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DANOS CARDIOVASCULARES E ESTRESSE OXIDATIVO CAUSADOS POR ETANOL – PAPEL DO HIDROLISADO DE CLARA DE OVO

DISSERTAÇÃO DE MESTRADO

Tatiane Inácio Cavallini

Uruguaiana, RS, Brasil
2022

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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
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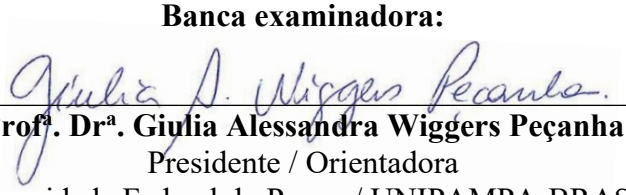

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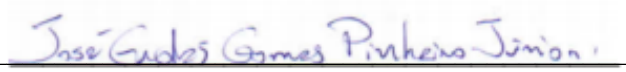

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“Feliz aquele que transfere o que sabe e aprende o que ensina.
Eu sou aquela mulher a quem o tempo muito ensinou.
Ensinou a amar a vida e não desistir da luta, recomeçar na derrota,
renunciar a palavras
e pensamentos negativos. Acreditar nos valores humanos e ser otimista.”

CORA CORALINA

Dedico este trabalho a
minha mãe Valma (*in memoriam*) e
a minha filha Antonella Helena.
Minha mãe por nunca me deixar desistir,
mesmo quando era ela que estava precisando de cuidados e
se mostrava mais forte do que realmente estava,
me dando força, que nem eu sabia que tinha.
A milha filha, manancial de amor genuíno e inesgotável.
Para que você saiba que a mulher pode o que ela quiser.

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PARTE II

LISTA DE ABREVIATURAS

ACh – Acetilcolina

ADH- Álcool desidrogenase

ANP- Agência nacional do petróleo

AT1 – Receptor para Angiotensina II

AUDIT- Alcohol use disorders identification Test

AVC- Acidente Vascular Cerebral

CISA- Centro de informações e saúde e álcool

COX-2 – Isoforma 2 da Enzima Ciclooxigenase

DHA- Doença hepática alcólica

ECA - Enzima conversora da angiotensina

EPM- Erro padrão da média

FAEE- Esteres etílicos de ácidos graxos

GABA- Ácido gama-aminobutírico

IAM- Infarto agudo do miocárdio

HCO – Hidrolisado de clara de ovo

H_2O_2- Peróxido de Hidrogenio

HDL- Lipoproteina de alta densidade

mg/kg/dia – miligramas por quilograma por dia

MEOS- Sistema mitocondrial de oxidação do etanol

MRA- *Mesenteric Resistance Artery* = Artéria mesentérica de resistência

NIAAA- Instituto nacional de abuso de ácool e alcolisno dos Estados Unidos

NAD- Nicotinamida Adenina Dinucleotídeo

NADPH oxidase – enzima Nicotinamida Adenina Dinucleotídeo Fosfato oxidase

NaOH – Hidróxido de Sódio

NE – Noraepinefrina

NO – Nitric Oxide

$O_2^{\cdot -}$ - ânion superóxido

OMS- Organização Mundial da Saúde

PAS- Pressão arterial sistólica

ROS – Especies reativas de oxigênio

SHR – Ratos espontaneamente hipertensos

PARTE I

RESUMO

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

DANOS CARDIOVASCULARES E ESTRESSE OXIDATIVO CAUSADOS POR ETANOL – PAPEL DO HIDROLISADO DE CLARA DE OVO

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Orientadora: Giulia Alessandra Wiggers
Co-orientador: Franck Maciel Peçanha
Local e Data da defesa: Uruguaiana, 08 de novembro de 2022

O consumo exagerado de etanol afeta o sistema cardiovascular. O hidrolisado de clara de ovo (EWH), obtido pela hidrólise enzimática com pepsina por 8 horas, produz peptídeos com funções biológicas com efeitos benéficos sobre o sistema cardiovascular~~es~~. Investigamos se o hidrolisado de clara de ovo (EWH) atua nos danos cardiovasculares causados pelo etanol em ratos. Ratos Wistar machos (CEUA 27/2021) tratados por 42 dias: I) Controle (Ct) - água potável ad libidum e via gavagem; II) EWH - água potável e EWH 1 g/kg/dia via gavagem; III) Etanol (Et) - 20% de etanol em água ad libidum e H_2O por gavagem) e IV) EtEWH - ambos os tratamentos. Semanalmente foi controlado o peso, consumo de líquidos e ração, e mensurada a pressão arterial sistólica (PAS) por pletismografia caudal; A reatividade vascular das artérias mesentéricas de resistência (MRA) foi investigada em miógrafo de tensão. A expressão de NOX-1 e COX-2 foi visualizada por imunofluorescência em MRA, e medida de peroxidação lipídica, espécies reativas e capacidade antioxidante foram analisadas em plasma, rim e fígado e MRA. Os resultados estão expressos como média ± EPM, comparados por ANOVA 2vias co. post-hoc de Bonferroni, com $p < 0,05$. O consumo de etanol reduziu o consumo de ração e líquido e o tratamento EWH não alterou este parâmetro. Os grupos tratados com etanol reduziram o peso corporal (em g: Controle: 470,2 ± 11,7; Et: 399,4 ± 76*; EWH: 448,9 ± 14,7; EtEWH: 407,9 ± 12,8* -* vs Controle). A PAS não foi alterada pelos tratamentos, porém a exposição ao etanol aumentou a resposta vasoconstritora á NE e reduziu o relaxamento a ACh e o EWH foi capaz de normalizar a disfunção endotelial promovida pelo etanol. Este efeito foi mediado pela ação antioxidante com redução da ativação da via da NADPHoxidase, em especial da NOX-1 e ação antiinflamatória com redução da expressão de COX-2 vascular. O aumento de espécies reativas (ROS) foi reduzido pelo tratamento com EWH no plasma e MRA, e não foi alterado no rim e fígado. Assim, o

EWH é um alimento funcional capaz de proteger as disfunções vasculares promovidas pelo consumo de etanol.

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

ABSTRACT

Masters Dissertation
Program of Post-Graduation in Biochemistry
Federal University of Pampa

CARDIOVASCULAR DAMAGE AND OXIDATIVE STRESS PROMOTED BY ETHANOL – ROLE OF EGG WHITE HYDROLYSATE.
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Advisor: Giulia Alessandra Wiggers
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Site and Date of defence: Uruguaiana, November 8th, 2022

Excessive consumption of ethanol affects the cardiovascular system. Egg white hydrolyzate (EWH), obtained by enzymatic hydrolysis with pepsin for 8 hours, produces peptides with biological functions that benefit the cardiovascular system. We investigated whether egg white hydrolyzate (EWH) acts on cardiovascular damage caused by ethanol in rats. Male Wistar rats (CEUA 27/2021) treated for 42 days: I) Control (Ct) - drinking water ad libidum and via gavage; II) EWH - drinking water and EWH 1 g/kg/day via gavage; III) Ethanol (Et) - 20% ethanol in water ad libidum and H_2O by gavage) and IV) EtEWH - both treatments. Weight, fluid, and food consumption were controlled weekly, and systolic blood pressure (SBP) was measured by caudal plethysmography; Vascular reactivity of mesenteric resistance arteries (MRA) was investigated using a tension myograph. The expression of NOX-1 and COX-2 was visualized by immunofluorescence in MRA, and lipid peroxidation, reactive species, and antioxidant capacity were analyzed in plasma, kidney, liver, and MRA. Results are expressed as mean ± SEM, compared by 2-way ANOVA and Bonferroni post-hoc test with $p < 0.05$. Ethanol consumption reduced feed and liquid consumption, and EWH treatment did not change this parameter. The ethanol-treated groups reduced body weight (in g: Control: 470.2 ± 11.7; Et: 399.4 ± 76*; EWH: 448.9 ± 14.7; EtEWH: 407.9 ± 12, 8* -* vs Control). The treatments did not alter SBP, but ethanol exposure increased the vasoconstrictor response to NE and reduced relaxation to ACh, and EWH was able to normalize the endothelial dysfunction promoted by ethanol. The antioxidant action mediated this effect with reduced activation of the NADPH oxidase pathway, especially NOX-1, and anti-inflammatory action with reduced vascular COX-2 expression. The increase in reactive species (ROS) was reduced by EWH treatment in plasma and MRA and was not altered in the kidney and liver. Thus, EWH is

a functional food capable of protecting vascular dysfunctions caused by ethanol consumption.

Keywords: Vascular Reactivity; Vascular Dysfunction; Oxidative Stress; Egg white hydrolyzate; Bioactive peptides.

INTRODUÇÃO

1 ETANOL

1.1 História do Etanol

Os primeiros registros históricos do uso e preparo de bebidas alcóolicas datam de 8000 anos a.C, em comunidades de agricultores. Na China, Mediterrâneo e Golfo Pérsico foram encontrados vasos contendo resíduos de bebida fermentada derivada de arroz, uva, mel e outras plantas (MCGOVERN et al., 2004; KHADERI, 2019).

O etanol é o resultado da fermentação de plantas ricas em açúcares ou amido. A cana-de-açúcar é a planta mais utilizada e produtiva, pois de uma tonelada se produz em média 70 litros de etanol. Sua descoberta, para extração de etanol, é atribuída a experiências de ingleses em colônias africanas. O etanol, pode ser usado como biocombustível, fonte de energia elétrica ou como matéria prima para gerar subprodutos. No Brasil, na década 20, impulsionado pela crise do setor açucareiro e para reduzir a dependência interna por petróleo, iniciou-se a manufatura da cana-de açúcar para produção de álcool, porém sua utilização foi concretizada e expandida com o uso em automóveis a partir da década de 80. Além disso, desde o tempo da colonização era utilizado como bebida alcólica (DIAZ et al., 2011, VANZELA et al., 2015). Vale ressaltar que o etanol presente em combustíveis não é próprio para o consumo humano, e a ingestão humana pode ser fatal.

O etanol e o álcool são a mesma substância, o termo “etanol”, passou a ser usado devido a uma resolução da Agência Nacional do Petróleo (ANP) de 23 de 2010 e, ainda hoje, é popularmente chamado de álcool.

O consumo de álcool etílico acompanhou a evolução dos povos e adquiriu características próprias ao longo dos tempos com benefícios farmacológicos, utilizado para limpezas, combustível e indústrias químicas (MCGOVERN et al., 2004). Devido às

sensações de prazer provocadas pelo consumo de bebida alcoólica, beber tornou-se um símbolo de comemoração presente na maioria das festividades. Na sociedade contemporânea, o álcool é usado de forma recreativa (BALTIERI; CORTEZ, 2005; KACHANI et al., 2008).

Segundo o Centro de Informações sobre Saúde e Álcool (CISA), uma dose padrão de bebida alcoólica no Brasil equivale a 14g de álcool puro (Tabela 1). De acordo com essas definições, é considerado consumo moderado no máximo duas doses em único dia ou 14 doses por semana para homens ou sete doses durante sete dias para mulheres. A Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico (Vigitel), do Ministério da Saúde, define consumo abusivo (Binge Drink) quando homens ingerem 5 ou mais doses ou mulheres bebem 4 ou mais doses em uma única ocasião no último mês. Para a Organização Mundial da Saúde (OMS), o *Binge Drink* está associado ao consumo único de 60 g de álcool em uma única ocasião. Em muitos países do mundo existe o AUDIT - Alcohol Use Disorders Identification Test, que é um instrumento que avalia a frequência e quantidade de álcool ingerido e classifica se a indicação de uso pesado (OMS, 2001; NIAAA, 2004; VIGITEL, 2019, 2020).



	CERVEJA/ CHOPP	**VINHOS**	**DESTILADO**	**Dose padrão (Álcool puro)**
OMS	330 ml	100 ml	30 ml	10-12 g
SENAD	340 ml	140 ml	44 ml	13,6 g
NIAAA	355 ml	150 ml	45 ml	14 g
CISA	350 ml	150 ml	45 ml	14 g

Tabela 1: Dose padrão, segundo agencias reguladoras. Fonte: Própria

Embora o consumo moderado de álcool, em torno de 9,6 g, esteja associado à diminuição de doenças cardiovasculares (OMS, 2018; NIAAA, 2013), melhora da sensibilidade à insulina e diminuição da incidência do resfriado em humanos, estes efeitos estão relacionados a presença de flavonoides, antioxidantes polifenólicos encontrados principalmente nos vinhos tintos e não ao etanol (KHORUTS et al., 1991; COHEN et al., 1993; TAKKOUCHE et al., 2002). O efeito oposto ocorre com o consumo excessivo, mesmo destas bebidas com propriedades benéficas, uma vez que os níveis e cronicidade de ingesta do etanol possuem relação direta com danos ao organismo (SURESHCHANDRA, 2019).

Neste contexto identificar os efeitos nocivos do etanol para o organismo humano é importante devido à abrangência social do consumo de bebidas alcoólicas (OMS, 2018; ROERECKE, 2021).

1.2 Propriedades físico e químicas, vias de passagem e dependência.

O álcool etílico ou etanol é um composto orgânico pertencente a família dos álcoois, cuja característica principal é a presença do grupo funcional hidroxila (─ OH) ligada a um ou mais carbonos saturados. Sua fórmula química C_2H_5OH é representada pela seguinte estrutura molecular (Figura 1):

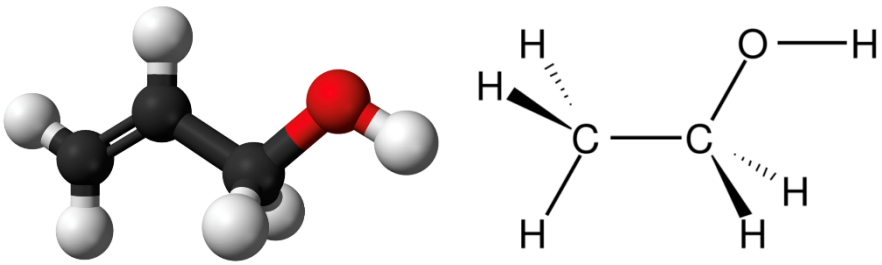


Figura 1: Representação esquemática da molécula de etanol em 3 dimensões e respectiva representação dos elementos.

(Fonte: free3d.com/techiescientist.com)

O grupo -OH na molécula é o que confere as propriedades químicas específicas de um álcool, que são a alta reatividade, caráter ácido e por isso reagem com metais, anidridos, cloretos de ácidos, metais alcalinos. O etanol é uma molécula de baixo peso molecular, polar, de pH neutro, com fusão a -114°C e ebulição aos 78°C. Apresenta-se na forma líquida e incolor, é hidrossolúvel, inflamável e tóxico.

O etanol faz parte do nosso cotidiano, pois é muito utilizado como combustível, e também possui utilidades funcionais para tintas e solventes, sobretudo como matéria-prima, que, quando hidratado, é usado para fabricar alimentos, ~~e~~ bebidas, produtos de limpeza, perfumes e medicamentos (CHAMPE et al., 2006; VANZELLA, 2015).

O etanol, quando ingerido, é rapidamente absorvido no trato gastrointestinal, incialmente no estômago (20%,), mas principalmente no intestino delgado (80%). Sua absorção é rápida inicialmente, no entanto, cai posteriormente, mesmo quando em alta concentração no estômago. O tempo de esvaziamento gástrico e o início da absorção intestinal podem ser considerados os principais fatores determinantes das taxas variáveis de absorção do álcool. Geralmente o pico de concentração plasmática ocorre de meia hora até uma hora e meia após a ingestão. No entanto, a presença de alimentos no estômago retarda a absorção de etanol neste órgão, porém, quando da sua chegada ao intestino delgado, sua absorção é rápida e completa, mesmo na presença de alimentos (OGA; SEIZE, 1996; CHAMPE et al., 2006).

O metabolismo do etanol ocorre essencialmente no fígado, por meio da ação de três enzimas: a álcool desidrogenase (ADH) que catalisa a oxidação a acetaldeído; a CYP2E1, principal componente do sistema microssomal hepático de oxidação do etanol (MEOS); e a catalase, localizada nos peroxissomas dos hepatócitos, responsável por apenas cerca de 10% de seu metabolismo. Vale ressaltar a via não oxidativa que envolve a esterificação

do etanol com ácidos graxos, que conduz à formação de ésteres etílicos de ácidos graxos (FAEE) e a via da catalase, onde nos perixomas, a oxidação do etanol da origem a aldeído, assim é necessário consumo de peróxido de hidrogênio transformado em água (OGA, 1996; CHAMPE, et al, 2006).

1.3 Efeitos tóxicos do etanol em diversos sistemas e órgãos.

O consumo excessivo de álcool episódico (*Binge Drink*), às vezes também chamado de consumo excessivo de álcool, não é claramente definido, no entanto, o *National Institute on Alcohol Abuse and Alcoholism* (NIAAA-EUA) estabelece como ocasiões episódicas de consumo excessivo de álcool como o consumo que eleva a taxa de concentração no sangue em pelo menos 0,08% (ou 0,08 g/dL de álcool) (ROERECKE, 2021; DAWSON, 2011; BIATIONE et al., 2017).

O primeiro levantamento relacionado ao *Binge Drink* no Brasil foi realizado em 2012 o qual estudou o comportamento de 3007 indivíduos e notou que 22,8% dos adultos jovens e adultos da população brasileira consumiam álcool em excesso pelo menos uma vez ao mês. Os autores observaram maior incidência do *Binge Drink,* em nosso país, no extrato da população com melhor poder aquisitivo (CASTRO et al., 2012).

O consumo contínuo ou excessivo de álcool tem sido relacionado a lesões multiorgânicas (SIMON et al., 2022) (Figura 2). Consequências graves deste tipo de consumo, em especial em órgãos como estômago, fígado, coração e cérebro foram reportadas, levando a doença hepática alcoólica (DHA) (SZABO; BALA, 2010) doenças cardiovasculares (DJOUSSE et al., 2007; MUKAMAL et al., 2011; MASIP et al., 2021) e alterações neurológicas como crises convulsivas, delirium, e neuropatia alcoólica (HAES et al., 2010). Doenças como a cirrose, gastrite, polineurite, anemia, pelagra, e

úlceras cutâneas e deficiência das vitaminas B1, B2, B6, B12 e C também estão correlacionadas com o uso de álcool (LANZA et al., 2021).

Estudos em humanos demonstram forte associação entre consumo excessivo de álcool e maior incidência de síndrome do estresse respiratório agudo (SDRA) (THAKUR et al., 2009), câncer (BAAN et al., 2007; FEDIRKO et al., 2011; GREWAL & VISWANATHEN, 2011), sepse (O'BRIEN et al., 2007). Além disso, deve-se ressaltar que ~~os~~ pacientes cirróticos apresentam maiores riscos para o desenvolvimento de doenças cardiovasculares, como a aterosclerose (SILVEIRA et al., 2017; LANZA et al., 2021).

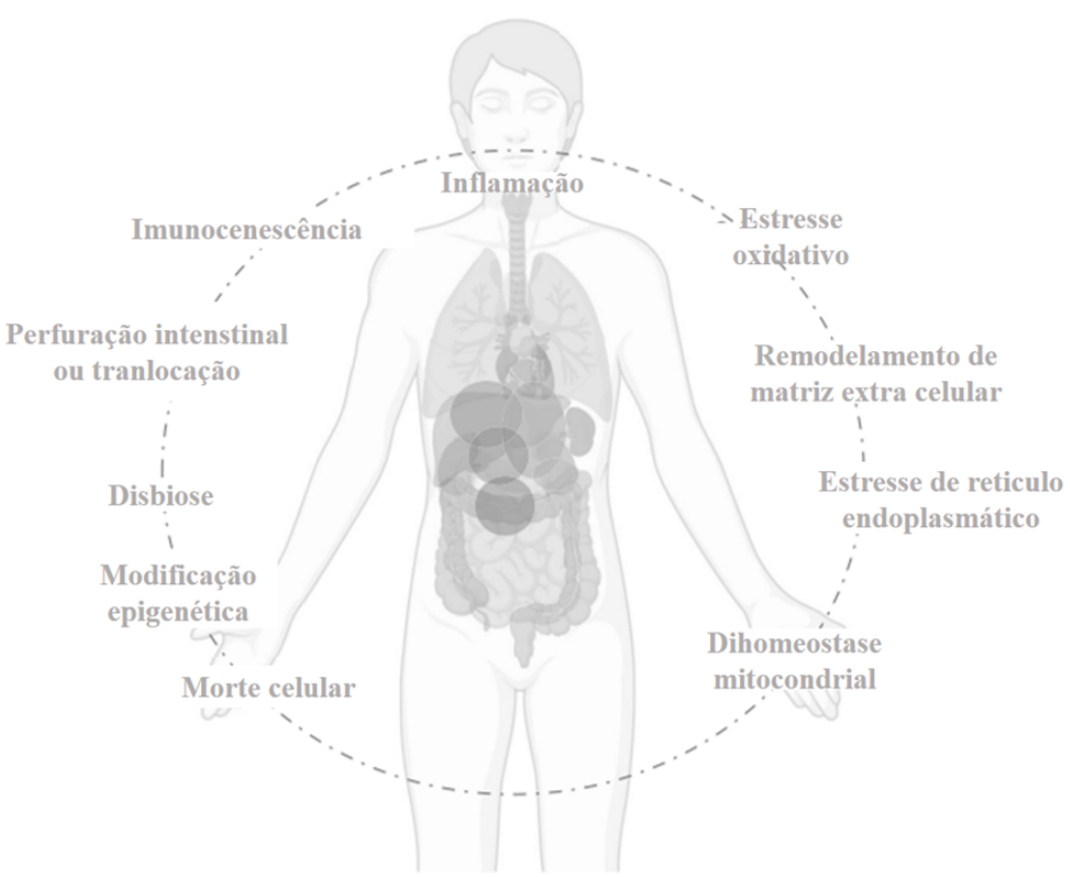


Figura 2: Principais mecanismos fisiopatológicos subjacentes à lesão tecidual induzida pelo álcool. (Fonte: adaptado de SIMON et al. 2022).

2 EFEITOS DO ETANOL NO SISTEMA CARDIOVASCULAR

O consumo baixo e moderado de álcool está associado com redução da mortalidade e da incidência de doenças cardiovasculares, incluindo acidente vascular cerebral (AVC), insuficiência cardíaca, e infarto agudo do miocárdio (IAM). O consumo de álcool versus a incidência de doença cardiovascular possui comportamento em forma de curva em J, onde bebedores leves a moderados apresentam menor risco de doença cardiovascular e menor mortalidade quando comparados a não bebedores ao passo que indivíduos que consomem grande quantidade de álcool apresentam maior risco em relação a bebedores moderados e não bebedores (DI CASTELNUOVO et al., 2006; MASIP et al., 2021)

Muitos dos benefícios cardiovasculares do álcool parecem estar relacionados com a melhora no perfil lipídico, agregação plaquetária, insulino-sensibilidade e de fatores vasculares endoteliais (BRIASOULIS, AGARWAL & MESSERLI, 2012). Também já foi observado que o consumo moderado de álcool está associado ao aumento do colesterol HDL, adiponectina, e apolipoproteina 1, enquanto diminui os níveis de plasminogênio e a concentração plasmática de proteína C-reactiva (PCR), que é um importante marcador inflamatório (GARDNER et al., 2015; MINZER et al., 2020; MASIP & LLUNCH, 2021).

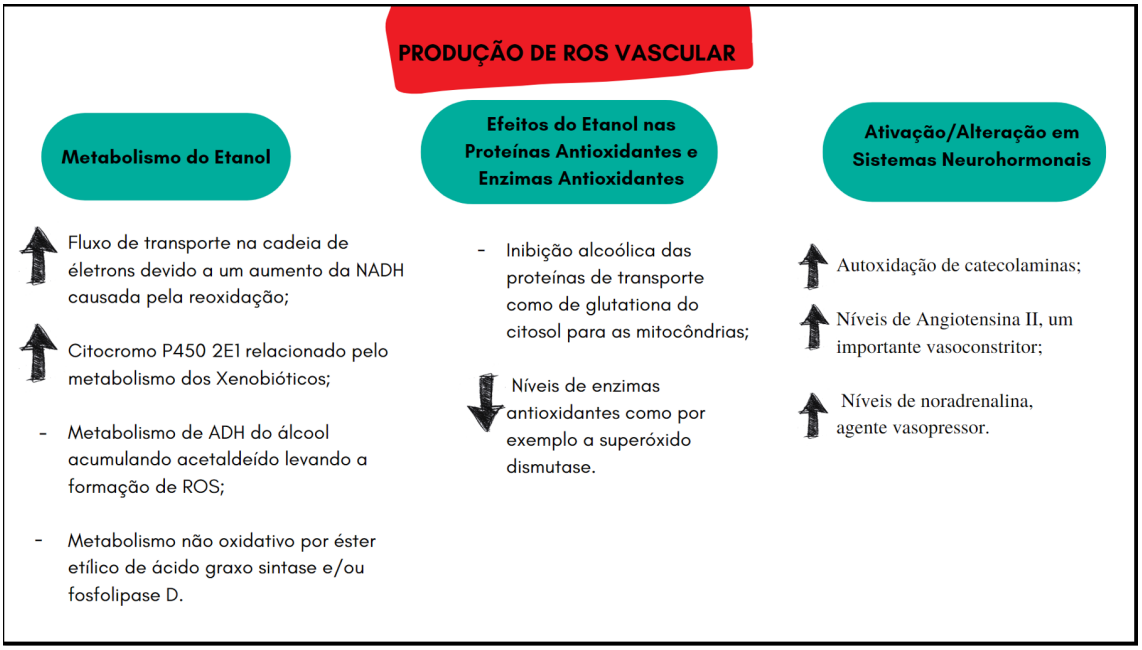
O abuso do álcool está relacionado diretamente com miocardiopatia dilatada, redução da fração de ejeção do ventrículo esquerdo, arritimias, alteração da síntese proteica mitocondrial, e aumento da metabolização de ácidos graxos e lipídios do organismo (VAN DE LUITGAARDEN et al., 2021; GRUBB et al., 2021).

No entanto estudos recentes, discutem que o álcool, mesmo em baixas doses pode trazer futuras alterações, já que continuam liberando, em quantidades menores, marcadores de estresse oxidativo e inflamatórios e é necessária melhor investigação nesse ponto já que existe ambiguidade nos efeitos em moderadas e baixas doses prara diferentes raças (PHILLIPIS et al., 2020; GRUBB et al., 2021; STĂTESCU et al., 2021).

2.1 Estresse Oxidativo e Processo Inflamatório

O consumo crônico de etanol é fator contribuinte para desenvolvimento da hipertensão arterial, e esse processo multifatorial envolve aumento da atividade simpática, estimulação do sistema renina-angiotensina-aldosterona, aumento do estresse oxidativo vascular e disfunção endotelial (TIRAPELLI et al, 2008; MARCHI et al, 2014; CERON et al, 2014; NORTH et al., 2019).

As células endoteliais vasculares que regulam a vasodilação e a vasoconstrição sofrem efeitos do estresse oxidativo que pode interferir diretamente na dinâmica dos vasos (ROCHA et al., 2012; SIMPLICIO et al., 2017; EBY et al., 2019). Quando do consumo do etanol e seu metabólito há redução de elétrons, levando ao desencadeamento de estresse oxidativo também em nível vascular, devido ao aumento de nicotinamina adenina dinucleotidio (NAD) que aumenta o Citocromo P450. Além disso, há maior acúmulo de acetaldeído, levando ao aumento do metabolismo de álcool desidrogenase e, por conseguinte, produção de ROS (CERON et al., 2014; PHILLIPS et al., 2020). Resumo esquemático de formação de ROS do sistema vascular:



Estudos com foco nos mecanismos moleculares demostraram ligação entre a superprodução de ROS na vasculatura e a hipertensão induzida pelo etanol (CERON et al., 2014). Os ROS gerados em células vasculares, ânion de superóxido ($O_2^{\cdot -}$) e peróxido de hidrogênio (H_2O_2), parecem ser especialmente importantes na elevação do cálcio intracelular ($[Ca_2^+]i$) (HAORAH et al., 2019; NORTH et al., 2019), biodisponibilidade de óxido nítrico reduzido (NO), disfunção endotelial e vasoconstrição. $O_2^{\cdot -}$ também pode atuar como uma molécula de sinalização vascular levando à contração vascular e comprometimento do relaxamento (KÄHÖNEN et al., 1999). Assim, através do aumento da geração de ROS e da ativação de vias sensíveis ao redox, o etanol pode induzir disfunção vascular e contribuir para o desenvolvimento de ~~a~~ hipertensão arterial.

Os efeitos do consumo excessivo de etanol resultam também em níveis elevados de mediadores pró-inflamatórios circulantes como TNFα, IL-1β e IL-6 (KHORUTS et al., 1991). Estudos in vitro com células mononucleares humanas saudáveis sugerem que a exposição a etanol, por curto ou longo prazo, tem efeitos opostos sobre respostas inflamatórias (BARR et al., 2016). Enquanto a exposição em curto prazo aumenta a produção de citocinas como a IL-10 e diminui a produção de fatores pró-inflamatórios como TNFα e IL-6 (BALA et al., 2016; MANDREKAR et al., 2007), a exposição a longo prazo aumenta a secreção de TNFα (MANDREKAR et al, 2009; PANG et al., 2009; BALA et al., 2012). Vale ressaltar que os resultados dos estudos *in vitro*, não levam em consideração os efeitos metabólicos e o impacto pleiotrópico do consumo do etanol, visto que, há um efeito sobre células do sistema imunológico que só podem ser observados com exposição *in vivo*.

Estas respostas inflamatórias exacerbadas, assim como o aumento do estresse oxidativo associados à ingestão crônica de etanol são relacionados à hipertensão e, por conseguinte, ao aumento do risco de doença cardiovascular (KLATSKY et al., 1977;

CRIQUI et al., 1981; STROGATZ et al., 1991; TIRAPELL, et al., 2008; PASSAGLIA et al., 2015). A ingesta de etanol também tem sido relacionada ao desenvolvimento de remodelamento vascular por ativação de diferentes vias de sinalização provocadas pelo aumento da produção de ROS que afetam as células adventícias, endoteliais e musculares lisas (MONTEZANO; TOUYZ, 2004; MARCHI et al., 2016; CERON et al., 2017).

Estudo de Haorah et al. (2011) evidencia o aumento dos níveis de espécies reativas de oxigênio (ROS, superóxido e radical hidroxila) e do óxido nítrico (NO) nas células endoteliais do cérebro, ativando a NADPH oxidase e a óxido nítrico sintase no consumo excessivo de etanol. Além disso, estudos da administração aguda de etanol em concentrações equivalentes aos níveis sanguíneos encontrados em indivíduos dependentes, isto é, três vezes o valor do limite legal que é de aproximadamente 9,6 g, promove a contração em artérias cerebrais de maneira independente do endotélio e ativada pela inibição dos canais de potássio dependente de voltagem e canais de cálcio (NORTH et al., 2019).

Além da ativação de agentes vasoconstritores (STRICKLAND; WOOLES 1988; HATTON et al., 1992; MARCHI et al., 2016) também é relatado comprometimento do relaxamento vascular (KÄHÖNEN et al., 1999) relacionado ao aumento de espécies reativas, e redução da biodisponibilidade de NO via mecanismos dependentes da ativação de receptor AT1. Estes mecanismos implicam em aumento de pressão arterial sem alteração de frequência cardíaca em ratos expostos ao etanol (PASSAGLIA et al., 2015).

3. ALTERNATIVAS TERAPÊUTICAS PARA OS DANOS CAUSADOS PELO ETANOL

3.1 Tratamento convencional e alternativos para dependência alcoólica

Atualmente, a farmacoterapia é um método bastante utilizado para tratamento de pacientes alcoólatras com o objetivo de reduzir os efeitos deletérios induzidos pelo uso do álcool no organismo e de reintegrar o indivíduo à sua vida social. A terapia farmacológica pode ser associada a grupos de apoio chamados de Alcoólicos Anônimos como perspectiva de maior aderência e sucesso no tratamento (MINISTÉRIO DA SAÚDE, 2001).

Dentre os medicamentos mais utilizados destacam-se os inibidores de enzimas como a acetaldeído desidrogenase (dissulfiram), os inibidores GABA (acamprosato), e antagonistas opioides (naltrexona) onde o uso é determinado pelas equipes de multiprofissionais, que avaliam e definem de maneira individualizada a indicação farmacológica (MINISTÉRIO DA SAÚDE, 2001; VARELLA; JARDIM, 2009).

No entanto, o uso destes medicamentos possui efeitos colaterais adversos, como a hepatite que já foi associada, mesmo que de forma rara, com o uso de dissulfiram. Cefaléia, sintomas gastrointestinais (dor abdominal, náuseas e vômitos), dermatológicos (prurido, rash máculo-papular e reações bolhosas), assim como confusão mental, sonolência e alteração de libido foram relatados em usuários de acamprostato; assim como náuseas são sintomas comuns após ingesta de naltrexona. Esse tratamento farmacológico exige o controle rotineiro da função hepática através de exames bioquímicos (CASTRO; BALTIERI, 2004).

No Brasil, existem tratamentos que preveem internação que pode ser voluntária, involuntária ou compulsória. No entanto, existe um movimento de especialistas em dependência química que incentivam o uso intervenções que não isolem o alcoolista do seu círculo familiar e da sua rotina normal (MINISTÉRIO DA SAÚDE, 2001; LARANJEIRA, 2004).

Sabendo-se disto, há a necessidade de encontrar formas não farmacológicas para se evitar os efeitos deletérios e prevenção dos efeitos do etanol. Uma alternativa é agregando compostos aos alimentos para auxiliar, durante o uso do etanol, a sua não absorção, ou interferência em seu metabolismo ou em seus efeitos sobre os órgãos, levando a proteção dos tecidos, redução de efeitos adversos com vistas a redução do uso e dependência desta substância danosa ao organismo e a sociedade.

3.2. Alimentos Funcionais

O uso de alimentos como fonte de saúde e seu uso medicinal data da antiguidade (ANJO, 2004). A grande incidência de doenças crônicas relacionadas em parte ao estilo de vida sedentário e a alimentação inadequada abriu portas para um nicho antes pouco estudado. Nas últimas décadas a comunidade científica tem dado atenção a incorporação de alimentos enriquecidos com propriedades benéficas conhecidas e saudáveis e/ou curativos na dieta, os chamados alimentos funcionais (COLLI, 1998; ROS, 2001; LEE et al., 2019; SHOBAKO; OHINATA, 2020; SAPWAROBOL et al., 2021).

O conceito de alimentos funcionais surgiu na década de 80 no Japão, por uma iniciativa governamental que pretendia por meio da alimentação saudável cuidar da sua população idosa (STRINGHETA et al., 2007). No Brasil a regulamentação deste conceito ocorreu somente depois de quase duas décadas com a publicação da Portaria 18/99 do Ministério da Saúde que definiu que alimentos funcionais são aqueles alimentos ou ingredientes que além das funções nutritivas básicas, quando consumidos como parte de uma dieta usual, produzem efeitos metabólicos e/ou fisiológicos, e/ou efeitos benéficos à saúde, devendo ser seguros para consumo sem supervisão médica (BRASIL, 1999).

Os efeitos dos alimentos funcionais se devem a seus compostos bioativos, que devem estar presentes em concentrações adequadas para efetuar o efeito desejado, sejam

eles vitaminas, minerais essenciais, proteínas, peptídeos isolados, ácidos graxos e ou fibras alimentares, dentre outros (PACHECO; SGARBIERI, 1999; VIDAL et al., 2012).

Estudos utilizando *Mucuna pruriens*, um tipo de feijão formando um suplemento natural, demonstrou que a ingesta auxilia na recaptação de dopamina melhorando quadros de depressão e já é utilizado como tratamento alternativo para Parkinson na Índia. Quando utilizado em ratos que fazem uso do álcool, promove melhora na reprodução de ratos machos reduzindo os danos reprodutivos (CHOOWONG-IN et al., 2021; TANGSRISAKDA et al, 2022). A capsaicina, derivada da pimenta, reduz o consumo de etanol em ratos C57BL/6 (HUH et al., 2022) e existem cogumelos que auxiliam no tratamento dos sintomas de abstinência do álcool prevenindo danos gastrointestinais (HOU et al., 2021).

O uso de diversos alimentos funcionais como os derivados de metabólicos fenólicos tem sido experimentado para reduzir os danos ou prevenir doenças cardiovasculares de diversas origens. Uma vez que os danos os danos vasculares causados pelo etanol são relacionados a vias inflamatórias e de aumento de estresse oxidativo, o uso de agentes antioxidantes e anti-inflamatórios advindos da dieta tem grande potencial terapêutico. Antioxidantes derivados de fontes vegetais como as vitaminas C e E, os carotenóides e os compostos fenólicos, especialmente os flavonoides (SILVA et al., 2010) inibem a cadeia de iniciação ou interrompem a cadeia de propagação das reações oxidativas (PODSEDEK, 2007). Além da ingestão de frutas e vegetais, que são recomendados como fontes de compostos antioxidantes, a suplementação dietética, contendo compostos antioxidantes, de origem vegetal ou animal, também podem ser úteis, como o uso do selênio para a melhora do sistema imunológico, homeostase antioxidante, ou liberação de mediador pró-inflamatório, em aves de consumo que são tratadas com selênio, segundo estudo de Michalczuk et al. (2021).

No entanto, há poucos estudos que utilizam a proteína animal derivada do ovo para o combate aos efeitos deletérios, sobre o sistema cardiovascular e outros sistemas, produzidos pelo consumo do etanol. Yan et al., (2021) utilizando um hidrolisado de feito a partir de pepsina frações de 0, 0.5, 1.5, 2.0, 2.5, 3.0, 3.5, e 4.0 h da proteína do ovo, utilizando a clara do ovo demonstrou hepatoproteção e ação antioxidante em ratos Wistar. A hepatoprotecção deve-se principalmente ao aumento das capacidades antioxidantes do fígado, baixando as frações de lípidio no fígado e a liberação de citocinas inflamatórias, bem como, a regulação do metabolismo lipídico, este mesmo tratamento foi utilizado em ratos machos para verificar o papel protetor do fígado por Lin et al. (2021).

3.3. O ovo

Dentre os mais variados tipos de alimentos com potencial para uso funcional temos o ovo da 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). Especificamente 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 proteína da clara do ovo faz com que ela seja uma rica fonte de valor biológico e seja considerada fonte de proteínas de referência para validar outras proteínas alimentares (INSTITUTO DE ESTUDIOS DEL HUEVO, 2009; YU et al., 2011; GARCÉS-RIMÓN et al., 2016b; LIAO et al., 2018).

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 & PIHLANTO-LEPPALA, 2002; SAMARANAYAKA & 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-RIMÓN et al., 2016), essa liberação ocorre através de hidrólise *in vitro* ou *in vivo* (LIAO et al., 2018).

3.4 Peptídeos bioativos derivados da clara do ovo

Desde 2006 por meio da hidrólise com pepsina por 8 horas da clara de ovo pausterizada foi desenvolvido um hidrolisado da clara do ovo, pelo Instituto de Investigación en Ciencias de La Alimentación - Consejo Superior de Investigaciones Científicas da Universidad Autónoma de Madrid (CIAL/SCIC), com o qual possuímos parceria, onde foi observada, *in vitro*, ação anti-inflamatória e antioxidante (MIGUEL et al., 2006), assim como, *in vivo,* foram observados efeitos benéficos em disfunções cardiovasculares e metabólicas (MIGUEL et al., 2006; GARCÉS-RIMÓN et al., 2016; 2018; MORENO-FERNANDEZ et al., 2018). Estes resultados com este hidrolisado da clara do ovo (HCO) feito via ação enzimática da pepsina por 8h mostrou ser capaz de reduzir a massa gorda corporal, aumentar a massa magra e acelerar a β-oxidação hepática

melhorando assim a síndrome metabólica e obesidade em modelos animais (GARCÉS-RIMÓN et al., 2016; 2018; MORENO-FERNANDEZ et al., 2018).

As sequências peptídicas responsáveis por esses efeitos já foram identificadas por Miguel et al. (2004) quando realizado hidrólise enzimática por 3 h, são elas: FRADHPFL, RADHPFL, YAEERYPIL, YRGGLEPINF, ESIINF, RDILNQ, IVF, YQIGL, SALAM e FSL. O hidrolisado de 8 hs contendo também essas sequências peptídicas mostrou atividade de modulação da microbiota intestinal de ratos Zucker (RAQUERA et al*.,* 2017). 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).

Em modelos experimentais de exposição a metais como mercúrio, alumínio e cádmio, o HCO demonstrou capacidade de prevenir os danos na memória e cognição (MARTINEZ et al., 2019a; RIZZETTI et al., 2016), no sistema reprodutor masculino (RIZZETTI et al., 2017; MARTINEZ et al., 2017; PINHEIRO et al., 2020), no sistema nervoso periférico (MARTINEZ et al., 2018), no tecido adiposo (RIZZETTI et al., 2019) e no sistema cardiovascular (MARTINEZ et al., 2019b; RIZZETTI et al., 2018).

Em animais espontaneamente hipertensos (SHR), peptídeos isolados do HCO utilizados aguda e cronicamente reduziram a pressão arterial (MIGUEL et al., 2007; MANSO et al., 2008). Este efeito foi atribuído em parte a proteção vascular por aumento

da síntese de óxido nítrico e diminuição da enzima conversora de angiotensina (ECA), evidenciadas em vasos de resistência em modelo agudo de exposição a sequências peptídicas extraídas do HCO (GARCÍA-REDONDO et al., 2010) devendo ser incentivadas como ingredientes funcionais na alimentação tanto para prevenção como para tratamento da hipertensão arterial.

Com base nas informações acima, hipotetizamos que o consumo de etanol está relacionado com dano cardiovascular, hipertensão e a geração de espécies reativas e a indução da ativação de vias de sinalização sensíveis a redox na vasculatura e que o HCO com reconhecido poder antioxidante e anti-inflamatório poderia ser uma importante estratégia terapêutica de proteção ao sistema cardiovascular contra os danos causados pelo uso do etanol.

4. JUSTIFICATIVA

O consumo excessivo de etanol é uma questão de saúde pública e apontado como causador de diversos danos ao organismo humano, bem como, um importante fator de risco para o desenvolvimento de doenças cardiovasculares (KUZKAYA et al., 2003). A ingestão crônica de etanol está associada à hipertensão (TIRAPELLI et al., 2008; PASSAGLIA et al., 2015) e a disfunção vascular relacionada ao aumento na geração de espécies reativas (CERON et al., 2014). A importância deste estudo reside em investigar uma alternativa terapêutica, não-farmacológica, o alimento funcional Hidrolisado de Clara de Ovo, para prevenção e / ou tratamento dos danos cardiovasculares causados pelo consumo de álcool em ratos, uma vez que há alta incidência de indivíduos em situação de dependência alcoólica e as alternativas farmacológicas vigentes ainda apresentam importantes diversos efeitos adversos.

5. OBJETIVOS

5.1. Objetivo geral

Avaliar os efeitos do tratamento com HCO sobre os danos cardiovasculares promovidos pelo consumo de etanol, em níveis similares ao consumo de um humano dependente.

5.2. Objetivo específico

Investigar os possíveis efeitos protetores do HCO sobre:

- A pressão arterial;
- As alterações na reatividade vascular de artérias de resistência promovidas pelo etanol e as possíveis vias envolvidas nesta resposta;
- As alterações de parâmetros de estresse oxidativo e alterações bioquímicas induzidos pelo uso de etanol;

PARTE II

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QUALIS CBII: A2

Role of Egg White Hydrolyzate Against Vascular Damage Promoted by Chronic Ethanol Consumption

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Highlights

- Egg white hydrolysate improves vasorelaxant response in vessels exposed to ethanol.

- The vascular effect was attributed to the antioxidant effect of EWH.

- EWH vascular improvement is mediated by reduction of NOX-1 and COX-2 activation.

- EWH did not protect oxidative stress promoted by ethanol in target organs.

Abstract

Ethanol is the licit drug consumed worldwide and affects the cardiovascular system. Enzymatic hydrolysis of egg white with pepsin for 8 hours produces peptides with biological functions with beneficial effects in cardiovascular disorders. We investigated whether egg white hydrolyzate (EWH) acts on cardiovascular damage caused by ethanol in rats. Male Wistar rats (CEUA 27/2021) treated for 42 days: I) Control (Ct) - drinking water ad libidum and via gavage; II) EWH - drinking water and EWH 1 g/kg/day via gavage; III) Ethanol (Et) - 20% ethanol in water ad libidum and H2O by gavage) and IV) EtEWH - both treatments. The animals were weighed weekly, ethanol content, fluid and feed consumption, systolic blood pressure (SBP) by caudal plethysmography; vascular reactivity of the mesenteric arteries by tension myography, immunofluorescence for NOX-1 and COX-2 and measurement of lipid peroxidation, antioxidant capacity in plasma, kidney and liver and ARM reactive species and other organs. Results are expressed as mean ± SEM, compared by 2v ANOVA with significance of $p < 0.05$. Ethanol consumption reduced feed and liquid consumption and EWH treatment did not change this parameter. The ethanol-treated groups reduced body weight (in g: Control: 470 ± 11.7; Et: 399.1 ± 76*; EWH: 448.9 ± 14.7; EtEWH: 407 ± 12.8* -* vs Control). SBP remained unchanged in all groups. There was an increase in the vasoconstrictor response to noradrenaline in the ethanol groups and worsening of relaxation in ACh in the Et group and improvement in the presence of co-treatment with EWH, accompanied by greater fluorescence in NOX-1 without reversion with the use of EWH and in attenuated COX-2 with use of EWH in these arteries. The increase in reactive species (ROS) in plasma and MRA was reduced by EWH. Ethanol promotes vascular changes that are attenuated by EWH that seem to be related to the production of ROS derived from NADPHoxidase and the bioavailability of NO in the vessel.

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

Abbreviations:

ACh – acetylcholine; MRA- Resistance mesenteric artery; COX-2 – Cyclooxygenase enzyme isoform 2; EWH – egg white hydrolysate; INDO – Indomethacin; FRAP- Ferric Reducing Antioxidant; KCl – Potassium chloride; MDA – Malondialdehyde; NADPH oxidase – Enzyme nicotinamide adenine dinucleotide phosphate oxidase; NaOH – Sodium hydroxide; NO – Nitric Oxide; NOS – Nitric oxide synthase; NOX-1; NE – Norepinephrine; ROS – Reactive oxygen species; SBP – Systolic blood pressure; SNP – Sodium

1 Introduction

Alcohol consumption has followed the evolution of people (McGovern et al., 2004). In contemporary society, the use of alcohol is recreational, and its consumption is growing (Baltieri & Cortez, 2005; Kachani et al., 2008). In countries with a high sociodemographic index, alcohol consumption is higher. However, ¼ of the world's alcohol consumption is not officially registered, and there are records of an increase in alcohol consumption in countries with low to medium economic levels (GBD, 2016; WHO, 2018; Massip & Luch, 2021).

The relationship between alcohol consumption and its effect on the cardiovascular system is binary and controversial. The harmful impact of alcohol consumption in high doses on the cardiovascular system is known (Hoek et al., 2022). However, for decades, it was still pointed out that moderate consumption leads to benefits to the cardiovascular system, such prevention of myocardial infarction, heart failure, and diabetes (Wood et al., 2018; Van de Luitgaarden et al., 2021). Recent studies point out that alcohol consumption can increase the risk of several cancers (mainly of the gastrointestinal tract and breasts), atrial fibrillation, and hypertension (Stătescu et al., 2021).

Chronic ethanol consumption leads to hypertension, and this process is a multifactorial event involving increased sympathetic activity, stimulation of the renin-angiotensin-aldosterone system with consequent increased vascular oxidative stress and endothelial dysfunction (Tirapelli et al., 2008; Marchi et al., 2014; Ceron et al., 2014; North et al., 2018).

Oxidative stress is a central mechanism in the development of changes promoted by ethanol, and the search for antioxidant and anti-inflammatory substances as non-

pharmacological therapeutic alternatives to counteract these cardiovascular effects is crucial (Phillips et al., 2020).

Several natural products with bioactive proprieties were investigated against the toxic effects of alcohol consumption on target organs such as the liver, kidney, and microbiota (Lee et al., 2020; Jedidi et al., 2022; Guo et al., 2022). Mostly, they had antioxidant and anti-inflammatory activity as potential benefits in common. Since the vascular effects of ethanol consumption are related to oxidative damage, we propose to investigate the use of an egg white hydrolysate obtained by hydrolysis with pepsin for 8 hours which has anti-inflammatory and antioxidant properties in an ethanol exposure model (Miguel et al., 2006 a, b; Garcés-Rimón et al., 2016; 2018; Moreno-Fernandez et al., 2018).

We hypothesized that the consumption of ethanol that causes vascular damage mediated by the generation of reactive species can be mitigated or prevented by using a functional food, an EWH, with recognized antioxidant and anti-inflammatory action.

2 Materials and Methods

2.1 EWH preparation

Pasteurized chicken egg white was treated with pepsin enzyme to produce egg white hydrolyzate (EWH). The liquid egg white was acidified with HCl and hydrolyzed with pepsin (E.C. 3.4.23.1 BC Pepsin - Biocatalysts - Cardiff, UK) for 8 h at 37 degrees. After this pepsin was inactivated with (NaOH) until reaching pH 7.0, the contents were centrifuged, and the supernatant lyophilized for use according to the protocol of Miguel et al. (2006).

2.2 *Animals*

Two-month-old male Wistar rats (260 ± 4.2 g) were obtained from the Central Animal Laboratory of the Federal University of Pelotas, 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 liquid 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: 27/2021).

Rats were divided into four groups (8 animals per group) and treated for 42 days with: a) Control- drinking water *ad libidum* and by gavage; b) EWH (drinking water and EWH – 1 g/ kg/day by gavage according to Miguel et al. (2006); c) Et - received ethanol 20% in drinking water and H_2O by gavage according to Tirapelli et al. (2006) and d) EtEWH – received both treatments. Animals of ethanol groups previously underwent 21 days of adaptation. The management of the animals was carried out following the appropriate safety measures and the general state of health, body weight, food and water intake were recorded once a week.

2.3 Cardiovascular Measurements

Indirect systolic blood pressure (SBP) was measured weekly from the adaptation period (21 days) to the end of treatment (42 days), measured by caudal plethysmography according to Buñag et al. (1977) (AD Instruments Pty Ltd, Bella Vista, NSW, Australia). At the end of treatment, the animals were anesthetized (ketamine and xylazine – 87 mg/kg and 13 mg/kg, respectively, i.p.) and euthanized for vascular reactivity experiments, blood collection and organs (liver and kidney) for analysis. Next, the mesenteric artery was carefully dissected and cleaned of adipose and connective tissue, divided into 2 mm long segments, and placed in 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), carbonated with 95% O_2 and 5% CO_2 (pH 7.4). The remaining MRA branches were

refrigerated and kept at -80 °C for further biochemicals assays. The MRA segments were mounted on a wire myograph (Multi Wire Myograph System, DMT620, ADInstruments do Brasil, São Paulo, SP, Brazil) to measure isometric tension, according to Wiggers et al. (2008). After an equilibration period of 30 minutes, the MRA was exposed to 120 mM KCl to verify the functional integrity of the vessel. Concentration-response curves to norepinephrine (NE, 0.1 nM - 3.5 mM) were performed after 30 min of a new stabilization period in rings with or without endothelium, and in the presence of Indomethacin (1 μM), a non-selective COX inhibitor. To investigate endothelium-dependent and independent relaxation, concentration-response curves to acetylcholine (ACh, 0.1 nM–3.5 mM) and sodium nitroprusside (SNP, 0.1 nM–3.5 mM) were performed in segments pre-contracted with NE at a concentration that produces approximately 50% of the contraction induced by K^+-KHS.

2.4 Biochemical Analyses

Biochemical studies of oxidative stress biomarkers were performed in plasma, MRA, liver and kidney. For that, tissues were homogenized in 50 mM TrisHCl, pH 7.4, centrifuged at 2500g 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) and the ROS 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 nmol MDA/g tissue.

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 mM Trolox equivalents.

2.5 Immunofluorescence for detection of NOX-1 and COX-2

Arterial segments were prepared and analysed according to Jimenez-Altayó (2005). The primary antibodies were against NOX1 (1:500) and COX-2 (1:500). The secondary antibody (Alexa 488-conjugated goat anti-mouse immunoglobulin G [IgG]) was diluted 1:400. We used DAPI (1:10,000) to stain nuclei. In the preparation of negative control sections, we omitted the primary antibody. Images were acquired using an EVOS® Floid® Cell Imaging Station (Life Technologies, Carlsbad, CA). For quantification, we analysed sections from five different animals per group with the same capture parameters. We performed the readings and calculated the mean fluorescence densities (histogram) using ImageJ. Data are expressed relative to the Control group.

2.6 Statistical analysis

Data are expressed as mean ± SEM. In the vascular reactivity experiments, vasoconstrictor responses of MRA were expressed as a percentage of the contraction induced by 120 mM of KCl. Results were analyzed using two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used. Values of $p < 0.05$ were considered significant.

3. Results

Ethanol consumption significantly reduced body weight from the 28th day of the exposure until the end of treatment (42nd day). Cotreatment with EWH did not interfere with ethanol-promoted weight loss (Fig. 1A). Likewise, the intake of feed and liquids (water or ethanol) decreased in both groups that received ethanol (Fig. 1B and C). The relative weight of target organs, such as the liver and kidney, was not altered by ethanol exposure or EWH cotreatment (Fig. 1D and E).

SBP was not affected by ethanol exposure in the adaptation period (21 days) or the treatment period (42 days), and EWH cotreatment did not affect this parameter either (Fig. 2). However, vascular reactivity was altered by ethanol. As shown in Figure 3, ethanol exposure increased vasoconstrictor responses to NE in the ARM, and EWH cotreatment did not reverse this effect (Fig. 3A). However, vascular response to ACh is significantly reduced in ethanol rat vessels, and EWH cotreatment completely recovered the endothelial dysfunction produced by ethanol (Fig. 3B). Endothelium-independent vascular relaxation was not affected by any treatment (data not shown). It is important to describe that the KCl response in the MRA of all groups did not differ (Control: 3.3 ± 0.2; EHW: 3.3 ± 0.2; Et: 3.3 ± 0.2; EtEHW: 3.4 ± 0.3 mN/mm) and MRA internal diameters were similar between groups (Control: 293.4 ± 5.7, EHW: 300.4 ± 5.4; Et: 304.7 ± 8.5; EtEHW: 291.9 ± 4.0 mN/mm).

We investigate the effect of endothelium removal on the vasoconstrictor response to NE in MRA segments. Endothelial modulation was impaired after Ethanol exposure, and EWH cotreatment could not reverse this response (Fig. 4 A-D). To verify the participation of prostanoids in the increase of the NE response in rats treated with Et and the possible role of EWH in this pathway, we used the non-selective COX inhibitor indomethacin (INDO). Indomethacin reduced the vasoconstrictor response to NE in MRA in the segments of both groups treated with Et, and this reduction was not avoided by cotreatment with EWH (Fig. 4 E-H).

Considering the previously described antioxidant property of this EWH/pepsin-8h, we also investigated the NOX-1 isoform by immunofluorescence. We observed a significant increase in ethanol-promoted NOX-1 fluorescence, and EWH reduced NOX-1 expression, indicating an antioxidant effect of EWH on vessels (Fig. 5A). Although the vascular function of EWH did not reverse the effect of ethanol, interestingly, the increased

COX-2 fluorescence in the arteries of ethanol rats was attenuated in the presence of EWH (Fig. 5B).

Corroborating these data, biochemical analyses showed elevated lipid peroxidation and ROS levels in MRA, plasma, liver, and kidney in rats exposed to ethanol (Fig. 6 A-F). In plasma and MRA, cotreatment with EWH reversed the oxidative stress promoted by ethanol (Fig. 6 A, B, G). However, in the liver and kidney, the target organs of ethanol effect increased lipid peroxidation, and ROS levels were not altered by EWH cotreatment (Fig. 6 E, F). In addition, the antioxidant status was different in the analyzed tissues; in plasma, there was a reduction in the antioxidant capacity in both groups exposed to ethanol; in the liver, there was an increase in the antioxidant capacity in both ethanol groups, and in the kidney, this parameter was not changed (Fig. 6 H, I, J).

These results demonstrate that ethanol exposure leads to increased vascular reactivity and reduced relaxation is mediated by increased oxidative stress and activation of the induced COX pathway, and vascular changes occur even before any blood pressure changes. Furthermore, EWH acts locally on MRA segments, reducing oxidative stress, inhibiting COX-2, and improving vasorelaxation.

4. Discussion

The present study provides evidence that EWH consumption partially prevents vascular dysfunction promoted by ethanol exposure for six weeks in resistance vessels through endothelium-dependent mechanisms mediated by the reduction of oxidative stress and the inflammatory pathway. Furthermore, these vascular changes are independent of changes in blood pressure.

Chronic ethanol consumption is related to the development of hypertension in humans (Fuchs & Fuchs, 2021, Coelho et al., 2021). In experimental models, different pressure behaviors are time and dose dependent. Tirapelli et al. (2007, 2017), using the

same time and dose of ethanol used in this study, found a significant increase in SBP that reached hypertension levels. However, as our results found, this same exposure model previously showed no change in SBP in Wistar rats (Baptista et al., 2014). Exposure to ethanol for more extended periods of 18 weeks reduced SBP (McCarron et al., 1992) or did not change this parameter at lower doses (Yuui et al., 2019). It is already known that EWH despite important in vitro ACE inhibitor and antioxidant activities (Garcés-Rimón et al., 2016) and in different models of hypertension, without affecting blood pressure in normotensive animals (Miguel et al., 2005, 2007), this fact could explain that in the present study, treatment with EWH did not modify blood pressure values.

Changes in the vasculature may precede pressure modifications (Phillips et al., 2019, Stătescu et al., 2021) and, in fact, our results point to vascular dysfunction observed in increased contractile response and reduced relaxation in the MRA, without changes in blood pressure. It is known that endothelial dysfunction is an early stage of vascular diseases and is an important prognostic indicator of cardiovascular and metabolic diseases such as hypertension, atherosclerosis and others (Huynh & Heo, 2019; Stătescu et al., 2021).

Several studies with ethanol exposition shows an increase in vascular reactivity in different vascular bed (aorta, MRA, cerebral artery) (Tirapelli et al., 2008; Yogi et al., 2012; North et al., 2018). Some antioxidants treatments, apocynin for example, was efficient to improve the enhanced vascular response to Phe in aorta (Marchi et al., 2016). The EWH used in this study showed great ability to reduce the increase in vascular contraction in arteries of conductance and resistance promoted by exposure to metals in high and low doses (Martinez et al., 2017). However, in this experimental model, this ability of this EWH was only observed in the reduction of vascular dysfunction induced in rats treated with ethanol. Yuui et al. (2019) using rats treated with 10% ethanol and

specific diet, Lieber di Carli, had a significant increase the acetylcholine response. Rizzetti et al. (2017) also most this increased reactivity when the animal was exposed to Hg and EWH was able to reverse this condition.

The vascular dysfunction generated by ethanol is related to its great oxidative and inflammatory capacity (Marchi et al., 2016). In fact, ethanol activated the COX pathway in vessels as shown in functional data and in COX-2 expression and EWH surprisingly did not alter vascular function but reduced COX-2 expression in situ. Activation of the ethanol-induced vascular inflammatory pathway is known in a rat model treated for two weeks or more in both aorta and mesentery on ethanol exposure (Tirapelli et al, 2008; Rocha et al, 2012; Ceron et al, 2014; North et al, 2019). Studies using antioxidants agents as apocynin in ethanol exposure model showed improvement in aortic vascular reactivity (Marchi et al., 2016). On exposure to different metals at the vascular level, this EWH had already demonstrated an effective ability to inhibit COX, especially COX-2 at low and high doses of Al and Cd exposure in the aorta and MRA (Martinez et al., 2019, Moraes et al., 2022).

However, the most crucial point of the effect of both ethanol on vessels and EWH protection lies in its effect on the redox balance process. Clearly ethanol has a vascular effect in stimulating both locally and systemically the oxidant system, as shown by the increased expression of NOX-1 and levels of reactive species in plasma and MRA. This effect had already been demonstrated by Marchi et al. (2016) with ethanol exposure in this same model, and as well as other systems such as liver (Yang et al., 2022). However, the antioxidant power of EWH was demonstrated over the effect of ethanol in reducing ROS levels in plasma and MRA and reducing NOX-1 expression in EWH co-treated arteries. Previous studies, with heavy metals, show improved NOX-1 expression when animals were exposed to aluminum and co-treated with EWH (Martinez et al., 2019).

The same effect could not be observed in the target organs, which maintained an increase in EO at the expense of cotreatment with EWH. However, a similar effect occurred in studies by Martinez et al. (2022), where EWH did not reverse the damage caused in the kidney with high doses of aluminum, nor did it reverse the lipid peroxidation process completely. In our study, this effect is possibly due to the damage caused by ethanol being greater, since the target organs studied are pathways and our route of administration was the same, so there may be a competition for receptors in organs such as liver and kidney.

The ethanol consumption in vivo models depends on the dose, frequency and duration of treatment (Abdel-Rahmanet et al., 1985; Abdel-Rahman & Wooles, 1987) and this factor may or may not alter the response in blood pressure and vascular response and that these effects such as redox imbalance are suggested as an early mechanism that is initiated by the use of ethanol, as observed in cardiac muscle and vessels (Piano et al., 2004; Dikalova et al., 2005; Tirapelli, 2006; Tan et al., 2012, Zhang et al., 2013, Silva et al., 2020).

In this work it was possible to analyze that even though there was no increase in blood pressure, our results already show the beginning of endothelial dysfunction with altered inflammatory response factors and oxidative stress level, evidencing that ethanol is an agent that uses these means to make important cardiovascular alterations. The EWH as already demonstrated in studies with metals (Martinez et al., 2019; Rizzetti et al., 2018), models of metabolic syndrome (Garcés-Rimón et al., 2016; 2018; Moreno-Fernandez et al., 2018) and hypertensive animals is shown to be effective when acting as co-treatment in endothelial dysfunction caused by inflammatory and redox system processes, promoting an improvement in the alterations of the cardiovascular system related to this process.

The effect of ethanol weight loss after five and six weeks of exposure was previously reported (Tirapelli et al., 2006; do Vale et al., 2021) and the reduction in food and liquid consumption accompanies the weight loss of the animals as corroborated by our data. Although not the focus of this study, it is widely recognized that alcohol consumption affects hepatic triglyceride and cholesterol metabolism and is associated with disturbance of lipid transporters (Zhang et al., 2019). It is also known that ethanol after oxidation by the enzyme Alcohol Dehydrogenase (EDH) provides 7.1 kcal per gram of metabolized ethanol and is a source of calories without vitamins or minerals, so chronic consumption of ethanol leads to malnutrition and decreased food intake, thus impairing absorption and causing weight loss (Molina et al., 2003; Kachani, et al., 2008).

Although liver and kidney weight were not affected by ethanol consumption or EWH supplementation, the negative effect on the imbalance of oxidative stress promoted by ethanol in these target organs is clear. Ethanol in the liver acts by decreasing the number of hepatocytes which may or may not be associated with mitochondrial oxidation (Simon et al., 2022). In addition to the possibility of ethanol being metabolized to acetaldehyde in hepatocytes inducing injury. Furthermore, the result of conversion to NADH, this re-oxidation of NADH to NAD+ in mitochondria has been associated with electron leakage from the mitochondrial respiratory chain and subsequent ROS production and there is also inhibition of expression of antioxidant enzymes (e.g. superoxide dismutase 1) and depleted levels of non-enzymatic antioxidants (e.g. glutathione), thus reducing the cellular ability to modulate oxidative stress (Yang et al., 2022, Simon et al., 2022). Our findings show an increase in lipid peroxidation levels and ROS in target organs such as the kidney, which corroborates previous findings (Silva et al., 2021).

According to Monma et al. (2015), alcohol accumulation and metabolism produce acetaldehyde and free radicals as ROS that cause cell membrane damage, with significant long-term functional impairment, changes in mitochondria and endoplasmic reticulum, and cell degeneration. Excess ROS, mainly NAD(P)H oxidase, decreasing the bioavailability of NO and inducing vascular dysfunction in brain, was demonstrated by Haorah et al. (2011).

There improvement in ROS levels and lipid peroxidation in hepatic and renal tissue was observed in the rats exposed to aluminum (Martinez et al., 2022). Treatment with EWH at the same doses used in this study also did not reverse this improvement in oxidative stress in target organs. And we hypothesized that the same route of administration (oral) may have compromised the absorption or passages of the substances used, since the same route was used for induction and treatment, which may also have happened in this study.

5. Conclusion

Our results suggest that co-treatment with EWH in rats showed a beneficial effect in protecting against ethanol-induced vascular dysfunction mediated by reducing the increase in COX-2 and NOX-1, showing its action as an antioxidant and anti-inflammatory agent.

These findings highlight that EWH shows preventive action against vascular effects caused by ethanol exposure and can be considered a useful functional food strategy easily accessible to reduce the damage caused by ethanol ingestion. Further investigation of the involved mechanisms of vascular protection promoted by the EWH in this model is needed.

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

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Figures

Fig. 1

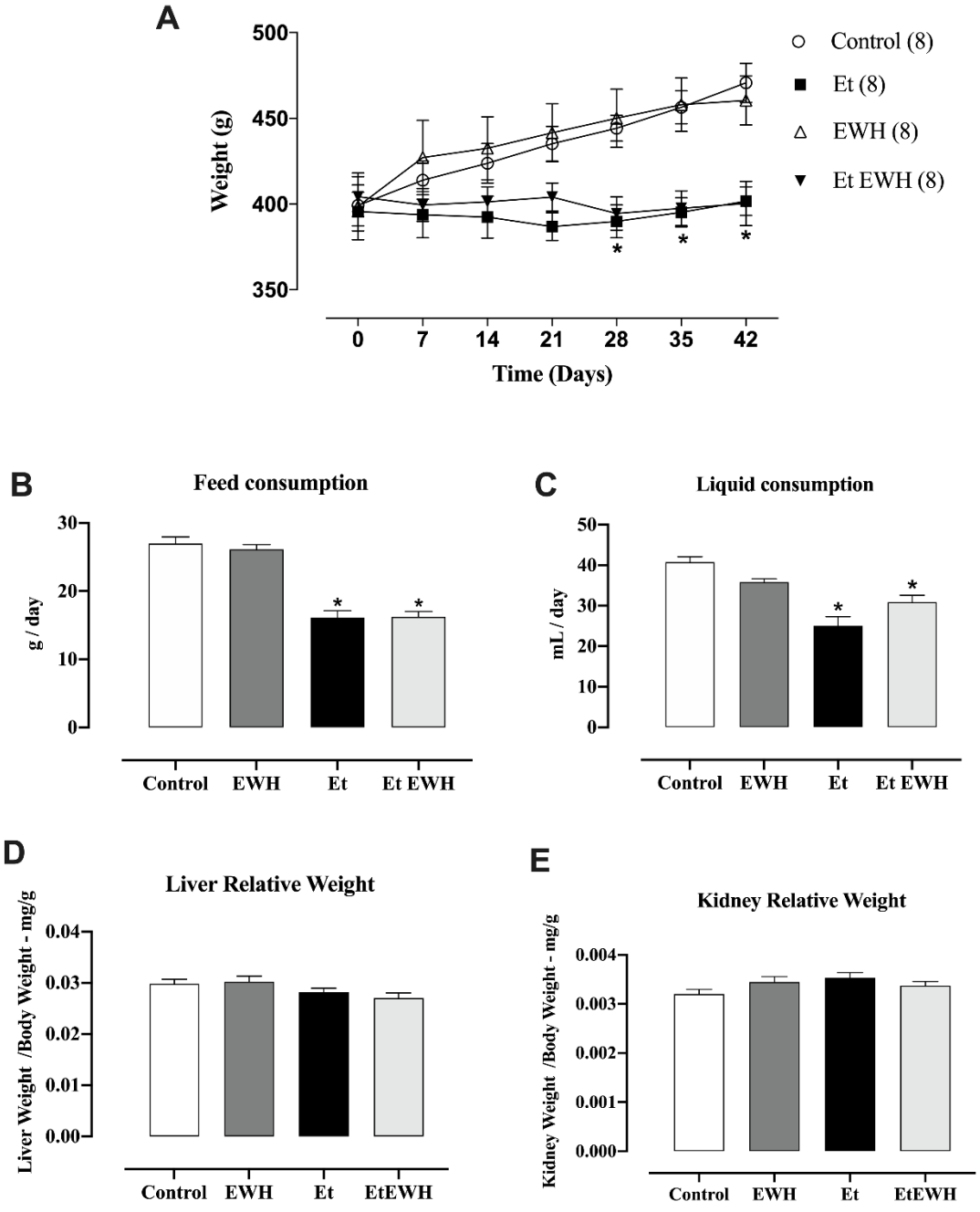


Fig. 2

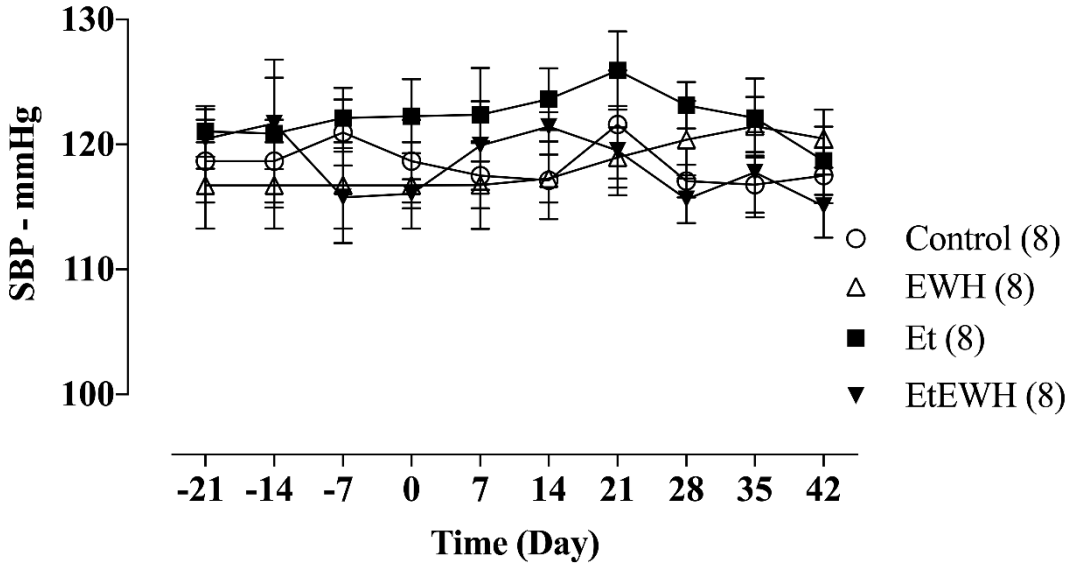

130
120
110
100
SBP - mmHg
Control (8)
EWH (8)
Et (8)
EtEWH (8)
-21 -14 -7 0 7 14 21 28 35 42
Time (Day)

Fig. 3

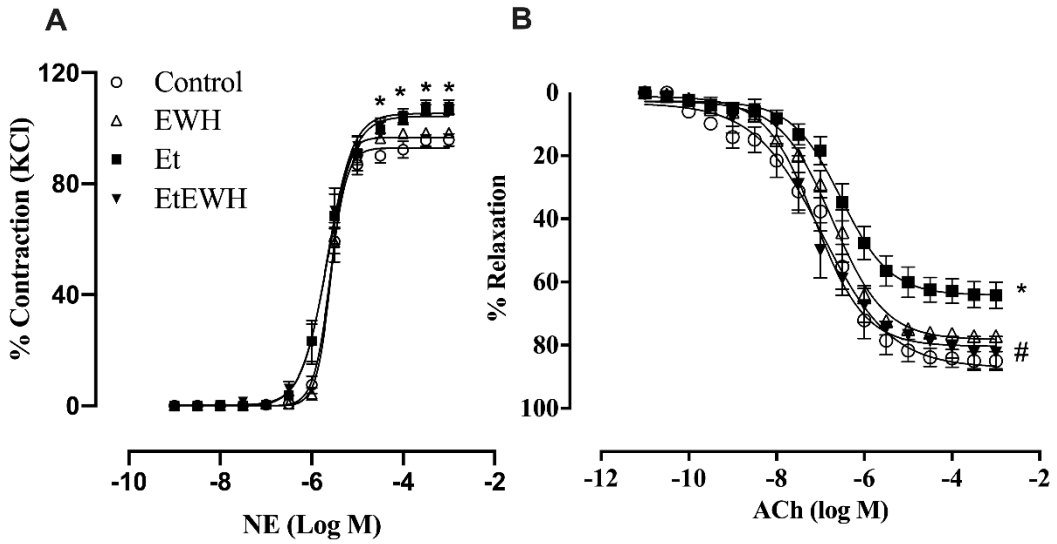
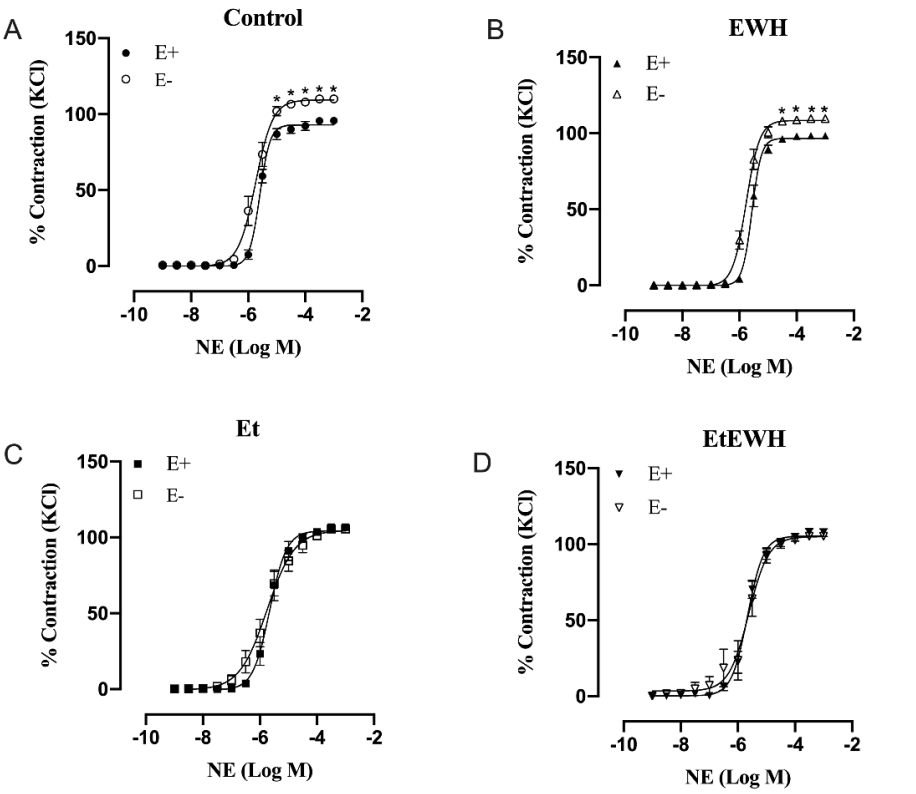

A
B
120
80
40
0
% Contraction (KCl)
Control
EWH
Et
EtEWH
* * * *
-10 -8 -6 -4 -2
NE (Log M)
0
20
40
60
80
100
% Relaxation
*
#
-12 -10 -8 -6 -4 -2
ACh (log M)

Fig. 4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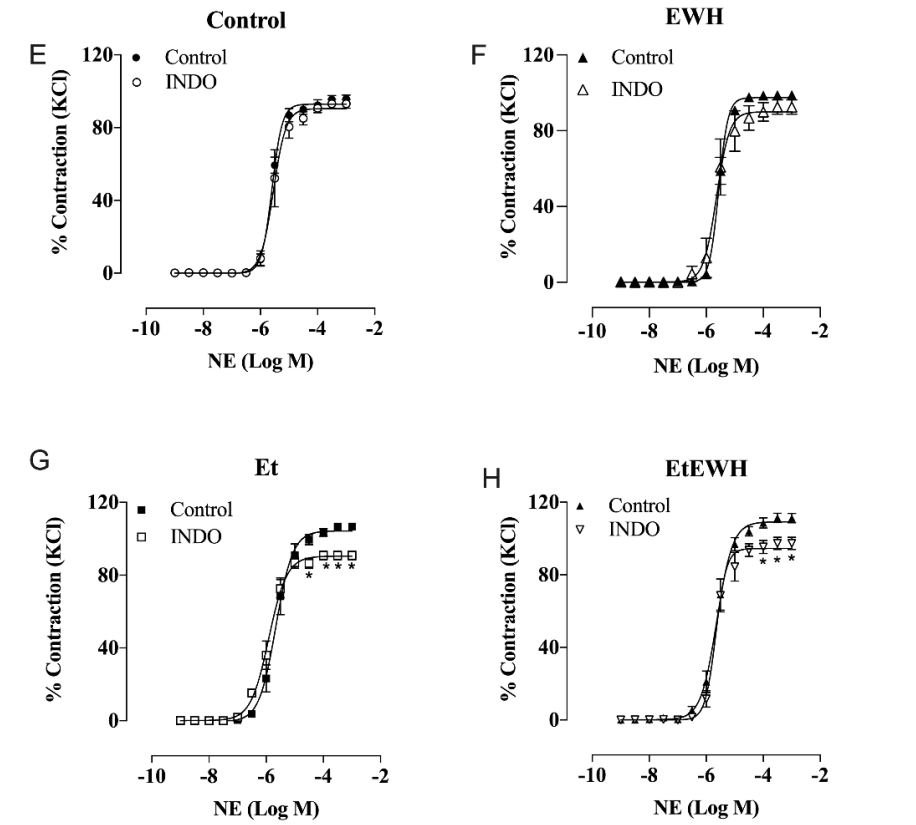
Control
E+
E-
% Contraction (KCl)
NE (Log M)
B
EWH
C
Et
D
EtEWH


E
Control
Control
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% Contraction (KCl)
NE (Log M)
F
EWH
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EtEWH

Fig. 5

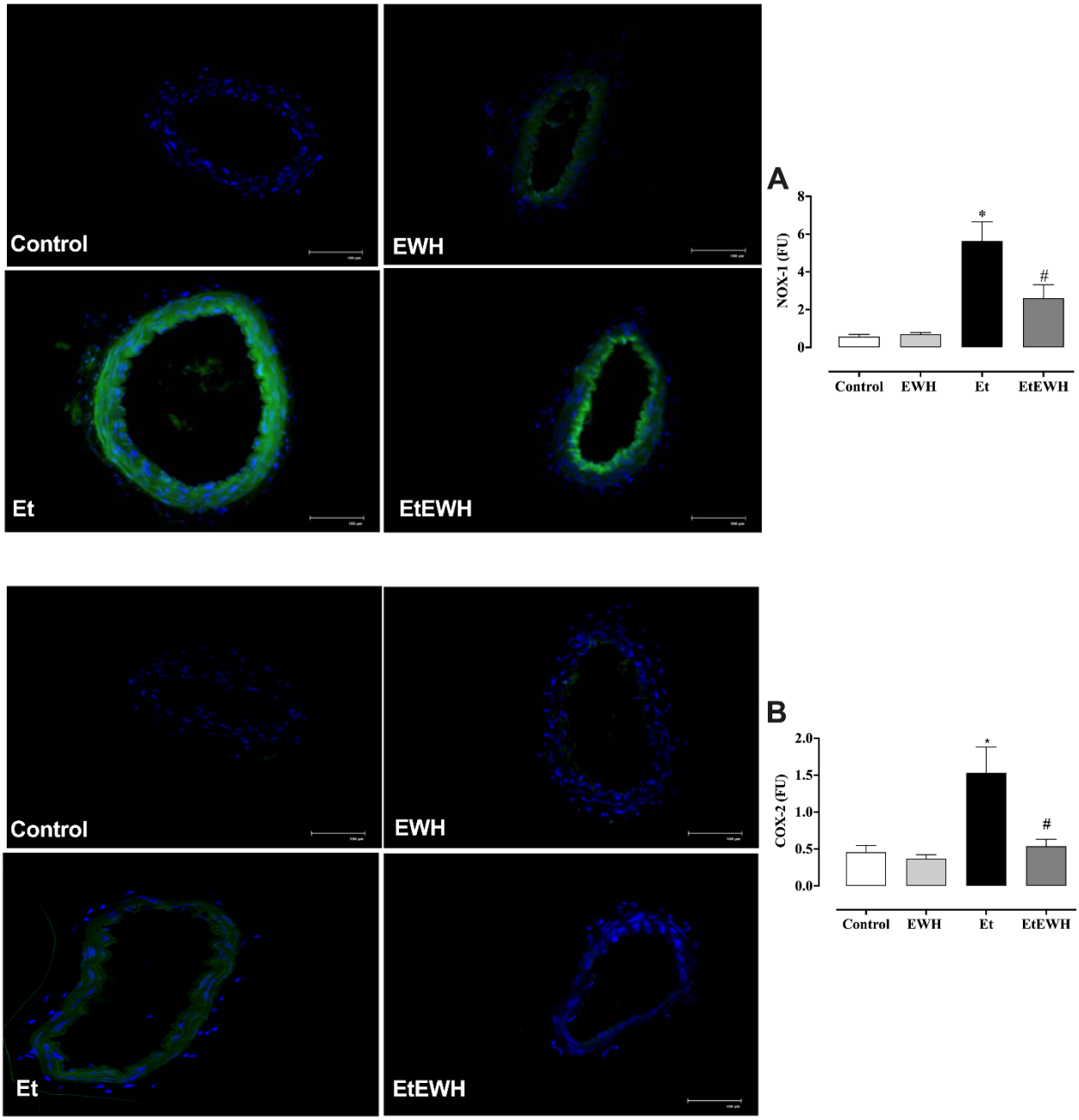
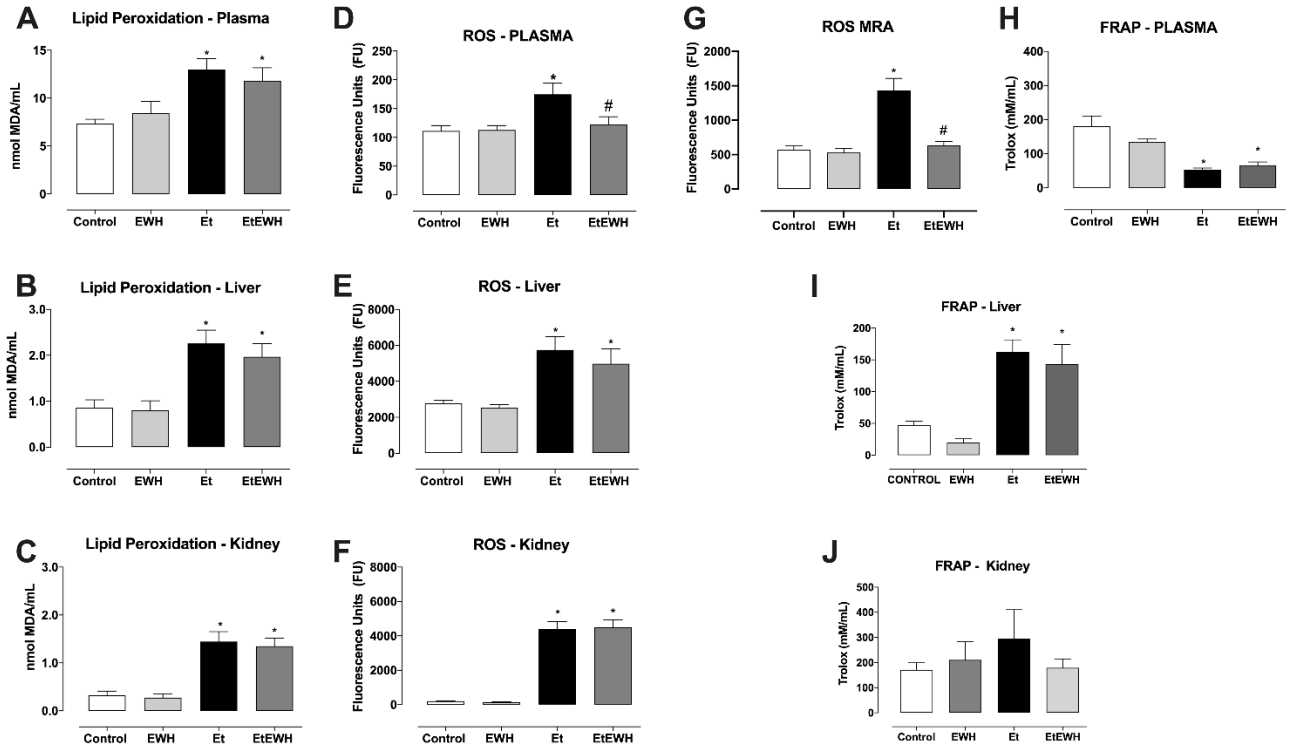

Control
EWH
Et
EtEWH
A
NOX-1 (FU)
0
2
4
6
8
*
#
Control
EWH
Et
EtEWH
Control
EWH
Et
EtEWH
B
COX-2 (FU)
0.0
0.5
1.0
1.5
2.0
*
#
Control
EWH
Et
EtEWH

Fig. 6


A
Lipid Peroxidation - Plasma
nmol MDA/mL
Control
EWH
Et
EtEWH
D
ROS - PLASMA
Fluorescence Units (FU)
G
ROS MRA
H
FRAP - PLASMA
Trolox (mM/mL)
B
Lipid Peroxidation - Liver
E
ROS - Liver
I
FRAP - Liver
CONTROL
C
Lipid Peroxidation - Kidney
F
ROS - Kidney
J
FRAP - Kidney

PARTE III

CONCLUSÕES

Os resultados sugerem, que o co-tratamento com hidrolisado de clara de ovo em ratos expostos a etanol por 42 dias:

- Embora o uso do HCO não tenha modificado a pressão arterial aumentada pela exposição a etanol, ele foi capaz de melhorar a função vascular;
- Apresentou efeito benéfico na proteção contra a disfunção vascular em artérias de resistência promovido pelo etanol.
- Reduziu o aumento de COX-2 e NOX-1 produzido pelo consumo de etanol mostrando sua a ação de agente antioxidante e anti-inflamatória neste modelo.

Esses achados evidenciam que o hidrolisado de clara de ovo apresenta ação preventiva contra os efeitos vaculares causados pela exposição ao etanol, podendo ser considerado uma estratégia alimentar funcional útil de fácil acesso para reduzir os danos causados pela ingestão de etanol.

É necessário aprofundar aind mais a investigação dos mecanismos envolvidos na roteção da vasculatura promovido pelo hidrolisado neste modelo.

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ANEXOS

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

CERTIDÃO

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 027/2021

Título: CONSUMO CRÔNICO DE ETANOL E EFEITOS CARDIOVASCULARES – USO DE HIDROLISADO DE CLARA DE OVO NA PREVENÇÃO DE DANOS

Data da aprovação: 20/08/2021

Período de vigência do projeto: 20/10/2022

Pesquisadores(a): Giulia Alessandra Wiggers Peçanha

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Finalidade	**( ) Ensino (X) Pesquisa**
Espécie / Linhagem / Raça	Ratos Wistar
N° de animais	48
Peso / Idade	200-300g/8 semanas
Sexo	Machos
Origem	Biotério da Universidade Federal de Pelotas e/ou do Biopampa

Assinado eletronicamente por **CATIA ALINE VEIVERBERG**, **PROFESSOR DO MAGISTERIO SUPERIOR**, em 25/08/2021, às 14:20, conforme horário oficial de Brasília, de acordo com as normativas legais aplicáveis.

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Referência: Processo n° 23100.014086/2021-68 SEI n° 0596521

ANEXO II - Normas da revista - *Food Chemistry*

FOOD CHEMISTRY

AUTHOR INFORMATION PACK

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ISSN: 0308-8146

DESCRIPTION

Food Chemistry has three open access companion journals: *Food Chemistry: X*, Food Chemistry: Molecular Sciences and Food Chemistry Advances.

The Aims and Scope of *Food Chemistry* are assessed and modified on an annual basis to reflect developments in the field. This means that research topics that have been deemed in scope previously may now fall outside of the scope of the journal as our scientific and technical understanding of the fields evolve and topics become less novel, original or relevant to *Food Chemistry*.

Food Chemistry publishes papers dealing with the advancement of the chemistry and biochemistry of foods or the analytical methods/approach used. All papers should focus on the novelty of the research carried out.

Research advancing the theory and practice of molecular sciences of foods or cure/prevention of human diseases will not be considered for inclusion in *Food Chemistry*.

Topics featured in *Food Chemistry* include:

- Chemistry relating to major and minor **components of food**, their nutritional, physiological, sensory, flavour and microbiological aspects;
- **Bioactive constituents** of foods, including antioxidants, phytochemicals, and botanicals. Data must accompany sufficient discussion to demonstrate their relevance to food and/or food chemistry;
- Chemical and biochemical composition and structure changes in molecules induced by processing,distribution and domestic conditions;

- **Effects of processing** on the composition, quality and safety of foods, other bio-based materials, by-products, and processing wastes;

–Chemistry of **food additives**, **contaminants**, and other agro-chemicals, together with their metabolism, toxicology and food fate.

Analytical papers related to the microbiological, sensory, nutritional, physiological, authenticity and origin aspects of food. Papers should be primarily concerned with new or novel methods (especially instrumental or rapid) provided adequate validation is described including sufficient data from real samples to demonstrate robustness. Papers dealing with significant improvements to existing methods, or data from application of existing methods to new foods, or commodities produced in unreported geographical areas, will also be considered.

For Analytical Papers, especially those dedicated to the development and validation of methods, authors are encouraged to follow internationally recognized guidelines, such as EURACHEM - for chemical compounds (https://www.eurachem.org/index.php/publications/guides/mv) or FDA - for microbiological data (https://www.fda.gov/downloads/ScienceResearch/FieldScience/ UCM298730.pdf) and proper statistical methods should be applied. Special attention should be given to linearity, selectivity, determination of LOD/LOQ, repeatability and reproducibility of the analysis. Authors should also pay attention to trueness and, when possible (quantitative methods), determine the uncertainty of measurement. Overall, real samples should be analyzed by the state-of-the-art and the newly developed method for validation purposes.

- Methods for the determination of both major and minor components of food especially nutrientsand non-nutrient bioactive compounds (with putative health benefits) will be considered.

- Results of method inter-comparison studies and development of food reference materials for usein the assay of food components;

- Methods concerned with the chemical forms in food, nutrient bioavailability and nutritional status;

- General authentication and origin [e.g. Country of Origin Labelling (COOL), Protected Designationof Origin (PDO), Protected Geographical Indication (PGI), Certificate of Specific Character (CSC)] determination of foods (both geographical and production including commodity substitution, and verification of organic, biological and ecological labelling) USING CHEMICAL MARKERS, providing sufficient data from authentic samples should be included to ensure that interpretations are meaningful.

Food Chemistry will not consider papers that focus on purely clinical or engineering aspects without any contribution to chemistry; pharmaceutical or non-food herbal remedies; traditional or folk medicines; or survey/surveillance data.

Papers on therapeutic application of food compounds/isolates for treatment, cure or prevention of human diseases will not be considered for inclusion in *Food Chemistry*.

AUDIENCE

Food technologists, scientists and chemists

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