derivam da sua clara, provavelmente devido a facilidade na obtenção desta parte do ovo e ao seu alto conteúdo proteico, onde as proteínas, em especial a ovoalbumina demonstram importante potencial antioxidante (MIGUEL e ALEIXANDRE 2006; SUN *et al.,* 2014; GARCÉS-RIMÓN *et al.,* 2016b; JOVANOVIC´ *et al.*, 2016).

A hidrólise de proteínas da clara do ovo realizada a partir de diferentes enzimas digestivas, dão origem a hidrolisados com capacidades anti-hipertensivas, antioxidantes e com propriedades de inibição da ECA *in vitro*, sendo que um dos primeiros hidrolisados estudados que demonstrou atividade potente foi o tratado com pepsina por três horas, esse hidrolisado apresentou capacidade de inibir a ECA mais elevada que os demais estudados até este ponto (DÁVALOS *et al.,* 2004; LIU *et al*., 2010; MIGUEL *et al.* 2014). Posteriormente esse hidrolisado foi testado *in vivo,* e comprovou-se sua alta capacidade anti-hipertensiva, promovendo redução tanto da pressão arterial sistólica quanto diastólica (MIGUEL *et al.,* 2005; MIGUEL *et al.,* 2006; MIGUEL *et al.,* 2007), aumento da capacidade antioxidante e redução da peroxidação lipídica em plasma e aorta de ratos espontaneamente hipertensos (SHR) (MANSO *et al.,* 2008). Sugerindo que a associação de atividades anti-hipertensivas e antioxidante contribui de forma positiva na redução da pressão arterial desse modelo.

Vale ressaltar que vários fatores podem levar a potencial atividade biológica de peptídeos quando administrados via oral, incluindo a resistência das enzimas gastrointestinais ao pH e a biodisponibilidade (absorção, transporte e capacidade de chegar nos seus lugares de ação) (LIAO *et al.,* 2018; SANTOS-HERNÁNDEZ *et al.,*

2018). Por esse motivo estudos que simulam a digestão gastrointestinal do hidrolisado da clara do ovo já foram realizados e neles se observou que peptídeos anti-hipertensivos YAEERYPIL e RADHPFL se hidrolisam durante o processo de digestão *in vitro*. Assim, os produtos derivados da hidrólise dessas sequências de peptídeos podem ser os responsáveis diretos do efeito anti-hipertensivo (MIGUEL *et al.*, 2007).

Recentemente estudos que analisaram a capacidade biológicas *in vitro* do hidrolisado da clara do ovo, obtidos a partir de diferentes graus de hidrólise, demonstraram que hidrólise por oito horas com pepsina agrega ao hidrolisado atividades mais potentes que as estabelecidas anteriormente para de três horas (GARCÉS-RIMÓN *et al.*, 2016). Esse hidrolisado mostrou efeitos potentes também *in vivo*, apresentando atividades inibidora da ECA, vasodilatadoras, antidiabéticas, hipocolesterolêmica, antiinflamatórias, antioxidantes e antihipertensivas, em ratos tratados com dieta de cafeteria e em ratos Zucker (GARCÉS-RIMÓN *et al.*, 2016; MORENO *et al.*, 2018). As sequências peptídicas responsáveis por esses efeitos já foram identificadas por Miguel *et al.* (2004), são elas: FRADHPFL, RADHPFL, YAEERYPIL, YRGGLEPINF, ESIINF, RDILNQ, IVF, YQIGL, SALAM e FSL. O hidrolisado contendo essas sequências peptídicas também mostrou atividade de modulação da microbiota intestinal de ratos Zucker (RAQUERA *et al.*, 2017) e reverter alterações cardiovasculares e neurológicas associadas ao estresse oxidativo gerado pela exposição ao mercúrio durante 60 dias, esse efeito vem sendo associado a propriedades quelantes do hidrolisado sobre esse metal pesado, que através da sua capacidade antioxidante evita o depósito de mercúrio no organismo de ratos Wistar (RIZZETTI *et al.*, 2016a; RIZZETTI *et al.*, 2016b; RIZZETTI *et al.*, 2017a; RIZZETTI *et al.*, 2017b).

Todos esses achados demonstram a eficácia do tratamento com hidrolisado de clara de ovo com pepsina durante oito horas em disfunções metabólicas e cardiovasculares tanto de origem genética como adquiridas, no entanto estudos com diferentes metais pesados como por exemplo o modelo de desordens induzidas pela exposição ao cádmio, ainda são necessários, visto que os metais possuem via de ação distintas e o tratamento com hidrolisado pode apresentar resultados diferentes frente a algumas alterações.

3. JUSTIFICATIVA

O contato do ser humano com o Cd é inevitável, pois além desse metal ser encontrado em baixas concentrações no ambiente, a sua larga utilização pela indústria fez com que ele se tornasse um contaminante comum de água e alimentos, além disso é muito utilizado na produção de baterias, pigmentos e plástico. Uma das principais fontes de exposição à este metal é a fumaça de cigarro, atualmente a população fumante no mundo ultrapassa a marca de um bilhão e a estimativa para 2030 é de dois bilhões de fumantes, sendo este um dos fatores que nos faz acreditar que o nível de exposição ao cádmio aumentará gradativamente nos próximos anos, gerando consequentemente aumento no número de pessoas com doenças provocadas pelo metal. Embora existam limites estabelecidos para a exposição ao Cd, sabe-se que mesmo exposições abaixo desse limite já causam efeitos deletérios em diversos órgãos e sistemas e esse metal tem efeito cumulativo e é associado a meia vida longa e baixos níveis de excreção. Desta forma a realização de pesquisas que viabilizem meios para o conhecimento de substâncias capazes de prevenir ou de tratar os efeitos danosos decorrentes dessa intoxicação torna-se fundamental para a saúde humana.

Além de políticas públicas para o controle da exposição ambiental ao Cd, substâncias terapêuticas de fontes naturais e de fácil acesso à população podem ser uma ferramenta positiva para redução de danos à saúde induzidos pelo metal. O hidrolisado de clara de ovo mostra-se como uma excelente alternativa terapêutica para disfunções metabólicas, neurológicas, reprodutivas e cardíacas associada a outros metais pesados, devido a suas propriedades, anti-hipertensivas, antioxidantes, antiinflamatórias, anti-hiperglicêmica e anti-hiperinsulinêmica observadas em outros modelos experimentais.

Embora existam evidências de que o hidrolisado possui efeitos benéficos sobre os malefícios causados pela exposição a metais pesados, a literatura carece de comprovação dos seus efeitos sobre um modelo de exposição ao cádmio, assim como da compreensão dos mecanismos utilizados para seus efeitos positivos. Esclarecendo sua eficiência e os mecanismos utilizados para tal, podemos estabelecer um meio natural e acessível para combater desordens ocasionadas pela exposição ao Cd.

4. OBJETIVOS

4.1. Objetivo geral

Avaliar os efeitos do co-tratamento com hidrolisado de clara de ovo sobre os danos cardiovasculares causados pela exposição ao cloreto de Cd por 14 dias.

4.2. Objetivo específico

Verificar se o co-tratamento com hidrolisado da clara de ovo é capaz de promover efeitos benéficos sobre:

- Disfunções hemodinâmicas
- Alterações vasculares e vias envolvidas em artéria de condutância
- Desequilíbrio de biomarcadores de estresse oxidativo

promovidos pela exposição ao cádmio no sistema cardiovascular.

PARTE II

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Protective effect of Egg White-derived peptides on vascular damage induced by cadmium exposure in rats.

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Abstract

This study aimed to investigate whether egg white hydrolysate (EWH) could be used as a protective agent in cardiovascular system disorders caused by exposure to cadmium (Cd) in rats. EWH prevented the increase in the systolic blood pressure (SBP) and vascular reactivity caused by Cd. These vascular improvements were related to the inhibition of COX-2 derived prostanoids by reducing the expression of this protein, inhibition of angiotensin II by reducing the activity of ECA and the consequent reduction of oxidative stress mediated by NADPH oxidase. In conclusion, the EWH could be considered as a therapeutic alternative for Cd-induced cardiovascular damage.

Keywords: Hypertension; Vascular Reactivity; Vascular Dysfunction; Oxidative Stress; Egg White Hydrolysate; Bioactive peptides; Cadmium.

Abbreviations:

µg - microgram; µg/kg - microgram per kilogram; mg - milligram; mg/kg - milligram per kilogram; g - gram; g/kg - gram per kilogram; ACh - acetylcholine; APO - Apocynin; Cd - cadmium; $CdCl_2$ - Cadmium chloride; COX-2 - Cyclooxygenase enzyme isoform 2; dAUC - Differences of area under the concentration-response curves; DCHF-DA-2'7' - dichlorofluorescein diacetate; DHE - dihydroethidium; ECA- Angiotensin converting enzyme; EDTA - Ethylenediaminetetraacetic acid; EWH - egg white hydrolysate; Fe^{2+} -Ferrous ion; Fe^{3+} - Ferric ion; H_3PO_4 - Phosphoric acid; INDO - Indomethacin; KCL - Potassium chloride; L-NAME - N(ω)-nitro-L-arginine methyl ester; LOS - Losartan; MDA - Malondialdehyde; MT - metallothionein; NADPH oxidase - Enzyme nicotinamide adenine dinucleotide phosphate oxidase; NaOH - Sodium hydroxide; NO - Nitric Oxide; NOS - Nitric oxide synthase; $O_2^{\cdot -}$ - Superoxide radical anion; Phe - phenylephrine; ROS - Reactive oxygen species; RNS - Reactive nitrogen species; SBP - Systolic blood pressure; SDS - Sodium dodecyl sulphate; SNP - Nitroprusside; TBA - thiobarbituric acid;

Highlights

- Cadmium exposure has serious consequences on human healthy.
- EWH showed antihypertensive effect in cadmium exposure rats.
- EWH showed antioxidant and anti-inflammatory properties in cadmium exposure rats.
- EWH could be used as a therapeutic alternative in cardiovascular diseases.

1 Introduction

Cadmium (Cd) is a toxic heavy metal present at low concentrations in the environment, however, its use in various industrial products increases its presence in the atmosphere, becoming a common contaminant of drinking-water and food (ATSDR, 2008; WHO, 2010). The human contamination is related to industrial activity, urban waste and food sources, being inhalation and oral the main exposure routes (Abu-Hayyeh, Sian, Jones, Manuel, and Powell, 2001; Satarug, Nishijo, Lasker, Eduards, Moore, 2006; Afrid, *et al.*, 2010; Ueno *et al.*, 2010; Rebelo & Caldas, 2016).

The World Health Organization establishes the limit for human exposure of 25 μg/kg of body weight/month (WHO, 2010) and the daily intake of adult individual is 0.30 to 0.35 μg/kg/day. The Cd exposure increases among smokers, since the amount of Cd absorbed from smoking on pack of cigarettes is about 1-3μg/day. Consequently, the blood concentration of cadmium from smokers are four times higher than nonsmokers, 1.58 μg/L and 0.38 μg/L, respectively (ATSDR, 2012). In addition to this, Cd has a half-life of 10 to 35 years in the human body and low rate of excretion (1-2 μg/day). This long half-life and excretion rate lead individuals to a long-term exposure to Cd, impacting human health at different organs and systems (Goering, Waalkes, and Klaasen, 1995; WHO, 2008). The cardiovascular system is one of impacted by Cd exposure where it is related to arterial hypertension (Eum, Lee, and Paek, 2008; Tellez-Plaza, Navas-Acien, Crainiceanu, and Guallar, 2008), atherosclerosis (Tinkov *et al.*, 2018), peripheral artery disease (Navas-Acien *et al.*, 2004), stroke and heart failure (Tellez-Plaza, *et al.*, 2012; Tellez-Plaza *et al.*, 2013).

In vitro and in vivo studies point to this metal as a toxic environmental factor linked to endothelial and vascular smooth muscle damages (Taniyama & Griendling, 2003; Touyz & Schiffrin, 2008; Gökalp, *et al.*, 2009; Almenara *et al.*, 2013). These

effects appear to be associated with a reduction in the bioavailability of nitric oxide and the increase on oxidative stress (Cuypers, *et al.*, 2010; Broseghini-Filho *et al.*, 2015; Vassallo *et al*, 2018).

In contrast to the increased exposure to environmental contaminants, in the last decades nutritional strategies have been explored for disease prevention using "functional foods" (Lobo, Patil, Phatak, and Chandra, 2010). Functional foods are considered safe with reduced adverse effects when compared to traditional pharmacological agents (Garcia-Mora, Penas, Frias, Gomez, and Martinez-Villaluenga, 2015; Manso, Miguel, Even, Hernandez, Aleixandre, Lopez-Fandino, 2008; Liu *et al.*, 2010).

Among functional compounds, peptides obtained from animal protein have been investigated in the form of bioactive peptides or hydrolyzed due to their high antioxidant potential (Mohamed, 2015; Sarmadi and Ismail, 2010). The egg white protein is a very rich source of bioactive peptides (Majumder; Liang; Chen; Guan; Davidge, and Wu, 2015; Nimalaratne, Bandara, and Wu, 2015; Sun, Chakrabarti, Fang, Yin, and Wu, 2016; Liu, Oey, Bremer, Carne, and Sikock, 2017). The egg hydrolyzate produces bioactive peptides with important functional capacity against oxidative stress induced by heavy metals (Abeyrathne, Lee, Ahn, 2013).

The EWH, through its antioxidant and anti-inflammatory capacities, is able to improve complications related to metabolic syndrome in genetic experimental models or in metabolic syndrome models induced by diet, such as body weight gain, abdominal obesity, peripheral neuropathy, increased plasma glucose levels, oxidative stress and inflammatory biomarkers (Garcés-Rimón *et al.*, 2016; Moreno-Fernández *et al.*, 2018). Specifically on cardiovascular system, the EWH shows ability to prevent vascular

damage induced by mercury exposure, normalizing blood pressure levels and vascular reactivity in aorta arteries (Rizzetti *et al.*, 2017).

Thus, the objective of our study was to investigate the potential beneficial effects of dietary supplementation with EWH on vascular disorders induced by Cd exposure, as well as to elucidate the mechanisms involved.

2 Materials and Methods

2.1 EWH preparation

EWH was prepared by pepsin hydrolysis of crude egg white (Garcés-Rimón, Lopez-Exposito, Lopez-Fandino, and Miguel, 2016a). Briefly, commercial pasteurized egg white was hydrolysed during 8 h with BC Pepsin 1:3000 (E.C. 3.4.23.1; from pork stomach, E:S: 2:100 w:w, pH 2.0, 38°C), purchased from Biocatalysts (Cardiff, United Kingdom). Enzyme inactivation was achieved by increasing the pH to 7.0 with 5N sodium hydroxide (NaOH). The hydrolysate was centrifuged at 2500g for 15 min and the supernatants were frozen and lyophilized for later use.

2.2 *Animals*

Three-month-old male Wistar rats (375.8 ± 8.9 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12 h light/dark), giving free access to water and fed with a standard chow 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 Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 017/2018).

Rats were divided into four groups (8 animals for group) and treated for 14 days with: 1) Untreated group - intraperitoneal injections (i.p.) of distilled water and tap water by gavage; 2) Cd group – i.p. injections of 1 mg/kg bw per day of cadmium chloride ($CdCl_2$) (Balaraman, Gulati, Bhatt, Rathod, and Hemavathi, 1989) and tap water by gavage; 3) EWH group - i.p. injections of distilled water and EWH at1g/kg/day by gavage (Miguel, Lopez-Fandino, Ramos, and Aleixandre, 2006); 4) Cd + EWH group. 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. The volume of injections and gavages of EWH and Cd were weekly adjusted according to the weight of each animal to ensure the receipt of the dose stipulated by the study.

$CdCl_2$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in distilled water. Salts and reagents were of analytical grade obtained from Sigma-Aldrich.

2.3 Cardiovascular Measurements

Indirect systolic blood pressure (SBP) was measured before and at the end of the treatment period (14 days), using non-invasive tail-cuff plethysmography according to Wiggers et al. (2008) (AD Instruments Pty Ltd, Bella Vista, NSW, Australia).

On the fifteenth day, animals were anaesthetized with a combination of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, i.p.) and euthanized for vascular reactivity experiments. Thereafter, the thoracic aorta was carefully dissected out and cleaned of fat and connective tissues, divided into segments of 2 mm in length and placed into Krebs-Henseleit solution (in mM: NaCl 118; KCl 4.7; $NaHCO_3$ 23; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 11 and EDTA 0.01), gassed with 95% O2 and 5% CO_2 (pH 7.4). The remaining aorta was kept at −80 °C for further

biochemical/biological assays. Segments of aorta were mounted in an isolated tissue chamber and maintained at a resting tension of 1.5 g at 37 °C. Isometric tension was recorded using an isometric force transducer (TSD125BX8, Biopac Systems, Inc, Santa Barbara, CA, USA) connected to an acquisition system (MP150WSW-SYS, Biopac Systems). After a 45-min equilibration period, aortic segments were exposed twice to 75mM KCl, first to check their functional integrity and again to assess the maximal tension developed. Afterwards, endothelial integrity was tested with acetylcholine (ACh, 10 μM) in segments that were previously contracted with phenylephrine (Phe) at a concentration that produced close to 50% of the contraction induced by KCl. After 60 min of washout, a single concentration response curve to Phe (0.01 nM – 300 μM) was performed. To evaluate the role of the endothelium in the vasoconstrictor responses to Phe, this vascular component was mechanically removed, and its absence was confirmed by the inability of ACh to induce relaxation greater than 10% of the previous contraction due to Phe. The effects of the following drugs were evaluated by its administration 30 min prior Phe concentration-response curve: the nonspecific nitric oxide synthase (NOS) inhibitor, N(ω)-nitro-L-arginine methyl ester (L-NAME, 100μM); the NADPH oxidase inhibitor, apocynin (0.3 μM,); the nonselective COX inhibitor, indomethacin (1 μM); the selective COX-2 inhibitor, NS398 (1 μM); and the AT-1 receptor blocker, losartan (10 mM). To evaluate the relaxation dependent and independent of the endothelium, concentration-response curves to acetylcholine (ACh) (0.1 nM − 300μM) and sodium nitroprusside (SNP, 0.1 nM−300μM) respectively, were performed in segments previously contracted with Phe.

2.4 *Biochemical Analyses*

Biochemical studies of oxidative stress biomarkers were performed in aorta. For that, vessels were homogenized in 50 mM TrisHCl, pH 7.4, centrifuged at 2400g for 10

min at 4°C. Levels of reactive species were determined by the spectrofluorometric method described by Loetchutinat *et al.* (2005) with modifications (Martinez *et al.*, 2017). This method is unspecific for reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The ROS and RNS levels were expressed as fluorescence units.

Lipid peroxidation was measured as malondialdehyde (MDA) levels using a colorimetric method, as previously described by Ohkawa *et al.* (1979), with modifications (Martinez *et al.*, 2017). The results were expressed as nanomoles of MDA per mg of protein.

We measured the total antioxidant capacity by Ferric Reducing Antioxidant Power (FRAP) assay described by Benzie & Strain (1996), with modifications (Martinez *et al.*, 2017). A standard dose-response curve of Trolox (50–1000 μM − water soluble analog of vitamin E) was prepared and the FRAP assay is described with particular reference to Trolox equivalents.

In situ detection of vascular superoxide radical anion production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* superoxide radical anion ($O_2^{\cdot-}$) production in aortic segments, as previously described (Briones *et al.*, 2009) with modifications (Martinez *et al.*, 2017). Fluorescence was detected with a 568 nm long-pass filter. For quantification, five rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the artery were calculated using NIH Image J software version 1.46r (http://rsbweb.nih.gov/ij/), using the same imaging settings in each case.

2.6 Determination of angiotensin converting enzyme (ACE) activity

ACE activity was measured according to fluorimetric method described previously (Silverstein, Friedland, Lyons, and Gourin, 1976; Miguel, Álvarez, Lópes-

Fandino, Alonso, and Salaices, 2007). Briefly, aliquots of plasma (3 μl) were incubated for 15 min at 37°C with assay buffer (40 μl) containing the ACE substrate 5 mM Hip-His-Leu (Sigma). The reaction was stopped by the addition of 0.35 N HCl (190 μl). The generated product, His-Leu, was measured fluorimetrically after 10 min incubation with 2%o-phathal dialdehyde in methanol (100 μl). Fluorescence measurements were carried out at 37°C in a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with 350 nm excitation and 520 nm emission filters. A calibration curve with ACE from rabbit lung (Sigma) was included in each plate.

2.7 Western blot analysis

To analyze the expression of COX-2 in aorta samples, proteins were separated via 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes before being incubated with mouse polyclonal antibody for COX-2 (1:1000, Cayman Chemical, Ann Arbor, MI, USA). After washing, membranes were incubated with an anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase (1:5000, Sigma Aldrich, St. Louis, MO, USA). Following a thorough washing, the immunocomplexes were detected using an enhanced horseradish peroxidase/luminal chemiluminescence system (ECL Plus, GE Healthcare, Buckinghamshire, UK). Image acquisition was carried out using ChemiDoc (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and signals on the immunoblot were quantified using the ImageJ computer program. The same membrane was used to determine the ponceau staining protein expression. Data are expressed as the ratio between signals on the immunoblot corresponding to the studied protein and Ponceau.

2.8 Statistical analysis

Data are expressed as mean ± SEM. In the vascular reactivity experiments, vasoconstrictor responses of aorta were expressed as a percentage of the contraction

induced by 75 mM. Vasodilator responses were expressed as a percentage of the previous contraction to Phe. To compare the effect of L-NAME, APO, INDO, NS398, LOS and on the response to Phe in segments from each group, some results were expressed as differences of area under the concentration-response curves (dAUC) in control and experimental situations, dAUCs were calculated from the individual concentration response curve plots; differences were expressed as the percentage of the dAUC of the corresponding control situation. Results were analyzed using unpaired Student's t-test or two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used to compare individual means. Results of biochemical experiments were analyzed using Student's t-test or one-way ANOVA. Values of $p < 0.05$ were considered significant.

3 Results

The initial weight of the animals was similar in all groups evaluated (in g - Untreated: 356.4 ± 10.3, Cd: 374.5 ± 12.4, EWH: 382.6 ± 9.1, CdEWH: 373.8 ± 14.7, n = 8). However, we observed that the body weight of both groups receiving Cd was lower in relation to the groups that did not receive the metal at the end of treatment but only it was significative in Cd group (in g - Untreated: 409.5 ± 13.5, Cd: 338.2 ± 16.0*, EWH: 396.0 ± 14.1, CdEWH: 348.0 ± 17.6, n=8, *p<0.05 *vs* Untreated). The water and feed intakes were also lower in the Cd and CdEWH groups (Feed in g/day - Untreated: 30.1 ± 1.9, Cd: 8.1 ± 1.8*, EWH: 24.9 ± 2.1, CdEWH: 15.3 ± 2.1*); (Water ml/day - Untreated: 66.7 ± 6.1, Cd: 29.6 ± 5.9 *, EWH: 78.6 ± 5.2, CdEWH: 36.6 ± 3.2*, n=8, *p<0.05 *vs* Untreated).

Cd exposure for 14 days showed a significant increased in SBP values reaching these animal a hypertensive condition. The co-treatment with EWH was able to prevent the increase on SBP, being the values of both groups that received EWH

similar to the untreated group (Untreated: 119.1 ± 2.1 mmHg, Cd 148.7 ± 5.0*, EWH: 124.4 ± 2.6, CdEWH: 123.2 ± 3.2# - n=8, *p<0.05 vs Untreated, #p<0.05 vs Cd).

Regarding vascular reactivity experiments, the response to KCl was similar in all treatments (in g, Untreated: 1.60 ± 0.04, Cd: 1.54 ± 0.06, EWH: 1.57 ± 0.06, CdEWH: 1.61 ± 0.06; n = 8). Cd treatment increased the contractile responses induced by Phe while, the co-treatment with EWH was able to prevent this increase (Table 1 and Fig 1A). The vasodilator response dependent and independent of the endothelium induced by ACh and NPS, respectively were not affected by Cd exposition or EWH (Fig 1B, C).

To investigate whether co-treatment with EWH modifies NO modulation in vasoconstrictor responses of rats exposed to Cd, we removed the endothelium and incubated the aortic rings with a NOS-inhibitor (L-NAME). The endothelial removal and NOS inhibition left shift the concentration response curve to Phe in all groups (Fig 2 – A, B, C, D, and a, b, c, d). However, dAUC values demonstrated that Cd exposure reduces endothelial modulation (Fig 2E) and NO bioavailability in the vasoconstrictor response to Phe (Fig 2e). The co-treatment with EWH has partially prevented the reduction in endothelial modulation and subsequently, vascular dysfunction induced by Cd exposure (Fig 2 E and e).

We have evaluated the effects of the EWH on oxidative stress in Cd-induced vascular dysfunction; we assessed the role of superoxide anion in Phe responses using apocynin (0.3 mM). Apocynin incubation reduced the vasoconstrictor response induced by Phe in aortas of all experimental groups, but this reduction was higher in Cd-treated rats, demonstrating an important participation of ROS in the increased vascular response (Fig 2 a', b', c', and d'). Supporting this data, we have found an increased ROS and lipid peroxidation levels in aorta tissue (Fig 3 A, B) and, an increased local

production of superoxide anion in aorta (Fig. 4E). The co-treatment with EWH has prevented the increased ROS participation in the vasoconstrictor response to Phe (Fig. 2 e'), the increased ROS levels in aorta tissues (Fig. 3 B), and, has partially prevented the increased lipid peroxidation (Fig. 3 A) and the *in situ* production of superoxide anion (Fig. 4 E). Cd exposure increased the antioxidant capacity and the EWH treatment has partially prevented these increased (Fig. 3 C).

To investigate the role of prostanoids in the increased vasoconstrictor response to Phe and, consequently, the involvement of the EWH in this pathway, we have used COX inhibitors indomethacin (1μM) and NS398 (1μM). The incubation with indomethacin and NS398 reduced the Phe vasoconstrictor responses in all groups. However, this reduction was greater in segments of aorta from Cd-treated rats, as shown by the dAUC (Fig. 5 E, e), indicating a role of contractile prostanoids in the increased vascular response in Cd-treated rats. Cd exposure has also increased COX-2 protein expression in aortas of exposed rats (Fig. 5 F). The increased participation of COX prostanoids and local COX-2 expressions were prevented by EWH treatment (Fig. 5 e and F).

Considering the importance of the RAAS in the control of the SBP, vascular reactivity and possible action of the EWH against the ACE we evaluated the action of EWH on the renin-angiotensin system in the vascular reactivity to Phe induced by Cd, blocking AT-1 receptors with losartan (10 mM). As expected, the incubation of vessels with losartan decreased the vasoconstrictor response to Phe. However, this reduction was greater in vessels of Cd-treated groups, suggesting an involvement of angiotensin II in the altered vascular function after Cd exposure (Fig. 6). Cd treatment also increased the plasmatic ACE activity, compared to untreated rats (mU ACE/mL Plasm - Untreated: 5361.9 ± 417.6; Cd: 9418.6 ± 943.7*; EWH: 6007.2 ± 462.9; CdEWH:

5911.4 ± 614.5$^{\#}$, n=8, one-way ANOVA, *p<0.05 *vs* Untreated and $^{\#}$p<0.05 *vs* Cd). The increased participation of angiotensin II in the vasoconstrictor response to Phe (Fig. 6) and, the increased plasmatic ECA activity were both prevented by the concomitant intake of EWH.

4. Discussion

Hypertension is the main risk factor for stroke and coronary heart disease and is also responsible for 13% of all deaths worldwide (Olsen & Spencer, 2017). Interestingly, it was seen that hypertensive patients have higher concentration of Cd in urine and that populations of contaminated area show higher incidence of atherosclerosis (Houtman, 1993, Madrigal, Ricardo, Persky, and Turyk, 2018). In this study, Cd exposure increased blood pressure, vascular reactivity, oxidative stress, inflammation and activation of renin-angiotensin system, . The present study has also demonstrated that the co-treatment with EWH could prevent cardiovascular alterations produced after Cd exposured. Concretely, the consumption of EWH was able to prevent the increase on SBP and vascular contractility, and these effect could be related to a reduction in oxidative stress mediated by NADPH oxidase and COX-2-derived prostanoids in aorta and plasma ACE activity. These biomarkers have shoen a significant increase after Cd exposure.

In other studies it was also observed that the exposure to lower or higher doses of Cd compared to that used in our study, contribute to the development of hypertension (Subramanyam, Bhaskar, and Govindappa, 1992; Lall, Peshin, Gulati, Khattar, Das, and Seth, 1997, Sangartit *et al.*, 2004, Almenara *et al*. 2013, Choudhary and Bodakhe, 2016). Several mechanisms have been suggested for Cd-induced hypertension, such as proximal tubulo-renal damage, salt retention, increased blood volume and oxidative

stress (Yiin, Chern, Sheu, and Lin, 1999, Satarug, Nishijo, Ujjin, Vanavanitkun, and Moore, 2005). Some studies suggest a role of Cd on NO production, reduction its production or availability, increasing vascular resistance, thus favoring the hypertensive process (Kolluru, Tamilarasan, Geeta-Priya, Durgha, and Chatterjee, 2006, Cannon III, 1998, Triagle *et al*., 2003, Kolluru, Siamwala, and Chatterjee, 2010).

The increased blood pressure due to Cd exposure was prevented by administration of 1 g/kg/day of EWH. Previous studies have showed the antihypertensive activity of EWH in different experimental conditions such as in spontaneously hypertensive rats (Miguel, López-Fandino, Ramos, and Aleixandre, 2005; Miguel, López-Fandino, Ramos, and Aleixandre, 2006, Miguel, Álvarez, López-Fanadino, Alonso, and Salaices, 2007), in animals exposed to mercury chloride (Rizzetti, *et al.*, 2017) or in animals exposed to aluminum (personal comunication)The main mechanism suggested by the antihypertensive effect of the EWH was the modulation of the ACE activity (Kawasaki *et al.*, 2002, Miguel, López-Fanadino, Ramos, and Aleixandre, 2006), which is the main mechanism implicated in drugs to reduce high blood pressure. This antihypertensive mechanism was also implicated in the present study. Moreover, some peptide sequences found in EWH showed tobe potent *in vitro* ACE inhibitors such as Tyr-Ala-Glu-Arg-Tyr-Pro-Ile-Leu (YAEERYPIL), Arg-Ala-Asp-His-Pro-Phe-Leu (RADHPFL) (Miguel, Recio, Gomes-Ruiz, Ramos, and Lopes-Fandino, 2004).

Regarding body weight, we observed in Cd and CdEWH groups showed a loss of weight of -9.7% and -7.6% respectively. This effect of Cd has been described in different type and dose of exposition and it is associated with increased levels of lipid peroxidation, as in the present study, but the mechanism involved is still not well established (Gökalp *et al*., 2009, Sompamit, Kukongviriyapan, Donpunha, Nakmareong, and Kukongviriyapan, 2010). Although EWH did not prevent such damage, it was able

to reduce the percentage of weight loss, feed and water consumption by 2%, 25% and 11%, respectively.

Pressure changes caused by exposure to Cd are also probably related with vascular changes promoted by the toxicity of this metal. In our study, EWH was able to prevent vascular dysfunction in aorta after exposure to Cd, probably by the reduction observed in ROS and local superoxide anion production, then, avoiding the negative effects on NO bioavailability. Similar results have been found in models of exposure to Hg at doses similar to those found in humans exposed to this metal and co-treated with EWH (Rizzetti, D. *et al.,* 2017). The antihypertensive effect of EWH in SHR rats was also related with the potent antioxidant capacity of these egg derived peptides, reducing lipid peroxidation in plasma and aorta affecting blood pressure levels of (Manso. Miguel, Even, Hernandez, Aleixandre, and López-Fandino, 2008). Therefore suggest that the association of the antihypertensive and antioxidant activities of EWH contributes to reduction of blood pressure and normalization of vascular reactivity.

Several factors may influence the potential activity of peptides when administered orally, including the physiological conditions to which these peptides will be subjected (Loião et al., 2008, Santos-Hernández, Miralles, Amigo, and Recio, 2018). A study with two of the peptides already cited in this study, YAEERYPIL and RADHPFL, showed that they hydrolyze during the gastrointestinal simulation process, leading them to believe that the hydrolyzed products derived from these sequences may be directly responsible for the antihypertensive effect (Miguel, Álvarez, Lópes-Fandino, Alonso, and Salaices, 2007). In addition, some peptide sequences after digestion did not present ACE-inhibitory activity, although they present vasodilatory activity (Miguel, *et al*, 2007), suggesting that other mechanisms could be implicated in their antihypertensive effect. Our data are in agreement with other studies using food protein derived peptides

that possess antioxidant, vasodilator and opioid activity (Yoshikawa, *et al.*, 1994, Fujita, *et al*., 1996, Sipola, *et al.*, 2002, Kuono, Hirano, Kuboki, Kasai and Hatae, 2005).

Among the reactive species O2-, ONOO- and OH- are the most unstable and reactive (Taniyama and Griendling, 2003), their formation is related to some vascular enzymes, such as NADPH oxidase, xanthine oxidase, eNOS, cytochrome isoenzyme P450, among others. NADPH oxidase is the main source of ROS in the endothelium, this enzyme uses NADH and NADPH as a substrate and can be activated by angiotensin II (Cai, 2005, Hamilton, Brosnam, Al-Benna, Berg and Dominiczack, 2002, Garrido & Griendling, 2009). In this study we observed that Cd increased the functional and biochemical activity of NADPH oxidase and in both cases the EWH prevented the increase of the participation of the ROS from this pathway in the contractile response to phenylephrine and the increase in the production of superoxide anion in the aorta arteries.

It was reported that the increase on local activity of the renin-angiotensin system and in the production of vasoconstrictor prostanoids promote an increase on ROS levels in arteries of normotensive and SHR rats (Álvarez, *et al.*, 2007). Our functional and biochemical data also show that the disturbances presented by Cd exposure are associated with increased participation of the renin-angiotensin system through increased ACE activity in the rat aorta exposed to Cd. therefore it could generates more angiotensin II and increases its participation in vascular reactivity to phenylephrine. It is also known that angiotensin II regulates the production of prostanoids from COX-2 and the expression of this protein, as well as the activation of NADPH oxidase (Griendling, Minieri, Ollerenshaw, and Alezander, 1994, Ohnaka, Numaguchi, Yamakawa, Inagami, 2000, Weseler & Bast, 2010). In our study Cd exposure also increased participation of vasoconstrictor prostanoids and COX-2 expression. Angeli, *et al.* (2013) observed that

the Cd induces the greater local release of angiotensin II and leads to the increase of COX-2 and the activity of NADPH oxidase, suggesting that this may be the mechanism for greater release of ROS and consequently the deleterious effects of the metal. The most important finding is that the administration of EWH prevented both disturbances in Cd exposure rats. These results have demonstrated, in turn, the antioxidant and anti-inflammatory properties of EWH. In fact, *in vitro* studies had previously indicated the antioxidants and antinflamamtory properties of EWH by reducing the intracellular ROS levels in t-BOOH challenged RAW 264.7 macrophages, without any effect oncell viability (Garcés-Rimon *et al.*, 2016a). Moreover, very recently we have demosntrated that in angiotensin II-stimulated adventitial fibroblasts some dervied peptides from EWH inhibited COX-2 expression and the production of pro-inflammatory prostanoids prostaglandin E2 (personal communication)

The binding of cadmium to metallothionein (MT) inhibits the action of the metal with other macromolecules and is considered a mechanism of protection of the organism, eventually preventing this metal from being excreted (Klaassen, Liu, and Diwan, 2009). When the complete MT-Cd2+ reaches the kidneys to be excreted it is filtered and reabsorbed by the cells of the proximal tubules, where the binding of this complex occur and the bivalent cadmium is released into the circulation. When the free cadmium concentration is greater than the yield or binding capacity of this protein, the concentration of this metal increases and causes deleterious effects on different organs and systems (Friberg, Elinder, Kjellström, and Nordberg, 1986, Klaassen, Liu, and Diwan, 2009, Cai, Liu, & Cherian, 2010). One of the factors due to the high affinity of cadmium to MT is the sulfhydryl groups (-SH) present in the structure of this protein

and where in fact the conjugation of both occurs. Cadmium has high affinity for -SH and this affinity makes possible its association with other molecules such as albumin, cysteine and glutathione (Klaassen, Liu, Diwan, 2009). Thus, the EWH, a potential source of binding to this metal. Egg white is a food matrix composed mostly of sulfur-containing proteins (Adham et al., 2013). Therefore, it is conceivable that the peptides contained in the egg white hydrolyzate could compete for their binding to Cd with Metalloproein, thus reducing the absorption of said metal and improving its natural excretion pathways.

In summary, EWH appears to have antihypertensive, anti-inflammatory and antioxidant properties against Cd-induced cardiovascular changes. These functional peptides could be considered as an alternative therapy to counteract the deleterious effects of Cd exposure.

Table 1: Effect of EWH on sensitivity (pD2) and maximal effect (Emax) to Phe in aortic rings of rats exposed to of $CdCl_2$ for 14 days.

	pD_2				E_{max} (%)			
	Untreated	**Cd**	**EWH**	**CdEWH**	**Untreated**	**Cd**	**EWH**	**CdEWH**
Ct	6.6 ± 0.06	6.5 ± 0.2	6.2 ± 0.1	6.4 ± 0.1	84.9 ± 1.5	101.6 ± 2.0 $^{\#}$	90.6 ± 1.5	91.7 ± 3.6
E-	7.7 ± 0.2	7.0 ± 0.2$^{\#}$	7.5 ± 0.1	7.0 ± 0.1	129.0 ± 2.5*	119.6 ± 4.4$^{*\#}$	127.8 ± 4.7*	108.0 ± 1.3*
L-NAME	6.7 ± 0.3	6.7 ± 0.1	6.9 ± 0.2	6.9 ± 0.1	130.3 ± 3.7*	126.7 ± 2.2$^{*\#}$	121.8 ± 4.2*	119.5 ± 5.0*
APO	6.4 ± 0.3	6.8 ± 0.2	6.4 ± 0.2	6.1 ± 0.1	54.0 ± 8.1*	41.7 ± 5.7$^{*\#}$	46.2 ± 7.6*	69.1 ± 1,4$^{*\&}$
INDO	6.4 ± 0.2	6.4 ± 0.3	6.0 ± 0.1	6.4 ± 0.2	64.9 ± 4.5*	46.2 ± 6.2$^{*\#}$	77.7 ± 4.7	41.0 ± 4.3 $^{*\#}$
NS398	6.3 ± 0.2	6.5 ± 0.1	6.3 ± 0.1	6.7 ± 0.3	57.8 ± 3.0*	37.1 ± 3.7$^{*\#}$	56.1 ± 4.5*	53.0 ± 3.9$^{*\&}$
LOS	6.4 ± 0.1	6.5 ± 0.1	6.1 ± 0.2	6.6 ± 0.2	69.9 ± 4.5*	40.9 ± 3.7$^{*\#}$	70.6 ± 3.2*	61,9 ± 6.8$^{*\&}$

Parameters of sensitivity (pD_2) and maximal effect (Emax) of the concentration-response curves to Phe in aortas from rats Untreated, treated with Cadmium, Hydrolysate and Hydrolysate plus Cadmium in intact (Ct) and endothelium removal (E-) segments and in the presence of L-NAME (100µM), Apocynin (0,3µM), Indomethacin (1µM), NS398 (1µM), and Losartan (10mM) incubation. Results are expressed as mean ± SEM. Emax: maximal effect (expressed as a percentage of maximal response induced by 75mM KCl) and pD_2 expressed as a -log one-half Rmax. n = 8, *$p < 0.05$ compared to the corresponding control in each group, $^{\#}p < 0.05$ compared with the Untreated group, $^{\&}p < 0.05$ compared with the Cd group (one-way ANOVA).

Legends

Fig 1. Effect of cadmium exposure on vascular reactivity. Concentration–response curves to (A) phenylephrine (Phe), (B) acetylcholine (Ach) and (C) sodium nitroprusside (SNP) in aorta segments. Data are expressed as mean ± SEM as a percentage of the response to 75 mmol/l KCl. n=8, *p<0.05 *vs* Untreated group and # *vs* Cd group (Two-Way ANOVA followed by Bonferroni).

Fig 2. Effect of EWH in cadmium exposure on NO and NADPH oxidase -mediated vascular response. Effect of endothelium removal (E−) (A, B, C and D), L-NAME (100μM) (a, b, c and d) and Apocynin (3.0μM) (a', b', c' and d') on the concentration response curve to Phe compared to control curves. The differences in the area under the concentration–response curves (dAUC) in (E) endothelium denuded and intact segments, in the presence and absence of L-NAME (e) and Apocynin (e') of the four experimental groups. Data are expressed as mean ± SEM as a percentage of the response to 75 mmol/l KCl, n= 8, *p< 0.05 vs control curve (Two-Way ANOVA followed by Bonferroni) *p<0.05 *vs* Untreated, # *vs* Cd (One-way ANOVA) in dAUC graphs.

Fig 3. Effects of EWH treatment on reactive species (A), lipid peroxidation (B) and total antioxidant capacity (C) in aorta tissue of rats of all groups. Data are expressed as mean ± SEM, n = 8, *p< 0.05 vs Untreated group, # vs Cd group (One-way ANOVA).

Fig 4. Effects of EWH on local anion superoxide production in aorta from rats Untreated (A), treated with Cadmium (B), Hydrolysate (C) or Cadmium plus

Hydrolysate (D). Representative images from aorta exposure to DHE ×20 objective, zoom 4×. n=8, *p< 0.05 vs Untreated; # vs Cd (one-way ANOVA).

Fig 5. Effect of the COX-derived prostanoids inhibitor indomethacin (1μM) (A, B, C and D) and selective COX-2 inhibitor (NS 398 - 1μM) (a, b, c and d) in vasoconstrictor responses to Phe in the aorta. The differences in the area under the concentration–response curves (dAUC) in the presence and absence of indomethacin (E) or NS 398 (e) and the COX-2 expression in aorta of all groups (F). Data are expressed as mean ± SEM as a percentage of the response to 75 mmol/l KCl, n = 8, *p< 0.05 vs control curve (Two-Way ANOVA followed by Bonferroni) and *p< 0.05 vs Untreated; # p<0.05 vs Cd in dAUC graphs and COX-2 protein expression (One-way ANOVA).

Fig 6. Effects of EWH on participation of local renin-angiotensin system in vasoconstrictor responses to Phe in aorta of rats exposed to Cd for 14 days. Concentration-response curve to Phe in the absence (Ct) and the presence of the AT-1 receptors blocker (Losartan 10 mM) in aortic segments of all groups (A, B, C and D). The differences in the area under the concentration–response curves (dAUC) in the presence and absence of Losartan (E). Data are expressed as mean ± SEM as a percentage of the response to 75 mmol/l KCl, n=8, *p< 0.05 vs control curve (Two-Way ANOVA followed by Bonferroni) *p< 0.05 vs Untreated; #p< 0.05 vs Cd in dAUC graphs (One-way ANOVA).

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Figure 1

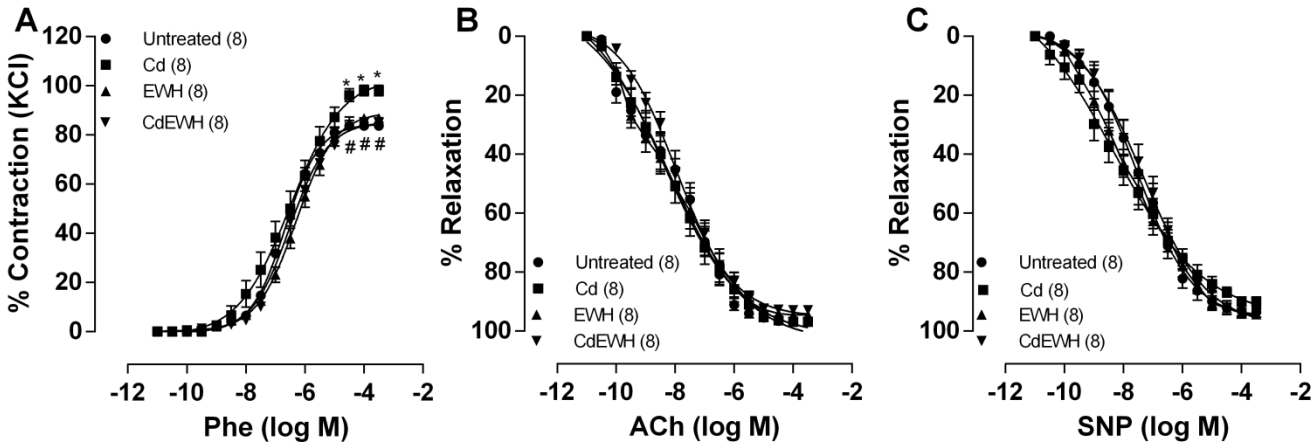

A
% Contraction (KCl)
120
100
80
60
40
20
0
Untreated (8)
Cd (8)
EWH (8)
CdEWH (8)
*
*
*
###
-12
-10
-8
-6
-4
-2
Phe (log M)
B
% Relaxation
0
20
40
60
80
100
Untreated (8)
Cd (8)
EWH (8)
CdEWH (8)
-12
-10
-8
-6
-4
-2
ACh (log M)
C
% Relaxation
0
20
40
60
80
100
Untreated (8)
Cd (8)
EWH (8)
CdEWH (8)
-12
-10
-8
-6
-4
-2
SNP (log M)

Figure 2

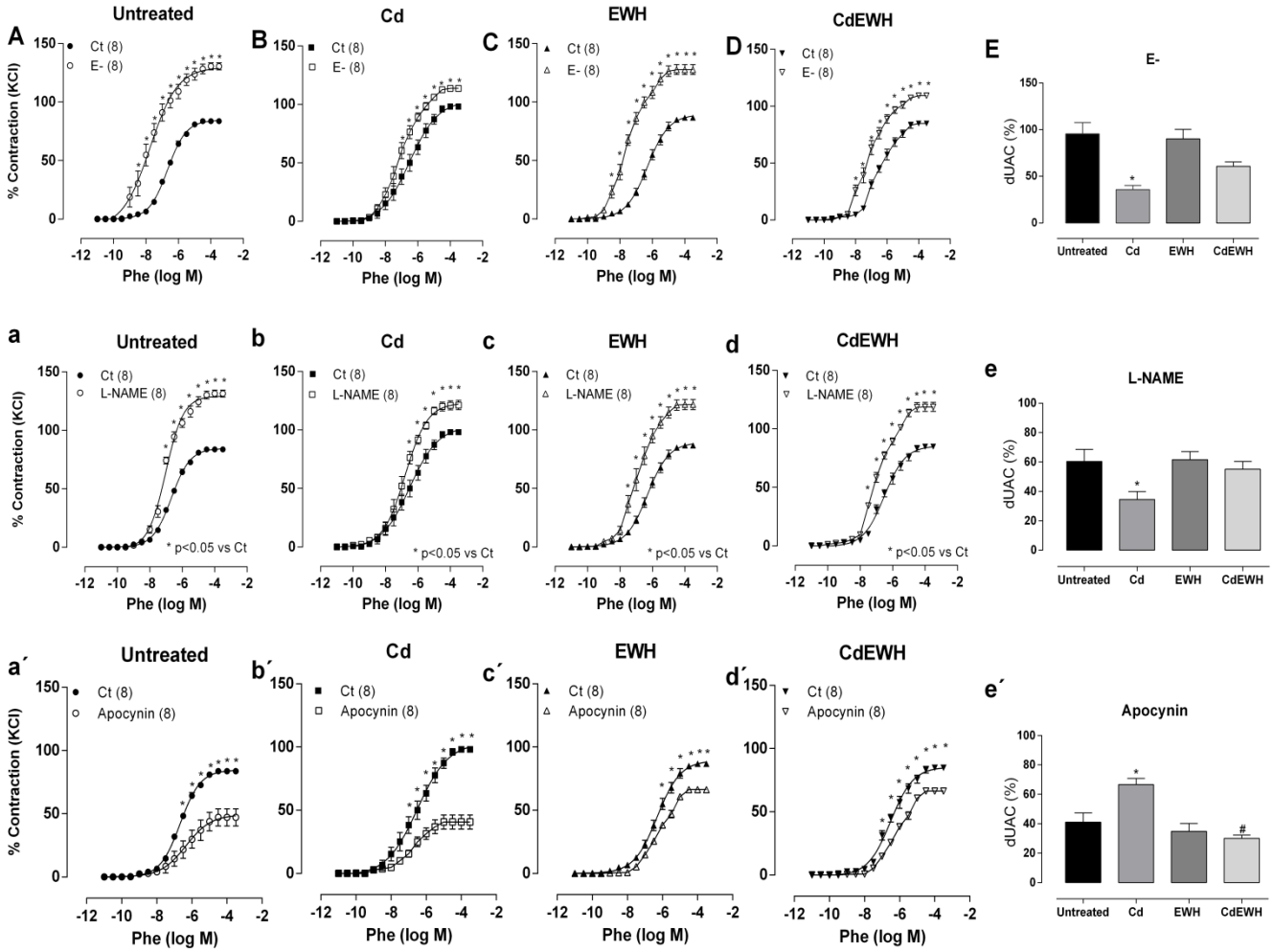

A
Untreated
Ct (8)
E- (8)
% Contraction (KCl)
Phe (log M)
B
Cd
C
EWH
D
CdEWH
E
E-
dUAC (%)
Untreated
Cd
EWH
CdEWH
a
L-NAME (8)
* p<0.05 vs Ct
b
c
d
e
L-NAME
a´
Apocynin (8)
b´
c´
d´
e´
Apocynin
#

Figure 3

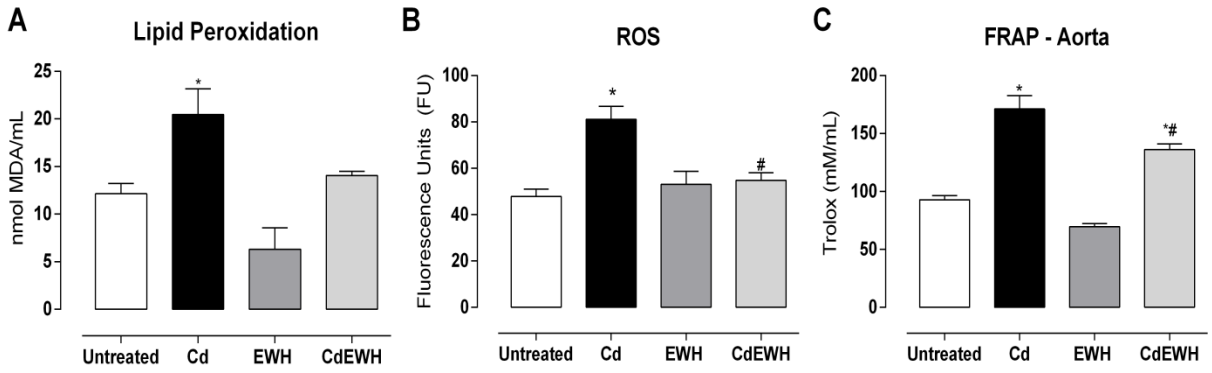

A
Lipid Peroxidation
nmol MDA/mL
25
20
15
10
5
0
*
Untreated
Cd
EWH
CdEWH
B
ROS
Fluorescence Units (FU)
100
80
60
40
20
0
*
#
Untreated
Cd
EWH
CdEWH
C
FRAP - Aorta
Trolox (mM/mL)
200
150
100
50
0
*
*#
Untreated
Cd
EWH
CdEWH

Figure 4

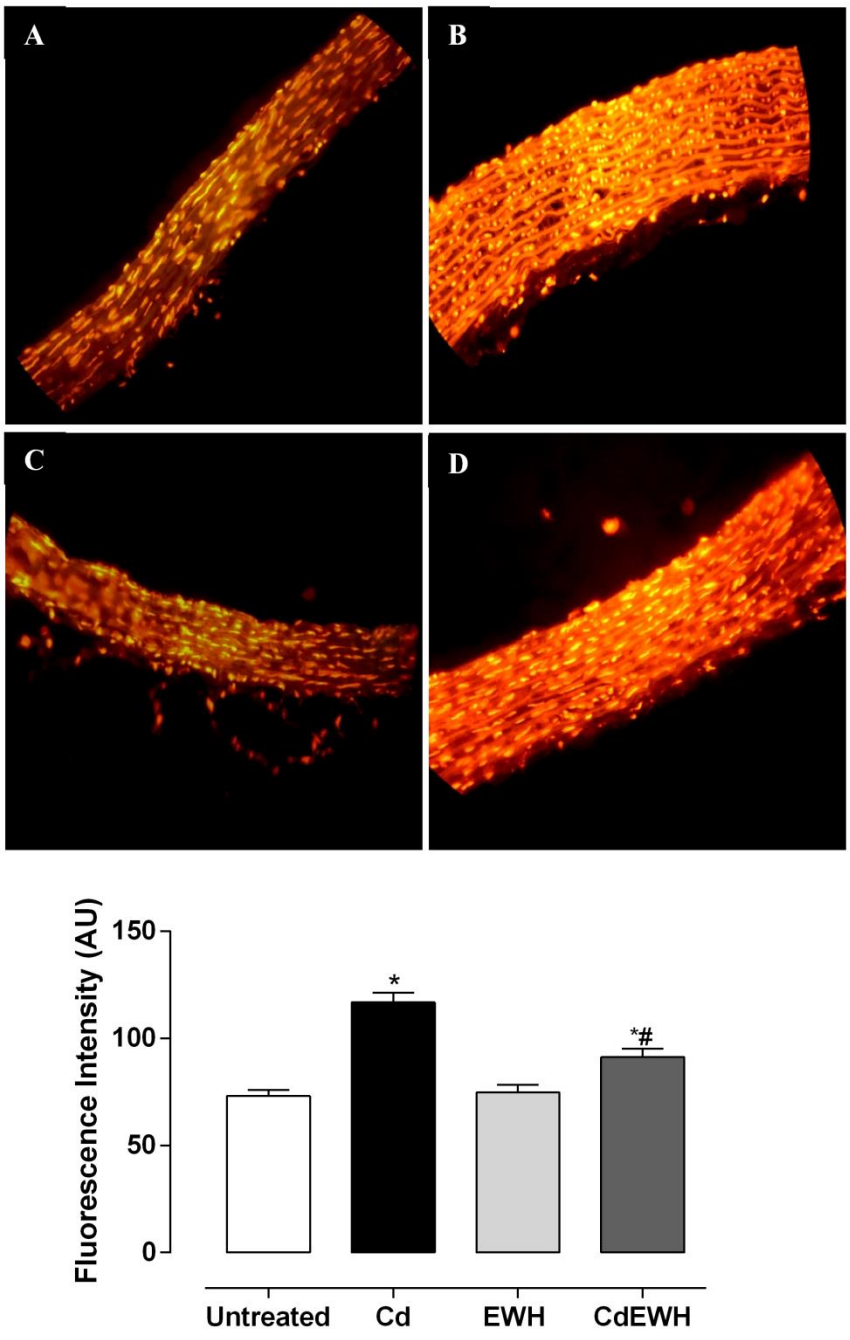

A
B
C
D
Fluorescence Intensity (AU)
150
100
50
0
*
*#
Untreated
Cd
EWH
CdEWH

Figure 5

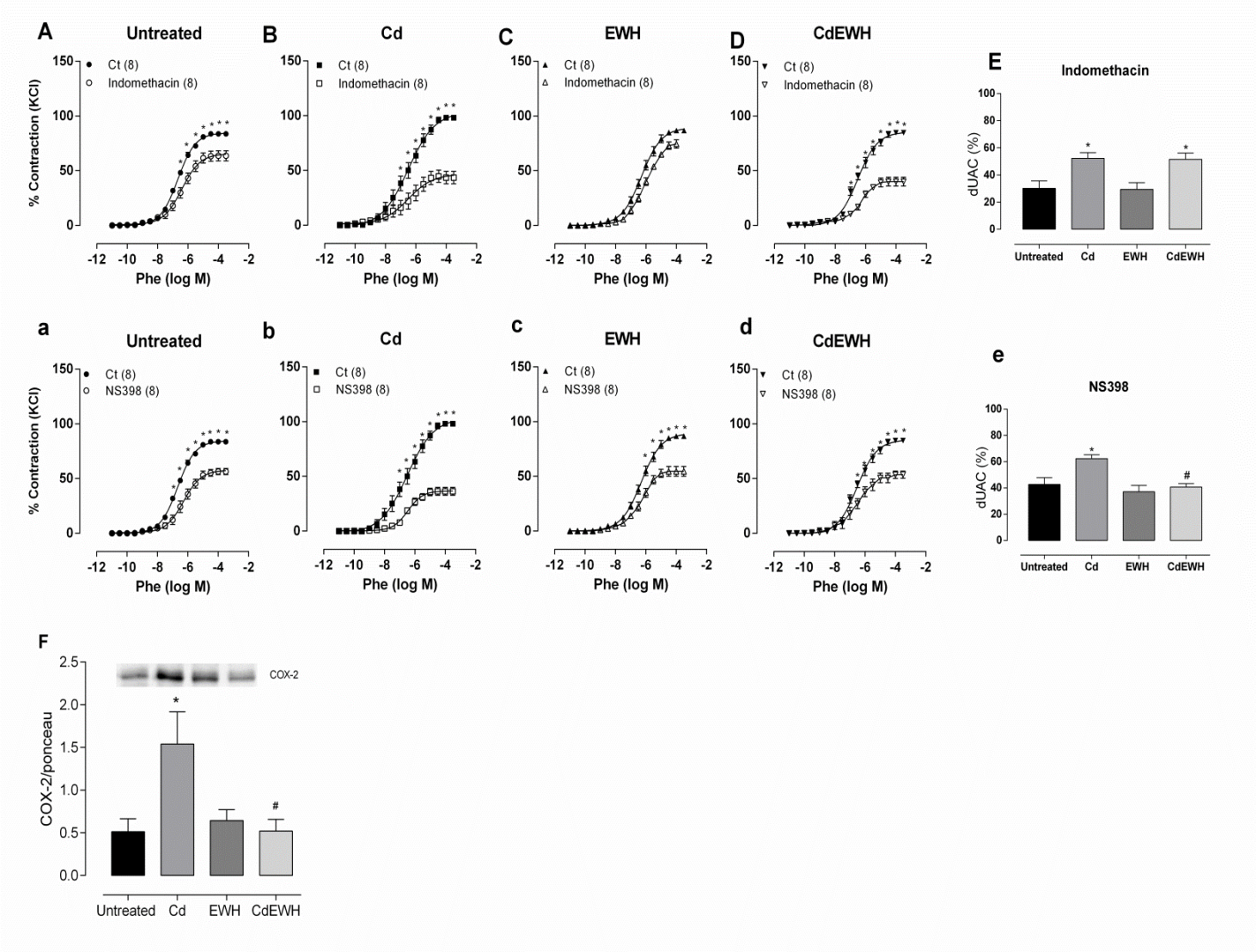
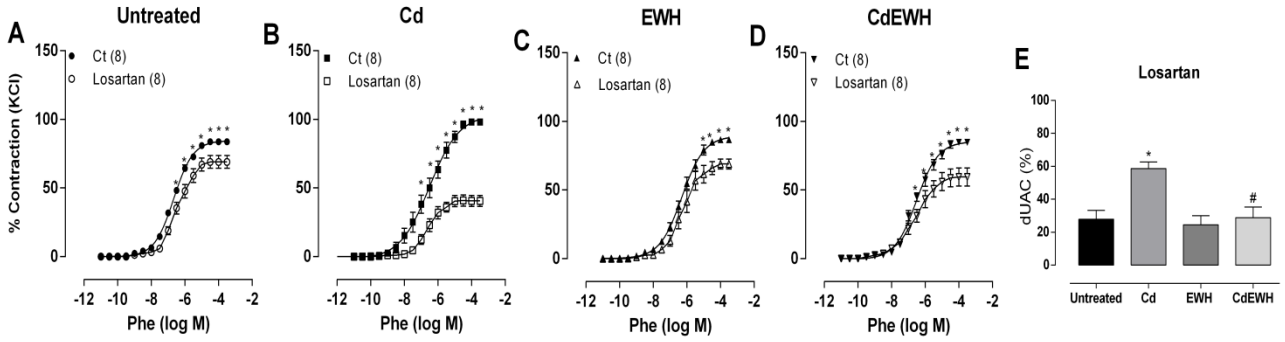

A
Untreated
Ct (8)
Indomethacin (8)
% Contraction (KCl)
Phe (log M)
B
Cd
C
EWH
D
CdEWH
E
Indomethacin
dUAC (%)
Untreated
Cd
EWH
CdEWH
a
Untreated
NS398 (8)
b
Cd
c
EWH
d
CdEWH
e
NS398
F
COX-2
COX-2/ponceau

Figure 6


A
Untreated
B
Cd
C
EWH
D
CdEWH
E
Losartan
Ct (8)
Losartan (8)
% Contraction (KCl)
Phe (log M)
dUAC (%)
Untreated
Cd
EWH
CdEWH

PARTE III

CONCLUSÕES

Nossos resultados sugerem, pela primeira vez, que o co-tratamento com hidrolisado de clara de ovo em ratos:

- Apresentou efeito benéfico na proteção contra a hipertensão arterial promovida pela exposição subcrônica ao $CdCl_2$ por 14 dias;

- Preveniu o aumento na reatividade vascular após exposição ao $CdCl_2$, agindo como um potente agente antioxidante e anti-inflamatório.

Esses achados evidenciam que o hidrolisado de clara de ovo apresenta uma forte ação preventiva contra os malefícios causados pela exposição ao Cd, podendo ser considerado uma forte estratégia de saúde pública, visualizando o amplo alcance que um alimento funcional de fácil acesso pode ter na vida da população.

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ANEXOS

Anexo I - Certificado de aprovação do Projeto pelo CEUA-UNIPAMPA

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CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 017/2018

Titulo: Efeito do hidrolisado da clara do ovo como possível protetor das alterações no sistema cardiovasculares e reprodutor promovidas pela exposição crônica ao cloreto de cádmio em ratos.

Data da aprovação: 12/06/2018

Período de vigência do projeto: 12/06/2020

Pesquisadores(a): Giulia Alessandra Wiggers Peçanha

Campus: Uruguaiana

Telefone: (55) 999147174

E-mail: giuliawp@gmail.com

Finalidade	**( ) Ensino (X) Pesquisa**
Espécie/Linhagem/Raça	Ratos Wistar
Nº de animais	80
Peso/Idade	300 g / 90 dias
Sexo	Machos
Origem	Biotério Central da UFSM

Profª. Drª. Vanusa Manfredini
Coordenadora CEUA/UNIPAMPA

Anexo II – Normas da revista *Journal of Functional Foods*

JOURNAL OF FUNCTIONAL FOODS

AUTHOR INFORMATION PACK

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ISSN: 1756-4646

DESCRIPTION

The *Journal of Functional Foods* aims to bring together the results of fundamental and applied research into healthy foods and biologically active food ingredients.

The Journal is centered in the specific area at the boundaries among food technology, nutrition and health welcoming papers having a good interdisciplinary approach.The journal will cover the fields of plant bioactives; dietary fibre, probiotics; functional lipids; bioactive peptides; vitamins, minerals and botanicals and other dietary supplements. Nutritional and technological aspects related to the development of functional foods and beverages are of core interest to the journal. Experimental works dealing with food digestion, bioavailability of food bioactives and on the mechanisms by which foods and their components are able to modulate physiological parameters connected with disease prevention are of particular interest as well as those dealing with personalized nutrition and nutritional needs in pathological subjects.

Papers will cover topics such as new food bioactives; efficacy and safety of bioactive compounds, and other healthy food constituents using genomic, chemical and biochemical technologies. Characterisation of healthy foods and functional constituents with reference to product development; preparation of natural and synthetic ingredients for use in foods, effects of

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