UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

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ÁCIDO ELÁGICO E HESPERIDINA COMO POTENCIAIS TERAPÊUTICOS EM DESORDENS NEUROINFLAMATÓRIAS

Santa Maria, RS
2020

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração Clínica Médica Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária.**

Orientador: Cinthia Melazzo de Andrade

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Dornelles, Guilherme Lopes
ÁCIDO ELÁGICO E HESPERIDINA COMO POTENCIAIS TERAPÊUTICOS EM DESORDENS NEUROINFLAMATÓRIAS / Guilherme Lopes Dornelles.- 2020.
120 p.; 30 cm

Orientadora: Cinthia Melazzo de Andrade
Coorientadora: Cássia Bagolin da Silva
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2020

1. Polifenóis 2. Antioxidantes 3. Anti-inflamatório 4. Estreptozotocina 5. Lipopolissacarídeos I. Melazzo de Andrade, Cinthia II. Bagolin da Silva, Cássia III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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Aprovado em 06 de março de 2020:

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Santa Maria, RS
2020

AGRADECIMENTOS

Agradeço à minha orientadora e supervisora profª. Cinthia Melazzo de Andrade, pela oportunidade concedida, por acreditar em mim, pelo carinho, ensinamentos, ajuda, apoio, orientação e compreensão. Foi graças à confiança depositada que esse trabalhado se tornou possível;

Aos meus pais pela oportunidade de estudo, ensinamentos, amor incondicional e por me apoiarem em todos os momentos, principalmente nos mais difíceis. Graças a isso, nunca desisti dos meus objetivos;

À toda minha família, em especial à minha avó Sonia, que foi minha mãe, avó e professora, pois sempre buscou me dar conselhos e transmitir conhecimentos, contribuindo para meu crescimento pessoal e profissional.

À Maria, secretária do PPGMV, por toda ajuda, carinho, conversas e paciência para responder à todas as minhas dúvidas;

Aos meus amigos que me apoiaram e foram presentes nos momentos mais difíceis, em especial à Juliana, Cássia, Luana e Raqueli que sempre estiveram dispostas a me ensinar;

Aos meus amigos Camila e Bernardo, que não mediram esforços para me auxiliar durante o experimento e alegrar o ambiente de trabalho;

À Liriani por me incentivar a seguir em frente e me ajudar a manter o foco;

Ao Erin John por todo apoio, ajuda, carinho e dedicação durante boa parte do doutorado. Sempre esteve disposto a me auxiliar em diversos experimentos, não importando o dia e a hora, além de me lembrar constantemente de que eu era capaz;

À toda equipe do LacVet e Bio-Ox pelo apoio, ajuda e carinho, em especial à Andressa Bueno pelos ensinamentos e risadas;

Aos meus amigos da Unicruz, pelo apoio e compreensão durante a etapa final do doutorado;

À CAPES, por possibilitar a execução deste trabalho através da bolsa a mim concedida;

Ao Programa de Pós-Graduação em Medicina Veterinária da UFSM, por permitir a realização de mais uma etapa de minha vida profissional;

A todos, MUITO OBRIGADO!

RESUMO

ÁCIDO ELÁGICO E HESPERIDINA COMO POTENCIAIS TERAPÊUTICOS EM DESORDENS NEUROINFLAMATÓRIAS

AUTOR: Guilherme Lopes Dornelles
ORIENTADORA: Cinthia Melazzo de Andrade

Neuroinflamação pode impactar negativamente o processo de neurogênese em adultos mamíferos, além de ser considerada fator de risco para déficit cognitivo e demência. Dentre os modelos experimentais de neuroinflamação, destacam-se os modelos que utilizam aplicações intrapeirtoneais (IP) de lipopolissacarídeos (LPS) ou administração intracerebroventricular (ICV) de estreptozotocina (STZ). Diversas pesquisas têm sido realizadas para buscar alternativas terapêuticas para desordens neuroinflamatórias. Os flavonoides, como o ácido elágico (AE) e a hesperidina (HES), provenientes principalmente de frutas, apresentam diversos efeitos benéficos ao organismo, como propriedades antioxidantes e anti-inflamatórias, e reduzida toxicidade. Nesse contexto, esta pesquisa teve por objetivo avaliar o potencial terapêutico do AE ou HES em modelos experimentais de neuroinflamação. Este estudo foi dividido em dois experimentos. No primeiro (MANUSCRITO I) o objetivo do estudo foi avaliar o potencial do AE em modelo experimental de neuroinflamação induzida por múltiplas aplicações de LPS. Foram utilizados 32 ratos machos Wistar, distribuídos em 4 grupos (n=8): controles (CTRL+SAL) e controle tratado com AE (CTRL+AE), e os grupos dos animais que receberam LPS (LPS+ SAL) e (LPS+AE). Os grupos LPS receberam 8 injeções IP diárias de LPS em 8 dias consecutivos na dose de 250mg/kg de peso corporal, dissolvida em salina 0,9% enquanto os grupos CTRL e AE receberam apenas o veículo salina 0,9% no mesmo volume. Duas horas após as aplicações (IP), os ratos do grupo AE e LPS+AE foram tratados com AE na dose de 100mg/kg por via oral durante os 8 dias de tratamento. Os testes de campo aberto e reconhecimento de objetos foram realizados nos dias seis, sete e oito do período experimental. No segundo experimento (MANUSCRITO II) o objetivo do estudo foi avaliar os efeitos da HES e sua associação com a rivastigmina (RIV) na memória e parâmetros oxidativos em um modelo de Doença de Alzheimer (DA) esporádica induzido por injeção intracerebroventricular de estreptozotocina (ICV-STZ). Foram utilizados 64 ratos Wistar, distribuídos em oito grupos (n=8): controle (CTRL), RIV, HES, RIV+HES, STZ, STZ+RIV e STZ+HES, STZ+RIV+HES. Os ratos receberam injeção ICV-STZ (3 mg/kg) ou solução salina e foram tratados diariamente, a partir do quarto dia, com 100 mg/kg de HES via oral, durante 30 dias. Aos 21 dias, pós injeção ICV-STZ, iniciou-se o tratamento oral com 2 mg/kg de RIV que teve duração de 13 dias. Realizou-se teste comportamental pelo labirinto aquático de Morris 30 dias após a injeção ICV-STZ. Em ambos estudos, os antioxidantes utilizados (AE ou HES) demonstraram potencial para reverter os efeitos deletérios do LPS ou STZ, a partir da redução do dano oxidativo, com incremento do sistema antioxidante e redução das espécies reativas de oxigênio (ERO), e melhora no potencial cognitivo dos animais. Em adição, no MANUSCRITO I, o AE demonstrou potencial imunomodulador, a partir de redução da expressão de células da glia, bem como capacidade de prevenir o aumento na atividade da acetilcolinesterase (AChE) e fosforilação da Tau. Esses resultados demonstram o potencial terapêutico do AE e HES em desordens cognitivas secundárias à neuroinflamação, o que torna esses antioxidantes potenciais candidatos para o tratamento de doenças neurodegenerativas.

Palavras-chave: Polifenóis. Antioxidantes. Anti-inflamatório. Estreptozotocina. Lipopolissacarídeos.

ABSTRACT

ELAGIC ACID AND HESPERIDINE AS POTENTIALS THERAPEUTIC FOR NEUROINFLAMMATORY DISORDERS

AUTHOR: Guilherme Lopes Dornelles
ADVISER: Cinthia Melazzo de Andrade

Neuroinflammation can negatively impact the process of neurogenesis in adult mammals in addition to being considered a risk factor for cognitive impairment and dementia. Among the experimental models of neuroinflammation, we highlight the models that use intraperitoneal (IP) applications of lipopolysaccharides (LPS) or intracerebroventricular administration (ICV) of streptozotocin (STZ). Several types of research have been carried out to search for therapeutic alternatives for neuroinflammatory disorders. Flavonoids, such as ellagic acid (EA) and hesperidin (HES), found mainly in fruits, have several beneficial effects on the body, such as antioxidant and anti-inflammatory properties, as well as reduced toxicity. In this context, this research aimed to evaluate the therapeutic potential of EA or HES in experimental models of neuroinflammation. This study was divided into two experiments. In the first one (MANUSCRIPT I), the objective of the study was to evaluate the potential of EA in an experimental model of neuroinflammation induced by multiple applications of LPS. Thirty-two male Wistar rats were used, distributed in 4 groups (n = 8): controls (CTRL+SAL) and control-treated with EA (CTRL+EA), and the groups of animals that received LPS (LPS+SAL) and (LPS+EA). The LPS groups received eight daily IP injections of LPS for eight consecutive days at a dose of 250mg/kg of body weight, dissolved in 0.9% saline while the CTRL and EA groups received only 0.9% saline vehicle in the same volume. Two hours after applications (IP), animals in the EA and LPS+EA group were treated with EA at a dose of 100mg/kg orally during the eight days of treatment. The open-field test and object recognition were performed at sixth, seventh, and eighth days of the experimental period. In the second experiment (MANUSCRIPT II), the study aimed to evaluate the effects of HES and its association with rivastigmine (RIV) on memory and oxidative parameters in a sporadic model of Alzheimer's Disease (AD) induced by ICV-STZ injection. 64 Wistar rats were used, divided into eight groups (n = 8): control (CTRL), RIV, HES, RIV+HES, STZ, STZ+RIV, and STZ, HES, STZ+RIV+HES. The rats received an ICV-STZ injection or saline solution (3 mg/kg) and were treated daily, from the fourth day, with 100 mg/kg of HES orally, for 30 days. Twenty-one days after ICV-STZ injection, oral treatment was started with 2 mg/kg of IVR that lasted for 13 days. Behavioral testing was performed by the Morris water maze 30 days after the ICV-STZ injection. In both studies, the antioxidants used (EA or HES) demonstrated the potential to reverse the harmful effects of LPS or STZ by reducing oxidative damage, increasing the antioxidant system, reducing reactive oxygen species (ROS), and improving in the animals' cognitive potential. Also, in MANUSCRIPT I, EA demonstrated immunomodulatory potential, from reduced expression of glial cells, as well as the ability to reduce acetylcholinesterase (AChE) activity and Tau phosphorylation. These results demonstrate the therapeutic potential of EA and HES in cognitive disorders secondary to neuroinflammation, which makes these antioxidants potential candidates for the treatment of neurodegenerative diseases.

Keywords: Polyphenols. Antioxidants. Anti-inflammatory. Streptozotocin. Lipopolysaccharides.

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LISTA DE ABREVIATURAS E SIGLAS

˙OH	Radical hidroxila
AcetilCoA	Acetil-coenzima A
ACh	Acetilcolina
AChE	Acetilcolinesterase
AD	Alzheimer's disease
AE	Ácido elágico
ATP	Adenosina trifosfato
BChE	Butirilcolinesterase
CAT	Catalase
ChAT	Acetiltransferase
CHT	Transportador de colina de alta afinidade
CO	Córtex cerebral
COX-2	Ciclo-oxigenase-2
CTRL	Controle/Control
DA	Doença de Alzheimer
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DNPH	2,4-dinitrofenil-hidrazina
EA	Ellagic acid
EDTA	Ácido etilenodiamino tetra-acético
ER	Espécies reativas
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
Fe^{2+}	Ferro na forma ferrosa
Fe^{3+}	Ferro na foram férrica
G-CSF	Fatores estimulantes de colônia-granulócitos
GFAP	Proteína fibrilar ácida da glia
GLUT2	Transportadores de glicose tipo 2
GM-CSF	Fatores estimulantes de colônia- granulócitos/macrófagos
GPx	Glutationa peroxidase
GSH	Glutationa
GSK3β	Glycogen synthase kinase 3β
H_2O	Água
H_2O_2	Peróxido de hidrogênio
HES	Hesperidina
HP	Hipocampo cerebral
Iba-1	Molécula adaptadora ligante de cálcio ionizado-1
ICV	Intracerebroventricular
ICV-STZ	Injeção intracerebroventricular de estreptozotocina
IGF-1	Fator de crescimento semelhante à insulina tipo 1
IL-12	Interleucina-12
IL-15	Interleucina-15
IL-18	Interleucina-18
IL-1β	Interleucina-1 beta
IL-6	Interleucina-6
IL-8	Interleucina-8
iNOS	Óxido nítrico sintase induzível

IP	Intraperitoneal
LPS	Lipopolissacarídeos
mAChR	Receptores colinérgicos muscarínicos
M-CSF	Fatores estimulantes de colônia-macrófagos
MDA	Malondialdeído
MIF	Fator inibitório de migração de macrófagos
MPO	Myeloperoxidase
MT	Microtúbulos
nAChR	Receptores colinérgicos nicotínicos
NAD^+	Dinucleótido de nicotinamida e adenina oxidado
NFTs	Neurofibrillary tangles
NF-κB	Fator nuclear kappa B
NO	Óxido nítrico
NO_2^-	Nitritos
NO_3^-	Nitratos
Nfr2	Fator nuclear (erythroid-derived 2)-like 2
NTA	Ácido nitrilotriacético
O_2	Oxigênio
$O_2^{\bullet-}$	Ânion superóxido
O_3	Ozônio
$ONOO^-$	Peroxinitrito
PAF	Fator de ativação plaquetária
PGE_2	Prostaglandina E_2
PLD	Potenciação de longa duração
PPA	Proteína percursora amiloide
ROS	Reactive oxygen species
SCNN	Sistema colinérgico não neuronal
SEM	Standard error of the mean
SNC	Sistema nervoso central
SNP	Sistema nervoso periférico
SOD	Superóxido dismutase
SP	Senile plaques
ST	Sulfotransferases
STZ	Estreptozotocina
Tau	Proteína tau
TBARS	Thiobarbituric acid reactive substances
TGF-β	Fator de transformação de crescimento beta
TLR4	Toll-like receptor 4
TNF- α	Fator de necrose tumoral alfa
T-SH	Tióis totais
TXA_2	Tromboxano A_2
UGT	Uridinodifosfoglucuronato glucuronosil transferase
UT	Urolitinas
VAChT	Transportador vesicular de acetilcolina
βA	Beta amiloide

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APRESENTAÇÃO

Os resultados e as metodologias que fazem parte desta tese estão apresentados sob a forma de dois manuscritos, os quais abordam a pesquisa de antioxidantes flavonoides como potenciais terapêuticos em desordens neuroinflamatórias. O experimento foi desenvolvido no biotério do setor de parasitologia da Universidade Federal de Santa Maria e as análises laboratoriais nos laboratórios de Análises Clínicas Veterinária e de Bioquímica e Estresse Oxidativo da mesma instituição, sob a coordenação e orientação da professora Drª. Cinthia Melazzo de Andrade.

Esse documento segue as normas do manual de dissertações e teses da UFSM (MDT – 2015). O item DISCUSSÃO, encontrado no final da tese, apresenta as interpretações sob um ponto de vista que buscou estabelecer uma conectividade entre os objetivos e resultados obtidos. As REFERÊNCIAS BIBLIOGRÁFICAS se referem somente às citações que aparecem no item INTRODUÇÃO.

Os artigos estão estruturados conforme as normas das revistas para as quais foram submetidos. Portanto, o MANUSCRITO I de acordo com as normas da *Neurochemical Research*, enquanto o MANUSCRITO II está descrito conforme as normas da revista *Metabolic Brain Disease*.

1 INTRODUÇÃO

1.1 NEUROINFLAMAÇÃO

Neuroinflamação aguda e crônica podem impactar negativamente o processo de neurogênese em adultos mamíferos. Ainda, o processo inflamatório no sistema nervoso central (SNC) é conhecido como fator de risco para déficit cognitivo e demência (BETTCHER; KRAMER, 2014). A ativação da micróglia e aumento na geração de citocinas pró-inflamatórias contribui significativamente para a neuroinflamação (VON BERNHARDI *et al.*, 2015), a qual é também observada no cérebro de pacientes afetados pela doença de Alzheimer (DA) e acidente vascular cerebral isquêmico. Diversos autores sugerem que a neuroinflamação é um importante componente em diversas desordens do sistema nervoso central, incluindo depressão, doença de Alzheimer, doença de Parkinson e injúria traumática cerebral (DELEGGE; SMOKE, 2008).

Estudo realizado em animais transgênicos sugeriu que a neuroinflamação possui um importante papel no processo de deposição cerebral de amiloide (GUO *et al.*, 2002). Foi demonstrado que citocinas inflamatórias, como interleucina-1 beta (IL-1β), interleucina-6 (IL-6), fator de necrose tumoral alfa (TNF-α) ou fator de transformação do crescimento beta (TGF-β) podem aumentar a expressão da proteína precursora amiloide (PPA) (HIROSE *et al.*, 1994) e formação de beta amiloide (βA) (BLASKO *et al.*, 1999). Além disso, as citocinas são capazes de transcricionalmente gerar a *up-regulation* da mRNA β-secretase, proteínas e atividade enzimática (SASTRE *et al.*, 2003). β-secretase é uma importante enzima limitante que desencadeia a formação de βA (VASSAR, 2002). Sem essa enzima, a formação de βA não ocorre ou pode ser considerada reduzida (WALTER *et al.*, 2001).

O aumento na produção de citocinas inflamatórias está associada ao aumento da ativação da micróglia e astrócitos (HOOGLAND *et al.*, 2015). Normalmente, as células da micróglia atuam fagocitando células mortas e debris celulares para manter a homeostase do SNC enquanto os astrócitos são responsáveis por preservar a função neurológica (ALMAD; MARAGAKIS, 2018). Os marcadores de células da glia incluem a proteína fibrilar ácida da glia (GFAP) e molécula adaptadora ligante de cálcio ionizado-1 (Iba-1). GFAP é um dos melhores marcadores de ativação de astrócitos decorrentes de injúrias ou estresse no SNC (SIRACUSA *et al.*, 2019). Iba-1 é uma proteína cuja expressão está restrita à micróglia e sua elevação está relacionada a diversas doenças cerebrais (HOOGLAND *et al.*, 2015). Desse modo, o aumento da frequência de células Iba-1 positivas (Iba-1^{+}) e GFAP positivas ($GFAP^{+}$)

no hipocampo indica, respectivamente, proliferação de micróglia e astrócitos resultante de um processo inflamatório, os quais, quando estimulados em excesso, poderão potencializar o efeito inflamatório, resultando em patogênese pela secreção de diversos mediadores pró-inflamatórios (HOOGLAND *et al.*, 2015). Essas interleucinas pró-inflamatórias afetam diretamente a função neuronal, como a potenciação de longa duração (PLD), liberação de glutamato, tráfego de receptores AMPA e ativação de vias de sinalização celular (BEATTIE *et al.*, 2002; LYNCH *et al.*, 2004; VEREKER *et al.*, 2000), os quais estão relacionado à plasticidade sináptica e neurotransmissão. Desse modo, poderá haver comprometimento de processos neuronais relacionados à cognição.

Estudo recente demonstrou que patologias sinápticas e microgliose podem ser as manifestações iniciais de neurodegeneração relacionadas a taupatias. Ainda, os autores observaram que a ativação proeminente da micróglia precede a formação de emaranhados neurofibrilares e a imunossupressão dos animais reduziu a patologia relacionada à proteína tau (Tau) e elevou a expectativa de vida dos animais. A relação causal entre a fosforilação da Tau e disfunção neuronal não está bem estabelecida, mas há duas hipóteses principais: a perda da função pode ser causada pela redução da ligação da Tau aos microtúbulos (MT), resultando em desestabilização dos MT e perturbação do transporte axonal; Tau hiperfosforilada resulta em agregação e efeitos tóxicos nas células neuronais. Estudos em modelos de camundongos transgênicos indicaram que perda neuronal e prejuízos na memória estão associados com a presença de proteína Tau solúvel altamente fosforilada (oligômeros), e supressão de sua expressão causa melhora na memória e aumento no número de conexões sinápticas (ROBERSON *et al.*, 2011; SANTACRUZ *et al.*, 2005; SYDOW *et al.*, 2011). Assim, concluiu-se que a neuroinflamação está relacionada à progressão precoce de taupatias.

Deve-se destacar o estresse oxidativo como uma via igualmente importante na patologia de desordens neuroinflamatórias. O alto consumo de oxigênio no cérebro (cerca de 20% do oxigênio proveniente da respiração) em comparação com outros órgãos, combinado com a alta natureza lipofílica do cérebro e seus baixos níveis de antioxidantes endógenos, leva ao acúmulo de espécies reativas de oxigênio e, deste modo, ao dano oxidativo no cérebro. Além disso, a elevada quantidade de ácidos graxos poli-insaturados nas membranas neuronais faz o cérebro particularmente suscetível à peroxidação lipídica. Os subprodutos dessa peroxidação podem induzir neurodegeneração e morte celular pelas vias apoptótica e necrótica (BHAT *et al.*, 2015).

1.2 ESTRESSE OXIDATIVO

O organismo produz constantemente diversas espécies reativas (ER), tais como as espécies reativas de oxigênio (ERO) e de nitrogênio (ERN), entre outras, as quais atuam fisiologicamente em funções como: fagocitose; sinalização celular; controle da pressão sanguínea; apoptose; e envelhecimento (FERNANDEZ *et al.*, 2007). O termo "espécies reativas'' refere-se a radicais livres e outras moléculas que são igualmente reativas, como por exemplo peróxido de hidrogênio (H_2O_2), ozônio (O_3), nitritos (NO_2^-) e nitratos (NO_3^-) (BARREIROS *et al.*, 2006). Os radicais livres são átomos ou moléculas que possuem número ímpar de elétrons em sua última camada, o que confere alta reatividade a esses átomos ou moléculas (FERREIRA; MATSUBARA, 1997).

Metais de transição, como ferro ou cobre, podem doar ou aceitar elétrons livres durante reações intracelulares, catalisando a formação de radicais livres. H_2O_2 pode reagir com o ferro (reação de Fenton) na sua forma ferrosa (Fe^{2+}) produzindo ferro na forma férrica (Fe^{3+}) e radical hidroxila ($^{\cdot}OH$), que é o radical livre mais reativo e nocivo e para o qual o organismo não possui mecanismo de defesa. A maior parte do ferro intracelular é Fe^{3+}, e por isso ele primeiro precisa ser reduzido a Fe^{2+} para participar da reação de Fenton (FERNANDEZ *et al.*, 2007; VASCONCELOS *et al.*, 2007).

Para equilibrar a produção de espécies reativas, o organismo possuiu mecanismos antioxidantes que são classificados como antioxidantes enzimáticos e não enzimáticos. Os enzimáticos são: a superóxido dismutase (SOD), que catalisa a dismutação do ânion superóxido ($O_2^{\bullet-}$) a H_2O_2 e oxigênio (O_2); a catalase (CAT) que decompõe H_2O_2 a O_2 e água (H_2O); e a glutationa peroxidase (GPx) que atua sobre peróxidos utilizando glutationa (GSH) como co-fator. O sistema antioxidante não enzimático é formado por diversas substâncias, dentre elas: GSH; tiois totais (T-SH); tocoferóis; ascorbato; proteínas de transporte de metais de transição, como a transferrina e a apoferritina (VASCONCELOS *et al.*, 2007).

Quando houver excesso de produção destas espécies reativas ou depleção do sistema antioxidante, ocorrerá o estresse oxidativo, o qual resultará em lesões celulares e consequentemente no surgimento de doenças crônicas (FERNANDEZ *et al.*, 2007; FERREIRA; MATSUBARA, 1997). O dano celular resulta da ação de ERO e ERN sobre as macromoléculas, tais como açúcares $(CHOH)_n$, DNA, proteínas e lipídeos (VASCONCELOS *et al.*, 2007). Todos os organismos aeróbios estão suscetíveis ao estresse oxidativo, pois durante a respiração mitocondrial pequenas porções do oxigênio consumido (aproximadamente 2%) são convertidas em espécies altamente reativos: $O_2^{\bullet-}$ e H_2O_2 (PAPA; SKULACHEV, 1997).

A peroxidação lipídica inicia quando as ERO agem sobre ligações duplas ou triplas de ácidos graxos poli-insaturados alterando sua conformação química inicial e, após estas reações iniciarem, elas se autoperpetuam. Como consequências, ocorrem alterações na coesão, na fluidez, na permeabilidade e nas funções metabólicas das células (CHIHUAILAF *et al.*, 2002). Esse dano é avaliado por meio dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) (ESTERBAUER, 1993). Um aumento na peroxidação lipídica provoca dano tecidual e está envolvido em diversas condições patológicas (HALLIWELL; CHIRICO, 1993). A determinação de grupos carbonil nas proteínas oxidadas tem se tornado um dos marcadores de estresse oxidativo mais utilizados na investigação do dano oxidativo proteico (PRATICÒ; TROJANOWSKI, 2000).

Vários autores documentaram a relação entre estresse oxidativo e neuroinflamação. A inflamação induz estresse oxidativo e danos ao DNA, o que desencadeia uma produção exacerbada de ERO por micróglia e macrófagos. Os danos causados pelo estresse oxidativo, como proteínas oxidadas, produtos glicados e peroxidação lipídica, resultam em degeneração neuronal frequentemente relatada em distúrbios cerebrais (POPA-WAGNER *et al.*, 2013). As células danificadas pelo dano oxidativo produzem grande quantidade de mediadores inflamatórios que promovem o envelhecimento da microglia (WU *et al.*, 2016). Além do dano oxidativo nas macromoléculas, as ERO também podem desencadear respostas inflamatórias, estimulando vários genes que regulam a cascata de sinalização inflamatória. Assim, os processos de inflamação e envelhecimento agudos e crônicos são os principais gatilhos para a produção excessiva de ROS.

1.3 SISTEMA COLINÉRGICO

O sistema de neurotransmissão colinérgica é composto pela acetilcolina (ACh), seus receptores e o aparato enzimático responsável por sua síntese e degradação (VENTURA *et al.*, 2010). A ACh é um mediador químico de sinapses presente nos sistemas nervosos central (SNC) e periférico (SNP), e na junção neuromuscular (BRUNEAU; AKAABOUNE, 2006). Esse neurotransmissor é sintetizado pelos neurônios colinérgicos e podem ser liberados por neurônios não colinérgicos, como neurônios do gânglio da raiz dorsal (BERNARDINI *et al.*, 2004; TATA *et al.*, 2014) e por células não neuronais, como linfócitos queratinócitos e células endoteliais (GRANDO *et al.*, 2006; KAWASHIMA; FUJII, 2004). Em células não neuronais o sistema de enzimas sintetizadoras de ACh, transportadores, receptores e enzimas de degradação é denominado sistema colinérgico não neuronal (SCNN).

A atividade da ACh no cérebro é determinada pela ação hidrolítica das colinesterases. Há pelo menos duas colinesterases: a acetilcolinesterase (AChE), uma colina esterase específica que hidrolisa predominantemente ésteres da colina e está presente em grande quantidade no cérebro, nervos e eritrócitos; e a butirilcolinesterase (BChE), que é uma colina esterase não específica (pseudocolinesterase), a qual hidrolisa outros ésteres além dos ésteres da colina. Está presente no soro sanguíneo, pâncreas, fígado, e SNC (DAS, 2007). O sistema colinérgico está fortemente associado com a via colinérgica anti-inflamatória. A AChE sanguínea e BChE plasmática são capazes de interromper a via colinérgica anti-inflamatória por hidrólise da ACh, podendo ser utilizadas como marcadores inflamatórios. Assim, com os estudos dessa via, os efeitos da BChE se mostraram relevantes visto que essa colinesterase desempenha um papel mais importante no sangue que no cérebro (POHANKA, 2014).

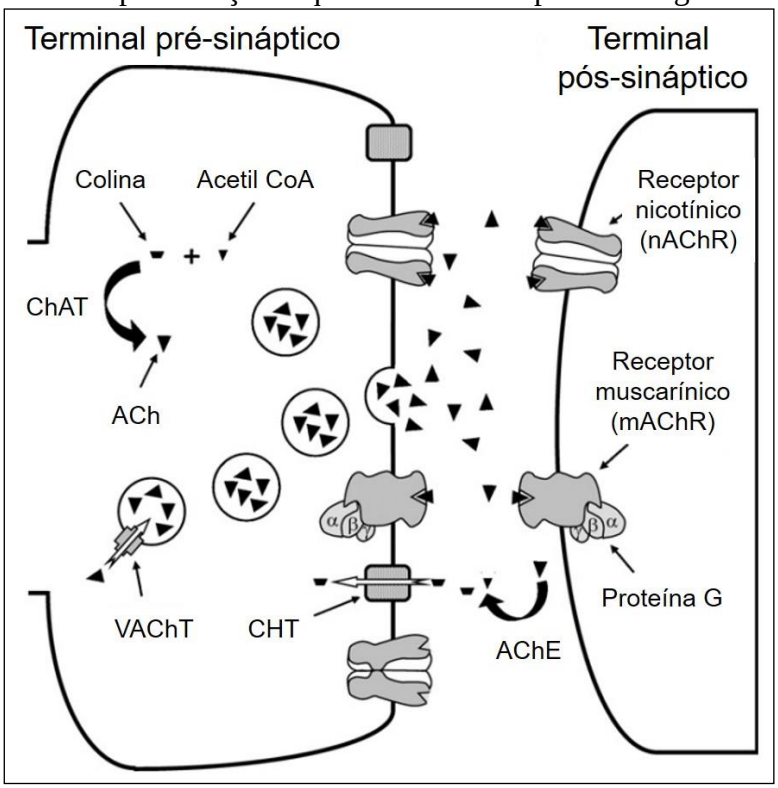
A ACh é amplamente distribuída no SNC e está envolvida em múltiplas funções neuromoduladoras, além de promover o desenvolvimento neuronal, regula a descarga neuronal, afeta a transmissão sináptica, promove plasticidade sináptica, e modula os circuitos neurais (RAMANATHAN *et al.*, 2015). É produzida a partir de acetil-coenzima A (acetilCoA) e colina pela ação da colina acetiltransferase (ChAT), e armazenada em vesículas pré-sinápticas pelo transportador vesicular de ACh (VAChT), permanecendo armazenada até que o terminal pré-sináptico seja ativado (VENTURA *et al.*, 2010). Quando liberada na fenda sináptica, a ACh liga-se a dois diferentes tipos de receptores: receptores colinérgicos nicotínicos (nAChR) e receptores colinérgicos muscarínicos (mAChR). Na fenda sináptica a ação da ACh cessa quando é hidrolisada pela AChE em colina e acetato. Cerca de 50% desta colina é recuperada por um transportador de colina de alta afinidade (CHT) auxiliando na produção e liberação contínua de ACh (Figura 1) (AMENTA; TAYEBATI, 2008).

A ChAT é expressa em praticamente todas as células. No SNC, possui uma extensa distribuição neuronal, estando presente nos corpos celulares, dendritos, axônios e terminais axônicos. Em células não neuronais, assume-se que a ACh sintetizada pela ChAT é constantemente liberada em pequenas quantidades no espaço extracelular para manter a homeostase celular, regulando diversas funções celulares básicas, como mitose, diferenciação celular, organização do citoesqueleto, interações celulares e regulação da funções imunológicas (WESSLER; KIRKPATRICK, 2008).

Os receptores de ACh são expressados no SNC de forma distinta, desempenhando funções na modulação da proliferação e sobrevivência celular, diferenciação neuronal, regulação da expressão gênica, formação e maturação de sinapses e liberação de neurotransmissores (ABREU-VILLACA *et al.*, 2011). Os nAChR localizados no cérebro

distribuem-se nas regiões pré-, pós-, peri- e extrassinápticas, podendo modular a liberação de neurotransmissores e, consequentemente, a atividade sináptica neuronal. Regulam a liberação e a ativação de neurotransmissores nas regiões pré- e pós-sinápticas, respectivamente, podendo controlar a eficácia da transmissão sináptica. Esses receptores estão relacionados a diversos processos, como o aprendizado e memória, o desenvolvimento neuronal e participa do sistema de recompensa (GOPALAKRISHNAN *et al.*, 1997). Os mAChR também são amplamente distribuídos por diversos sistemas biológicos. No SNC estão envolvidos no controle da função extrapiramidal, vestibular, em funções cognitivas como memória, aprendizado e atenção, em respostas emocionais, na modulação do estresse, no sono e na vigília. A ativação desses receptores no sistema nervoso periférico tem ações que incluem a redução da frequência e força da contração cardíaca, o relaxamento de vasos sanguíneos periféricos e a constrição das vias respiratórias (brônquios e bronquíolos) (VENTURA *et al.*, 2010).

Figura 1 – Representação esquemática de sinapse colinérgica.



Fonte: Adaptado de ABREU-VILLACA *et al.* (2011).

1.4 MODELOS DE NEUROINFLAMAÇÃO

Modelos animais são críticos para o descobrimento de novas drogas e proporcionam mecanismos para avaliação de terapias (MISHRA *et al.*, 2018). Avanços nos estudos sobre neuroinflamação baseados em modelos animais contribuíram para elucidar os mecanismos patofisiológicos envolvidos em doenças inflamatórias no SNC e novas estratégias terapêuticas (HAMASAKI *et al.*, 2018). Os modelos animais de neuroinflamação compreeendem modelos baseados em desafios imunes, a partir da administração de lipopolissacarídeos (LPS) ou polinossínico: ácido policitidílico (poli I:C); administração de neurotoxinas, como estreptozotocina (STZ), ácido ocadaico e colchicina; e modelos geneticamente modificados (NAZEM *et al.*, 2015). Dentre esses, destacam-se os modelos de LPS e STZ amplamente estudados por diversos autores (ABBAS *et al.*, 2019; GAO *et al.*, 2020; LYKHMUS *et al.*, 2019; MISHRA *et al.*, 2018; ROSTAMI *et al.*, 2020).

1.1.1 Lipopolissacarídeos

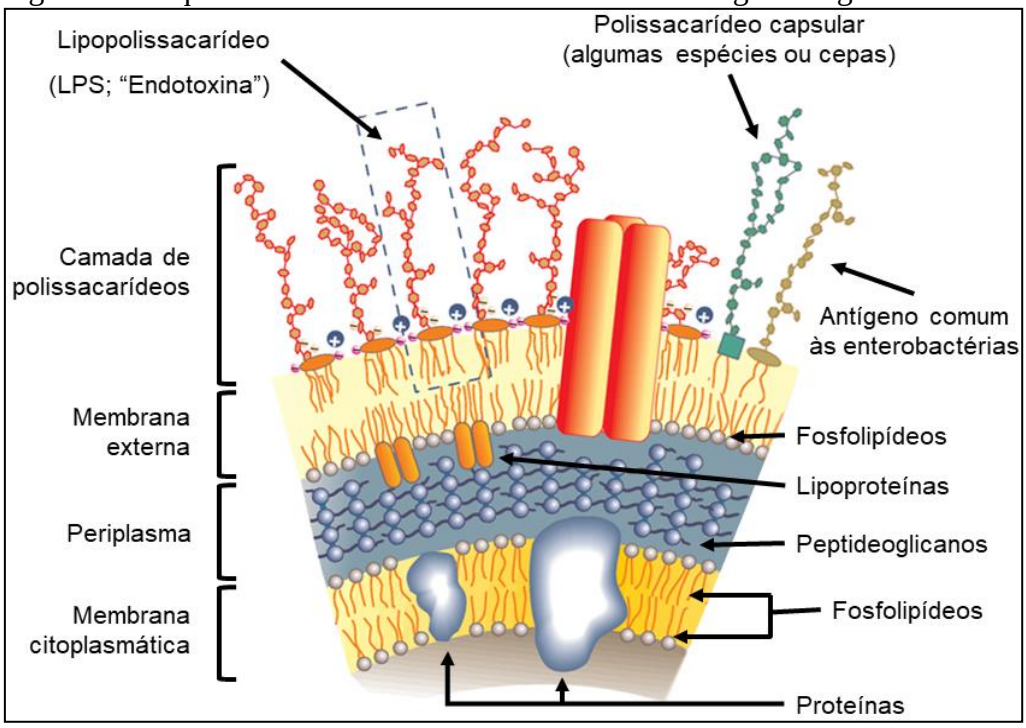
A membrana de bactérias gram-negativas é caracterizada pela presença de duas bicamadas lipídicas, a membrana externa e a membrana interna ou citoplasmática, que são separadas pelo espaço periplasmático contendo uma rede tridimensional de peptideoglicanos. A membrana externa de bactérias gram-negativas comuns, como a *Escherichia coli*, é construída de forma assimétrica, pois a porção lipídica da membrana externa é formada predominantemente por regiões de lipídeo A de moléculas de LPS. Em adição ao LPS, a camada de polissacarídeos da membrana externa de enterobactérias é formado por antígenos comuns às enterobactérias e, em algumas espécies e cepas, também por polissacarídeos capsulares. Além disso, camada interna da membrana celular externa, bem como a camada de lipídeos da membrana citoplasmática, é predominantemente composta de fosfolipídeos (Figura 3) (ALEXANDER; RIETSCHEL, 2001; DMITRIEV *et al.*, 1999).

LPS são moléculas anfifílicas extremamente termoestáveis, compostas de uma região lipídica, lipídeo A e uma porção covalente hidrofílica de poli ou oligossacarídeo (HOLST *et al.*, 1996). Estão presentes no folheto externo da membrana celular de diversas bactérias gram-negativas e desempenham diversas funções na sua biologia (ALEXANDER; RIETSCHEL, 2001). Aproximadamente $2x10^6$ moléculas de LPS cobrem em torno 75% da superfície celular. A camada de LPS, estabilizada por cátions divalentes associados, representa uma efetiva barreira da célula bacteriana contra fatores externos de estresse. Muitos compostos, como

polimexinas, proteínas catiônicas, peptídeos ou poliaminas, e queladores, como ácido etilenodiamino tetra-acético (EDTA) ou ácido nitrilotriacético (NTA), atuam aumentando a permeabilidade da membrana externa desestabilizando a interação entre LPS e cátions na superfície celular (HANCOCK; SCOTT, 2000).

Nas últimas duas décadas, preparações altamente purificadas de LPS, derivados de uma vasta quantidade de espécies de bactérias gram-negativas, têm sido caracterizadas quimicamente, fisiologicamente e biologicamente. Essa molécula tem se mostrado uma das mais potentes classes de imunoestimuladores, conhecidos por fisiologicamente funcionar como indicadores específicos de infecções por bactérias gram-negativas. Todas as formas de LPS conhecidas consistem de um lipídeo A, que ancora a molécula à membrana externa bacteriana, ligado a uma porção covalente de polissacarídeo ou oligossacarídeo. O polissacarídeo é composto pela região proximal do núcleo do lipídeo A e pela cadeia terminal do antígeno O formado por até 50 unidades de repetição. De acordo com a composição de carboidratos na estrutura nuclear, poderá ser distinguida uma região nuclear interna e uma externa. A porção do lipídeo A representa o centro imunoestimulatório primário do LPS, determinando a endotoxicidade da enterobactéria em espécies mamíferas (Figura 4) (ALEXANDER; RIETSCHEL, 2001).

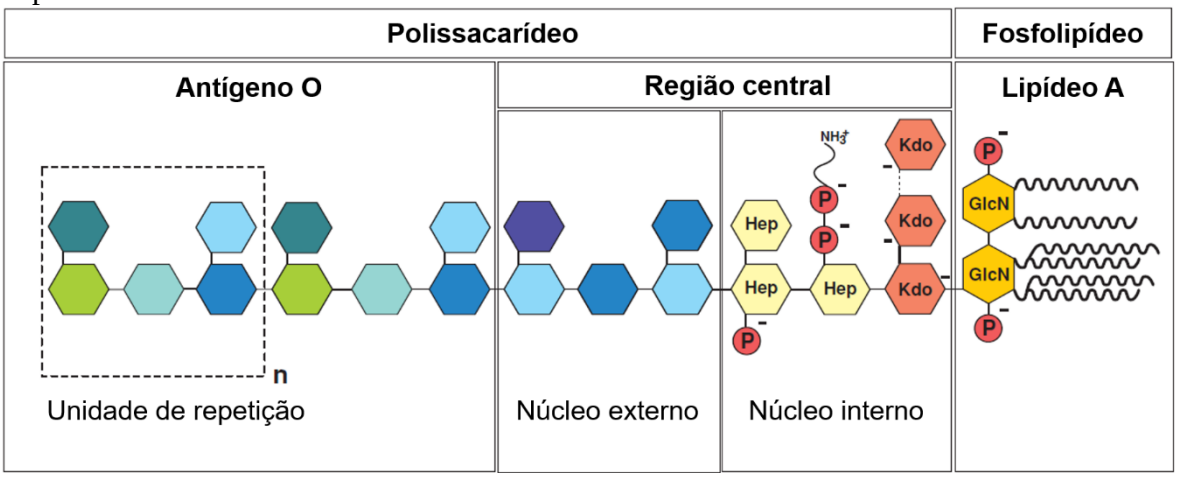
Figura 2 – Arquitetura da membrana celular de bactérias gram negativas.



Fonte: Adaptado de ALEXANDER e RIETSCHEL (2001).

Na grande maioria das estruturas de LPS, a região do antígeno O é caracterizada por uma variabilidade estrutural extremamente alta e suas regiões externas frequentemente atuam como escudo protetor contra o acesso dos agentes antibacterianos do hospedeiro, como ácidos biliares e peptídeos catiônicos ou proteínas de reconhecimento do lipídeo A, aos sítios mais conservados do núcleo interno e região do lipídeo A (MORAN *et al.*, 1996). No geral, a região do lipídeo A representa o centro imunorreativo primário do LPS devido ao reconhecimento específico desta estrutura bacteriana lipídica por numerosos componentes celulares e humorais da imunidade inata (HOFFMANN *et al.*, 1999; MEDZHITOV, RUSLAN; JANEWAY, CHARLES, JR., 2000; MEDZHITOV, R.; JANEWAY, C., JR., 2000).

Figura 3 – Estrutura química geral de LPS de enterobactérias gram-negativas. No núcleo interno da região central e lipídeo A, os fosfatos e resíduos de etanolaminas estão adicionalmente indicados. Abreviações dos resíduos de monossacarídeos: GIcN, glucosamina; Kdo, "2-keto-3-deoxyoctulosonic acid" (3-deoxy-D-manno-octulosonic acid); Hep, D-glycero-D-manno-heptose.



Fonte: Adaptado de ALEXANDER e RIETSCHEL (2001).

Somente certas formas de LPS, caracterizadas por estruturas químicas similares na região do lipídeo A, desencadeiam fortes atividades imunoestimulatórias (agonistas). Em adição, os efeitos clássicos (endotóxicos) de preparações agonistas de LPS são causados indiretamente, pela indução de uma resposta imune desequilibrada e excessiva (ALEXANDER; RIETSCHEL, 2001). As células alvo primárias do LPS são os fagócitos da imunidade inata – monócitos periféricos, neutrófilos e macrófagos teciduais – os quais expressam CD14 ligado à membrana, bem como receptor *tool-like* 4 (TLR4) (ZHANG *et al.*, 1999).

A ativação de células mononucleares *in vitro* pelas formas endotóxicas do LPS ou por

lipídeo A livre resulta na secreção de diversos mediadores endógenos, como fator de necrose tumoral alfa (TNF-α), fator inibitório de migração de macrófagos (MIF), interleucinas-1 beta (IL-1β), interleucina-6 (IL-6), interleucina- 8 (IL-8), interleucina-12 (IL-12), interleucina-15 (IL-15), interleucina-18 (IL-18), fatores estimulantes de colônia-macrófagos (M-CSF), granulócitos (G-CSF), granulócitos/macrófagos (GM-CSF), fator de ativação plaquetária (PAF), prostaglandina E_2 (PGE_2), tromboxano A_2 (TXA_2) ou leucotrienos, bem como espécies reativas de oxigênio e nitrogênio, como ânion superóxido ($O_2^{\bullet-}$), radical hidroxila ($^{\bullet}OH$) ou óxido nítrico (NO). Além disso, LPS pode causar uma ativação rápida e intensa do sistema complemento, pelas vias clássica e alternativa ou da lectina, ativadas pela região do lipídeo A e porção do polissacarídeo, respectivamente (ALEXANDER; RIETSCHEL, 2001).

A administração intraperitoneal de LPS pode induzir um aumento imediato, exacerbado e persistente de citocinas pró-inflamatórias, as quais exercem efeitos neurobiológicos, sugerindo que a inflamação sistêmica também pode alterar a condição neurobiológica (BLUTHÉ *et al.*, 2000). Deste modo, o LPS estimula a cascata de sinalização pró-inflamatória através de proteínas da membrana celular tais como o receptor TLR4, acarretando em superprodução de citocinas pró-inflamatórias (SUN *et al.*, 2015), além de estresse oxidativo (JANGRA *et al.*, 2016), aumento na atividade da aceticolinesterase (AChE) (MING *et al.*, 2015a), ativação da micróglia e astrócitos e elevação da liberação de mediadores pró-inflamatórios, tais como TNF-α e IL-1β (WANG, X.; WANG, C.*; et al.*, 2014; ZHAO *et al.*, 2011), *down-regulation* do fator nuclear (*erythroid-derived 2)-like 2* (Nrf2) e *up-regulation* do fator nuclear kappa B (NF-κB) (ZHOU *et al.*, 2015). A administração sistêmica de uma única dose de LPS por via intraperitoneal é capaz de induzir neuroinflamação que persiste por dez meses e resulta em perda progressiva de neurônios dopaminérgicos na substância negra (QIN *et al.*, 2007).

LEE *et al.* (2008) observaram que repetidas aplicações de lipopolissacarídeos (LPS) (3 ou 7 vezes) resultaram em acúmulo de βA_{1-42} no hipocampo e córtex cerebral de camundongos. Isso se deve ao aumento da atividade de β- e γ-secretase acompanhados pela elevação da expressão da PPA, fragmento C-terminal associado à membrana contendo 99 aminoácidos (C99), geração de βA_{1-42}, bem como a ativação de astrócitos, que resulta em morte de células neuronais e com isso, déficit cognitivo.

1.1.2 Estreptozotocina

A estreptozotocina (STZ) é um derivado de glucosamina-nitrosouréia, originalmente identificada em 1959 como um antibiótico (LEWIS; BARBIERS, 1959), obtido a partir de *Streptomyces achromogenes*. A STZ tem sido comumente utilizada como indutora de diabetes melito tipo I em modelos animais devido sua toxicidade contra células β-pancreáticas (FURMAN, 2015), além de promover resistência à insulina (SZKUDELSKI, 2012). Diversas espécies, incluindo camundongos, ratos, coelhos e macacos, são sensíveis aos efeitos citotóxicos β-pancreáticos deste composto (WU; HUAN, 2008). A STZ é captada pelas células β-pancreáticas via transportadores de glicose tipo 2 (GLUT2) e, uma vez dentro da célula, o grupamento metil-nitrusurea promove a alquilação do DNA, o que leva sucessivos eventos, resultando em depleção de dinucleótido de nicotinamida e adenina oxidado (NAD+) com consequente diminuição dos estoques de adenosina trifosfato (ATP) e necrose das células β-pancreáticas (LENZEN, 2008).

Receptores de insulina estão amplamente distribuídos pelo cérebro, predominantemente no hipocampo, uma região neurogênica envolvida no aprendizado e memória (SCHULINGKAMP *et al.*, 2000). A redução nos níveis de insulina cerebrais (SARTORIUS *et al.*, 2015) e neurogênese (KEMPERMANN *et al.*, 2002) observadas durante o processo de envelhecimento, sugerem que a insulina esteja relacionada com os processos de neurogênese (MISHRA *et al.*, 2018). Estudos demonstraram que infusão intracerebroventricular de fator de crescimento semelhante à insulina tipo 1 (IGF-1) atenuou a redução da neurogênese relacionada ao processo de envelhecimento (LICHTENWALNER *et al.*, 2001) e indução seletiva da neurogênese no hipocampo de ratos adultos foi observada após infusão periférica de IGF-1 (ABERG *et al.*, 2000). A co-localização de genes envolvidos na regulação da neurogênese e sequências indispensáveis para a transcrição do gene de insulina fortalecem o conceito de que as vias de sinalização de insulina regulam a neurogênese (SHARMA *et al.*, 1999).

Nesse contexto, estudos têm demonstrado que a injeção intracerebroventricular de estreptozotocina (ICV-STZ) pode prejudicar o metabolismo energético cerebral e reproduzir características moleculares e patológicas observadas em doenças neurodegenerativas. A ICV-STZ induz alterações nos receptores de insulina no cérebro e na sua sinalização, promovendo um estado cerebral de resistência à insulina, com diminuição do aporte de glicose cerebral (CHEN *et al.*, 2013). Evidências clínicas sugerem ainda, que a inibição da sinalização da insulina contribui para a neurodegeneração, por ter potencial influência sobre o metabolismo do peptídeo βA, sendo o aumento da expressão da PPA, bem como do peptídeo βA relatados,

sobretudo em regiões do córtex cerebral e hipocampo após 3 semanas da injeção ICV-STZ (STANLEY *et al.*, 2016). Além disso, devido ao distúrbio da transdução do sinal da insulina, a ICV-STZ pode levar a uma desregulação da fosforilação de quinases, e assim ocasionar a hiperfosforilação da proteína Tau, o que induz a formação de emaranhados neurofibrilares (GRUNBLATT *et al.*, 2007).

Diversos estudos relataram neuroinflamação acentuada nos modelos experimentais de doença de Alzheimer induzidos pela ICV-STZ (CHEN *et al.*, 2013; JAVED *et al.*, 2012; KRASKA *et al.*, 2012; RAJASEKAR *et al.*, 2017). A neuroinflamação possivelmente ocorre como consequência da lesão neuronal gerada pelo estresse oxidativo, visto que a molécula de STZ, após decomposição, origina peróxido de hidrogênio e óxido nítrico. Estas moléculas irão gerar espécies reativas de oxigênio e nitrogênio, respectivamente, o que causa pronunciado estresse oxidativo nos modelos STZ (CHEN *et al.*, 2013; EJAZ AHMED *et al.*, 2013; ISHRAT *et al.*, 2009). O processo inflamatório neuronal culmina em gliose reativa e aumento de marcadores pró-inflamatórios (KRASKA *et al.*, 2012; RAJASEKAR *et al.*, 2017). Dados sugerem que a ativação da micróglia e aumento de citocinas pró-inflamatórias possam ser responsáveis pela inibição da neurogênese, o que contribui para o declínio cognitivo observado em doenças neurodegenerativas (BASSANI *et al.*, 2018). Ainda, o declínio cognitivo relatado em roedores no modelo ICV-STZ também está relacionado ao aumento na atividade da AChE (AGRAWAL *et al.*, 2009), redução da síntese de adenosina trifosfato (ATP) e acetil-CoA, os quais resultam em disfunção da homeostase colinérgica (DE LA MONTE; WANDS, 2008).

1.5 FLAVONÓIDES

Diversos estudos têm demonstrado as propriedades anti-inflamatórias e efeitos imunomoduladores dos compostos fenólicos (HANDA *et al.*, 2002; ROGERS *et al.*, 2005; WATSON *et al.*, 2005). Especificamente, o ácido elágico (AE) possui seus efeitos anti-inflamatórios pela óxido nítrico sintase induzível (iNOS), ciclo-oxigenase-2 (COX-2), TNF-α, *down-regulation* da IL-6 devido inibição do NF-κB e IL-1β (MASAMUNE *et al.*, 2005; UMESALMA; SUDHANDIRAN, 2010).

Os flavonoides são um grupo de compostos sintetizados por plantas como uma resposta adaptativa a condições de estresse, protegendo a planta de ataques bióticos e abióticos (WEN *et al.*, 2017). Os flavonoides diferem entre si pela sua estrutura química e características particulares. A grande maioria apresenta uma organização genérica composta por 15 átomos de carbonos arranjados em dois anéis aromáticos (A e B) e um heterocíclico oxigenado (anel C)

(AHLENSTIEL *et al.*, 2003). A diversidade estrutural dos flavonoides contribui para as diferenças na sua eficácia biológica, com diferenças sutis que afetam sua biodisponibilidade (WILLIAMSON; CLIFFORD, 2010). Na natureza, os flavonoides podem ocorrer na forma livre, ou seja, não conjugado com nenhum heterosídeo, como por exemplo, quercetina ou então, na forma conjugada, ligado a uma unidade glicosídica, como o ácido elágico (LARROSA *et al.*, 2006a) e hesperidina, sendo desta forma, considerados substâncias lipossolúveis e hidrossolúveis, respectivamente (KRUEGER, 2002).

Visto que dietas ricas em flavonoides têm sido relacionadas com baixa incidência de doenças cardiovasculares, neurodegenerativas e oncológicas (ROTHWELL *et al.*, 2017), esses compostos têm atraído o interesse de pesquisadores, que relatam a ação antioxidante (JEONG *et al.*, 2007) e imunomoduladoras (WEN *et al.*, 2017) desses compostos. Neste contexto, produtos naturais tem sido estudados como fontes para o desenvolvimento de novas drogas (NEWMAN; CRAGG, 2007).

1.1.3 Ácido elágico

O ácido elágico (AE - 4,4′,5,5′,6,6′-ácido hexahidroxidifênico 2,6,2′,6′-dilactona) é quimicamente caracterizado como uma dilactona derivada do HHDP, com peso molecular de 338,2 g/mol, altamente termoestável devido aos seus quatro anéis na molécula, que representam dominância lipofílica, e aos quatro grupos fenólicos e duas lactonas, representando a zona hidrofílica (BALA *et al.*, 2006). Essas propriedades do AE resultam em alta insolubilidade em água. No entanto, é solúvel em metanol acidificado (LEI *et al.*, 2003), etanol (SHI *et al.*, 2005) e dimetil sulfóxido (BALA *et al.*, 2006). É um polifenol não-flavonoide presente em altas concentrações em vegetais e frutas, tais como romã, morango, framboesa e nozes (LARROSA *et al.*, 2010). Em alimentos, esse composto está comumente conjugado com uma unidade glicosídica, como a glicose, ou formando parte da estrutura química de elagitaninos (ETs) poliméricos (LARROSA *et al.*, 2006b), que são ésteres de HHDP e monossacarídeos, geralmente beta-D-glicose. Devido às ligações de éster, os ETs hidrolisam relativamente devagar durante os processos de digestão e absorção, o que causa prolongada secreção de AE no intestino (LARROSA *et al.*, 2010).

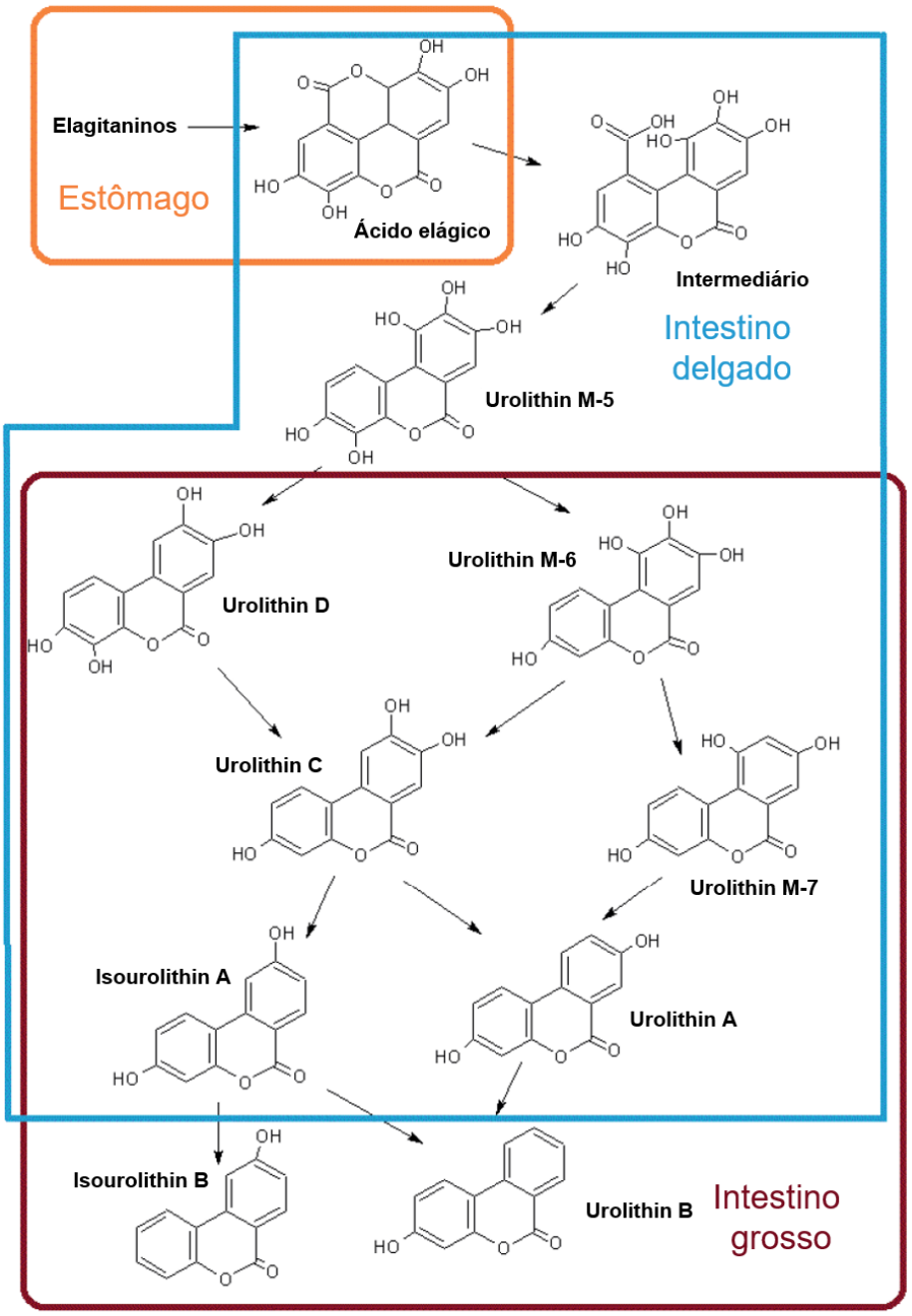
ETs são relativamente estáveis no pH fisiológico do estômago, no qual possivelmente não são hidrolisados. Podem ser catabolizados pela microbiota intestinal para liberar AE, e o subsequente metabolismo deste pelos microorganismos do intestino irá produzir urolitinas

(UT), aparentemente pela via de hidrolise dos anéis de lactona e descarboxilação, seguida por dehidroxilação (Figura 6) *(GARCIA-MUNOZ; VAILLANT, 2014)*. As UT passam pela circulação entero-hepática *(CERDA et al., 2004)* com concomitante conjugação no fígado via uridinodifosfoglucuronatoglucuronosiltransferase (UGT) ou sulfotransferases (ST) aos correspondentes glucuronídeos ou sulfatos, respectivamente *(OTAKE, 2002; VAIDYANATHAN; WALLE, 2002)*. O AE é absorvido rapidamente e atinge máxima concentração plasmática uma hora após ingestão *(SEERAM et al., 2004; STONER et al., 2005)*. Em contrapartida, as UT são observadas em máxima concentração plasmática entre 24 e 48 horas pós ingestão de ETs. Os conjugados do AE e/ou de UT são responsáveis pelos efeitos benéficos das frutas ricas em elagitaninos*(ESPIN et al., 2013; PIWOWARSKI et al., 2014)*.

O AE possui atividades anti-carcinogênica (KIM *et al.*, 2009; UMESALMA; SUDHANDIRAN, 2010; 2011), antiviral (GOODWIN *et al.*, 2009), antibacteriana (NOHYNEK *et al.*, 2006), anti-inflamatória (ROGERIO *et al.*, 2008), gastroprotetora (BESERRA *et al.*, 2011), cardioprotetora (IAKOVLEVA *et al.*, 1998), além de possuir ação inibitória de β-secretase, uma protease de ácido aspártico relacionada à patogênese da Doença de Alzheimer (KWAK *et al.*, 2005). Estes efeitos benéficos podem ser atribuídos, ao menos parcialmente, à atividade antioxidante do AE (KAHKONEN *et al.*, 2012; QIU *et al.*, 2013), proporcionada pelas quatro hidroxilas e dois grupos funcionais de lactona que atuam como receptores e doadores de hidrogênio respectivamente, possibilitando a eliminação de $O_2^{\bullet-}$, $^{\bullet}OH$, peróxido de hidrogênio (H_2O_2) e peroxinitrito ($ONOO^-$) (NUGROHO *et al.*, 2014). Esse composto é eficiente em inibir a peroxidação lipídica, mesmo em concentrações micromolares (ZAFRILLA *et al.*, 2001). Em adição, o AE pode possuir atividade antioxidante similar à outros compostos, como a vitaminas E e C (PRIYADARSINI *et al.*, 2002).

Tem sido relatado que os metabólitos do AE, as UT, também possuem atividade antioxidante, ou seja, sua capacidade de eliminar espécies reativas não é reduzida após metabolização (QIU *et al.*, 2013). Deste modo, esse composto promove proteção contínua contra o estresse oxidativo através eliminação de radicais livres, o que é um comportamento raro e muito desejável (GALANO *et al.*, 2014). Além disso, em estudo realizado *in vitro*, observou-se que as UT possuem todos os critérios para atravessar a barreira hematoencefálica e assim exercer neuroproteção a partir de seus efeitos antioxidantes (YUAN *et al.*, 2016).

Figura 4 – Metabolismo dos elagitaninos e ácido elágico pela microbiota gastrointestinal.



Fonte: Adaptado de LIPIŃSKA *et al.* (2014).

1.1.4 Hesperidina

Dentre os flavonoides, destaca-se a hesperidina (HES) (3',5,7-tri-hidroxi-4'-metoxi-flavanona-7-ramnoglucosídeo), uma flavanona glicolisada de ocorrência natural, predominantemente encontrada em frutos cítricos (PARHIZ *et al.*, 2015). A laranja (*Citrus sinensis*) e o limão (*C. limon var. criolo*) são os representantes majoritários das flavanonas

(JUSTESEN *et al.*, 1997). A HES é hidrolisada pelas enzimas glicosidases da microflora colônica do intestino. As agliconas livres liberadas são então captadas e conjugadas pelas enzimas de fase II no intestino e no fígado. Como resultado, ocorre a liberação da hesperetina, que circula no sistema sistêmico em formas conjugadas (SPENCER; CROZIER, 2012).

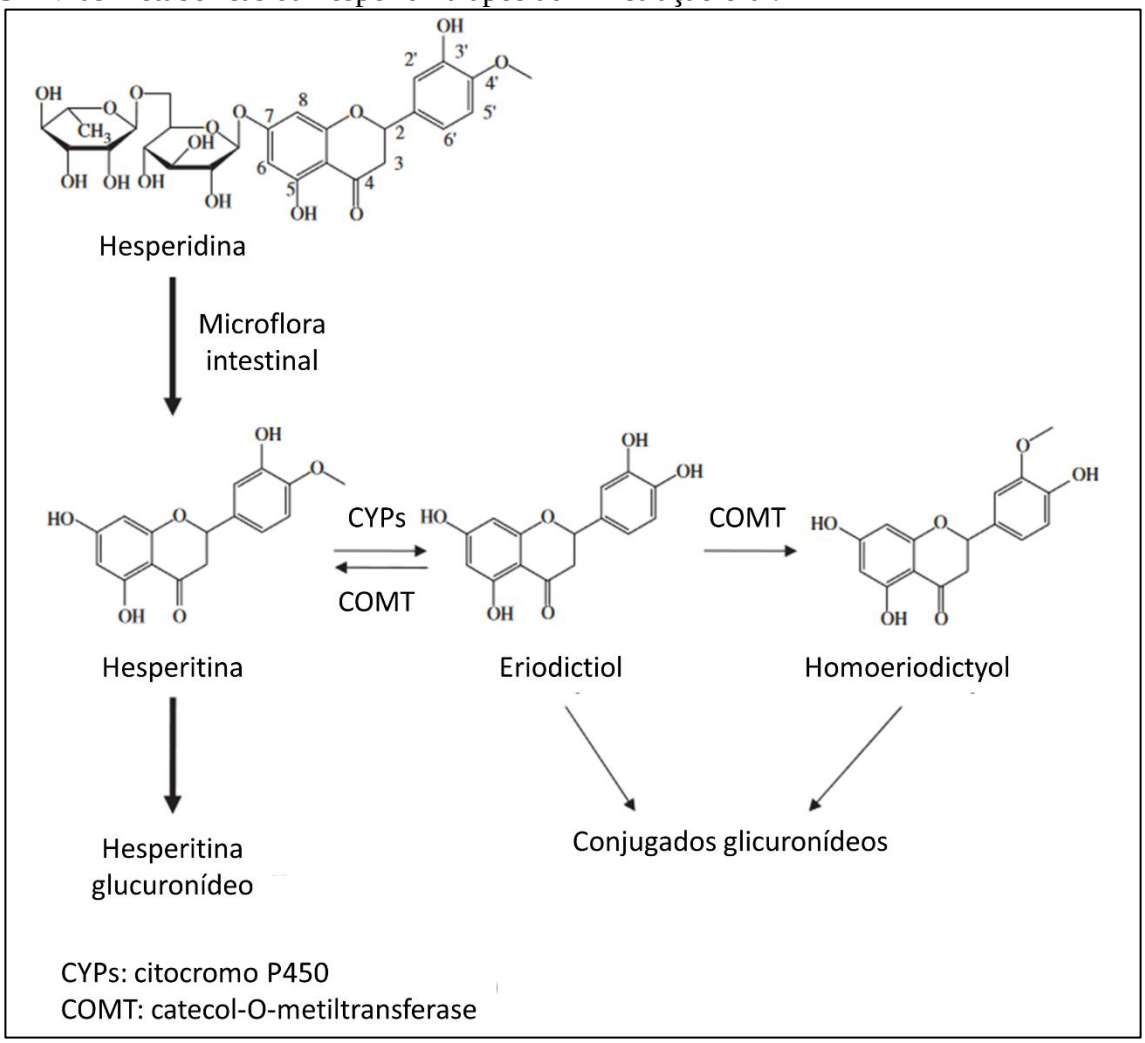
A HES possui biodisponibilidade limitada devido ao rutinosídeo ligado ao flavonoide (NIELSEN *et al.*, 2006). O tempo para atingir máxima concentração plasmática em ratos é de aproximadamente 0,75h e a meia vida varia em torno de 10 h (HU *et al.*, 2015). Após administração oral, a HES é convertida em hesperitina, uma aglicona da HES, pela β-glucosidase presente na microflora intestinal e então absorvida pelo trato gastrointestinal (figura 5) (MATSUMOTO *et al.*, 2004). Após a hidrólise dos glicosídeos flavonoides pelas bactérias do trato gastrointestinal, a reação procede para a degradação da cadeia flavonoide em numerosos produtos fenólicos e de ácido carboxílico (WALLE, 2004). Posterior a absorção da HES, o composto é imediatamente metabolizado para formar produtos conjugados com glicuronídeo no epitélio intestinal e fígado (MANACH *et al.*, 2003). Desse modo, o metabolismo bacteriano intestinal representa um papel fundamental na absorção desse antioxidante, visto que alterações na microflora podem alterar a absorção da HES e consequentemente sua farmacocinética, culminando em alterações na sua atividade biológica (JIN *et al.*, 2010).

A HES possui diversos efeitos farmacológicos, como atividade anti-aterogênica, atividade antialérgica, anti-inflamatória, antimutagênica e neuroprotetora (BORRADAILE *et al.*, 1999; GALATI *et al.*, 1994; PARHIZ *et al.*, 2015; WILCOX *et al.*, 2001). Além de atuar sobre o metabolismo da glicose (UMENO *et al.*, 2016) e ter efeitos positivos sobre a resposta imune (CAMPS-BOSSACOMA *et al.*, 2017). Em diversos modelos animais, a HES demonstrou capacidade de elevar os níveis de GSH e atividade enzimática de antioxidantes, como SOD, CAT e GPx (EL-SAYED EL *et al.*, 2008; KUMAR *et al.*, 2013). Essas propriedades da HES são responsáveis por seus efeitos benéficos em diversas doenças, como doenças cardiovasculares, neoplasias e distúrbios induzidos por irradiação (LI; SCHLUESENER, 2017). Além disso, estudos observaram o potencial dessa molécula em atravessar a barreira hematoencefálica, destacando-se assim a atividade da HES sobre neurônios de importantes regiões para processos cognitivos e de memória como hipocampo e córtex cerebral de ratos (DIMPFEL, 2006; JUSTIN THENMOZHI *et al.*, 2015), o que demonstra seu potencial para utilização em desordens neurológicas (LI; SCHLUESENER, 2017).

Nesse contexto, esta pesquisa teve por objetivo avaliar o potencial terapêutico do AE ou HES em modelos experimentais de neuroinflamação. Para isso, avaliou-se os efeitos do AE em

múltiplas aplicações intraperitoneais (IP) de LPS e os efeitos da HES após administração intracerebroventricular de estreptozotocina (ICV-STZ).

Figura 5 – Vias metabólicas da hesperidina após administração oral.



Fonte: adaptado de Jin et al. (2010).

2 CAPÍTULO I – MANUSCRITO I – ELLAGIC ACID INHIBITS NEUROINFLAMMATION AND COGNITIVE IMPAIRMENT INDUCED BY LIPOPOLYSACCHARIDES

Artigo submetido para: *Neurochemical Research.*

Ellagic acid inhibits neuroinflammation and cognitive impairment induced by lipopolysaccharides

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ABSTRACT

Neuroinflammation is a predisposing factor for the development of cognitive impairment and dementia. Among the new molecules that are currently being studied, ellagic acid (EA) has stood out for its neuroprotective properties. The present study investigated the effects of ellagic acid in the object recognition test, oxidative stress, cholinergic neurotransmission, glial cell expression, and phosphorylated Tau protein expression. For this, 32 male Wistar rats received an intraperitoneal (IP) application of lipopolysaccharides (LPS) at a dose of 250 µg/kg or 0.9% saline solution (SAL) for eight days. Two hours after the IP injections, the animals received 100 mg/kg of EA or SAL orally (P.O.). Behavioral parameters (open field test and object recognition) were performed on days five, six, and seven of the experimental periods. The results showed that the treatment with EA in the LPS group was able to inhibit cognitive impairment, modulate the immune system response by significantly reducing glial cell expression, attenuating phosphorylated Tau and oxidative damage with consequent improvement in the antioxidant system, as well as preventing the increase of acetylcholinesterase (AChE) activity. Thus, the neuroprotective effects of EA and its therapeutic potential in cognitive disorders secondary to neuroinflammation were demonstrated.

Keywords: Antioxidant; oxidative stress; acetylcholinesterase; microglia; astrocytes; tau protein.

INTRODUCTION

Neuroinflammation is a characteristic of several neurological disorders, including Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, and acute traumatic brain injury (BERGOLD, 2016; GRIGORIADIS; VAN PESCH, 2015; LATTA *et al.*, 2015; ROCHA *et al.*, 2015). Systemic administrations of lipopolysaccharides (LPS) have been described as experimental models that mimic the pathological disorders of these diseases, including AD-associated cholinergic neuronal degeneration. LPS can impair the consolidation of specific memory processes. Acute administration of LPS before training impairs the contextual fear conditioning test, a learning paradigm dependent on the hippocampus (PUGH *et al.*, 1998), while chronic LPS infusions affect spatial memory (HAUSS-WEGRZYNIAK *et al.*, 2000) and induce impairments in memory and learning analogous to cognitive impairment observed in

AD (LEE *et al.*, 2008). In contrast, systemic administration of LPS results in damage to the hippocampus-dependent memory on object discrimination, but not on spatial memory (CZERNIAWSKI *et al.*, 2015).

Intraperitoneal (IP) injections of LPS cause cognitive impairment in laboratory animals through the activation of microglia, which stimulates the production of pro-inflammatory mediators. This mechanism is apparently due to the communication pathways between the immune system and the brain (DELEGGE; SMOKE, 2008). In response to the production of pro-inflammatory cytokines, several reactive oxygen species (ROS) are produced, which culminates in oxidative stress (COZZI *et al.*, 1995; MASHHADIZADEH, SHAHRAM *et al.*, 2017). Increased production of ROS promotes rapid changes in the antioxidant system, through the induction or depletion of cellular antioxidant reserves (TYAGI *et al.*, 2008). Also, excessive activation of the microglia perpetuates the inflammatory cycle (TANSEY *et al.*, 2007), prolonging inflammation (SCHMID *et al.*, 2009), which predisposes to the development of several neurodegenerative diseases (BLOCK; HONG, 2005), damage to the vascular endothelium, depletion of redox-glutathione, and mitochondrial respiratory dysfunction, which culminates in a reduction in the consumption of ATP and O2 (SUGINO *et al.*, 1987).

The tau protein (Tau) is related to several physiological processes in neurons. When hyperphosphorylated, Tau monomers detach from microtubules and tend to aggregate into neurofibrillary tangles. This process is observed in several neurodegenerative disorders, called tauopathies (LUPPI *et al.*, 2019). The neurodegenerative process in these diseases is characterized by an amyloid cascade with consequent formation of amyloid plaques, Tau phosphorylation, neuroinflammation, and neuronal death. It is believed that the formation of amyloid oligomer (A) is the first step towards neurodegeneration, initiating the amyloid cascade (HARDY; HIGGINS, 1992). In a brain inflammatory microenvironment, the production of cytokines by microglia and astrocytes can potentiate the amyloid cascade, which demonstrates the relationship between tauopathies and neuroinflammation (ACOSTA *et al.*, 2017; DZAMBA *et al.*, 2016).

Drugs for improving cognition such as memantine, aniracetam, piracetam and cholinesterase inhibitors such as galantamine are used to improve memory, mood, and behavior, but their side effects limit the use of these agents. Thus, other possibilities, including plant derivatives, have been considered and evaluated as therapeutic alternatives (PARK *et al.*, 2011). There are several evidences to support the potential of antioxidants in the prevention and treatment of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. Furthermore, evidences in the literature confirms the ability of components with antioxidant

properties to protect neurons against the harmful effects of ROS, preventing, or delaying the development of neurodegenerative diseases (GILGUN-SHERKI *et al.*, 2003; KELSEY *et al.*, 2010). Among these antioxidants, ellagic acid (EA) stands out, which is relatively stable under physiological conditions in the stomach and can be a potential phytotherapeutic candidate for the development of neuroprotective drugs that can be administered orally. This antioxidant has multiple pharmacological properties that are useful in the treatment and maintenance of disorders of the central nervous system. It can regulate several molecular signaling pathways, in order to normalize mitochondrial dysfunctions that result in the generation of free radicals and thus attenuate neurodegeneration (AHMED *et al.*, 2016). The antioxidant action of EA occurs due to its direct property of free radicals scavenging and potentiating endogenous antioxidants (COZZI *et al.*, 1995). EA can protect the brain from inflammation through down-regulation of the expression of several pro-inflammatory cytokines (such as TNF-α) (MASHHADIZADEH, SHAHRAM *et al.*, 2017). The suppression of microglial responses represents the therapeutic effect of EA in AD. Also, in vivo and in vitro studies have shown a reduction in the release of inflammatory cytokines by microglia and amyloid plaques induced by EA (ROJANATHAMMANEE *et al.*, 2013).

Thus, the present study aimed to evaluate the action of EA in the cerebral cortex and hippocampus by recognizing memory and oxidative stress parameters such as ROS, lipid peroxidation, protein carbonylation, and T-SHs and GSH levels in an experimental model of neuroinflammation induced by multiple applications of LPS in rats. The study also aimed to investigate the effect of EA on AChE activity and expression of neural and phosphorylated proteins in this experimental model.

MATERIALS AND METHODS

Animals

This work was approved by the Ethics Committee on the Use of Animals of the Federal University of Santa Maria under number 5580160118. Thirty-two male Wistar rats with 6 to 7 weeks old (200 - 230g), from the Central Bioterium of the Federal University of Santa Maria, were used. Animals in this age group have been chosen as they are more anxious and show more exploratory behavior than rats aged 16 weeks (300 - 320 g) commonly used in several experimental models (RAY; HANSEN, 2005).

Four animals were housed per box with food and water available ad libitum. The rats were kept in an environment with controlled temperature and humidity (22º - 24 ° C; 70% RH), light/dark cycle (7:00 a.m. - 7:00 p.m.), and previously acclimated for two weeks. The animals were randomly divided into four groups, containing eight animals each: control (CTR+SAL), control treated with ellagic acid (CTRL+EA), lipopolysaccharide (LPS+SAL) and lipopolysaccharide treated with ellagic acid (LPS+EA). The animals in the LPS groups (LPS+SAL and LPS+EA) received, for eight consecutive days, a daily application (IP) of LPS at a dose of 250 µg/kg dissolved in 0.9% saline, while the control groups (CTRL+SAL and CTRL+EA) received only injections (IP) of 0.9% saline solution (SAL) in the same volume and period. One hour after the IP injections, the animals received orally (P.O.) EA at a dose of 100 mg/kg (CTRL+EA and LPS+EA) or 0.9% of saline in the same volume and route (CTRL+SAL and LPS+SAL). The animals were weighed daily to adjust the dose of the compounds to be used (Fig. 1).

Lipopolysaccharide

Systemic administration of LPS is a model widely used to induce neuroinflammation, as it results in increased levels of cerebral cytokines and activation of microglia (Qin et al., 2007; Henry et al., 2008). In this context, to induce the neuroinflammatory response, lipopolysaccharides from Escherichia coli (Sigma-Aldrich, O111-B4) diluted in saline and injected intraperitoneally at a dose of 250 µg/kg, once a day, for eight days were used. This dose was selected according to previous studies (LEE *et al.*, 2008; ZHU *et al.*, 2014).

Ellagic acid

Ellagic acid (Sigma-Aldrich) was used in doses of 100 mg/kg, orally, once daily, one hour after application of LPS. The treatment lasted eight days. The EA was suspended in saline and administered via gavage. The suspension was homogenized in a sonicator before each administration to obtain a homogeneous solution. This treatment protocol is based on previous studies with this polyphenol (BHARATHI; JAGADEESAN, 2014; BHARATHI *et al.*, 2014; FARBOOD *et al.*, 2015a; GUADA *et al.*, 2017; HASSAAN *et al.*, 2014; JAGADEESAN; BHARATHI, 2014; MASHHADIZADEH, S. *et al.*, 2017; UEDA *et al.*, 2004; UZAR *et al.*, 2012).

Open field test

This test was performed to identify changes in the locomotor and exploratory capacity of the animals, as previously described by (ZANIN; TAKAHASHI, 1994) and was performed on day 5 (Fig. 1). The apparatus consists of a wooden box covered with waterproof material with dimensions 70 x 70 x 30 cm. The floor was divided into 16 squares measuring 12 x 12 cm each to assess the open field. The session lasted five minutes and was recorded for further processing by an automated activity monitoring system (AnyMaze, Stoelting, USA) to assess the total distance covered; mobile or immobile time; time in the central zones, walls or corners; and number of entrances or exits in the central zones, walls or corners.

Object recognition test

The object recognition task was used to study recognition memory in rats (LUEPTOW, 2017). The animals were submitted to training on day 6 (Fig.1), where they were individually placed in the open field containing two similar objects (A1 and A2) being allowed to explore them freely for 5 minutes. For the evaluation of short-term memory 2 hours after the training session the animals were individually reintroduced into the open field, where one of the objects presented during training was replaced by a new object with different size and shape (A1 and B). To assess long-term memory the same procedure was performed 24 hours after the training session, replacing object B with a new object of different size and shape (object C). This task consists of the spontaneous and differential exploration of familiar and new objects, and the recognition performance is derived from the time spent exploring the two stimuli. Exploration of objects was considered by was considered by animal's snout directing at a distance $\leq$ 2 cm from the object and sniffing or touching the object with the snout. Climbing or sitting on objects was not classified as exploratory behavior. The results were expressed as preference index (percentage of time = new object/[new object+family object] x100) ± SEM, which evaluates the percentage of time exploring the new object, and total exploration time (total time = new object)+familiar object) ± SEM.

Brain tissue preparation

At the end of the behavioral assessments, the animals were euthanized. After opening the skull, the brain was removed and separated into the cerebral cortex and hippocampus and

homogenized in a solution of 10 mM Tris-HCl (pH 7.4), under ice, in a proportion of 1:10 (weight/volume). After centrifugation, the aliquots resulting from the homogenates of the brain structures were used to determine the parameters of oxidative stress and acetylcholinesterase activity.

The protein of brain structures was previously determined through a range varying for each structure: cerebral cortex (0.7 mg/ml) and hippocampus (0.8 mg/ml), as determined by the Coomassie blue method(BRADFORD, 1976).

Determination of acetylcholinesterase activity in the brain

The AChE enzymatic activity was determined by the ELLMAN *et al.* (1961) method as modified by ROCHA *et al.* (1993). This method is based on formation of the yellow 5-thio-2-nitrobenzoic acid, which was measured spectrophotometrically at 412 nm for 2 minutes at 25°C. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) and the AChE enzyme (40–50 μg of protein), which was pre-incubated for 2 minutes. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). The experiment was carried out in triplicate, and enzyme activity was expressed as μmol AcSCh/h/mg of protein.

Measurement of reactive oxygen species (ERO)

The 2'-7'-dichlorofluorescein fluorescence assay was used to measure the production of hydrogen peroxide and other reactive species (MYHRE *et al.*, 2003). 50 ml aliquots of the brain structure homogenate supernatant were added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and DCFH DA 2'-7'-dichlorofluorescein-diacetate (1 mM). After adding DCFH-DA, the medium was incubated in the dark for 1 hour until fluorescence measurement (excitation at 488 nm and emission at 525 nm, with both slit widths at 1.5 nM). Dichloro-oxidized fluorescein was determined using an oxidized dichlorofluorescein standard curve, and the results are expressed as DCFH-DA Fluorescence.

Thiobarbituric acid reactive substances (TBARS) measurement

The levels of thiobarbituric acid reactive substances (TBARS) were determined according to JENTZSCH *et al.* (1996) by measuring the concentration of malondialdehyde

(MDA) as a product of lipid peroxidation through reaction with thiobarbituric acid (TBA). Briefly, the reaction mixture containing 200 µL of supernatant from the brain structure or standard homogenate (0.03mMMDA), 1 ml of 0.2 M orthophosphoric acid, and 250 µL thiobarbituric (0.1 M) was heated to 95 ° C for 120 min. Absorbance was measured at 532 nm. Serum TBARS levels are expressed in nmol MDA/mg protein.

Protein carbonyl levels

Protein carbonyl was determined by the method of LEVINE *et al.* (1990) and modified by REZNICK e PACKER (1994) and LIEBEL *et al.* (2011). A medium containing 2,4-dinitrophenylhydrazine (DNPH) 10 mmol and hydrochloric acid (HCl) was added to the protein precipitate and incubated at room temperature for one h. During the incubation, samples of the supernatant from the brain structure homogenate were mixed vigorously every 15 min. Then, 500 µL of denaturation buffer (3% sodium dodecyl sulfate (SDS) plus 2000 µL of ethanol and 2000 µL of heptane were added. Resuspended in 1000 µL of denaturation buffer and placed in the maria for about 20 minutes (40 or 50 ° C) until the pellets are dissolved. The reading was performed at 370nm on the UV-VIS spectrophotometer. The results are expressed as nmol/mg of protein.

Determination of total thiols (T-SH) and reduced glutathione (GSH)

The total number of thiol groups was analyzed spectrophotometrically using the method of ELLMAN (1959) and BOYNE e ELLMAN (1972), with some modifications. A 200 µL aliquot of the brain structures homogenate supernatant in a final volume of 900 µL of the solution was used for the reaction. The reaction product was measured at 412 nm after adding 50 µL of 10 mM 5-5-dithiobis (2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and it will be expressed as nmol of T-SH/ml of serum. GSH was measured spectrophotometrically with Ellman's reagent. An aliquot of 200 µL of serum in a final volume of 900 µL of the solution was used for the reaction. The reaction product was measured at 412 nm after adding 50µL of 5-5-dithiobis (2nitrobenzoic acid) (DTNB). A standard curve using cysteine was added to calculate the content of non-protein thiol groups in samples and expressed as nmol of GSH serum/ml.

Flow cytometry analysis of neural marker proteins and phosphorylated proteins

Flow cytometry experiments for measurement of p-Tau and Iba-1 were performed as previously described (PILLAT *et al.*, 2016). Briefly, cells from hippocampus were fixed for 10 min by adding 4% PFA. Primary staining was performed with monoclonal antibodies against the phosphorylated Tau (1:200; Sigma-Aldrich) and Iba-1 (1:200; Wako) for 30 min followed by addition of secondary Alexa-Fluor-488 antibodies (1:500; Life Technologies). The measurements were performed on a Calibur Cytometer (BD Biosciences) and analyzed with Flowjo V10 software (Flowjo, Ashland, OR). The results are expressed as percentage (%) of positive cells.

Statistical analysis

All data were analyzed using two-way ANOVA followed by Tukey's post hoc test in a statistical program (GraphPad Prism 8). The data were expressed as mean ± SEM, and a statistically significant difference was considered $p < 0.05$.

RESULTS

LPS promotes a reduction in body weight after the first application

The activation of the innate immune system by bacterial products such as LPS induces a group of symptoms known as sickness behavior, which includes, among others, lethargy or immobility, drowsiness, and reduced consumption of water and food (DANTZER *et al.*, 2008; GIBB *et al.*, 2009). Thus, to assess the systemic effects of LPS or EA, the bodyweight of the rats was measured (Fig. 2). Thus, a slight reduction in mean body weight was observed in the groups that received IP injection of LPS (LPS+SAL) on the second day of the experimental period, with a significant reduction ($p < 0.05$) in the bodyweight of the animals in the LPS+SAL group on days 3-5 when compared to the control groups (CTRL+SAL and CTRL+EA). The animals in the present study showed a gradual increase in body weight during the experimental period. This fact was attributed to the growth phase of the animals.

LPS and EA did not alter locomotor activity

In this experiment, the effects of repeated applications of LPS were evaluated, as well as the treatment with EA on the locomotor activity of the rats in an open field test, since the memory test can be affected by locomotor changes. There were no significant differences ($p < 0.05$) between groups in the total distance covered; mobile or immobile time; time in the central zones, walls or corners; and number of entrances or exits in the central zones, walls or corners indicating that the compounds did not promote changes in the animals' locomotor activity and, therefore, the results observed in the memory recognition test are not related to locomotor impairment (Fig. 3).

Ellagic acid reverses cognitive impairment induced by LPS

Knowing that systemic applications of LPS promote cognitive impairment through several pathways (HOOGLAND *et al.*, 2015) and to assess whether the EA has a neuroprotective effect, the object recognition test was performed. A significant reduction ($p < 0.05$) in the preference index of the new object was observed in 2 hours (short term memory) and 24 hours (long term memory) in the group that received multiple applications (IP) of LPS when compared to the control group. However, the group treated with 100 mg/kg of EA demonstrated a significant improvement in memory retention when compared to the LPS group in both short- and long-term memories, indicating that treatment with EA prevents cognitive impairments induced by LPS. Also, there was no significant difference ($p < 0.05$) between groups in the exploration time of both objects during the training phase, 2 hours, and 24 hours (Fig. 4).

EA prevents LPS-induced increased AChE activityc

AChE hydrolyzes acetylcholine (ACh), which is involved in the processes of memory and learning (SKALICKA-WOZNIAK *et al.*, 2018). Also, ACh is known to inhibit the production of pro-inflammatory cytokines produced by macrophages (BOROVIKOVA *et al.*, 2000) and microglia (SHYTLE *et al.*, 2004). In this context, there was a significant increase ($p < 0.05$) in AChE activity in the cerebral cortex (CO) and hippocampus (HP) in the LPS group when compared to the control group. In contrast, treatment with EA in the LPS group (LPS+EA) was able to prevent an increase in the activity of this enzyme (Fig. 5).

EA prevents LPS-induced increased oxidative damage

Oxidative stress is defined as the imbalance between the production of ROS and its elimination by protective mechanisms, which culminates in chronic inflammation (HUSSAIN *et al.*, 2016). Also, oxidative stress in the brain culminates in several deleterious effects that negatively affect brain functions. Knowing the potential of LPS to promote higher production of free radicals in the nervous system, we seek to assess whether EA has the potential to reverse these deleterious effects (SALIM, 2017). In this context, there was a significant increase ($p < 0.05$) in ROS levels in the cerebral cortex (CO) (Fig. 6A) and hippocampus (HP) (Fig. 6B) in the LPS group compared to group control. As a consequence of the increased production of these reactive species, it was also possible to observe a significant increase ($p < 0.05$) in lipid peroxidation, demonstrated by the high levels of substances reactive to thiobarbituric acid (TBARS) (Fig. 6C and 6D), and protein damage, evidenced by the elevation of the protein carbonyl in CO and HP (Fig. 6E and 6F). On the other hand, compared to the LPS group, the treatment with EA (LPS+EA) was able to inhibit the oxidative damage caused by ROS in CO and HP, as evidenced by Figs 6A-F.

EA prevents LPS-induced depletion of total (T-SH) and non-protein (GSH) thiols

The antioxidant system plays a crucial role in maintaining the redox balance in the brain. The thioredoxin and glutathione systems are active in several brain regions and are considered critical antioxidant defense mechanisms in the central nervous system (REN *et al.*, 2017). Since we observed a reduction in the production of ROS and related damages after treatment with EA in the group that received LPS (LPS+EA), we evaluated the levels of antioxidants to better understand the mechanisms involved in the neuroprotection performed by EA. Thus, a significant ($p < 0.05$) reduction in the levels of total thiols (T-SH) and non-protein thiols (GSH) levels was observed in CO and HP in the LPS+SAL group when compared to the CTRL+SAL group. However, treatment with EA (LPS+EA) was able to prevent the reduction of T-SH and GSH in both brain structures when compared to the LPS+SAL group (Fig. 7).

EA inhibits LPS-induced neuroinflammation

Astrocytes and microglia actively modulate neuronal activity and brain functions. The increase in the frequency of Iba-1 positive (Iba-1^{+}) and GFAP positive ($GFAP^{+}$) cells in the

hippocampus indicates, respectively, proliferation of microglia and astrocytes resultant from an inflammatory process, which, when stimulated in excess, may enhance the inflammatory effect, resulting in pathogenesis by the secretion of several pro-inflammatory mediators (HOOGLAND *et al.*, 2015; SIRACUSA *et al.*, 2019). In this context, a significant increase ($p < 0.05$) was observed in the percentage of Iba-1^+ and $GFAP^+$ cells in the LPS+SAL group compared to the control group (CTRL+SAL) (Fig. 8). In contrast, the groups treated with EA (CTRL+EA and LPS+EA) had a low frequency of glial cells when compared to the LPS group (LPS+SAL), suggesting that this compound inhibits the neuroinflammatory process triggered by LPS.

EA suppresses LPS-induced phosphorylation of tau protein (P-Tau)

The tau protein (Tau) is responsible for stabilizing microtubules in neurons. Abnormal forms of the Tau caused by hyperphosphorylation (P-Tau) alters the stabilization of microtubes, which impairs the shape and functionality of neurons, resulting in long-term memory loss and cognitive disorders (BARANOWSKA-WOJCIK; SZWAJGIER, 2020). In the present study, a significant ($p < 0.05$) reduction in the percentage of P-Tau^+ cells were observed in the groups treated with EA (CTRL+EA and LPS+EA), indicating a neuroprotective effect of this compound. Although there is no statistically significant difference between the control and untreated LPS groups (CTRL+SAL and LPS+SAL), there is an increase in the frequency of P-Tau^+ in the LPS+SAL group (Fig. 9).

DISCUSSION

This study aimed to demonstrate the effects of EA on LPS-induced neuroinflammation through memory-related assessments, such as object recognition test and AChE activity. The percentage of Iba-1^+, $GFAP^+$, and p-Tau^+ cells was quantified to evaluate the neuroinflammatory effect, the redox profile was assessed by ROS generation, lipid peroxidation and protein carbonylation, as well as levels of non-enzymatic antioxidants. Also, the effects of multiple LPS applications on the animals' body weight and locomotor activity, assessed through the open field test, were evaluated. The results of this study demonstrated that EA was able to prevent cognitive impairment caused by multiple applications of LPS, as well as modulate the immune system response by significantly reducing the expression of glial cells, attenuating oxidative damage caused by the action of endotoxins.

The animals in the present study showed a reduction in body weight from the first application of LPS (LPS+SAL and LPS+EA), becoming significant ($p < 0.05$) on day 3 in the LPS+SAL group. From the fourth day on, there was a gradual increase in the body weight of animals in the LPS groups (LPS+SAL and LPS+EA). Also, no statistically significant differences were observed in the open field test, performed on the sixth day of the experimental period. Corroborating with the results obtained by other authors (ENGELAND *et al.*, 2003), which performed an IP application of LPS (100 or 200 mg/kg) on days 1, 4, and 7 in female and male rats and evaluated locomotor activity, body weight, and hormone levels. The authors reported a reduction in locomotor activity and in the body weight of the animals after the first application of LPS. In contrast, there was a reduction in the deleterious behavioral effects of LPS after a second exposure to LPS in male and female rats, being more evident in females. After the third administration of LPS, no behavioral changes were observed. The authors attributed the findings to the mechanism of tolerance to LPS, which after multiple sublethal injections, results in less responsiveness to the compound and, consequently, higher survivability to the subsequent lethal dose of endotoxins. This low responsiveness has been called tolerance (CROSS, 2002; LIU *et al.*, 2017; WEST; HEAGY, 2002) and comprises an adaptation of the organism to limit excessive inflammation, trough less production of pro-inflammatory cytokines (LIU *et al.*, 2017). Consequently, there is a reduction in sickness behavior, since this mechanism is mediated mainly by the action of macrophages and cytokines on the periphery, as well as mechanisms of transduction of inflammation from the periphery to the brain (CLARK *et al.*, 2015). Thus, it is suggested that the weight gain observed from the third day of the experimental period is a consequence of the inhibition of sickness behavior, which possibly resulted in higher food and water intake by the groups treated with LPS (LPS+SAL and LPS+AND THE). The same can be attributed to the absence of changes in the locomotor activity of the animals, evidenced by the open field test.

Although the effect of tolerance to multiple IP applications of LPS has been well described in the literature (SEELEY; GHOSH, 2017), several authors have reported cognitive impairment (JI *et al.*, 2020; KHAN *et al.*, 2019; LEE *et al.*, 2020; WANG *et al.*, 2020) and elevation in pro-inflammatory cytokines in the central nervous system. Chen et al. (CHEN *et al.*, 2005) demonstrated, after multiple applications of LPS, that the expression of cytokines in response to this endotoxin can be regulated in different ways between the peripheral immune system and the CNS. The increase in the production of pro-inflammatory cytokines is associated with an increase in the activation of microglia and astrocytes (HOOGLAND *et al.*, 2015). Usually, microglia cells act phagocyting dead cells and cellular debris to maintain CNS

homeostasis, while astrocytes are responsible for preserving neurological function (ALMAD; MARAGAKIS, 2018). However, when stimulated in excess, microglia and astrocytes significantly increase neuroinflammation, resulting in pathogenesis by the secretion of several pro-inflammatory mediators (ALMAD; MARAGAKIS, 2018; BAUER *et al.*, 2001; YANGUAS-CASAS *et al.*, 2014).

In the present study, a significant increase in the percentage of positive glial cells (Iba-1^+ and $GFAP^+$) was observed in the LPS+SAL group. These findings can be attributed to the action of LPS, a potent stimulator of microglia and astrocyte activation that can cause harmful neuroinflammatory responses through the production of TNF-α, IL-6, IL-1β, iNOS and COX-2 (LONG-SMITH *et al.*, 2009; LULL; BLOCK, 2010). In contrast, in the group treated with EA (LPS+EA), less expression of Iba-1^+ and $GFAP^+$ cells were observed. These results are in agreement with that described by other authors (ROJANATHAMMANEE *et al.*, 2013), who observed that the EA is able to inhibit microglial activation via attenuation of Nuclear factor of activated T-cells (NFAT) activity. Still, it is believed that polyphenols acts extracellularly by capturing cytokines to attenuate the stimulation of glial cells, thus exerting their anti-inflammatory function (HOLLEBEECK *et al.*, 2012). Thus, an anti-inflammatory effect of EA was observed, since this antioxidant reduced the expression of Iba-1^+ and $GFAP^+$ cells in the hippocampus of the LPS+EA group rats, which suggests that this compound can mitigate the deleterious effects observed in neurodegenerative disorders.

As previously described, the activation of microglia and astrocytes results in the cerebral release of cytokines. These pro-inflammatory interleukins directly affect neuronal function, such as long-term potentiation (LTP), glutamate release, AMPA receptor trafficking, and activation of cell-signaling pathways (BEATTIE *et al.*, 2002; LYNCH *et al.*, 2004; VEREKER *et al.*, 2000), which are related to synaptic plasticity and neurotransmission. Therefore, there may be impairment of neuronal processes related to cognition.

In the present study, the animals in the LPS+SAL group showed significantly lower performance in object recognition in the short- and long-term memory tests when compared to the other groups. This cognitive impairment is due to the high density of receptors for cytokines in the hippocampus, particularly in the dentate gyrus (SCHOBITZ *et al.*, 1992), indicating that this structure may be particularly vulnerable during neuroinflammation (CZERNIAWSKI *et al.*, 2015). Consequently, the administration of immunogenic stimuli, such as LPS, can compromise hippocampus-dependent memory and learning processes (BARRIENTOS *et al.*, 2002). In contrast, there was a protective effect of EA in the short and long-term memory test, in which the LPS+EA group had a significantly higher performance than the LPS+SAL group.

Several authors have reported the beneficial effects of EA on memory in models of cognitive impairment (DOLATSHAHI *et al.*, 2015; FARBOOD *et al.*, 2015b; MANSOURI *et al.*, 2016; MASHHADIZADEH, S. *et al.*, 2017), which occurs from the action of this antioxidant at the molecular level through the attenuation of oxidative stress, reduced AChE activity and modulation of the pathway of nuclear factor kappa B (NF-κB), nuclear factor erythroid 2–related factor 2 (Nfr2) and Toll-like receptor (TLR4) signaling, which are related to the neuroinflammation mechanism induced by LPS. This endotoxin binds to TLR4 on the surface of the microglia. It activates several transduction pathways, which result in the activation of NF-κB, which will mediate the production of pro-inflammatory cytokines, chemokines and inducible enzymes, such as inducible synthase oxide (iNOS) and COX-2, culminating in neuroinflammation (GLASS *et al.*, 2010; PARK *et al.*, 2011), as observed by the increased expression of positive glial cells (Iba-1^{+} and GFAP^{+}) in the LPS+SAL group. These findings demonstrate the potential of EA to reverse cognitive impairments secondary to neuroinflammatory processes. This hypothesis is supported by the reduction in the expression of positive glial cells observed in the LPS+EA group observed in the present study and improved performance in the object recognition test compared to the untreated group (LPS+SAL).

Also, the cognitive impairment produced by systemic administration of LPS may be involved with the dysregulation of the cholinergic system, evidenced by the reduction in levels of acetylcholine (Ach), a neurotransmitter involved in the processes of memory and learning (HOUDEK *et al.*, 2014; MING *et al.*, 2015b). Previous studies have shown that LPS causes depletion in brain ACh levels as a consequence of inducing AChE activity (EDUVIERE *et al.*, 2016; MING *et al.*, 2015b; TYAGI *et al.*, 2008), which degrades ACh. Also, the expression of AChE increases in response to IL-1 (LI *et al.*, 2000) and oxidative stress (BOND; GREENFIELD, 2007; BOND *et al.*, 2006) induced by LPS. This pattern was observed in the present study, in which the animals that received LPS (LPS+SAL) showed a significant increase in AChE activity compared to the animals in the control group (CTRL+SAL). In contrast, the increased AChE activity was prevented in animals treated with EA (LPS+EA). It is believed that this prevention occurs through changes in the gene expression profile involved in the synthesis of AChE (JHA *et al.*, 2018). These results corroborate with previous studies (JHA *et al.*, 2018; KIASALARI *et al.*, 2017). Thus, it is suggested that the improvement in cognitive performance may also be related to the reduced activity of AChE in the LPS+EA group compared to the LPS+SAL group since the reduction in the activity of this enzyme promotes an increase in the concentration of ACh. This hypothesis is supported by studies that have

observed that AChE inhibition promotes learning and memory improvement in animals (JHA *et al.*, 2018; PEPEU; GIOVANNINI, 2010).

Several authors have documented the relationship between oxidative stress and inflammation. Inflammation induces oxidative stress and DNA damage, which triggers an exacerbated production of ROS by microglia and macrophages. Damage from oxidative stress, such as oxidized proteins, glycated products, and lipid peroxidation, results in neuronal degeneration frequently reported in brain disorders (POPA-WAGNER *et al.*, 2013). Cells damaged by oxidative damage produce a large number of inflammatory mediators that promote the aging of the microglia (WU *et al.*, 2016). In addition to the oxidative damage of ROS in macromolecules, these reactive species can also trigger inflammatory responses by stimulating several genes that regulate the inflammatory signaling cascade. Acute and chronic inflammation and aging processes are the primary triggers for excessive ROS production.

We observed significantly high levels of ROS, TBARS, and protein carbonylation (carbonyl) in the cerebral cortex and hippocampus in the LPS+SAL group compared to the CTRL+SAL group. Studies have shown that LPS activates astrocytes and microglia that secrete gliotransmitters, such as glutamate and adenosine triphosphate (ATP), which play the role of substrate for the production of extracellular adenosine and neurotoxic molecules, such as free radicals (GAO *et al.*, 2002; QIN *et al.*, 2004), which justifies the results found by our group, since there was an increase in the expression of positive glial cells in the LPS+SAL group as previously described. Furthermore, there was a depletion of the intracellular antioxidant system, demonstrated by the significant reduction in the levels of GSH and T-SH in the cerebral cortex and hippocampus of the LPS+SAL group compared to the CTRL+SAL group. These results suggest exhaustion of the antioxidant system, due to the progression of the inflammatory reaction, which may contribute to the neurodegeneration process (HALLIWELL, 2006). In contrast, the EA promoted a reduction in oxidative parameters (ROS, TBARS, and carbonyl) in the cerebral cortex and hippocampus through its antioxidant action, which occurs due to its direct property of free radical scavenging (COZZI *et al.*, 1995). The hydroxyl group and the lactone ring present in the EA directly detoxify superoxide, hydroxyl radicals, hydrogen peroxide, and peroxynitrite (GARCIA-NINO; ZAZUETA, 2015). Furthermore, this compound has a potentiation effect of endogenous antioxidants such as GSH, SOD, catalase, glutathione reductase and glutathione peroxidase (COZZI *et al.*, 1995), which can be evidenced by the significant increase in the levels of GSH and T-SH in the cerebral cortex and hippocampus in the LPS+ EA group compared to the LPS+SAL group. Herewith, we can relate the neuroprotective effects of EA to its anti-inflammatory potential by reducing the expression of

positive glial cells and its antioxidant properties, as evidenced by the increase in the antioxidant system and consequent reduction in the generation of ROS and its by-products.

A recent study has shown that synaptic pathologies and microgliosis may be the initial manifestations of neurodegeneration related to tauopathies. Furthermore, the authors observed that the prominent activation of the microglia precedes the formation of neurofibrillary tangles, and the immunosuppression of the animals reduced the pathology related to Tau and increased the life expectancy of the animals. The causal relationship between Tau phosphorylation and neuronal dysfunction is not well established, but there are two main hypotheses: the loss of function may be caused by a reduction in the binding of Tau to microtubules (MT), resulting in destabilization of TM and transport disruption axonal; Hyperphosphorylated Tau results in aggregation and toxic effects on neuronal cells. Studies in transgenic mices have indicated that neuronal loss and impairment in memory are associated with the presence of soluble and highly phosphorylated Tau (oligomers), and suppression of its expression causes improved memory and increased number of synaptic connections (ROBERSON *et al.*, 2011; SANTACRUZ *et al.*, 2005; SYDOW *et al.*, 2011). Thus, it was concluded that neuroinflammation is related to the early progression of tauopathies.

In this context, in the present study, a significant reduction in the percentage of p-Tau^{+} cells were observed in the groups that received EA (CTRL+EA and LPS+EA) when compared to the LPS+SAL group. Zhong et al. (ZHONG *et al.*, 2018) demonstrated that the potential of EA to inhibit hyperphosphorylation of Tau is related to the reduction in the activity of glycogen synthase kinase 3β (GSK3β), which is involved in the phosphorylation of Tau. However, the authors point out that several other kinases may be involved in this mechanism. These results demonstrate the potential of EA to reduce the deleterious effects caused by the hyperphosphorylation of Tau, which includes the formation of neurofibrillary tangles with consequent cognitive impairment.

The results of this study demonstrated that EA was able to prevent cognitive impairment caused by multiple applications of LPS, as well as, modulate the immune system response by significantly reducing the expression of glial cells and phosphorylated Tau, attenuating oxidative damage caused by the action of endotoxins and prevent the increase in AChE activity. Thus, this study demonstrated the beneficial effects of EA on memory, neuroinflammation, and restoring redox balance. These effects are the consequence of the anti-inflammatory and antioxidant action of this compound. With these results, the therapeutic potential of EA in cognitive disorders secondary to neuroinflammation was demonstrated.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support given by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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FIGURE CAPTIONS

Fig. 1 Experimental protocol

Fig. 2 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the weight (A) and body temperature (B) of rats. The data are expressed as mean of the weights ± SEM N = 8 animals/group. # Significant difference ($p < 0.05$) compared to the groups CTRL+SAL and CTRL+EA; * Significant difference ($p < 0.05$) compared to the CTRL+SAL group

Fig. 3 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the locomotor activity of rats. The behavioral test was performed two hours after treatment (P.O.) with EA 100 mg/kg or saline, which occurred one hour after IP injection of LPS 250 µg/kg or saline. Data are expressed as mean ± SEM N = 8 animals/group. There were no statistically significant differences ($p < 0.05$) between groups

Fig. 4 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the short- and long-term memory of rats submitted to the memory recognition test. The results are expressed as% of the exploration time of the new object (percentage of time = new object/[new object+familiar object] x100) ± SEM (A) and total exploration time of both objects (total time = new object+familiar object) ± SEM (B). N = 8 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups

Fig. 5 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the activity of AChE in the cortex and hippocampus of rats. Data are expressed as mean ± SEM N = 8 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups Fig. 5 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the levels of reactive oxygen species (ROS), lipid peroxidation (TBARS) and protein carbonylation in the cerebral cortex (CO) and hippocampus (HP) of rats. Data are expressed as mean ± SEM N = 8 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups Fig. 4 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the activity of AChE in the cortex and hippocampus of rats. Data are expressed as mean ± SEM N

= 8 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups

Fig. 6 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the levels of reactive oxygen species (ROS), lipid peroxidation (TBARS) and protein carbonylation in the cerebral cortex (CO) and hippocampus (HP) of rats. Data are expressed as mean ± SEM N = 8 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups

Fig. 7 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the levels of total unions (T-SH) and non-protein unions (GSH) in the cerebral cortex (CO) and rat hippocampus (HP). Data are expressed as mean ± SEM N = 8 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups

Fig. 8 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the expression of positive GFAP (A) and positive Iba-1 cells (B) in rat hippocampus. Data are expressed as mean ± SEM N = 5 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups

Fig. 9 Effect of multiple applications (IP) of LPS 250 µg/kg and treatment (P.O.) with EA 100 mg/kg on the expression of positive P-Tau cells in the hippocampus of rats. Data are expressed as mean ± SEM N = 5 animals/group. Different letters indicate a statistically significant difference ($p < 0.05$) between groups

FIGURES

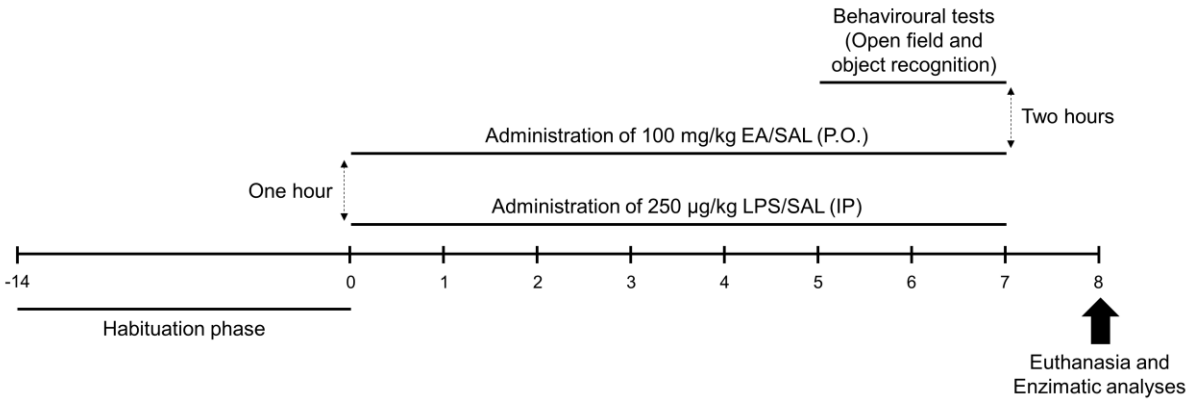

Fig. 1

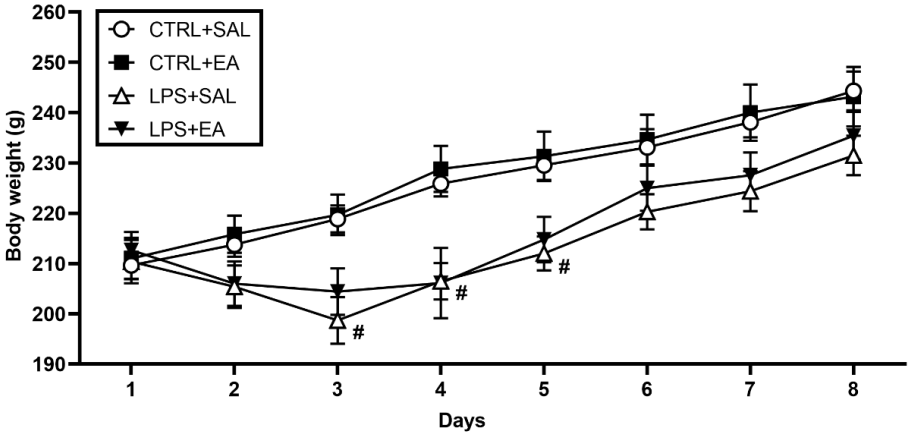
CTRL+SAL
CTRL+EA
LPS+SAL
LPS+EA
Body weight (g)
260
250
240
230
220
210
200
190
1
2
3
4
5
6
7
8
Days
#
#
#

Fig. 2

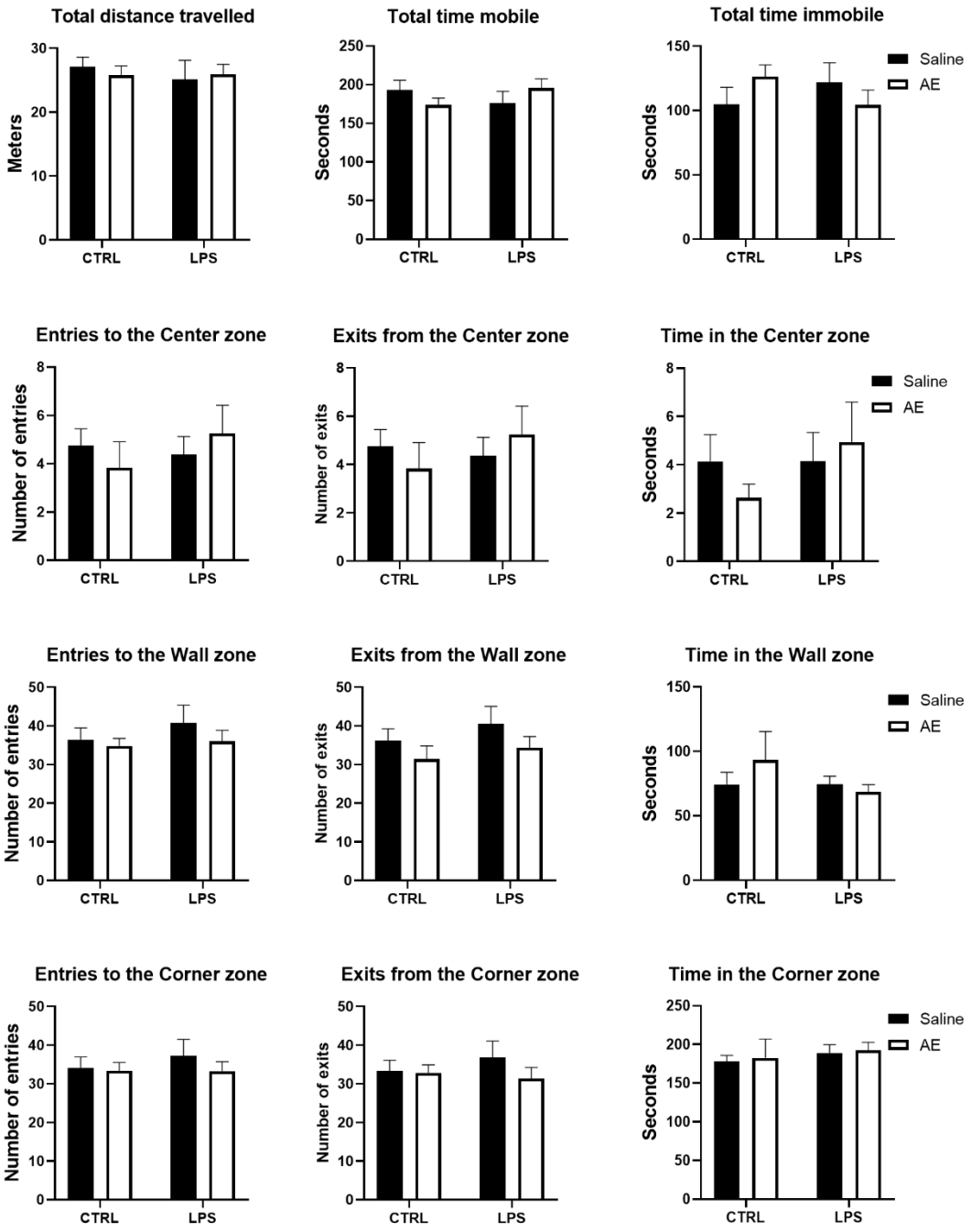
Total distance travelled
Meters
30
20
10
0
CTRL
LPS
Total time mobile
Seconds
250
200
150
100
50
0
CTRL
LPS
Total time immobile
Seconds
150
100
50
0
CTRL
LPS
Saline
AE
Entries to the Center zone
Number of entries
8
6
4
2
0
CTRL
LPS
Exits from the Center zone
Number of exits
8
6
4
2
0
CTRL
LPS
Time in the Center zone
Seconds
8
6
4
2
0
CTRL
LPS
Saline
AE
Entries to the Wall zone
Number of entries
50
40
30
20
10
0
CTRL
LPS
Exits from the Wall zone
Number of exits
50
40
30
20
10
0
CTRL
LPS
Time in the Wall zone
Seconds
150
100
50
0
CTRL
LPS
Saline
AE
Entries to the Corner zone
Number of entries
50
40
30
20
10
0
CTRL
LPS
Exits from the Corner zone
Number of exits
50
40
30
20
10
0
CTRL
LPS
Time in the Corner zone
Seconds
250
200
150
100
50
0
CTRL
LPS
Saline
AE

Fig. 3

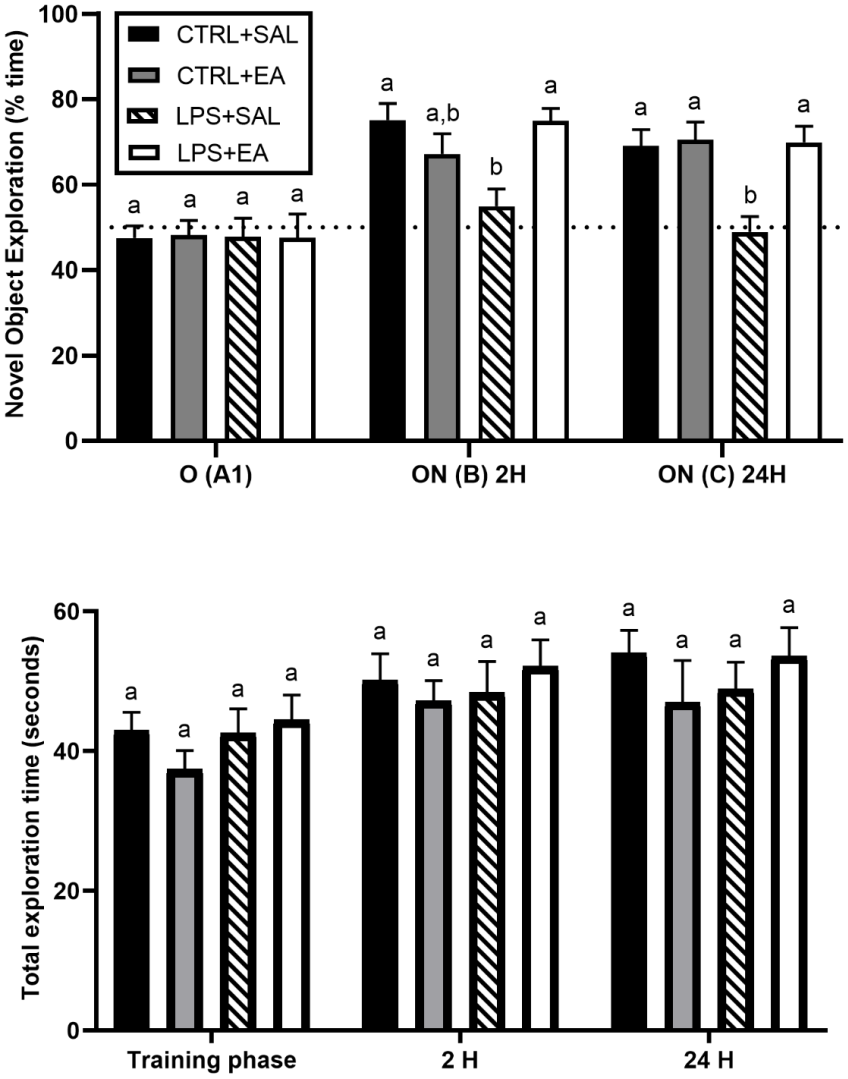
Novel Object Exploration (% time)
100
80
60
40
20
0
CTRL+SAL
CTRL+EA
LPS+SAL
LPS+EA
a
a
a
a
a
a,b
b
a
a
a
b
a
O (A1)
ON (B) 2H
ON (C) 24H
Total exploration time (seconds)
60
40
20
0
a
a
a
a
a
a
a
a
a
a
a
a
Training phase
2 H
24 H

Fig. 4

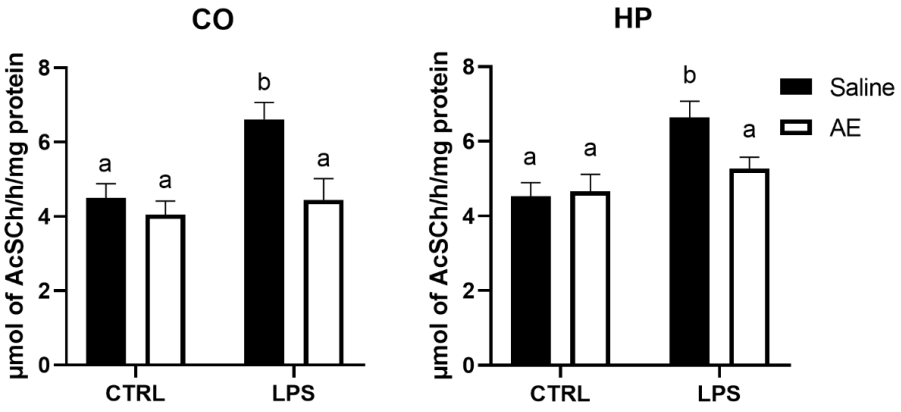

Fig. 5

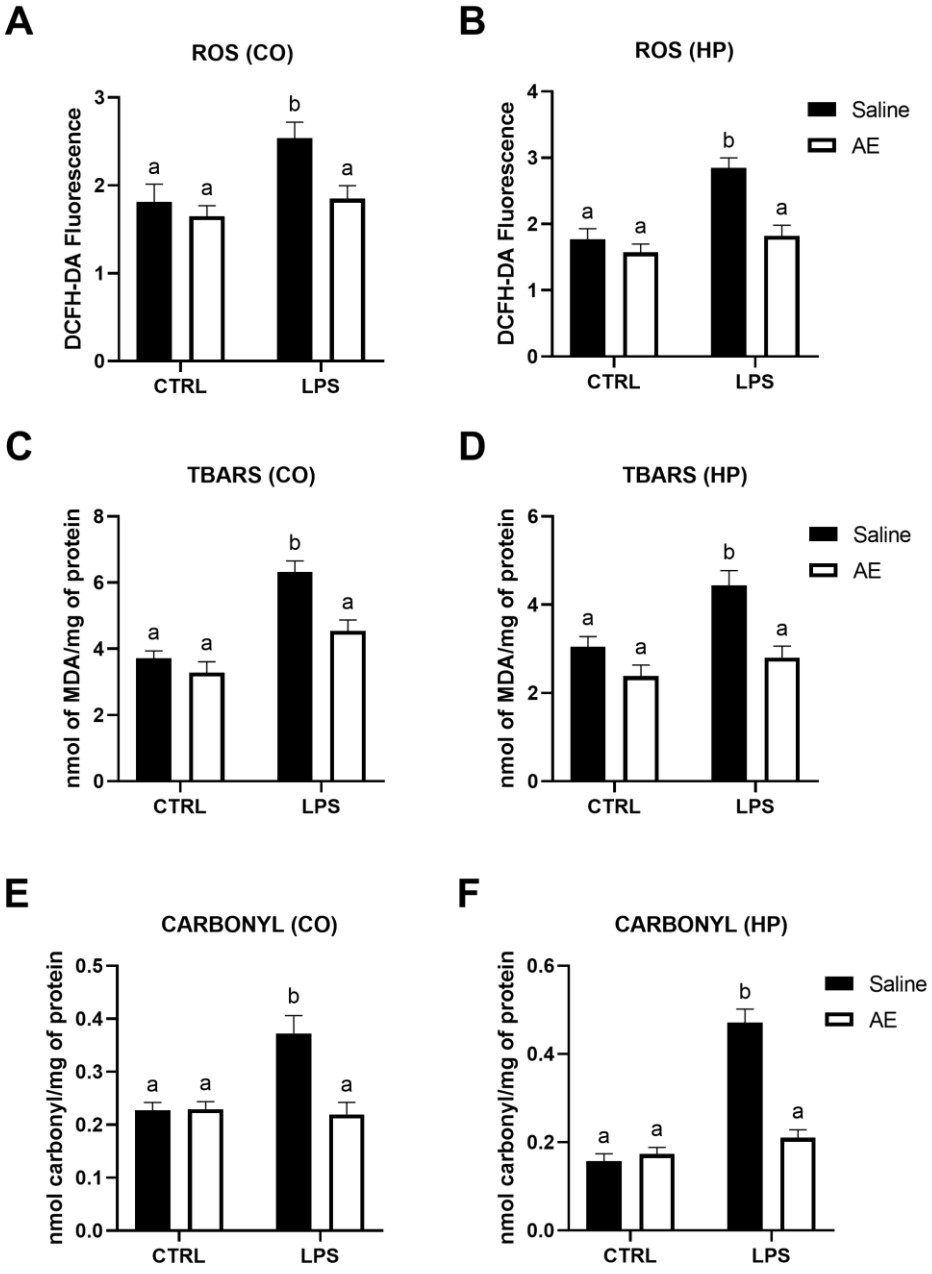
A
ROS (CO)
DCFH-DA Fluorescence
CTRL
LPS
B
ROS (HP)
Saline
AE
C
TBARS (CO)
nmol of MDA/mg of protein
D
TBARS (HP)
E
CARBONYL (CO)
nmol carbonyl/mg of protein
F
CARBONYL (HP)

Fig. 6

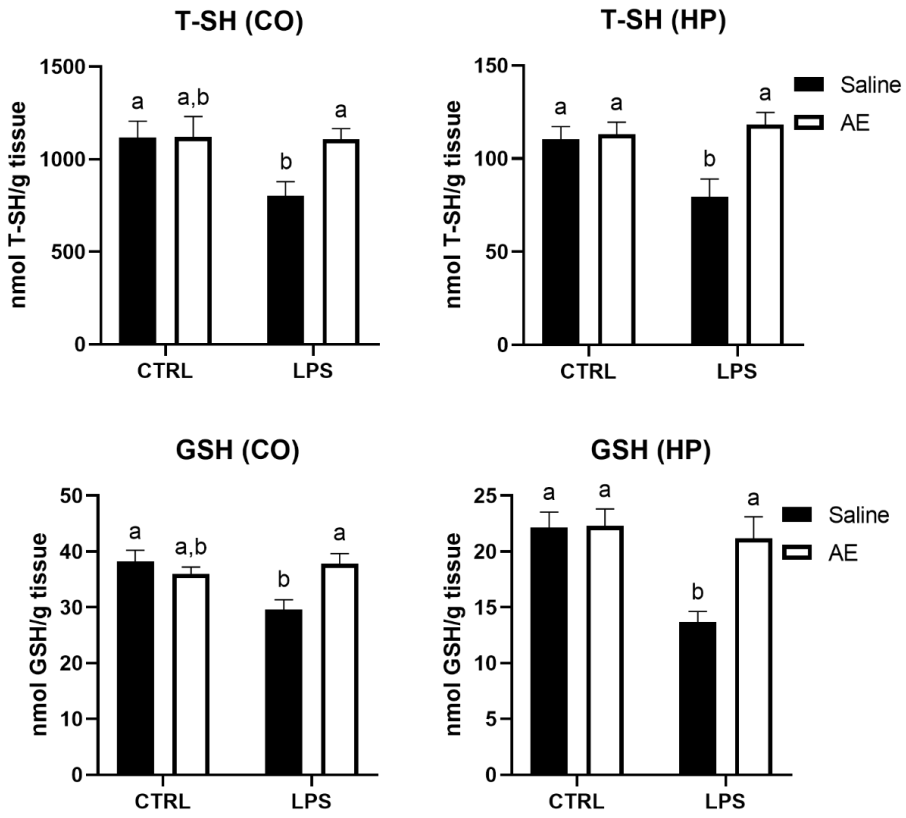
T-SH (CO)
T-SH (HP)
GSH (CO)
GSH (HP)
nmol T-SH/g tissue
nmol GSH/g tissue
CTRL
LPS
Saline
AE
a
a,b
b

Fig. 7

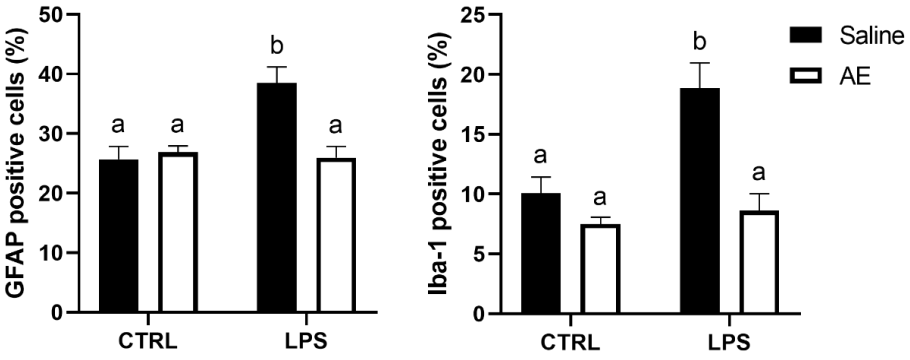
GFAP positive cells (%)
50
40
30
20
10
0
a
a
b
a
CTRL
LPS
Iba-1 positive cells (%)
25
20
15
10
5
0
a
a
b
a
CTRL
LPS
Saline
AE

Fig. 8

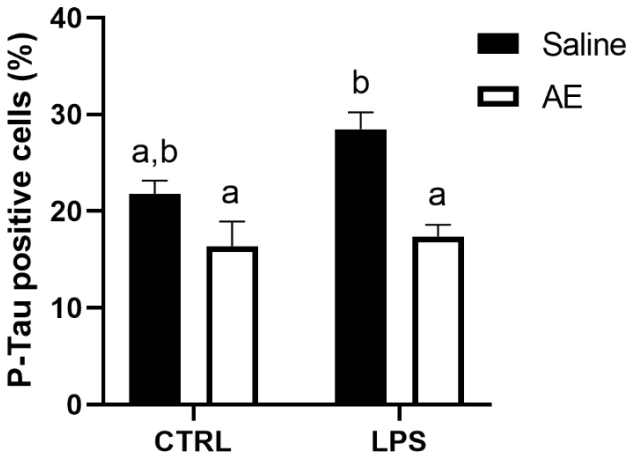

Fig. 9

3 CAPÍTULO II – MANUSCRITO II – HESPERIDIN AS AN ADJUVANT IN THE TREATMENT OF EXPERIMENTAL SPORADIC ALZHEIMER'S DISEASE: EFFECTS ON MEMORY AND OXIDATIVE PARAMETERS

Artigo submetido para: *Metabolic Brain Disease.*

Hesperidin as an adjuvant in the treatment of experimental sporadic Alzheimer's disease: effects on memory and oxidative parameters

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Abstract

The study aimed to evaluate the effects of hesperidin (HES) and its association with rivastigmine (RIV) on memory and oxidative parameters in a sporadic Alzheimer's Disease (AD) model induced by intracerebroventricular injection of streptozotocin (ICV-STZ). 64 Wistar rats were used for this study, divided into eight groups (n = 8): control (CTRL), RIV, HES, RIV+HES, STZ, STZ+RIV, STZ+HES, and STZ+RIV+HES. The rats received an ICV-STZ injection or saline solution (3 mg/kg) and were treated daily, from the fourth day, with 100 mg/kg of HES orally, for 30 days. At 21 days after ICV-STZ injection, oral treatment was started with 2 mg/kg of RIV that lasted for 13 days. Morris water maze was performed 30 days after the ICV-STZ injection. The levels of reactive oxygen species (ROS), lipid peroxidation (TBARS), glutathione (GSH) and total thiol content (T-SH) were measured in samples of the cerebral cortex and hippocampus, and the activity of myeloperoxidase (MPO) was evaluated in blood plasma samples. The results showed that treatment with HES and/or RIV attenuated the cognitive impairment and promoted an improvement in the antioxidant system, increasing the levels of GSH and T-SH and significantly reducing the levels of ROS, TBARS, and MPO activity of the ICV-STZ rats. Therefore, the results of this study provide a greater understanding of the effects of HES, as well as its association with RIV, suggesting that HES has the potential to be used in addition to conventional therapy in AD.

Keywords: rivastigmine; intracerebroventricular; antioxidant; memory.

Introduction

Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disease, characterized as the leading cause of dementia (YE *et al.*, 2011). People with AD have a progressive loss of cognitive skills, behavioral disorders, and loss of functional autonomy (CAI *et al.*, 2016). Although the cause of AD is not well established, morphologically it is characterized by specific neuropathological changes, such as deposition of senile plaques (SP) followed by neurofibrillary tangles (NFTs), causing neuronal degeneration and synaptic loss (KIM *et al.*, 2014; REITZ *et al.*, 2011). Senile plaques are extracellular deposits of fibrillar and amorphous aggregates of beta-amyloid peptide (βA), while NFTs are intracellular fibrillar aggregates of hyperphosphorylated microtubes associated with the tau protein (MATTSON *et al.*, 2004). The formation of SP and NFTs in brain regions compromises memory and learning

functions. Also, the brain of patients with AD show significant loss of synapses, reactive gliosis, and inflammatory processes (PRATICÒ; TROJANOWSKI, 2000). Genetic and environmental factors are considered to be risk factors for the development of this disease (YE *et al.*, 2011).

Oxidative stress should be highlighted as an essential pathway in the pathology of neurodegenerative disorders. The high consumption of oxygen in the brain (about 20% of oxygen from respiration) compared to other organs, combined with the high lipophilic nature of the brain and its low levels of endogenous antioxidants, leads to the accumulation of reactive oxygen species (ROS) and, thus, to oxidative damage. Also, the high amount of polyunsaturated fatty acids in neuronal membranes makes the brain particularly susceptible to lipid peroxidation. Its by-products can induce neurodegeneration and cell death via apoptotic and necrotic pathways (BHAT *et al.*, 2015). Thus, high oxidative stress and mitochondrial dysfunction result in synaptic and neuronal dysfunction and neurodegeneration (DEMURO *et al.*, 2010; JOMOVA *et al.*, 2010). Lesions in the hippocampus are considered the leading causes of the development of cognitive dysfunction in AD, including impaired memory and learning (FERREIRA-VIEIRA *et al.*, 2016; PLOWEY; ZISKIN, 2016).

Intracerebroventricular administration of streptozotocin (ICV-STZ) has been used as an experimental model of sporadic AD (SALKOVIC-PETRISIC *et al.*, 2013), which corresponds to 95% of AD cases (LECANU; PAPADOPOULOS, 2013). ICV-STZ administration generates a state of insulin resistance that is restricted to the brain. Also, changes in cerebral glucose metabolism, oxidative stress, high amyloidogenesis, hyperphosphorylation of tau protein, accumulation of βA peptides, cholinergic neuronal degeneration, and memory impairments occur (SALKOVIC-PETRISIC *et al.*, 2013).

There is no definitive treatment for AD, as the signaling pathways of this disease are complex, and the initial definitive causes are unknown. Existing therapies promote the improvement of some behavioral symptoms but hardly mitigate cognitive impairments. Still, the drugs available for the treatment of AD are palliative, have numerous adverse effects and high cost, which impairs treatment adherence (ATUKEREN *et al.*, 2017; DANI *et al.*, 2017).

Rivastigmine (RIV) is one of the first-line drugs for the treatment of AD, being classified as a pseudo-irreversible inhibitor of brain acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), selectively inhibiting its activities, consequently increasing the effect of acetylcholine (ACh) to stimulate brain nicotinic and muscarinic receptors (GROSSBERG, 2003; NOETZLI; EAP, 2013). However, several side effects related to the use of this medication are reported, such as gastrointestinal problems, muscle weakness, loss of appetite, weight loss, dizziness, and extrapyramidal symptoms (DIAZ; ROSALES, 2015). For

this reason, it is essential to research alternative treatments capable of alleviating symptoms and cognitive impairments in AD.

In this context, some plants are an essential source for the discovery of new active components for the treatment of various diseases. Studies suggest that photo composites naturally found in fruits, vegetables, and nuts, can potentially delay neurodegeneration, improving memory and cognitive functions (DANI *et al.*, 2017; OMAR *et al.*, 2017). Hesperidin (HES), a flavonoid glycoside commonly found in citrus fruits, has antioxidant, anti-inflammatory, antifungal, antiviral, and anticancer properties (GALATI *et al.*, 1994). Several studies have demonstrated its antioxidant activity and free radical scavenging property in vitro (AGATI *et al.*, 2012; RAMFUL *et al.*, 2010; WILMSEN *et al.*, 2005) and in vivo (ARAFA *et al.*, 2009; CHOI, 2008). It has been reported that flavonoids can transpose and tighten the membrane bilayer, thus reducing the membrane's interaction with Aβ peptides in vitro (TEDESCHI *et al.*, 2010). Moreover, HES has shown an improvement in the use of cerebral glucose and the protection of cortical neurons against neuronal lesions induced by βA (HUANG *et al.*, 2012). Thus, HES has the potential to slow the progression of neurodegenerative diseases (WANG, D. *et al.*, 2014).

Given the limitations of conventional AD treatment and the potential of natural compounds for the treatment of various diseases, the present study aimed to evaluate the effects of HES and its association with RIV on memory and oxidative parameters in ICV-STZ rats. For this, the Morris water maze test, the concentration of reactive oxygen species (ROS), lipid peroxidation (TBARS), glutathione (GSH) and total thiol content (T-SH) were evaluated, as well as the myeloperoxidase activity (MPO).

Matherials and methods

Animals

Sixty-four male Wistar rats with approximately 90 days old and weighing 300-350g, from the Central Bioterium of the Federal University of Santa Maria, were used. The animals remained in an environment with a controlled temperature of 25 ± 2 °C and relative humidity of 45-55% with 12h of light/dark cycle and with free access to food and water. The project was submitted and approved by the Animal Ethics Committee of the Federal University of Santa Maria, under number 1786040216.

Intracerebroventricular injection of streptozotocin

The rats were anesthetized with ketamine and xylazine (0.5 mg/kg), intraperitoneally, and placed in a stereotaxic device. The skull was exposed by an incision in the sagittal midline. Two profiles were drilled through the skull for bilateral placement of a microinjector into the lateral ventricles using the following coordinates according to (PAXINOS; WATSON, 1986): 0.8 mm anterior to posterior to bregma; 1.5 mm lateral to the sagittal suture; and 4.0 mm ventral surface of the brain. Through a hole in the animal's calvaria, a 10 μL Hamilton® 28-gauge syringe attached to the stereotaxic was manually displaced through a piston in each lateral ventricle. The animals in the STZ group received 3mg/kg ICV-STZ. Also, the rats in the control group received ICV the same volume of saline. Immediately after surgery, an opioid analgesic (tramadol 5 mg/kg body weight) was administered subcutaneously every 12 hours for three days.

Hesperidin and rivastigmine

Eight different groups (n = 8) were used for this study: control (CTRL), rivastigmine 2 mg/kg (RIV), hesperidin 100 mg/kg (HES), rivastigmine 2 mg/kg + hesperidin 100 mg/kg (RIV+HES), streptozotocin (STZ), streptozotocin + rivastigmine 2 mg/kg (STZ+RIV), streptozotocin + hesperidin 100 mg/kg (STZ+HES), streptozotocin + rivastigmine + HES 2 mg/kg 100 mg/kg (STZ+RIV+HES). Four days after induction of the sporadic AD experimental model, all rats were treated with saline or HES at a dose of 100 mg/kg dissolved in saline for 30 days. HES and saline were administered orally using the gavage method, in a volume of 1 ml/kg.

Twenty-one days after the ICV-STZ injection, treatment with Rivastigmine at a dose of 2 mg/kg was started for 13 days. The control group received only vehicle (saline). The HES, STZ+HES, and STZ+RIV+HES groups continued to be treated with HES. The solutions were administered orally by the gavage method, in a volume of 1 ml/kg (fig 1).

Morris water maze

Thirty days after the ICV-STZ injection, the Morris water maze test was performed (fig 1). This test is used to assess spatial memory and learning according to the MORRIS (1984) method. The water maze consists of a circular container made of black plastic (150 cm in

diameter x 60 cm high x 30 cm deep) with an automatic heater to keep the water temperature at 25 ± 2 ° C. The water maze is placed in a room with several visual clues outside the labyrinth. The platform is hidden 2 cm below the water level, where it remains during the test days. Rats can climb onto that platform to avoid swimming. The animals were submitted to the test with four trials per day (starting in the north quadrant, after east, south, and, finally, west) during four consecutive days. After the animals found the platform, they remained on it for 40 seconds after each test. When the animal was unable to reach the escape platform within 1 minute, it was manually placed on the platform. The time taken to reach the platform (latency), and the time spent in each quadrant was calculated as the average of the four trials each day.

Blood samples and brain structures preparation

At the end of the behavioral evaluation (fig 1) the rats were anesthetized in an anesthetic chamber with isofluorane. The blood was collected through the intracardiac route and stored in tubes containing ethylenediaminetetraacetic acid (EDTA), which were centrifuged at 1800 x g for 10 minutes to obtain the plasma and later evaluate the MPO activity.

After blood collection, the animals were euthanized. The brain was removed, and the cerebral cortex and hippocampus were separated and stored separately in a 10 mM Tris-HCl (pH 7.4) solution under the ice. The brain structures were homogenized separately in a glass Potter. The supernatants resulting from the homogenates of the brain structures (S1) were separated through centrifugation at 3,550 RPM for 10 min and stored at -20 ℃ until assays.

Oxidative stress indicators

Plasma myeloperoxidase activity

MPO activity was analyzed spectrophotometrically by an assay system coupled with modified peroxidase involving phenol, 4-aminoanthypyrine (AAP), and H_2O_2 (METCALF *et al.*). Briefly, 390 ml of AAP, 2.5 mM phenol, and 20 mM were placed in each tube, followed by 450 μL of H_2O_2 (1.7 mM), in the presence of H_2O_2 as an oxidizing agent, catalyzed by MPO the oxidative coupling of phenol and AAP obtaining a colored product, quinoneimine, with a maximum absorbance at 500 nm. The results were expressed in micromolar of the quinone imine produced in 30 min.

Reactive oxygen species (ROS) levels

The fluorescence assay with 2'-7'-dichlorofluorescein was used to measure the production of hydrogen peroxide and other reactive species (MYHRE *et al.*, 2003). 50 μL aliquots of S1 were added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and DCFH-DA 2'-7'-dichlorofluorescein-diacetate (1 mM). After adding DCFH-DA, the medium was incubated in the dark for one hour until fluorescence measurement (excitation at 488 nm and emission at 525 nm, with both slit widths at 1.5 nM). Dichloro-oxidized fluorescein was determined using an oxidized dichlorofluorescein standard curve, and the results were expressed as DCFH-DA Fluorescence.

Lipid peroxidation

The levels of thiobarbituric acid reactive substances (TBARS) were determined according to JENTZSCH *et al.* (1996), by measuring the concentration of malondialdehyde (MDA) as a product of lipid peroxidation through reaction with thiobarbituric acid (TBA). Briefly, the reaction mixture containing 200 μL of S1 or standard (0.03 mM MDA), 1 ml of 0.2 M orthophosphoric acid, and 250 μL of TBA (0.1 M) was heated to 95 ° C for 120 min. Absorbance was measured at 532 nm. Serum TBARS levels were expressed in nmol MDA/mg protein.

Protein determination

The protein of brain structures was determined through a range varying for each structure: cerebral cortex (0.7 mg/ml) and hippocampus (0.8 mg/ml), as determined by the Coomassie blue method (BRADFORD, 1976).

Determination of total thiols (T-SH) and reduced glutathione (GSH)

The total thiol groups were analyzed spectrophotometrically using the method ofELLMAN (1959) and BOYNE e ELLMAN (1972), with some modifications. A 200 μl aliquot of S1 in a final volume of 900 μL of the solution was used for the reaction. The reaction product was measured at 412 nm after adding 50 μL of 10 mM 5-5-dithiobis (2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was added to calculate the content of thiol groups in samples and will be expressed as nmol of T-SH/ml of serum. GSH was measured

spectrophotometrically with Ellman's reagent. An aliquot of 200 μL of serum in a final volume of 900 μL of the solution was used for the reaction. The reaction product was measured at 412 nm after adding 50 μL of 5-5-dithiobis (10 mM 2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was added to calculate the content of non-protein thiol groups in samples and was expressed as nmol of GSH serum/ml.

Statistical analysis

All data were analyzed by two-way ANOVA, followed by Tuckey's post-hoc test using the GraphPad Prism® 6.01 statistical program. Data were expressed as mean ± standard error, and a statistically significant difference was considered when $p < 0.05$.

Results

Morris water maze

The results are shown in fig 2. During the training phase, all rats learned the location of the platform, as observed by the decrease in the latency period to locate the submerged platform. However, compared to the CTRL group, the STZ and RIV group showed a learning impairment, evidenced by the more significant latency to find the platform. In contrast, in the STZ, STZ+HES, STZ+RIV and STZ+RIV+HES groups, treatment with HES and/or RIV reversed the learning impairment, evidenced by the significant reduction ($p < 0.05$) in the latency period. The association of the compounds (STZ+RIV+HES group) was more effective in reversing the damage to memory when compared to the other STZ groups since the latency of the STZ+RIV+HES group was significantly ($p < 0.05$) lower than the groups STZ+RIV and STZ+HES.

Myeloperoxidase activity

Plasma myeloperoxidase activity is shown in fig 3. The ICV-STZ rats (STZ, STZ+HES, STZ+RIV, and STZ+RIV+HES) showed significantly ($p < 0.05$) higher MPO activity than the CTRL group. Animals that were treated with RIV or RIV associated with HES (STZ+RIV and STZ+RIV+HES) showed a significant reduction ($p < 0.05$) in MPO activity compared to the STZ group. However, in the groups, as mentioned earlier (STZ+RIV and STZ+RIV+HES),

MPO activity was significantly ($p < 0.05$) higher when compared to the group that was treated only with HES (STZ+HES).

Reactive oxygen species

Fig 4 shows the levels of ROS in the cerebral cortex (A) and hippocampus (B). STZ rats showed higher levels ($p < 0.05$) of ROS compared to CTRL groups. Treatment with HES or RIV significantly reduced ($p < 0.05$) ROS levels in the hippocampus and cerebral cortex when compared to the STZ group. The association of treatments (STZ+RIV+HES group) significantly reduced ($p < 0.05$) the levels of ROS in the cerebral cortex, when compared to the STZ+HES group, and in the hippocampus, when compared to the STZ group.

Lipid peroxidation

Fig 5 shows the levels of lipid peroxidation in the cortex (A) and hippocampus (B), respectively. TBARS levels were higher ($p < 0.05$) in the ICV-STZ groups (STZ, STZ+HES, STZ+RIV and STZ+RIV+HES) in both studied structures when compared to the CTRL, RIV and HES groups, except for the STZ+HES group in the cortex (fig 4A). The treatments with HES and/or RIV significantly reduced ($p < 0.05$) the levels of lipid peroxidation when compared to the STZ group. Also, there was a significant reduction ($p < 0.05$) in TBARS levels in the STZ+HES and STZ+HES+RIV group in the hippocampus, and STZ+HES cerebral cortex when compared to other treatments in the ICV-STZ groups (STZ, STZ+HES, STZ+RIV and STZ+RIV+HES).

Reduced glutathione

Figures 6A and 6B demonstrate the levels of GSH activity in the cortex and hippocampus, respectively. GSH levels were significantly ($p < 0.05$) lower in the STZ group compared to the control group. Treatment with RIV or HES showed a significant increase ($p < 0.05$) of GSH in the cortex and hippocampus of rats that received ICV-STZ (STZ+RIV and STZ+HES) compared to the STZ group. In addition, the association of HES and RIV (STZ+RIV+HES) significantly increased the levels of GSH in the cortex, when compared to the STZ group, and hippocampus when compared to the STZ+RIV and STZ+HES groups.

Total thiols

T-SH levels in the cortex and hippocampus are shown in Fig. 7A and B, respectively. T-SH levels in rats in the STZ group were lower ($p < 0.05$) compared to the CTRL group. In contrast, treatment with HES and/or RIV significantly increased the levels of total thiols in the ICV-STZ groups compared to the STZ group. In the cerebral cortex, the association of the two treatments in the STZ+RIV+HES group showed a significant increase ($p < 0.05$) in T-SH levels when compared to the other treatments in the ICV-STZ groups (STZ+RIV and STZ+HES).

Discussion

Although it is difficult to establish an experimental animal model that mimics the development of AD, injections of STZ in rats and mice have been described as an appropriate model for sporadic AD (SALKOVIC-PETRISIC *et al.*, 2013). The ICV-STZ injection possibly desensitizes neuronal insulin receptors, which causes a reduction in cerebral energy metabolism, inhibiting the synthesis of adenosine triphosphate (ATP) and acetyl-CoA, with a consequent deficiency in cholinergic transmission in the brain of rats (SONKUSARE *et al.*, 2005). This model promotes multiple changes similar to those found in AD patients, such as the decrease in cerebral glucose metabolism, oxidative stress, reduction in cholinergic signaling, neuroinflammation, neuronal loss, impaired learning and memory (KAMAT *et al.*, 2016; SALKOVIC-PETRISIC *et al.*, 2013; SALKOVIC-PETRISIC *et al.*, 2014). Thus, it has been shown that the impairment in cholinergic transmission can potentially influence cognitive and behavioral aspects, including information processing in the regions of the hippocampus and cerebral cortex (BENTLEY *et al.*, 2011). Thus, compensatory strategies are sought to increase the synaptic levels of ACh, delaying the effects of AD and thus highlighting the neuroprotective potential of flavonoids, such as HES (ANTUNES *et al.*, 2014; HUANG; MUCKE, 2012). In addition, regarding experimental models of AD, studies have shown improvements in the spatial memory of mice after two weeks of treatment with HES with action on the cholinergic system by modulating the enzyme AChE in the cerebral cortex (JAVED *et al.*, 2015).

In the present study, attenuation of the deleterious effects on memory and learning of ICV-STZ animals treated with RIV and/or HES was observed, according to a reduction in escape latency in repeated tests in the Morris water maze. Among these, the STZ+RIV+HES group stands out, which had lower latency when compared to the other ICV-STZ groups, suggesting that HES can be used as an adjunctive treatment to RIV. These effects on memory

and learning occur through several mechanisms, such as the increase in levels of the brain-derived neurotrophic fator (DONATO *et al.*, 2014; GAUR; KUMAR, 2010). Still, our results are similar to those obtained by other researchers, who demonstrated that HES improved learning and memory in neurodegenerative diseases (KHERADMAND *et al.*, 2018; THENMOZHI *et al.*, 2017). However, animals in the RIV group showed significantly higher latency than the CTRL group. Knowing that the animals in the RIV group did not receive an ICV-STZ injection, it is suggested that the results obtained stem from the inhibitory effects of AChE and BChE by rivastigmine, increasing ACh levels. This hypothesis is supported by studies that demonstrated that high levels of ACh alter memory consolidation in cholinergic infusions in the medial septum after training in rats (BUNCE *et al.*, 2004) and by the use of AChE blocking physostigmine in humans (GAIS; BORN, 2004).

Elevated plasma MPO activity, as observed in the ICV-STZ rats in this study, have been reported in patients with AD, indicating a clear relationship between MPO and neurodegeneration (SCHREITMÜLLER *et al.*, 2013; TZIKAS *et al.*, 2014). Under various neuropathological conditions, substantial amounts of hypochlorous acid (HOCl) is released by the MPO from peripheral leukocytes and micróglia (CHANG *et al.*, 2011) and passively diffuses through the brain parenchyma (BRECKWOLDT *et al.*, 2008). This acid reacts with H_2O_2 (MIYAMOTO *et al.*, 2006) and $O_2^{\bullet-}$ (CANDEIAS *et al.*, 1993) to produce highly reactive ROS (RAY; KATYAL, 2016) and with nitrite forming reactive nitrile chloride (EISERICH *et al.*, 1996), that may contribute to tissue damage. Also, HOCl can significantly inhibit intracellular NAD levels by inhibiting mitochondrial respiration with a consequent decrease in ATP, NAD, and GSH levels (RAY; KATYAL, 2016). MPO and its oxidizing products can promote protein nitrification and lipid peroxidation in AD, contributing to neuronal dysfunction and memory loss (ZHANG *et al.*, 2002). Thus, the increase in MPO activity may be related to the high levels of ROS and TBARS in the groups that received ICV-STZ injection.

ICV-STZ administration in rats induced oxidative stress in the hippocampus and cerebral cortex, as observed by an increase in TBARS and ROS and a reduction in GSH and T-SH in the groups that received an ICV-STZ injection. Oxidative stress is one of the main factors triggering neurotoxicity induced by ICV-STZ(JAVED *et al.*, 2012; SOFIC *et al.*, 2015). Also, the ICV-STZ injection can cause depletion of other components of the antioxidant system, increase in protein carbonylation levels, a decline in ATP levels and mitochondrial dysfunction (KAMAT *et al.*, 2016; SALKOVIC-PETRISIC *et al.*, 2013). Oxidative stress occurs initially in the pathogenesis of AD and possibly plays a fundamental role in the pathophysiology of this disease (CORREIA *et al.*, 2012; GRAMMAS, 2011; WANG, X.; WANG, W.*; et al.*, 2014),

since oxidative stress is involved in several mechanisms that culminate in neuronal death and, consequently, cognitive impairments (KAMAT *et al.*, 2016).

The treatment with RIV and/or HES in ICV-STZ rats promoted an improvement in the antioxidant system by the increase in the levels of GSH and T-SH, in addition, a significant increase in the activity of antioxidant enzymes, such as superoxide dismutase, is described in the literature, as well as GSH levels. Also, both substances have antioxidant properties that include reduced levels of MDA (KHERADMAND *et al.*, 2018; MAHDY *et al.*, 2012; SHAFIEY *et al.*, 2018), significantly reducing TBARS levels in the hippocampus and cerebral cortex of ICV-STZ rats, in addition to the ERO levels and MPO activity.

In the proposed experimental model, the ICV-STZ injection caused a cognitive impairment associated with higher activity of MPO, and higher levels of lipid peroxidation and ROS. In contrast, HES was able to mitigate the cognitive impairment oxidative damage caused by the ICV-STZ injection. Also, it was observed that HES acted adjuvant to RIV, significantly improving the performance of ICV-STZ rats in the memory test, as well as attenuating oxidative damage and improving the antioxidant profile in the analyzed parameters. Thus, HES has demonstrated the potential to be used in addition to conventional therapy for the treatment of this disease.

Acknowledgements

The authors acknowledge the financial support given by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Figures

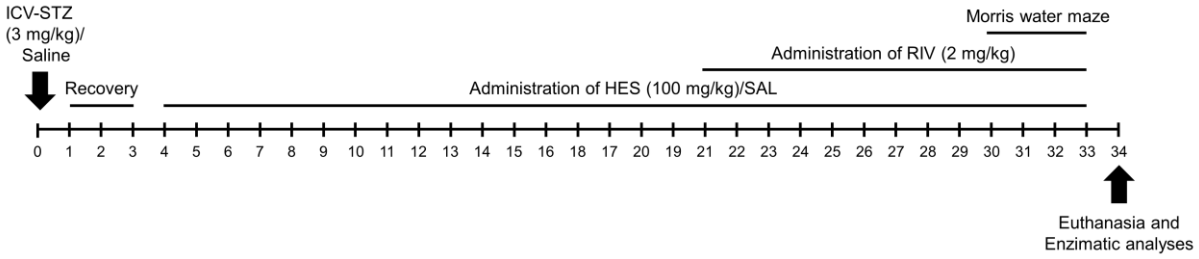

Fig. 1 Experimental protocol

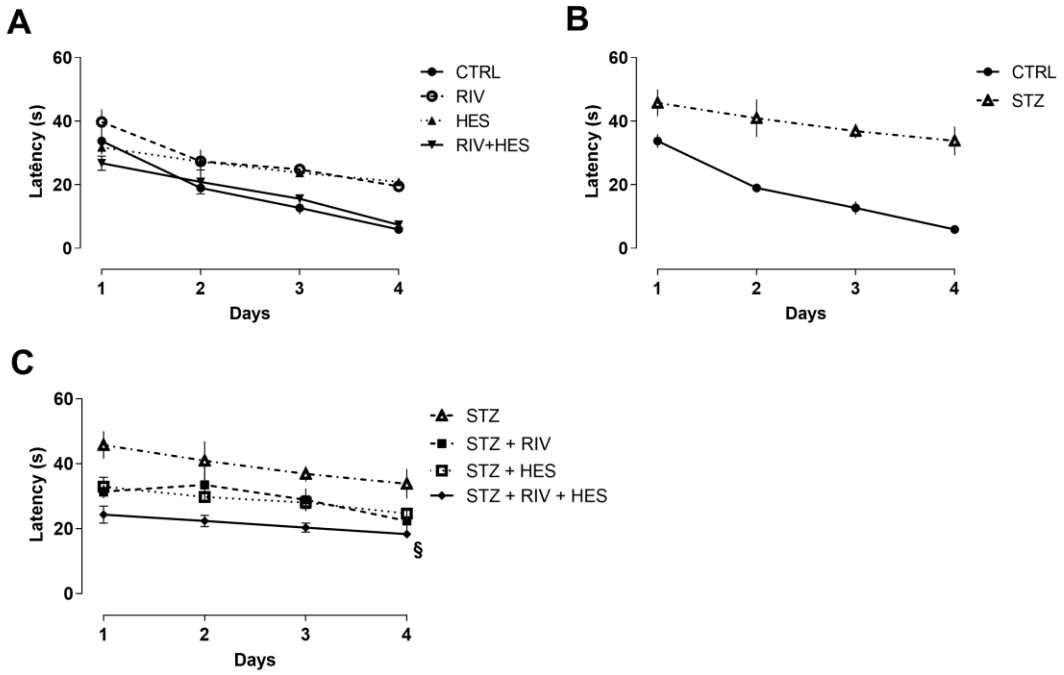

Fig. 2 Effects of intracerebroventricular injection of streptozotocin (ICV-STZ), rivastigmine (RIV) and hesperidin (HES) in the Morris water maze test. Comparison between the animals' latency in the non-ICV-STZ groups (A), control group compared to the ICV-STZ group (B), and treated ICV-STZ groups (C). The data are expressed as mean ± standard error. * $p < 0.05$ when compared to the CTRL group. #$p < 0.05$ when compared to the STZ group. §$P < 0.05$ when compared to the STZ+RIV and STZ+HES groups

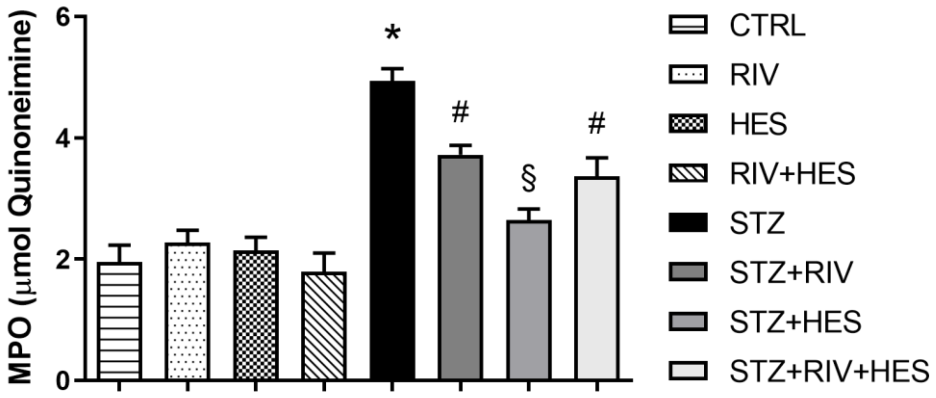

Fig. 3 Effects of intracerebroventricular injection of streptozotocin (ICV-STZ), rivastigmine (RIV) and hesperidin (HES) on myeloperoxidase activity in the plasma of rats. The data are expressed as mean ± standard deviation. * $p < 0.05$ when compared to the CTRL group. #$p < 0.05$ when compared to the STZ group. §$P < 0.05$ when compared to the STZ+RIV and STZ+HES groups

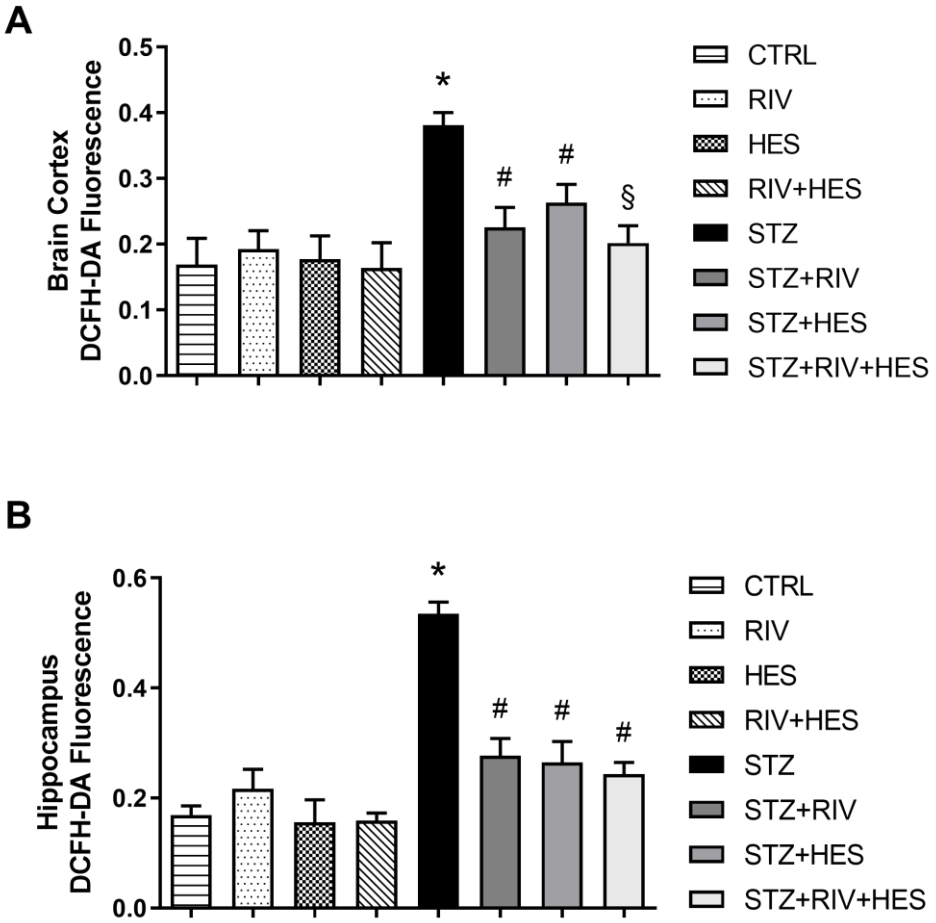

Fig. 4 Effects of intracerebroventricular injection of streptozotocin (ICV-STZ), rivastigmine (RIV) and hesperidin (HES) in reactive oxygen species in the cerebral cortex (A) and hippocampus (B) of rats. The data are expressed as mean ± standard deviation. * p <0.05 when compared to the CTRL group. #p <0.05 when compared to the STZ group. §P <0.05 when compared to the STZ+HES group. DCFH-DA: 2′-7′- dichlorofluorescein diacetate

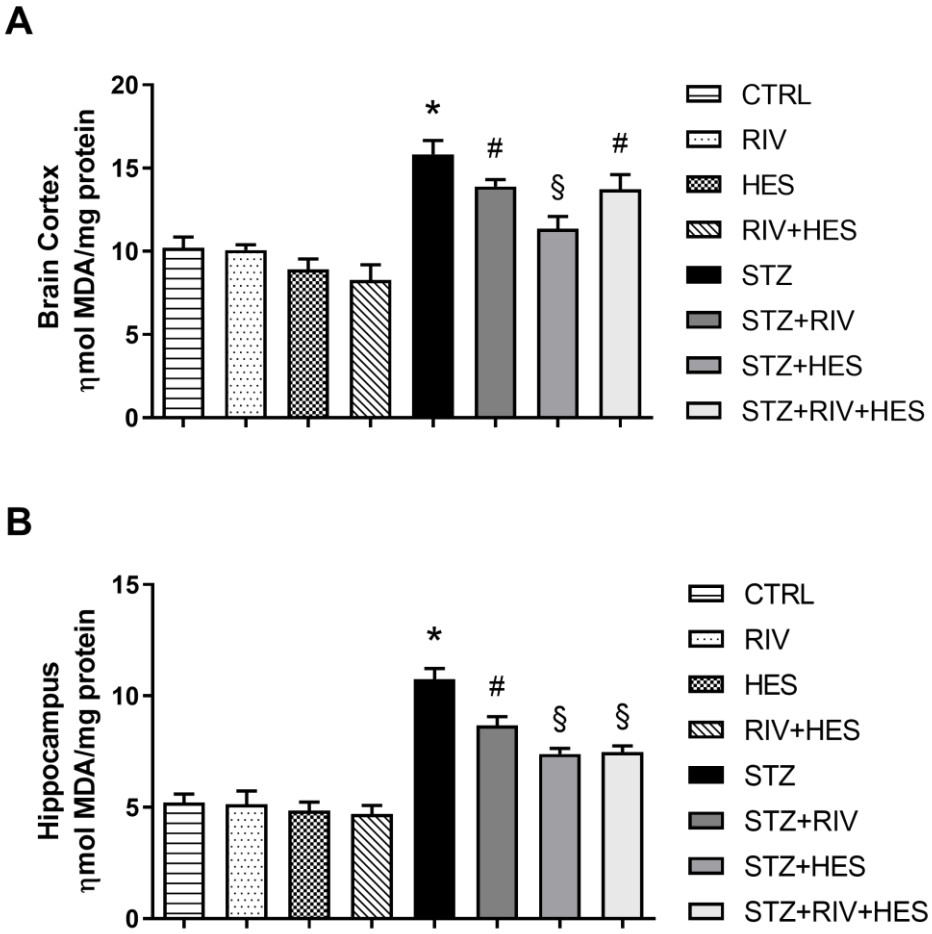

Fig. 5 Effects of intracerebroventricular injection of streptozotocin (ICV-STZ), rivastigmine (RIV) and hesperidin (HES) in substances reactive to thiobarbituric acid (TBARS) in the cerebral cortex (A) and hippocampus (B) of rats. The data are expressed as mean ± standard deviation. * p <0.05 when compared to the CTRL group. #p <0.05 when compared to the STZ group. §P <0.05 when compared to the STZ+HES group. MDA: Malondialdehyde

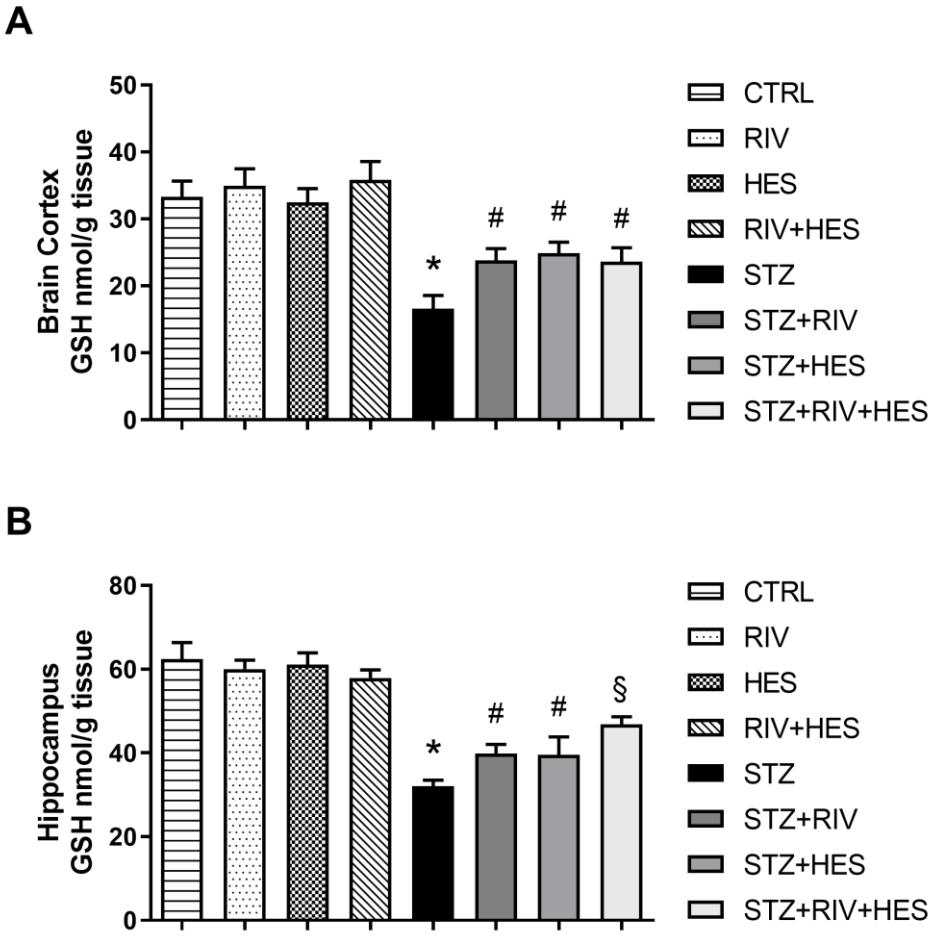

Fig. 6 Effects of intracerebroventricular injection of streptozotocin (ICV-STZ), rivastigmine (RIV) and hesperidin (HES) on the levels of glutathione (GSH) in the cerebral cortex (A) and hippocampus (B) of rats. The data are expressed as mean ± standard deviation. * $p < 0.05$ when compared to the CTRL group. #$p < 0.05$ when compared to the STZ group. §$P < 0.05$ when compared to the STZ+RIV and STZ+HES groups

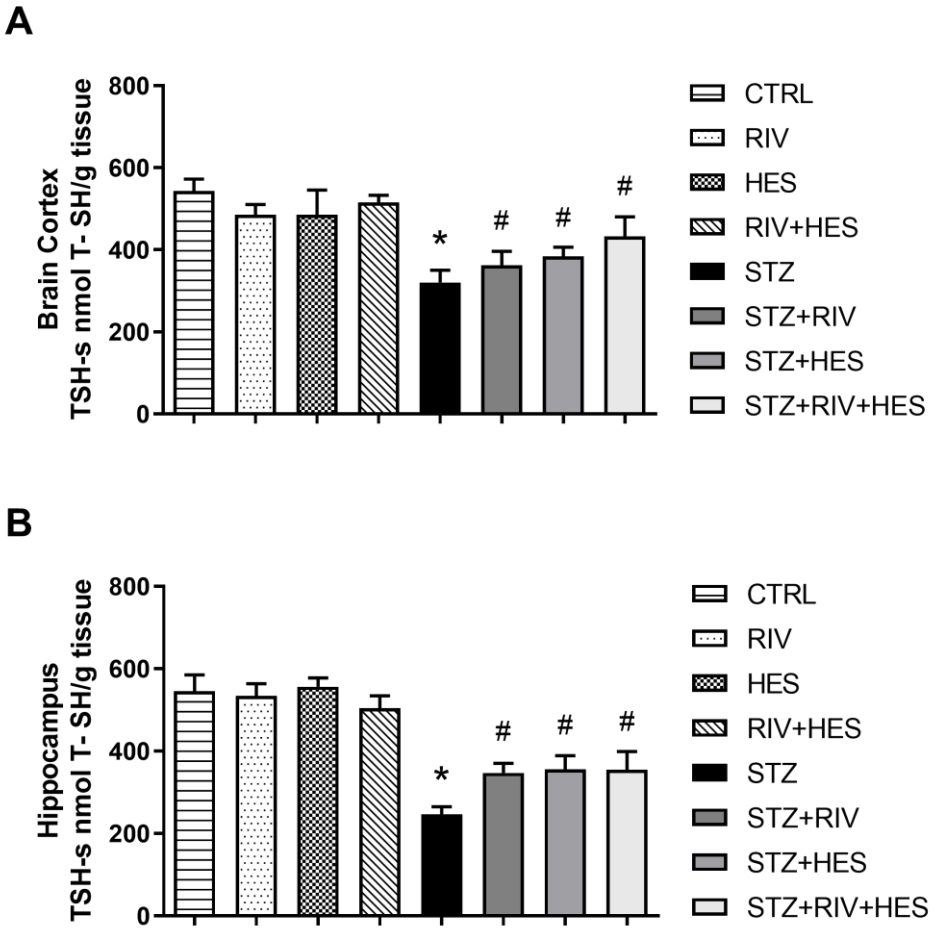

Fig. 7 Effects of intracerebroventricular injection of streptozotocin (ICV-STZ), rivastigmine (RIV) and hesperidin (HES) on the levels of total thiols (T-SH) in the cerebral cortex (A) and hippocampus (B) of rats. The data are expressed as mean ± standard deviation. * $p < 0.05$ when compared to the CTRL group. #$p < 0.05$ when compared to the STZ group

4 DISCUSSÃO

Nos últimos anos, diversos estudos têm sido realizados para elucidar os processos fisiopatológicos envolvidos com a neuroinflamação, visto que esses processos inflamatórios culminam no desenvolvimento de diversas doenças neurodegenerativas, como doença de Parkinson e a doença de Alzheimer (DA). Sabe-se que inúmeros processos inflamatórios sistêmicos podem culminar em desordens neuroinflamatórias. Assim, durante os anos, desenvolveu-se diversos modelos experimentais de neuroinflamação, destacando-se os modelos que utilizam aplicações intraperitonel (IP) de lipopolissacarídeo (LPS) ou administração intracerebroventricular de estreptozotocina (ICV-STZ). Esses modelos são amplamente utilizados devido a sua fácil aplicabilidade, mecanismos de ação bem estabelecidos e por mimetizarem as alterações fisiopatológicas observadas em pacientes humanos com doenças neurodegenerativas.

Grande parte dos estudos com modelos de neuroinflamação tem por objetivo buscar alternativas terapêuticas para essa enfermidade. Diversas pesquisas têm sido realizadas com produtos naturais. Dentre os compostos, destacam-se os flavonoides, provenientes principalmente de frutas, que apresentam diversos efeitos benéficos ao organismo, como propriedades antioxidantes e anti-inflamatórias, e reduzida toxicidade. Neste estudo, utilizou-se o ácido elágico (AE) e a hesperidina (HES), que possuem como vantagem a capacidade de exercer seus efeitos benéficos após administração por via oral. Isso ocorre, pois após sofrerem metabolização pelas bactérias do trato gastrointestinal, atingem a circulação e seus subprodutos possuem potencial para atravessar a barreira hematoencefálica.

Nesse contexto, este estudo teve por objetivo avaliar o potencial terapêutico de dois antioxidantes flavonoides (AE e HES) em modelos de neuroinflamação.

No MANUSCRITO I observou-se, após múltiplas aplicações IP de LPS, déficit cognitivo relacionado à maior ativação de células da glia, fosforilação da Tau, elevação na atividade da AChE, bem como no estresse oxidativo. Em contrapartida, o AE foi capaz de prevenir o déficit cognitivo causado por múltiplas aplicações de LPS, bem como, modular a resposta do sistema imune através da redução significativa na expressão de células da glia e fosforilação da Tau, preveniu o aumento na atividade da AChE, além de atenuar o dano oxidativo causado pela ação das endotoxinas. Deste modo, este estudo demonstrou os efeitos benéficos do AE na memória, neuroinflamação e reestabelecimento do equilíbrio redox a partir da ação imunomoduladora e antioxidante desse composto.

No MANUSCRITO II, que teve como proposta mimetizar um modelo experimental de doença de Alzheimer esporádica (DAE), avaliou-se a ação da HES *per se* e em associação a rivastigmina (RIV), medicamento comumente utilizado para o tratamento sintomático da DA. Desse modo, no modelo experimental proposto, a injeção ICV-STZ ocasionou déficit cognitivo associado ao aumento na atividade da MPO, na peroxidação lipídica e nos níveis de ERO. Em contrapartida, a HES foi capaz de atenuar o déficit cognitivo danos oxidativos causados pela injeção ICV-STZ. Além disso, observou-se que a HES atuou de forma adjuvante a RIV, melhorando significativamente o desempenho dos ratos ICV-STZ no teste de memória, bem como atenuando os danos oxidativos e melhorando o perfil antioxidante nos parâmetros analisados.

Observou-se nos modelos de neuroinflamação utilizados, déficit cognitivo e dano oxidativo, evidenciado pelo aumento das ERO e TBARS e depleção do sistema antioxidante. Ainda, no MANUSCRITO I, houve aumento na atividade da AChE e ativação de células da glia e fosforilação da Tau. Esses resultados estão de acordo com os mecanismos fisiopatológicos de neuroinflamação desencadeados pelos compostos utilizados já descritos na literatura. Assim, em ambos os estudos, houve sucesso no desenvolvimento de processo inflamatório no sistema nervoso.

Ainda, em ambos estudos, os antioxidantes utilizados (AE ou HES) demonstraram potencial para reverter os efeitos deletérios do LPS ou STZ, a partir da redução do dano oxidativo, com incremento do sistema antioxidante e redução das ERO com consequente melhora no potencial cognitivo dos animais. Em adição, no MANUSCRITO I, o AE demonstrou potencial imunomodulador, a partir de redução da expressão de células da glia, bem como capacidade de prevenir o aumento na atividade da AChE e fosforilação da Tau. Esses resultados demonstram o potencial terapêutico do AE e HES em desordens cognitivas secundárias à neuroinflamação, o que torna esses antioxidantes potenciais candidatos para o tratamento de desordens cognitivas.

5 CONCLUSÕES

Baseados nos dados do Manuscrito I e II conclui-se que em ambos modelos de neuroinflamação propostos, os antioxidantes utilizados apresentaram resultados promissores na busca por compostos naturais para o tratamento de desordens neuroinflamatórias. Desse modo, futuros estudos em humanos devem ser considerados para avaliação dos flavonoides e seus metabólitos no desenvolvimento de estratégias neuroprotetoras.

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