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Luana Pereira Magni

EFEITO DA HESPERIDINA SOBRE O DÉFICIT COGNITIVO E O SISTEMA COLINÉRGICO DE RATOS SUBMETIDOS A UM MODELO DE DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER

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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Profª Dra. Cinthia Melazzo de Andrade
Co-orientadora: Profª Dra. Fátima Husein Abdalla

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Aprovado em 16 de outubro de 2017:

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À UFSM e ao Programa de Pós-graduação em Ciências Biológicas- Bioquímica Toxicológica.

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RESUMO

EFEITO DA HESPERIDINA SOBRE O DÉFICIT COGNITIVO E O SISTEMA COLINÉRGICO DE RATOS SUBMETIDOS A UM MODELO DE DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER

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CO-ORIENTADORA: Fátima Husein Abdalla

A Doença de Alzheimer (DA) é considerada a principal causa de demência no mundo e a busca por novas intervenções terapêuticas para seu tratamento é de grande relevância. Estudos pré-clínicos sugerem a utilização da hesperidina (HES), um flavonóide da subclasse flavonona com ações antioxidantes, anti-inflamatórias e neuroprotetoras. Deste modo, o objetivo deste estudo foi investigar os efeitos do tratamento com a HES e, também, seu efeito como adjuvante no tratamento com rivastigmina sobre parâmetros de aprendizagem e memória e atividades das enzimas acetilcolinesterase (AChE) e butirilcolinesterase (BuChE) em um modelo de demência esporádica do tipo Alzheimer induzido por estreptozotocina (STZ) em ratos. Foram utilizados 64 ratos Wistar machos, distribuídos em oito grupos (n= 8): controle (CTR), rivastigmina 2 mg/kg (RIV), hesperidina 100 mg/kg (HES), rivastigmina + hesperidina (RIV+HES), estreptozotocina (STZ), estreptozotocina + rivastigmina (STZ+RIV), estreptozotocina + hesperidina (STZ+HES), estreptozotocina + rivastigmina + hesperidina (STZ+ RIV+ HES). Os ratos receberam uma injeção de STZ (3mg/kg) intracerebroventicular (ICV) e foram tratados com HES ou salina via oral durante 30 dias Os animais controle receberam ICV-salina e foram tratados com as mesmas soluções. As investigações comportamentais foram realizadas 21 dias (campo aberto, reconhecimento de objetos) e 30 dias (reconhecimento de objeto e tarefa de evitação passiva), após a injeção STZ. As atividades enzimáticas da AChE foram realizadas em sinaptossomas do córtex cerebral e hipocampo, bem como no sangue total, já a atividade da BuChE no homogeneizado do córtex cerebral e hipocampo. Os resultados revelaram que HES, e mais significativamente a HES em associação com a RIV, foi capaz de atenuar a perda de memória e o aumento nas atividades das enzimas do sistema colinérgico, demonstrando um efeito neuroprotetor sobre animais submetidos a este modelo de demência. Portanto, sugere-se que este composto pode ser um potencial candidato adjuvante ao tratamento convencional com a RIV na melhora do déficit de memória ocasionado na DA.

PALAVRAS-CHAVE: Doença Neurodegenerativa. Déficit de mémória. Acetilcolinesterase. Butirilcolinesterase. Rivastigmina.

ABSTRACT

EFFECT OF HESPERIDINE ON COGNITIVE DEFICIT AND THE COLINERGIC SYSTEM OF RATS SUBMITTED TO MODEL OF SPORADIC DEMENTIA OF ALZHEIMER TYPE

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Alzheimer's disease (AD) is considered the main cause of dementia in the world and the search for new therapeutic interventions for its treatment is of great relevance. Preclinical studies suggest the use of hesperidin (HES), a flavonoid of the subclass flavonone with antioxidant, anti-inflammatory and neuroprotective actions. Thus, the objective of this study was to investigate the effects of treatment with HES and also its effect as an adjuvant in the treatment with rivastigmine on learning and memory parameters and activities of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in a model sporadic dementia of streptozotocin-induced Alzheimer's disease (STZ) in rats. Sixty male Wistar rats were divided into eight groups (n = 8): control (CTR), rivastigmine 2 mg/kg (RIV), hesperidin 100 mg / kg (HES), rivastigmine + hesperidin (RIV + HES), streptozotocin , streptozotocin + rivastigmine (STZ + RIV), streptozotocin + hesperidin (STZ + HES), streptozotocin + rivastigmine + hesperidin (STZ + RIV + HES). The rats received an injection of STZ (3mg / kg) intracerebroventicular (ICV) and were treated with HES or saline orally for 30 days. The control animals received ICV-saline and were treated with the same solutions. The behavioral investigations were performed 21 days (open field, object recognition) and 30 days (object recognition and passive avoidance task) after the STZ injection. The enzymatic activities of AChE were performed in synaptosomes of the cerebral cortex and hippocampus, as well as in whole blood, and the activity of BuChE in the homogenate of the cerebral cortex and hippocampus. The results revealed that HES, and more significantly HES in association with RIV, was able to attenuate memory loss and increase in the activities of the enzymes of the cholinergic system, demonstrating a neuroprotective effect on animals submitted to this model of dementia. Therefore, it is suggested that this compound may be a potential candidate adjuvant to conventional treatment with RIV in the improvement of the memory deficit caused in AD.

KEY WORDS: Neurodegenerative disease. Deficit of memory. Acetylcholinesterase. Butyrylcholinesterase. Rivastigmine.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ACh	Acetilcolina
AChE	Acetilcolinesterase
AChEI	Inibidores da Acetilcolinesterase
APOE	Apolipoproteína E
APP	Proteína Precursora Amiloide
ATP	Adenosina Trifosfato
βA	β-amilóide
BACE1	Enzima de Clivagem de APP com Sítio Beta
BuChE	Butirilcolinesterase
ChAT	Acetiltransferase
DA	Doença de Alzheimer
DAE	Doença de Alzheimer Esporádica
HES	Hesperidina
ICV	Intracerebroventricular
ICV-STZ	Intracerebroventricular de Estreptozotocina
RIV	Rivastigmina
SNC	Sistema Nervoso Central
SNP	Sistema Nervoso Periférico
STZ	Estreptozotocina
TAChV	Transportador de Acetilcolina Vesicular

SUMÁRIO

1 INTRODUÇÃO

A doença de Alzheimer (DA) é uma doença neurodegenerativa irreversível e progressiva, caracterizada como a principal causa de demência (YE et al., 2011). De acordo com o World Alzheimer Report (2015), mundialmente aproximadamente 46,8 milhões de pessoas foram acometidas com a DA em 2015. Ressalta-se que este número tende a aumentar juntamente com o envelhecimento da população, com previsão estimada de 74,4 milhões de pessoas acometidas em 2030 e 131,5 milhões em 2050.

Embora a causa da DA não seja bem estabelecida, morfologicamente a DA é caracterizada por mudanças neuropatológicas específicas, tais como: deposição de placas neuríticas e emaranhados neurofibrilares. Estas alterações estão associadas com elevados níveis de peptídeo β-amilóide (βA) depositado extracelularmente e ao acúmulo intracelular da proteína tau hiperfosforilada, respectivamente (KIM et al., 2014; REITZ et al., 2011).

Além disso, um dos achados mais evidentes na DA é a atrofia cerebral, com redução do número de neurônios e sinapses com déficit colinérgico acentuado. Neste quadro, os níveis de acetilcolina (ACh), um neurotransmissor que atua na transmissão de funções cognitivas e consolidação da memória, são reduzidos devido à sua rápida hidrólise pela enzima acetilcolinesterase (AChE) (GARCÍA-AYLLÓN et al., 2011; SERRANO-POZO et al., 2011). Deste modo, é fato comumente aceito que a disfunção colinérgica tem uma forte correlação com a DA, especialmente nas áreas do encéfalo relacionadas com a aprendizagem, memória e respostas emocionais, sobretudo o córtex cerebral e o hipocampo (LADNER; LEE, 1998).

A injeção intracerebroventricular de estreptozotocina (ICV-STZ) em ratos tem sido amplamente utilizada como um modelo experimental de demência esporádica do tipo Alzheimer. Este modelo é bem aceito por ser capaz de mimetizar vários processos que ocorrem nesta doença, como o prejuízo no metabolismo energético de glicose, resistência à insulina no encéfalo, comprometimento da homeostase colinérgica (LESTER-COLL et al., 2006), agregação de βA e formação de emaranhados neurofibrilares (KNEZOVIC et al., 2015; LANNERT; HOYER, 1998), entre outros efeitos que levam a uma progressiva deterioração cognitiva caracterizada por déficits de aprendizado e memória (AWASTHI et al., 2010).

Embora não exista um tratamento curativo para DA, o tratamento sintomático envolve o uso de inibidores das colinesterases (CACABELOS et al., 2016). A Rivastigmina (RIV) tem sido uma das drogas de primeira linha para o tratamento da DA, sendo classificada como um inibidor pseudo-irreversível da AChE e butirilcolinesterase (BuChE) cerebral, inibindo seletivamente as suas atividades, aumentando assim, a capacidade da ACh de estimular os receptores nicotínicos e muscarínicos encefálicos (GROSSBERG, 2003; NOETZLI; EAP, 2013). No entanto, efeitos indesejáveis referentes ao uso deste medicamento são relatados, como problemas gastrointestinais, fraqueza muscular, perda de apetite, perda de peso, tontura e sintomas extrapiramidais (DIAZ et al., 2015).

Portanto, conforme acima mencionado e a partir dos avanços no tratamento de doenças através de compostos naturais, o estudo da fitoterapia parece promissor, podendo atuar como um adjuvante na terapêutica convencional para DA (HOWES; PERRY, 2011). Destaca-se assim a hesperidina (HES), que se caracteriza como um flavonoide da subclasse flavonona, sendo encontrada principalmente em frutos cítricos sendo classificada como um bioflavonóide cítrico, com múltiplas atividades farmacológicas, como efeitos anti-inflamatórios (PARHIZ et al., 2015), antioxidantes e neuroprotetores, no qual há evidências pré-clínicas da sua utilidade em várias doenças neurogenegerativas, onde a mesma foi capaz de proteger as células neuronais, em doenças como de Huntington, Parkinson (ANTUNES et al., 2014; HUANG et al., 2012).

Além disso, uma característica de grande importância da HES é sua capacidade de atravessar a barreira hematoencefálica e assim ter ação sobre neurônios de regiões importantes para processos cognitivos e de memória como córtex cerebral e hipocampo de ratos (DIMPFEL, 2006; THENMOZHI et al., 2015), atuando desta forma na modulação da atividade da AChE, a qual apresenta-se aumentada após a ICV-STZ (JAVED et al., 2015; LESTER-COLL et al., 2006; TOTA et al., 2010).

Nesse contexto, tendo em vista as inúmeras ações benéficos relatados para a HES, torna-se relevante elucidar os efeitos deste composto sobre os parâmetros de aprendizagem e memória, bem como, na atividade de importantes enzimas do sistema colinérgico em sinaptossomas do córtex cerebral e hipocampo de ratos submetidos a um modelo de demência esporádica do tipo Alzheimer.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar os efeitos da HES sobre parâmetros de memória e aprendizagem, e atividade de enzimas do sistema colinérgico, bem como seu papel como adjuvante no tratamento com RIV em ratos submetidos ao modelo de demência esporádica do tipo Alzheimer.

2.2 OBJETIVOS ESPECÍFICOS

Em ratos controles e ratos submetidos a um modelo de demência esporádica

do tipo Alzheimer tratados com HES e/ou RIV foram avaliados:

- Parâmetros de aprendizagem e memória através dos testes de reconhecimento de objetos e tarefa de evitação passiva.
- A atividade da enzima AChE em sinaptossomas do córtex cerebral e hipocampo, além da atividade no sangue total.
- A atividade da enzima BUChE no córtex cerebral e hipocampo.

3 REVISÃO DE LITERATURA

3.1 CARACTERÍSTICAS NEUROPATOLÓGICAS DA DOENÇA DE ALZHEIMER

A demência é uma síndrome neurológica, geralmente crônica, caracterizada principalmente por uma progressiva perda da memória e da capacidade intelectual do indivíduo, de forma a interferir nas suas atividades sociais e ocupacionais. Além do prejuízo cognitivo, comprometimento da linguagem e da capacidade de compreensão e julgamento (BATURE et al., 2017; HELMES et a., 2002; STIX, 2010).

Sabe-se que existem cerca de 47 milhões de pessoas mundialmente diagnosticadas com algum tipo de demência e a DA é a principal enfermidade associada a este transtorno (WORLD ALZHEIMER REPORT, 2015), caracterizada pela primeira vez por Alois Alzheimer (ALZHEIMER, 1907). A DA representa atualmente a forma mais comum de demência em idosos (REITZ et al., 2011), afetando cerca de 11% de pessoas com mais de 65 anos e 50% daqueles com idade superior a 85 anos (SANCHEZ-MUT et al., 2014). Além disso, estatísticas mundiais demonstraram que esse número poderá duplicar a cada 20 anos, com uma expectativa de 74,7 milhões em 2030 e 131,5 milhões em 2050 (WORLD ALZHEIMER REPORT, 2015). Entretanto, não existe muita informação a respeito da incidência da DA no Brasil, mas estimou-se que um milhão de pessoas foram acometidas por esta doença em 2012 no país (FERRI, 2012).

Além disso, o aumento na expectativa de vida é um fenômeno que vem se manifestando de forma crescente em escala mundial (ARKING, 1998; NARAYAN, et al, 2014). Com o aumento da expectativa de vida há uma mudança no padrão de distribuição etária da população, acarretando um aumento na prevalência de doenças associadas ao envelhecimento, entre elas, as doenças associadas ao sistema nervoso como a Doença de Parkinson e a DA (BALIN et al., 1998; MACCIONE et al., 2009; NARAYAN, et al., 2014), sendo a expectativa de vida do paciente com a DA curta, pois o falecimento do indivíduo costuma ocorrer em cerca de 3 a 9 anos após o diagnóstico (QUERFURTH; LAFERLA, 2010).

Pode-se observar que em um estágio precoce, a DA é mais comumente caracterizada pelo declínio gradual da memória de curto prazo e da cognição, onde uma incapacidade de reter a informação recentemente adquirida é tipicamente a

apresentação inicial. Sinais como a perda da memória de longo prazo, deficiência em outras áreas do processo cognitivo (linguagem, raciocínio abstrato e função executiva ou de tomada de decisão), confusão, delírios, alterações de humor, perda de funções corporais são habitualmente observados após alguns anos, devido à progressão da DA (BEKRIS et al., 2010; SITX, 2010).

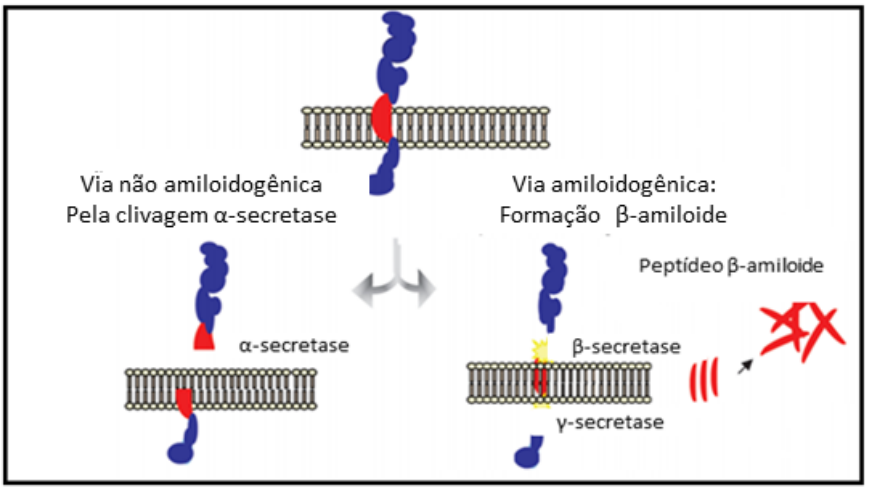
A DA pode ser diferenciada de duas formas: DA de início tardio, também denominado de Doença de Alzheimer Esporádica (DAE) e de início precoce, denominada de Doença de Alzheimer Familiar. A DA de início precoce representa de 5 a 10% dos casos de Alzheimer acometendo uma população em idade adulta inferior a 65 anos e evolui rapidamente (ASSOCIATION ALZHEIMER'S, 2015). Alguns genes são considerados como principal fator de risco para o desenvolvimento da DA, estes genes possuem modo autossômico dominante de transmissão (mutações nos genes codificadores da proteína precursora amilóide (APP), presenilina 1, presenilina 2 e apolipoproteína E. As mutações nesses genes podem resultar em alterações na produção da proteína β-amiloide, levando à apoptose de neurônios e à demência (BERTRAN; TANZI, 2005). Por outro lado, a DA de início tardio ou esporádico, principal forma da DA, que se manifesta em indivíduos com mais de 60 anos, tem sido associada a outros fatores, incluindo a idade, sexo, aumento do estresse oxidativo, falha no metabolismo energético, neuroinflamação e déficit em sistemas de neurotransmissão (FERRER, 2012). Desta forma, a DAE é considerada uma doença de caráter multifatorial, ao invés de ter uma única causa predominante (WICHUR; MALAWSKA, 2015).

De maneira geral, observa-se as mesmas características patológicas em ambas as formas da DA, principalmente o comprometimento da capacidade cognitiva dos pacientes, o que tende a tornar-se mais significativo com o passar dos anos, sendo comumente, a memória recente a primeira a ser afetada (BEKRIS et al., 2010). Já no aspecto morfológico, a DA é caracterizada por mudanças neuropatológicas específicas, que incluem à atrofia cerebral com perdas neuronais e sinápticas envolvendo vários sistemas de neurotransmissão. Além disso, apresenta placas senis extracelulares compostas de agregados filamentosos da proteína βA e emaranhados neurofibrilares, formados principalmente pela hiperfosforilação da proteína tau (KIM et al., 2014; REITZ et al., 2011).

Os peptídeos βA são produtos naturais do metabolismo, originados através da proteólise de uma grande proteína transmembrana chamada de proteína precursora

β-amilóide, ou APP pela ação das enzimas α-secretase, β-secretase e γ-secretase. A α-secretase compõe a via não amiloidogênica, produzindo o peptídeo βA solúvel, por clivar o APP na região central do domínio amiloide, prevenindo assim, a geração e a liberação do peptídeo βA patogênico (WEINER, et al., 2012). As outras duas enzimas compõem a via amiloidogênica, por ocasionarem uma clivagem proteolítica anormal, produzindo o peptídeo βA neurotóxico; a β-secretase com atividade que se origina de uma aspartil protease chamada enzima de clivagem de APP com sítio beta (BACE1) a qual media a primeira clivagem da APP; e a γ-secretase, uma proteína com sítio catalítico complexado com presenilina que assume a segunda clivagem, resultando na liberação do peptídeo βA (Figura.1) (LAFERLA et al., 2007).

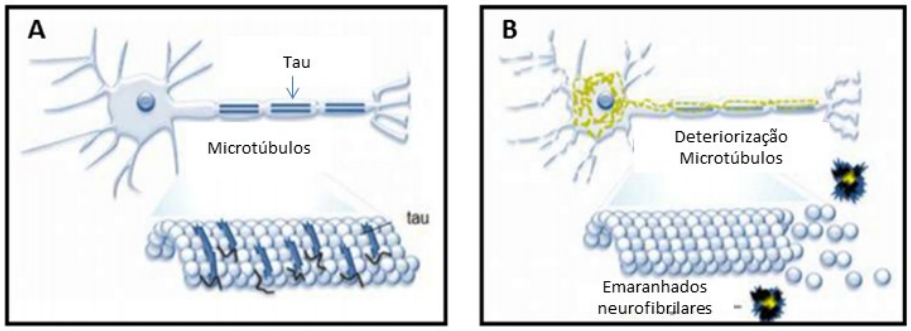
Figura 1: Geração do peptídeo β-amilóide pela via amiloidogênica



Adaptado de DE PAULA et al., 2009.

Adicionalmente, em segundo plano ocorre à formação dos emaranhados neurofibrilares, que são constituídos por proteína tau hiperfosforilada. Num estado normal, tau é uma proteína solúvel que promove a junção e estabilização dos microtúbulos dos axônios. A proteína tau hiperfosforilada, pelo contrário, exibe propriedades de solubilidade alteradas (LAFERLA; ODDO, 2005), provocando a desestabilização dos microtúbulos axonais, o que prejudica assim o transporte axonal, comprometendo a função neuronal e sináptica (IQBAL et al., 2005).

Figura 2: Diferenças entre um neurônio normal (A) em que a proteína tau normal mantém a rede de microtúbulos e um neurônio de um paciente com DA (B), em que a proteína tau hiperfosforilada desestabiliza os microtúbulos e forma os emaranhados neurofibrilares.



Adaptado de LIM et al., 2014.

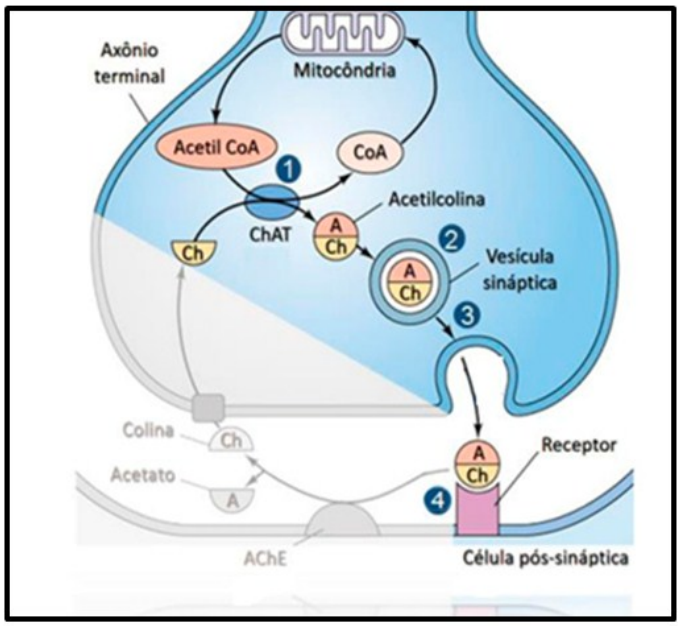
Para além destes agregados proteicos, outro achado evidente na DA é a perda da neurotransmissão colinérgica central. Neste quadro, os níveis de acetilcolina (ACh), um neurotransmissor que atua na transmissão de funções cognitivas e consolidação da memória são reduzidos, devido à alterações na atividade de enzimas responsáveis pela sua síntese e degradação, sendo esta hipofunção colinérgica associada com o declínio cognitivo observado nesta patologia (FERREIRA-VIEIRA et al., 2016; SCHLIEB; ARENT, 2011).

Nesse contexto, a hipótese do envolvimento da neurotransmissão colinérgica na DA é introduzida desde a década de 80 (BARTUS et al., 1982; COYLE et al., 1983), a qual postula que a escassez de ACh causa muitos dos sintomas da DA, especialmente os relacionados a dificuldade de aprendizado e memória. A hipótese colinérgica fornece até os dias atuais a base racional para abordagem terapêutica em pacientes com DA, com drogas direcionadas para a melhora dos níveis do neurotransmissor ACh (FERREIRA-VIEIRA et al., 2016). A ACh foi a primeira molécula a ser identificada como um neurotransmissor e passou a ser alvo de muitos estudos nas sinapses no Sistema Nervoso Central (SNC). Está molécula é considerada um mediador químico de sinapses do SNC e Sistema Nervoso Periférico (SNP), e também na junção neuromuscular. No SNP a ACh estimula o músculo esquelético ao ligar-se aos receptores nicotínicos presentes nas fibras musculares, abrindo assim os canais de sódio na membrana celular e produzindo a contração muscular (LI; COL, 2014). Já no SNC, ela desempenha um papel

fundamental em funções vitais especificamente na aprendizagem e memória, atuando em importantes áreas para esses processos como hipocampo e córtex cerebral (PICCIOTTO; HIGLEY; MINEUR, 2012). Além disso, a ACh circulante é conhecida como um modulador do sistema imune, a qual atual como uma molécula anti-inflamatória, uma vez que a medida das atividades das colinesterases têm sido descrita como marcadores de inflamação de baixo grau (DAS, 2007, REALE et al., 2014).

A síntese do neurotransmissor ACh (Figura 3) ocorre no citosol dos terminais dos neurônios a partir da acetil coenzima-A (acetil-CoA) e da colina. A acetil-CoA tem origem mitocondrial, ao passo que a colina é um importante produto do metabolismo dos lipídeos da dieta (PRADO et al., 2002). A combinação da acetil-CoA à colina é catalisada pela colina acetiltransferase (ChAT). Uma vez sintetizada a ACh é transportada pelo transportador de acetilcolina vesicular (TAChV) que armazena a ACh dentro de vesículas e liberam seu conteúdo por exocitose, após um influxo de cálcio no terminal nervoso (SARTER; PARIKH, 2005). A ACh pode se difundir no espaço extracelular, ser degradada a colina e acetato pela AChE e BuChE, ou ainda, difundir-se na fenda sináptica e ativar os receptores específicos de ACh, que são sensibilizados, causando a despolarização e propagação do potencial de ação na célula pós-sináptica (PRADO et al., 2002; SARTER; PARIKH, 2005). Assim, duas classes de receptores são sensíveis a ACh, os receptores muscarínicos, que agem via ativação de proteínas G principalmente associados a neurônios do SNC e SNP, além de outros tecidos ganglionares, e os receptores nicotínicos, que atuam por canais iônicos regulados por ligante e localizam-se, predominantemente, nas sinapses ganglionares (FERREIRA-VIEIRA et al., 2016).

Figura 3: A síntese de ACh a partir da colina e acetil CoA é catalisada pela ChAT (1), em seguida ACh é armazenada nas vesículas (2) e transportado à membrana para ser liberado (3), uma vez no espaço sináptico a ACh irá ligar-se ao receptor na célula pós-sináptica (4)



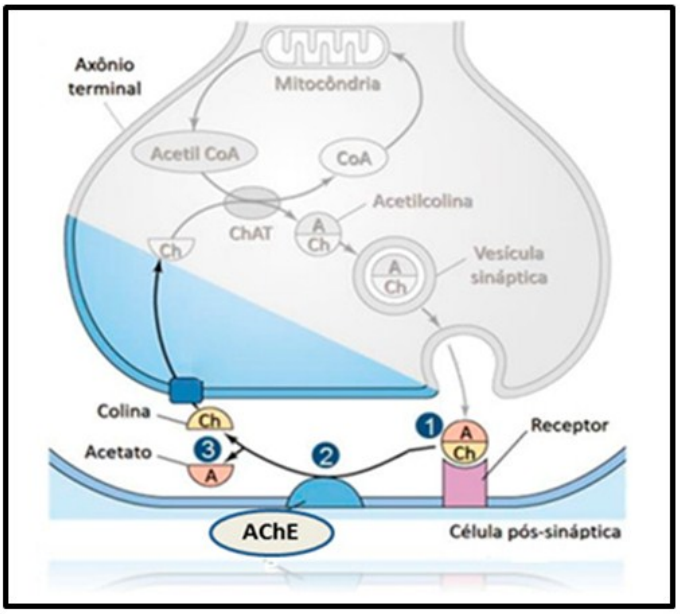
Adaptado de DA COSTA, 2016.

A AChE, ou colinesterase verdadeira, é predominantemente encontrada no encéfalo, junção neuromuscular e eritrócitos (MESULAM, et al, 2002). Enquanto que a BuChE, ou pseudocolinesterase, é uma enzima sérica, com ampla distribuição no organismo, encontrada, principalmente, no soro, rins, fígado, intestino, coração, e possui também uma distribuição neuronal, porém mais restrita em relação a AChE (COKUGRAS, 2003).

A enzima AChE, é uma glicoproteína globular, classificada como uma serina hidrolase, que hidrolisa, preferencialmente, ésteres com grupamento acetil, como a ACh, sendo degrada em acetato e colina (DVIR et al., 2010). Deste modo, a AChE modula a concentração de ACh nas sinapses colinérgicas (Figura 4), finalizando a transmissão do impulso. Esta enzima está presente em diversas formas moleculares no organismo, como a forma globular, que se apresenta como uma montagem homomérica de subunidades catalíticas, exibindo-se como monômeros, dímeros e tetrâmeros (G1, G2 e G4). Outra forma molecular da AChE é a assimétrica, que apresenta-se como uma montagem heteromérica das subunidades estrutural e catalítica (A4, A8, A12). As formas globulares são caracterizadas de forma solúvel

ou ancoradas à membrana, enquanto que as assimétricas estão incluídas na matriz extracelular. Entretanto, quaisquer das formas apresentam uma alta taxa de hidrólise enzimática (MASSOULIÉ et al., 2008; MESHORER; SOREQ, 2006). As formas globulares encontram-se, predominantemente, no SNC, enquanto que as formas assimétricas são encontradas, principalmente, no SNP e no músculo (ALDUNATE, et al., 2004; RAKONCZAY, et al., 2005). Além disso, é demonstrado que a principal forma da AChE encontrada no tecido nervoso é o tipo G4, ligada à membrana (NAVARATNAM et al., 2000;. PERRIER et al., 2002).

Figura 4: Após ligar-se ao seu receptor colinérgico (1), a ACh é hidrolisa pela AChE (2), formando como produtos o acetato e a colina (3)



Adaptado de DA COSTA, 2016.

Além da AChE, outro tipo de colinesterase encontrado no SNC é a BuChE, caracterizada como uma colinesterase não específica, por hidrolisar além da ACh, outros ésteres de colina, como a butirilcolina, os relaxantes musculares succinilcolina (DARVESH, et al., 2003). Ambas as enzimas são capazes de hidrolisar a ACh, mas a AChE tem uma atividade hidrolítica 10 vezes maior do que a da BuChE (COKUGRAS, 2003). No entanto, estudos sugerem que a AChE não é essencialmente necessária para o estabelecimento da via colinérgica, pois a BuChE pode substituir essa enzima ao recuperar a função colinesterásica na sua ausência (MESULAM et al., 2002).

Com base nessa relação, é demonstrado que déficits na transmissão colinérgica podem potencialmente influenciar os aspectos da cognição e comportamento, incluindo o processamento de informações em regiões do hipocampo e córtex cerebral (BENTLEY; DRIVER; DOLAN, 2011). Dessa maneira, diversos estudos estão desenvolvendo estratégias compensatórias, no intuito de promover o aumento dos níveis sinápticos de ACh, através da inibição das enzimas AChE e BuChE, retardando assim, os efeitos da doença (DE OLIVEIRA et al., 2016; KAMKWALALA ; NEWHOUSE, 2017; PINTON et al., 2010).

3.2 INIBIDORES COLINESTERÁSICOS

Ainda não existe um tratamento curativo para a DA, entretanto, o tratamento sintomático envolve o uso de inibidores colinesterásicos. Os inibidores da acetilcolinesterase (AChEI) foram os primeiros medicamentos aprovados e utilizados no tratamento dos sintomas dos pacientes com DA (SMALL; BULLOCK, 2011), os quais demonstram eficácia em atividades do cotidiano, comportamento e função cognitiva. Eles atuam impedindo a hidrólise da ACh, aumentando os níveis deste neurotransmissor na fenda sináptica (NOETZLI; EAP, 2013).

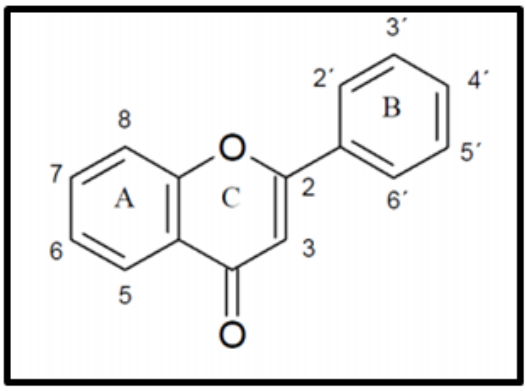
O primeiro AChEI introduzido na terapia contra a DA foi a tacrina (TUMIATTI et al., 2010), porém, apesar de um início muito promissor seu uso foi descontinuado devido a evidências de sua toxicidade ao tecido hepático. Dentre os AChEI atualmente aprovados para uso na DA, donepezil e galantamina inibem seletivamente a AChE, enquanto que a rivastigmina inibe tanto a AChE quanto a BuChE (NOETZLI; EAP, 2013).

No entanto, os inibidores das colinesterases são responsáveis por causar muitos efeitos indesejáveis, sendo os mais comuns náuseas, diarreia, vômitos, fraqueza muscular, perda de apetite, perda de peso, tontura, sonolência, dor de estômago e sintomas extrapiramidais (ANAND; SINGH, 2013; DHIKA; ANAND, 2013). Desse modo, são realizadas pesquisas que se concentram na seleção de plantas com atividade anticolinesterásica para o tratamento da DA (BUI; NGUYEN, 2017; DE OLIVEIRA, et al., 2016) com o intuito de diminuir os efeitos colaterais exibidos pelas drogas sintéticas comercializadas.

3.3 PROPRIEDADES FARMACOLÓGICAS DA HESPERIDINA

Os flavonoides são metabólitos secundários com ampla distribuição na natureza, que 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) (Figura 2), e o que permite os diferenciar em diferentes subgrupos é a estrutura de cada um a partir do núcleo fundamental, sendo classificados em: flavonóis, antocianidinas flavonas, flavanonas, flavonolignanas, flavana, isoflavonas, (SINGH et al., 2014).

Figura 5: Núcleo genérico de um flavonoide. Anéis aromáticos (A, B) e o anel heterocíclico (C)



Adaptado de COOK; SAMMMAN, 1996.

Estes compostos não podem ser sintetizados pelo metabolismo humano, sendo, portanto, adquiridos exclusivamente na dieta. São encontrados em frutas, legumes, vegetais, açúcares, grãos, vinho tinto, chás de ervas, café e chocolate, constituindo assim, uma ampla classe de substâncias de origem natural (MARTINEZ-VALVERDE et al., 2000).

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 a hesperidina, sendo desta forma, considerados substâncias lipossolúveis e hidrossolúveis, respectivamente (DEWICK, 2009). Sabe-se que a forma glicosilada é dificilmente absorvida no intestino delgado, necessitando de um processo de quebra, auxiliado

por enterobactérias, liberando a aglicona, uma molécula que pode ser absorvida mais facilmente pelas células epiteliais do intestino grosso devido a sua lipofilicidade que facilita a passagem pela camada fosfolipídica da membrana celular. Após a passagem destas moléculas no trato digestivo e metabolização no fígado, as mesmas encontram-se livres na corrente sanguínea, podendo exercer uma sucessão de efeitos no organismo (MENDEL et al., 2016; YAO et al., 2004).

Figura 6: Características e fontes das diferentes subclasses de flavonoides

Subclasses	Cor	Flavonoides representativos	Fontes
Antocianidina	Azul, vermelho, violeta	Cianidina	Frutas e flores
Flavanol	Incolor	Catequinas, epicatequinas	Maçãs, chá, cerveja
	Amarelo	Procianidina	Sucos de frutas, vinho
Flavanona	Incolor, amarelo	Hesperidina, Naringenina	Frutas cítricas
Flavona	Amarelo claro	Apigenina, luteolina	Cereais, frutas,flores,vegetais
Flavonol	Amarelo claro	Quercetina, Miricetina e rutina	Cebolas, maçãs,tomates,vinho tinto, trigo sarraceno, chá
Isoflavona	Incolor	Genisteína, daizeína	Legumes (derivados de soja)

Adaptado de PEDRIALI, 2005.

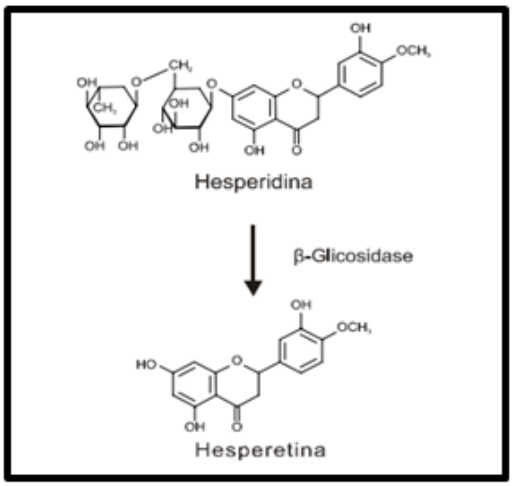
Estudos tem demonstrado a capacidade dos flavonoides de atravessar a barreira hematoencefálica, através da quantificação dos mesmos no SNC (JAGER; SAABY, 2011; THENMOZHI, et al., 2015), no qual o efeito neuroprotetor foi evidenciado, com potencial proteção sobre a morte celular de células neuronais em alguns modelos animais de doenças neurodegenerativas como a Doença de Parkinson, Huntington e DA. Além disso, foi relatada a ação destes compostos na modulação da atividade da enzima AChE cerebral (ANTUNES et al., 2014; HUANG et al., 2012; JAVED, et al., 2015).

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 por isso é classificada como um bioflavonóide cítrico (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). Em adultos a ingestão diária de flavanonas foi estimada

entre 2,7 e 78,0 mg (ERDMAN et al, 2007; PEREZ-JIMENEZ, 2011). Os sucos cítricos, em especial, o de laranja, é o mais consumido em todo o mundo, sendo este a principal fonte de flavonóides dietéticos. Neste suco, o teor médio de hesperidina varia entre 487-584 mg de hesperidina/L (TOMÁS-BARBERÁN; CLIFFORD, 2000). Entretanto, a fruta pode conter cinco vezes mais flavanonas do que o suco de laranja devido à presença do albedo (parte interna da casca), que possui grande quantidade de flavanonas (ERDMAN et al, 2007).

Em relação às características cinéticas, 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 (URPI-SARDA et al., 2012).

Figura 7: Hidrólise enzimática da hesperidina a hesperetina



Adaptado de NIELSEN, et al. (2006)

No estudo de Boonpawa et al. (2017), a biodisponibilidade da HES após administração oral de uma concentração de 50 mg/kg foi investigada em um modelo cinético baseado na fisiologia humana. O principal pico de concentração de hesperetina no plasma foi de 0.02 mg/mL em numa dose oral de 50 mg/kg de hesperidina. Além disso, outros estudos relataram que a HES foi detectada no plasma 6 horas após sua ingestão, sendo o pico máximo alcançado entre 9 e 12 horas (YAMADA et al., 2006), com excreção através da urina, em torno de 24 horas

após o consumo (VALLEJO et al., 2010). Ainda, o estudo de Yamada et al. (2006) relata que a HES não é tóxica, mesmo em tratamento com altas doses e por períodos prolongados.

Diversos efeitos farmacológicos têm sido descritos para HES, como atividade anti-aterogênica, atividade antialérgica, antioxidante, anti-inflamatória, anti-mutagênica e neuroprotetora (BORRADAILE et al, 1999; GALATI et al, 1994; PARHIZ et al., 2015; WILCOX et al, 2001;). Além disso, a HES pode agir sobre o metabolismo da glicose (JUNG et al., 2004; UMENO et al., 2016) e ter efeitos positivos sobre a resposta imune (CAMPS et al., 2017).

Desta maneira, enfatiza-se efeitos promissores da HES sobre o SNC, a qual apresenta uma característica de grande importância que é capacidade de atravessar a barreira hematoencefálica e assim ter ação sobre neurônios de importantes regiões para processos cognitivos e de memória como hipocampo e córtex cerebral de ratos (DIMPFEL 2006; THENMOZHI et al., 2015). Em modelos experimentais de doenças neurodegenerativas que levam ao prejuízo da memória foi relatado efeitos positivos da HES sobre este parâmetro nas doses de 50 mg/kg e 100 mg/kg após 21 e 28 dias de tratamento (KUMAR et al., 2013; THENMOZHI et al., 2015). Além disso, no que se referem em especial a modelos de DA, estudos demonstraram melhora sobre a memória espacial em camundongos após duas semanas de tratamento com HES nas doses de 50 mg/kg e 100 mg/kg (JAVED et al., 2015) com ação sobre o sistema colinérgico, através da modulação da enzima AChE.

Posto isso, estudos recentes tem dado atenção à influência da suplementação dietética na terapêutica para muitas patologias. Neste contexto, tendo em vista as inúmeras propriedades relatadas para a HES, o estudo da fitoterapia parece promissor, podendo atuar como um adjuvante nos tratamentos convencionais para DA.

3.4 MODELO EXPERIMENTAL COM INJEÇÃO INTRACEREBROVENTRICULAR DE ESTREPTOZOTOCINA

Modelos experimentais que mimetizam os sinais clínicos de pacientes com a DA para animais, são ferramentas bastante relevantes na busca de novas alternativas para terapêutica desta patologia. Desta maneira, a administração intracerebroventricular de estreptozotocina (ICV-STZ) vem sendo utilizada em modelos animais para estudar a DAE, por mimetizar diversos aspectos das anormalidades da DA (SANTOS et al., 2012; SHOHAM et al., 2006).

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*. Esta tem sido comumente utilizada como indutora de diabetes melito em modelos animais, sendo administrada perifericamente, causando lesão às células β-pancreáticas e resistência à insulina (SZKUDELSKI, 2012). 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 NAD^+ com consequente diminuição dos estoques de ATP e necrose das células β-pancreáticas. (LENZEN, 2008).

Por outro lado, estudos têm demonstrado que a injeção ICV-STZ em doses subdiabetogênicas pode prejudicar o metabolismo energético cerebral e reproduzir características moleculares e patológicas observadas na DAE. 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 esta inibição da sinalização da insulina contribui para a neurodegeneração decorrente da DAE, 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; MACAULEY; HOLTZMAN, 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 (GRÜNBLATT,et al., 2007).

Além disso, é relatado que a ICV-STZ ocasiona déficits cognitivos e comprometimento da memória em roedores, devido a redução da síntese de adenosina trifosfato (ATP) e acetil-CoA, os quais resultam em disfunção da homeostase colinérgica (DE LA MONTE; WANDS, 2008). Estudos demonstram ainda, que o prejuízo na disfunção colinérgica neste modelo ocorre principalmente pelo aumento na atividade da AChE (AGRAWAL et al., 2009).

Dessa maneira, no que se refere ao uso deste modelo para DAE, dados condizentes são apresentados em relação a déficits cognitivos, de aprendizagem e de memória de curto e longo prazo avaliados após a administração ICV-STZ em animais, utilizando-se testes como o labirinto aquático de Morris, esquiva inibitória e reconhecimento de objetos (ISHRAT et al., 2006; SANTOS et al., 2012; SHOHAM et al., 2003). Em adição, o comprometimento da memória de roedores parece ser evidenciado já em torno de 14 dias após ICV-STZ (AGRAWAL et al., 2009), sendo mais pronunciado aos 21 dias (DESHMUKH et al., 2009) e, permanecendo até 14 semanas após a ICV-STZ (MEHLA; PAHUJA; GUPTA, 2013).

Assim, pelo fato da ICV-STZ produzir distúrbios metabólicos, neuropatológicos e comportamentais em roedores, semelhantes aos encontrados em pacientes com a DA, este modelo tem sido amplamente aceito para experimentação relativa à DA. Sendo desta forma, utilizado na investigação das possíveis causas do desencadeamento da doença, bem como na busca de novas alternativas terapêuticas para o tratamento da DA.

4 MANUSCRITO CIENTÍFICO

A metodologia, os resultados, discussão e referências desta dissertação apresentam-se sob a forma de um manuscrito científico. O manuscrito encontra-se nas normas da revista Journal of Alzheimer's Disease.

As referências citadas ao final da dissertação referem-se somente às citações que aparecem nos itens INTRODUÇÃO e REVISÃO DE LITERATURA desta dissertação.

HESPERIDIN IMPROVES MEMORY DECLINE AND THE CHOLINERGIC SYSTEM CHANGED OF RATS SUBMITTED TO SPORADIC DEMENTIA OF ALZHEIMER'S TYPE INDUCED BY STREPTOZOTOCIN

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Abstract

Recent attention is given to the influence of dietary supplementation on the therapy of many pathologies. The present study aimed to investigate the effects of hesperidin (HES) on learning memory, acetylcholinesterase (AChE) activity in cerebral cortex and hippocampal synaptosomes and in peripheral blood, and butyrylcholinesterase (BuChE) activity in the cortex and hippocampus in an experimental intracerebroventricular streptozotocin (ICV-STZ) model induced in rats. Sixty four male Wistar rats were randomly divided into eight groups (n=8): control (CTR), 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 2 mg/kg + hesperidin 100 mg/kg (STZ + RIV +HES). Rats were injected with ICV-STZ or saline solution (3 mg/kg), and daily oral HES treatment started on day 4 over in a period of 30 days. The behavioral investigations were performed after 21 days (open field, object recognition) and 30 days (object recognition and step-down passive avoidance task), after STZ injection. Analysis of AChE and BuChE activity was performed on samples from the cerebral cortex and hippocampus. HES administration attenuated cognitive impairment and prevented the increase of cholinesterases activities the cerebral cortex and hippocampus induced by ICV-STZ. In addition, concomitant treatment with RIV and HES significantly reduced memory impairment, as well as, the AChE and BuChE activities in the cerebral cortex and hippocampus. The results of this study provide a better understanding of the effect of HES, as well as its use in association with conventional treatment with RIV in the brain of rats exposed to ICV-STZ. Thus, we suggest that HES may serve as a potential dietary flavonoid to be used in novel therapeutic adjuvant strategies in the treatment of sporadic dementia of Alzheimer's disease.

Keywords: Alzheimer's disease; Cognitive deficit; Acetylcholinesterase. Butyrylcholinesterase; Rivastigmine.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, being the most common cause of dementia in the elderly [1]. Mainly characterized by cognitive decline, loss of intellectual capacity and memory. AD affects 47 million people worldwide, and it is estimated that this number doubles every 20 years [2], being considered a public health problem.

Although the etiology of AD is not well established, specific neuropathological changes that occur involve the deposition of neurofibrillary plaques and neurofibrillary tangles [3], resulting in loss of neuronal function and synaptic damage. In addition to, the dysfunction of cholinergic transmission, with marked reduction of acetylcholine (ACh) levels in the synaptic process, as well as inflammation oxidative damage and ultimately neuronal death, particularly in the cerebral cortex and hippocampus [4]. It is well known that there is a correlation between the intensity of clinical symptoms of dementia and the reduction of cholinergic activity, such as levels of choline acetyltransferase, muscarinic receptors and Ach [5]. However therapeutic strategies based on the inhibition of the enzyme AChE, seems to be the one that presents better results in patients with AD helping to maintain and increase the levels of ACh in the neuronal synapses [6, 7].

In addition, severe changes in brain glucose and energetic metabolism, where insulin-resistant brain state, interfere with signal transduction of the insulin receptor, contribute to late sporadic AD onset [8, 9]. Intracerebroventricular injection of streptozotocin (ICV-STZ), a glucosamine-nitrosourea compound ($C_8H_{15}N_3O_7$) derived from soil bacteria, has been described as an appropriate model of sporadic Alzheimer's type dementia (sAD). ICV-STZ desensitizes neural insulin receptors and reduces neuronal energy metabolism [10, 11]. In addition, this model of sAD may to cause hyperphosphorylation of tau [12], increase of oxidative stress and AChE activity [13], with accumulation of the β-amyloid peptide [10], and finally apoptosis, especially in hippocampal neurons [14].

The current treatment for AD involves the use of anticholinesterase drugs that lead to a modest clinical improvement of the symptoms of the disease, but none of the drugs can prevent or reverse the pathology [15]. In this way, the research of new therapeutic agents capable of fully attending to the multifactorial disease is necessary. Thus, some research has been focused in natural and dietary compounds in the AD combat [16].

Among the natural compounds we highlight the hesperidin (HES), a subclass of flavonoids, found mainly in citrus fruits such as oranges and lemons. HES has multiple therapeutic effects, such as anti-inflammatory effects, antioxidant [17], analgesic [18], and antiatherogenic properties [19] and neuroprotective properties [20]. The effectiveness of the neuroprotection properties of this compound in several neurodegenerative processes is described in Ischemia, Parkinson's disease and Huntington's disease [21-23]. Thus, we emphasize the effects of HES on the CNS, which presents a characteristic of great importance that is to cross a blood brain barrier and thus to act on neurons of regions important for cognitive and memory processes like hippocampus and cerebral cortex of rats [24, 25].

Taking into account the advances in the treatment of diseases through natural compounds, the aim of the present study was to investigate the effect of HES on memory and cholinergic deficiency in ICV-STZ injected rats. Thus, we evaluated the open field tests, object recognition and passive evasion, as well as the activity of AChE in synaptosomes of the cerebral cortex and hippocampus and in the peripheral blood, as well as, the BuChE activity in the cerebral cortex and hippocampus. .

MATERIAL AND METHODS

Chemicals

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), butyrylthiocholine, Tris- (hydroxymethyl)- aminomethane, ouabain octahydrate, Coomassie Brilliant Blue G, Trizma Base, streptozotocin (STZ) and HES were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rivastigmine (S)-N-ethyl-3-[(1-dimethyl-amino)ethyl]-N-methyl-phenylcarbamate hydrogentartrate (Novartis Pharma). All other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

Male Wistar rats weighing 300 - 350 g were obtained from the Central Animal House of the Federal University of Santa Maria (UFSM) for the study. The animals were maintained at a constant temperature (23 ± 1 °C) and under a 12 h light/12 h dark program, with free access to food and water. All animal procedures were approved by the Animal Ethics Committee for the care and use of laboratory animals (protocol number: 1786040216).

Experimental protocol - Administration of drugs to animals

Intracerebroventricular of streptozotocin (ICV-STZ) or saline administration

The animals were anesthetized with ketamine and xylazine (0.5 mg / kg) given intraperitoneally. The head was placed in a stereotaxic apparatus and exposed skull. Two profiles were drilled through the skull for bilateral placement of a microinjector into the lateral ventricles using the following coordinates: 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 [26]. STZ (3 mg/kg, body weight) was dissolved in saline, the dose chosen was based on work already done by the research group, which demonstrated that ICV-STZ (3 mg / kg, body weight) caused a memory and learning in the spatial memory test and caused an increase in the activity of the enzyme AChE. In addition, the rats in the control group received ICV injection of the same volume of saline as in the ICV-STZ group [27]. The animals were allowed to recuperate from the surgery for three days. On the fourth day, oral administration of HES was performed.

Hesperidin and rivastigmine administration

Three days after the induction of the model of sporadic Alzheimer's type dementia (sAD) in rats, the treatment with HES (100 mg / kg body weight and -1 ml/kg) was started daily for 30 days in order to evaluate its effects on memory and the activity of important enzymes of the cholinergic system. After three weeks of ICV-STZ application, the Object Recognition test was performed to evaluate if memory impairment occurred in the ICV-STZ rats. The memory deficit was confirmed the

treatment with RIV was initiated at the dose of 2 mg/kg for 13 days. In this study, the rats were randomly divided into eight different groups, with eight animals per group, including: control (CTR), 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 2 mg/kg + hesperidin 100 mg/kg (STZ + RIV +HES). The HES was dissolved in saline and dose selection was based on literature on the safety of the compound [25]. In addition, studies conducted at this dose have shown that HES has the ability to cross the blood-brain barrier [28] and thus modulate AChE activity [29], thus showing positive effects on memory loss [30]. Rivastigmine is one of the first-line drugs for the treatment of AD in patients [6]. The choice of dose of RIV was based on studies by Singh and Chopra (2014) that reported sustained effects of this dose on memory improvement, as well as the non-toxicity of this dosage [31]. The control group will receive only vehicle (saline). Both solutions were administered orally in the volume of 1 ml/kg. The control group received only vehicle (saline). Behavioral investigations were carried out 21 days after ICV-STZ (object recognition) and 30 days (open field, object recognition and avoidance passive), after the ICV-STZ (Figure 1). During the behavioral tests the animals received the treatments normally. After the end of the tests the rats were euthanized to obtain the biological samples.

Behavioral procedure

Open Field Test

This test was performed to identify changes in locomotory and exploratory [32] abilities that can influence the other test such of the object recognition test and Inhibitory avoidance task. The animals were transferred to a box of 70 x 70 x 30 cm3, with the floor divided into 16 squares measuring 12x12 cm each for the evaluation of the open field. The session lasted for five minutes during which an observer who does not have the knowledge of animal treatment recorded the number of crossing and rearing. Locomotory activity was defined as the total number of areas crossed by

four legs and exploratory activity is was defined as total number of investigations of rats.

Novel object recognition test

In order to evaluate the learning and memory of short and long duration the test of object recognition was performed [33]. The object recognition task occurred in an open field (70 x 70 x 30 cm) as described [34]. All animals received a habituation session, where they were allowed to freely explore the open field arena for 5 min. No objects were placed in the box during the habituation test. Twenty-four hours after the habituation session, the animals were allowed to explore two identical objects, (objects 1 and 2) were positioned in two adjacent corners, for a total of 5 minutes (training session). The short-term memory test (STM) started 1.5 h after training, the rats explored the open field for 5 minutes in the presence of a familiar (object 1) and a new object (object 3). All objects displayed textures, colors and sizes, but different shapes. Memory retention was assessed during the test session and the percentage of the total time of exploration that the animal investigated each object was the measurement memory of recognition. Between experiments, the objects were washed with 30% ethanol solution. In the long term (LTM), performed 24 hours after training, the same rats explored the field for 5 minutes in the presence of object 1 and a new object (object 4). Exploration was defined as smelling or touching the object with nose and /or front legs.

Step-down passive avoidance task

The step-down passive avoidance task has been used to study non-spatial long-term memory [35]. The apparatus consisted of a single box where the floor was made of a metal grid connected to a shock scrambler and in its side, there was a safe platform. During the training session (acquisition trial), each rat was placed on the platform, and usually the stepped down off the platform to explore the box. When it stepped down and placed its four paws on the grid floor, an electric Shock (0.5 mA) was delivered for 3 s. Some seconds later, the rat was removed from the step-down passive avoidance apparatus and returned to its home cage. The retention trial was performed 24 h after training; each rat was again placed on the platform and the

transfer latency time (time it took the step to step off the platform) was measured in the same way as in the acquisition trial, but foot shock was not delivered and the transfer latency Time was recorded to a maximum of 300 s. The criterion for learning was taken as an increase in the transfer latency time on the retention (2nd) trial as compared to the acquisition (1st) trial. Thus, short transfer latencies indicate poor retention.

Brain tissue preparation and isolation of synaptosomes of cerebral cortex and hippocampus

After the behavioral tests, the animals were submitted to euthanasia for exsanguination of the cardiac puncture. The brain was excised and the cerebral cortex and hippocampus were dissected to isolate the synaptosomes [36]. Synaptosomes are artificial structures characterized as membranous sacs, which contain synaptic components obtained from homogeneous fractions of synaptic vesicles [37]. Thus, the cerebral cortex and hippocampus were homogenized separately in a 0.25 M sucrose medium containing 10 mM HEPES (pH 7.4). The homogenate was centrifuged for 3 min at 2000 x g at 4 ° C and the supernatant was again centrifuged at 9500 x g for 13 min. The granules were then resuspended in 2 ml of 0.25 M sucrose, 10 mM HEPES (pH 7.4) and placed on 3 ml Percoll gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 dithiothreitol mM and 3.10 or 23% Percoll, pH 7.4. The gradients were centrifuged at 25,000 xg for 11 min at 4 ° C. Synaptosomes were collected between 10 and 23% bands and diluted with 15 ml of HEPES (140 mM NaCl, 5 mM KCl, 5 mM NaHCO 3, NaH 1, 2 mM 2 PO 4, 1 mM MgCl 2, 10 mM glucose and 10 mM HEPES, pH 7.4). After centrifugation at 22,000 x g for 11 min at 4 ° C, the pellet was collected as the synaptosomes. Synaptosomes were prepared fresh daily and maintained at 0 ° C to 4 ° C throughout the procedure and used to measure AchE enzyme activity.

Lactate dehydrogenase

In order to evaluate the integrity of the synaptosomes preparations, was determined the lactate dehydrogenase (LDH) activity, which was obtained after synaptosomes and platelet lysis with 0.1 % Triton X-100 and comparing it with an intact preparation, using the Labtest kit (Labtest, Lagoa Santa, MG, Brazil).

Brain tissue preparation

At the end of the behavioral test, on day 34 the animals were euthanized. The cranium was opened and the structures were gently removed and separated into the cerebral cortex and hippocampus. These structures are important encephalic regions involved in memory, learning and cognition [38]. Thus, the brain structures were homogenized in a glass potter in a solution of 10 mM Tris-HCl, pH 7.4, on ice, at a ratio of 1:10 (w/v). The resulting homogenate was used to determine the activity of the enzyme BuChE.

Determination of acetylcholinesterase activity in synaptosomes in cerebral cortex and hippocampus

The AChE enzymatic activity was determined [39] method as modified [40]. 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.

Determination of butyrylcholinesteraseactivity in brain

The enzymatic assay of BuChE was determined by the modified spectrophotometric method described [40] and previously described [39]. The method is based on the formation of the yellow anion, 5,5'-dithio-bis-acid-nitrobenzoic anion, measured at 412 nm at 25 ° C for 2 min. The reaction mixture contained 100 mM K + 217 phosphate buffer (pH 7.5), 5,5'-dithiobis (2-nitrobenzoic acid 1 mM) and the enzyme (40-50 µg protein) preincubated for 2 min. The reaction was then started by adding 0.8 mM butyrylthiocholine (BCTh). The experiment was performed in triplicate and the enzymatic activity was expressed in protein µmol BCTh/h/mg protein.

Determination of acetylcholinesterase activity in whole blood

The AChE enzymatic activity was determined [39] method as modified [40]. 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). This enzymatic assay enables the rapid and precise determination of AChE activity in whole blood without sample pre-treatment, and it guarantees high levels of accuracy and reproducibility. The experiment was carried out in triplicate, and enzyme activity was expressed as µmol AcSCh/h/mg of protein.

Statistical analysis

All data were analyzed by two-way ANOVA followed by Tukey's post hoc test, using GraphPad software. Data were presented as mean ± SEM, and $p < 0.05$ was considered to be statistically significant. The body weight of animals was evaluated by repeated-measures ANOVA, in which $p< 0.05$ was considered to be significant. Pearson's correlation coefficient was used to investigate the correlation between some results.

RESULTS

HES and in combination with RIV reestablishes the body weight of ICV-STZ rats

First, we evaluated whether the experimental model (ICV-STZ), HES and/or RIV treatment could influence the loss or gain of body weight in rats, and the results are presented in the Figure 2. There was a decrease in rat body weights in all groups in the first week after of ICV-STZ / saline. However, it was gradually reestablished in the CTR, HES, RIV or HES + RIV / kg groups, as well as in the ICV-STZ group treated HES and / or RIV from the eighth day until the last day of the experiment. However, there was a significant reduction of body weight in the STZ group when compared to the CTR group, being consistent until the thirty-third day. In addition, there was no significant difference in body weight of ICV-STZ rats treated with HES and RIV when compared to the CTR group.

Effect of ICV-STZ on locomotor and exploratory activities in the open field test

Figure 3 shows that the open field test demonstrated that there was no significant difference in exploratory (3A) and locomotor (3B) activities among both groups, suggesting that neither STZ or HES did not cause severe motor impairment during object recognition and avoidance tests.

HES and its association with RIV ameliorate memory decline induced by STZ in rats on object recognition test

In order to confirm whether STZ was able to cause memory impairment in rats, we performed the object recognition before to start the RIV treatment (Figure 4). There were no significant differences among groups in the time spent in both objects (1 and 2) during the training session (Figure 4A).

In contrast, our data revealed that the STZ group did not show a significant preference for the new object when compared to the CTR group (Figure 4B), demonstrating that ICV-STZ leads to deficiency in memory. However, the STZ + HES

group showed a significant preference for the new objective, which demonstrated that STM memory deficits in rats was improved by HES on the twenty-first day of treatment.

As shown in Figure 4C, a significant difference was also observed between the STZ group treated with HES and the STZ group in the LTM test. Control rats had a significant preference for exploration of the new object, while ICV-STZ rats continued to explore the old object. More importantly, similar to the STM test, HES treated animals had a preference over the new object in the LTM test. After the twenty-first day of ICV-STZ or saline, to assess the adjuvant protective effect of HES we administered together the RIV which is considered one of the standard medications in patients with AD. The figure 5 shows the STM and LTM tests performed in the 27 day. The results were similar to the first test, where there was no exploratory difference between the groups by the objects (1 and 2) during the training session (Figure 5A). However, were observed a significant preference of exploration for the new objects in the control group in relation to STZ group in the STM and LTM tests (Figure 5B and Figure 5C). In addition, rats receiving ICV-STZ and treated with HES and/or RIV demonstrated a preference for the new subject. These findings support the hypothesis that HES alone, and more potentially when administered concomitantly with RIV, is capable of improving and recovering memory in ICV-STZ rats.

HES and HES + RIV improves non-spatial memory impairment in rats

Learning in step-down passive avoidance task is based on contextual memory in an adaptive response to a stressful experience (TSUJI et al., 2003). During the acquisition, there was no difference in latency to leave the platform between groups (Figure 6). By contrast, STZ rats decreased the latency time of transfer in the retention assay when compared to the CTR group. However, the treatment with HES and RIV, as well as, both (RIV + HES) was able to prevent this decrease and thus was effective to improve the memory of rats that received ICV-STZ. Moreover, the treatment concomitant of RIV + HES in animals ICV-STZ was more effective in the improvement of memory when compared to animals ICV-STZ that receive just treatment with RIV.

HES combined with RIV attenuates the increase of AChE activity in synaptosomes of the cerebral cortex and rat hippocampus

One of the most obvious findings in AD is the degeneration of cholinergic neurons. Thus, ACh levels are reduced due to their rapid hydrolysis in the synaptic cleft by the AChE enzyme. For this reason, we evaluated the activity of this enzyme and the effect of the treatment with RIV or HES and HES + RIV (Figure 7). Figures 7A and 7B show AChE activities in the cerebral cortex and hippocampus, respectively. AChE activity was significantly higher in the cerebral cortex (146.25%) and in the hippocampus (246%) of the STZ group than to the CTR group (59.39%, 71,09% respectively). Treatment with HES and more significantly with HES + RIV decreased AChE activity in the cerebral cortex (51,41%) and hippocampus (40.81%) of rats receiving ICV-STZ when compared to rats of STZ group. In addition, there was a significant difference in AChE activity in the hippocampus of STZ rats treated with HES + RIV when compared to those treated with HES alone (52.50%). There was no significant difference in AChE activity observed in CTR, RIV, HES and RIV + HES.

HES attenuates the increase of butyrylcholinesterase activity in cerebral cortex and hippocampus of rats

BuChE is an important enzyme in the CNS, its role is also to hydrolyze the neurotransmitter ACh in the CNS. Experiments suggest that BuChE provides a supporting for AChE in the hydrolysis function of ACh [41]. Thus, in order to confirm the increase in BuCh activity in AD, we evaluated the activity of this enzyme in the cerebral cortex and hippocampus of rats (Figure 8). Figure 8A and 8B show a significant increase in BuChE activity in the cerebral cortex (52.41%) and hippocampus (45.88%) of the STZ group compared to the CTR group (34.37%, 31.45% respectively). However, a significant decrease in the BuChE activity in rats treated with HES was observed in the cerebral cortex (34.96%) and hippocampus (30,04%). In addition, a significant decrease in this enzyme activity was demonstrated in the cerebral cortex (30,84%) and hippocampus (30.79%) in animals receiving STZ + RIV + HES when compared to those in the STZ group.

ICV-STZ increases acetylcholinesterase activity in whole blood

Besides AChE activity in the cerebral cortex and hippocampus, we evaluated the activity of this enzyme and the effect of the treatments with RIV or HES and HES + RIV in the peripheral blood (Figure 9). Peripheral AChE activity was higher in the STZ group (51.67%) than in the CTR group (33.77%). However, treatment with HES (38.77%) and more significantly with HES + RIV (34.06%) was able to decrease the peripheral AChE activity of rats receiving ICV-STZ when compared to the STZ group. In addition, there was no significant difference in peripheral AChE activity observed in both treatment control groups.

Correlation between acetylcholinesterase activity in brain regions and passive avoidance latency

In order to verify the existence of a correlation between ACh levels and the intensity of dementia symptoms in ICV-STZ rats, we analyzed the possible correlation of AChE activity and memory test (Figure 10). There was a negative correlation between AChE activity in both regions of the cerebral cortex (Figure 10A) and hippocampus (Figure 10B) with transfer latency in the step-down passive avoidance task test, where increased activity of AChE is related to the decrease in transfer latency time in the memory test. This suggests the involvement of AChE activity in memory deficit in ICV-STZ rats and demonstrates that treatments with HES and/or RIV + HES were effective in improving memory.

Correlation between acetylcholinesterase activity in brain regions and activity peripheral acetylcholinesterase

In order to evaluate a possible correlation between AChE enzyme activity in the cerebral cortex and hippocampus with AChE peripheral enzyme activity, we correlate these two parameters (Figure 11). There was a positive correlation between AChE activity in regions of the cerebral cortex (Figure 11A) and hippocampus (Figure 11B) with peripheral enzymatic activity. Thus, it can be inferred that the increase in the peripheral activity of the enzyme AChE may reflect the increase found in the activity of this enzyme in the CNS.

Discussion

Injection of STZ has been described as an appropriate animal model for sAD by mimicking many of the processes found in this diseasein humans [42], yet it is difficult to establish an experimental animal model that closely mimics the development of SAD. However, studies have shown that ICV-STZ induces AD-like neuropathological characteristics in rats and mice [27, 42, 43]. ICV-STZ injection possibly desensitizes neuronal insulin receptors, which causes a reduction in brain energy metabolism, inhibiting the synthesis of adenosine triphosphate (ATP) and acetyl CoA, with consequent deficiency of cholinergic transmission in rat brain [44].

Based on this relationship, it is demonstrated that deficits in cholinergic transmission may potentially influence aspects of cognition and behavior, including information processing in hippocampal and cerebral cortex regions [45]. Therefore, compensatory strategies are sought in order to promote the increase of the synaptic levels of ACh, thus delaying the effects of AD. Thus, the neuroprotective potential of the dietary flavonoid HES in neurodegenerative pathologies has been supported [15, 21, 23]. In addition, in what refers in particular to DA models, studies have shown improvement in spatial memory in mice after two weeks of treatment with HES [46] with action on the cholinergic system by modulating the enzyme AChE in the cerebral cortex.

In this way, this ICV-STZ model allowed us to evaluate the therapeutic potential of HES in relation to memory restitution in ICV-STZ rats. Furthermore, we emphasize that, to our knowledge, this is the first study to report the effects of HES at a dose of 100 mg/kg in rats ICV-STZ, as well as its adjuvant effect on RIV in memory tests and AChE activity in synaptosomes of cerebral cortex and hippocampus.

Therefore, we evaluated in the present study the possible neuroprotective effect of HES in rats and their adjuvant use to a standard drug in the treatment of AD. Among the main findings, we highlight the best performance of HES treated ICV-STZ rats and in association with RIV in memory evaluation tests, such as object recognition and the passive step-down prevention task, as well as positive effects of these treatments on the activity of AChE and BuChE enzymes.

In this study, initially the weight of the animals was monitored after the intracerebroventricular (ICV) surgery until the end of the experiment. Rats receiving ICV-STZ and/or saline and both HES and/or RIV treatments showed lower body weight after ICV. These findings agree with studies that used the same ICV-STZ model, and also showed weight loss after ICV-STZ in rats and mice [6, 27, 47]. This decrease may be related to food-related changes, where hippocampal damage is related to loss of signs of food deprivation [48]. On the other hand, it is important to note that treatment with HES and RIV does appear to interfere with body weight loss.

Moreover, an important fact to be observed is the relation with spontaneous and exploratory locomotor activities, in which no significant differences were observed during the open field test between the ICV-STZ group and the HES treatment. Thus, this result excludes the interference of this parameter in the step-down passive avoidance task and the object recognition tests.

According to previous studies that demonstrated that the administration of STZ in rat brain induces memory deficits [27, 49, 50], we demonstrated that STZ injection impaired the STM and LTM object recognition memory at 21 days after ICV-STZ and remained until the end of the experiment. It is emphasized that there were no differences between STZ and CTR rats at the time of the object exploration during the training phase, confirming the absence of locomotion deficits or levels of exploitation of the animals. In contrast, behavioral data showed that HES improved the performance of ICV-STZ rats in the task of recognition of STM and LTM objects at 18 days of treatment. Our findings were consistent with other studies reported, in which HES administration is effective in aluminum-mediated memory deficits [51].

Furthermore, emerging evidence indicates that HES attenuates neurodegenerative processes and related complications through its antioxidant properties by modulating oxidative stress in an animal model of Huntington's disease, which demonstrated that the concentration of 100 mg/kg p.o for 21 days of HES was able to improve memory retention in the high labyrinth paradigm, as well as to restore the activity of antioxidant enzymes superoxide dismutase and reduced glutathione and to reverse inversely the lipid peroxidation [52].The present study revealed that HES per se had the potential to increase the time spent in animals by the new object relative to the CTR group, which allows inferring that HES has the ability to improve memory in apparently healthy animals. Also, a significant improvement in rat performance in the STM and LTM object recognition test was observed after 30 days

of ICV-STZ in animals receiving RIV + HES adjuvant treatment when compared to ICV-STZ animals receiving only the treatment with HES. Thus, we observed that there were significant differences in the performance of ICV-STZ rats treated with HES and HES + RIV, which demonstrates that HES alone avoids the deleterious effect of ICV-STZ on memory in rats and that this effect can be potentiated in the treatment adjuvant with RIV.

The long-term non-spatial memory was assessed using the step-down passive avoidance task. Similarly, ICV-STZ rats had impaired performance in the passive avoidance test, as evidenced by the decrease in transfer latency in memory retention when compared to the CTR group. However, treatment with HES and HES + RIV in STZ rats revealed a significant increase in transfer latency. This finding not only corroborates previous studies, which show that HES at a concentration of 100 mg kg orally during 42 days of treatment was able to reverse the learning disability in rats [51], but also evidence that the treatment with HES may serve as a promising adjuvant to conventional treatment with RIV in improving memory deficit.

It is well recognized that the cerebral cholinergic system is important for learning and memory and is closely related to the pathogenesis of AD [53]. AChE is an enzyme that hydrolyzes the neurotransmitter ACh and is responsible for the modulation of the cholinergic system [41]. In addition, BuChE accounts for approximately 20% of ACh hydrolysis in healthy brain, and is also shown to be increased during AD [54]. There isrecords of its observed increase in senile plaques compared to normal brain, suggesting that BuChE could facilitate the formation of senile plaque in AD [55, 56]. Thus, the reduction of ACh levels, either by increasing cholinesterases or by decreasing their synthesis, are considered the main factor of deterioration of learning and memory [57]. Therefore, to avoid the lack of ACh in the case of neurodegenerative diseases such as AD, AChE was considered an attractive target in the treatment of AD in order to minimize the degradation of ACh by keeping it active longer in the synaptic cleft.In this study, we investigated the effects of HES on AChE activity in synaptosomes of the cerebral cortex and hippocampus of ICV-STZ rats. The AChE activity was showed to be increased in the synaptic terminals of the STZ groups in relation to CTR groups. Our findings on AChE activity were complementary to other studies reporting increased activity in the cerebral cortex and hippocampus of ICV-STZ rats [27, 31, 47]. Interestingly, administration of HES alone has already attenuated the STZ-induced increase in AChE in both cerebral cortex

and hippocamppus synaptosomes, but a still more significant reduction was demonstrated in the treatment with HES + RIV, where a similarity occurred with enzyme activity in the CTR groups.

In this sense, our results are consistent with previous studies reporting that HES inhibits AChE activity in the cerebral cortex of ICV-STZ rats [58], but the mechanism of action of HES in the inhibition of AChE enzyme activity is not established. However, inhibition caused by the enzyme AChE may reduce amyloid beta (Aβ) peptide aggregation and formation of neurotoxic fibrils in AD [59]. Thus, it is suggested that the decrease found in the AChE activity may be one of the mechanisms responsible for the better cognitive performance of STZ rats treated with HES and HES associated with RIV.

Similarly, BuChE activity in the cerebral cortex and hippocampus was shown to be increased in ICV-STZ rats when compared to the CTR group, confirming that BuChE is also related to AD, further damaging cholinergic transmission. However, treatments of ICV-STZ rats with HES and co-administration of HES plus RIV reduced the activity of this enzyme when compared to the STZ group, these findings confirm the efficacy of RIV as a competitive inhibitor of AChE and BuChE [60, 61]. However, to our surprise, HES demonstrated to have great influence on this parameter, with a remarkable inhibitory affinity on the activity of BuChE in the CNS, given the fact that there were no significant differences between the treatments with HES and HES plus the pattern drug for AD in the ICV rats -STZ compared to the STZ group.

For this purpose, correlation studies were performed to evaluate the possible relationship between AChE activity and long-term non-spatial memory tests. A negative correlation was observed between AChE activity in the cerebral cortex and hippocampus with transfer time of the animals in the step-down passive avoidance task test. The results of the present study showed that the cholinergic dysfunction promoted by ICV-STZ is closely related to the impairment of cognitive functions, and is in agreement with other studies that emphasize that the cholinergic system is involved In the amplification of LTM induction, and consequently in the consolidation of learning and memory [13, 62].

In addition, we correlate the activity of the cerebral AChE enzyme with the peripheral activity, in order to verify the possible involvement of the peripheral enzyme activity in AD. There was a positive correlation between the activity of the enzyme in the cerebral cortex and hippocampus with peripheral activity. Previous

studies have reported the increase in AChE activity around amyloid plaques in the brain [63, 64], where AChE accelerates the assembly of Aβ1-40, favoring the amyloidogenic pathway [65]. In vivo experiments have shown that when AChE has been incorporated into amyloid complexes, a stronger neurodenegerative response occurs than β-amyloid (Aβ) fibrils alone [66, 67].

Another important finding is that the inflammatory response triggered in the brain by Aβ deposition may peripherally affect AChE activity. The inflammatory stimuli induce the vagus nerve to increase release of ACh neurotransmitter, which in turn acts as an anti-inflammatory agent [68]. Therefore, increased AChE activity and Aβ plaques in the brain may reflect increased peripheral AChE activity.

Thus, in view of the behavioral deficits associated with cholinergic impairment that occur in ICV-STZ imitated AD, our current results indicate that HES and RIV associated neurotransmission in ICV-STZ rats in important regions such as the cerebral cortex and hippocampus (Figure 12). We therefore believe that these findings may have important implications for the administration of HES as a useful intervention in the treatment of sporadic dementia of Alzheimer's disease, so that this compound has been shown to be a potent neuroprotective adjuvant in conventional RIV therapy. However, greater understanding is needed for the precise mechanism of hesperidin neuroprotection.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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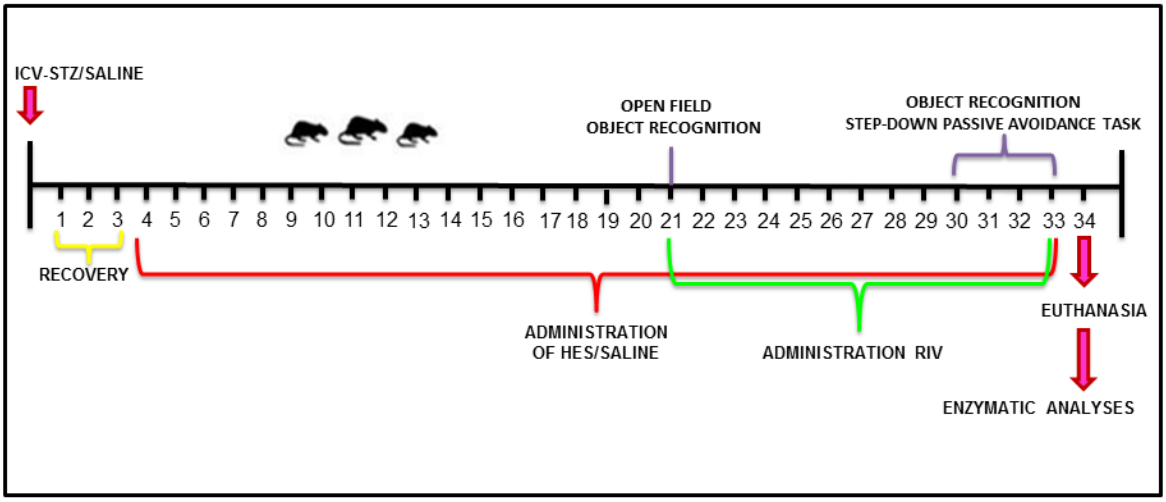

Figure 1: Experimental protocol

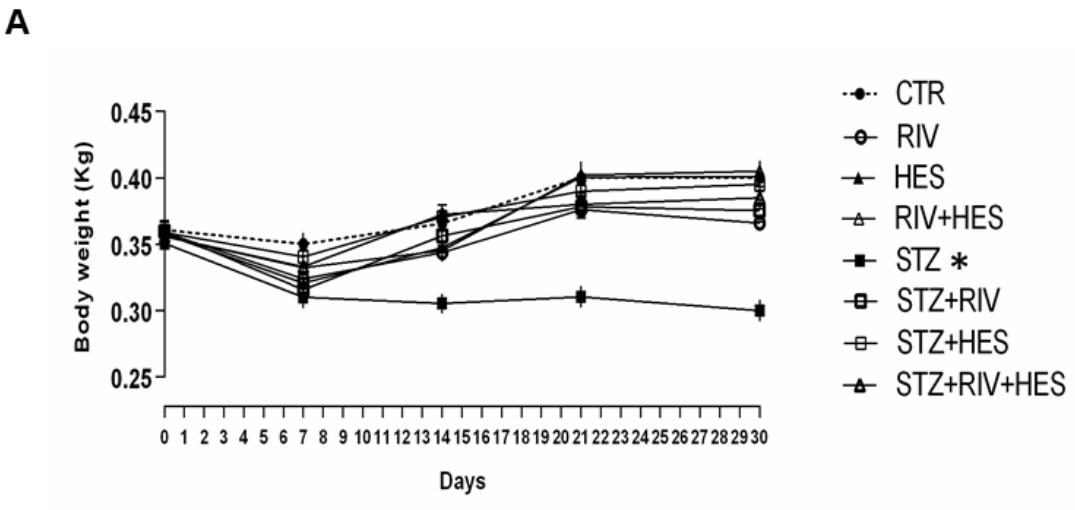

Figure 2: Effect of intracerebroventricular streptozotocin (ICV-STZ or saline), hesperidin (HES) and rivastigmine (RIV) oral treatment on body weight. Data values are expressed as mean body weight in Kg ± SEM (n = 8). *p<0.05, when compared to the CTR group.

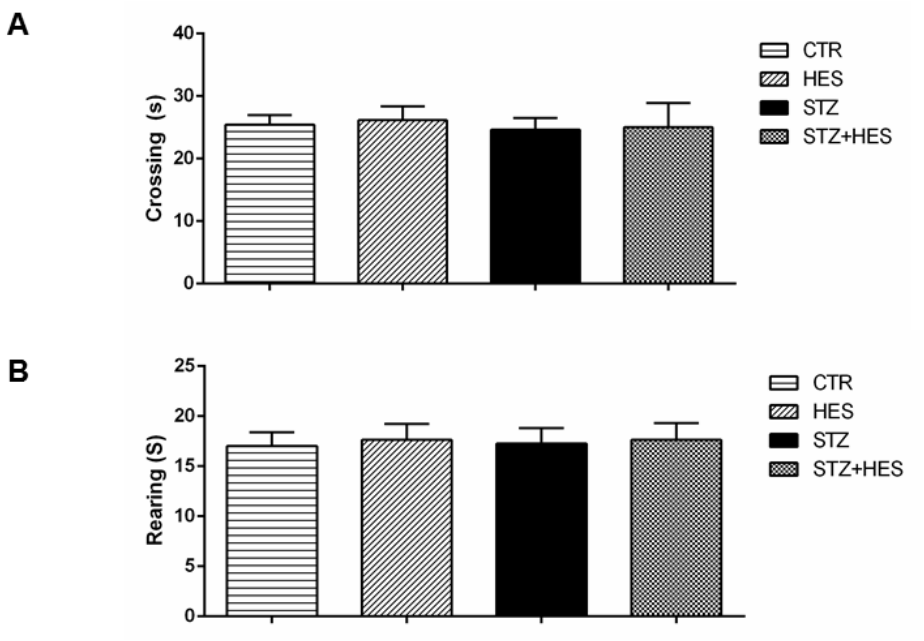

Figure 3: Effect of intracerebroventricular streptozotocin (ICV-STZ or saline) and the treatment with hesperidin (HES) on locomotor and exploratory activities. (A) Crossing, (B) Rearing. Data values are expressed as mean ± SEM (n = 8).

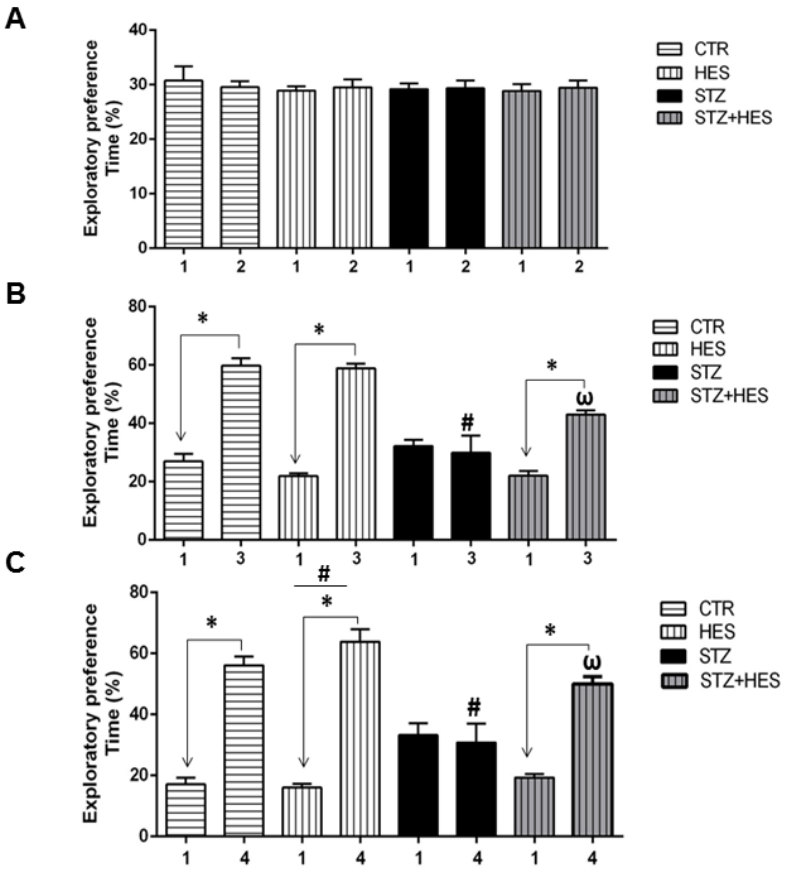

Figure 4: Effect of streptozotocin (STZ) and hesperidin (HES) on a new object recognition task after 21 days of ICV-STZ. A) Exploration Preference during training (two identical objects, 1 and 2). B) Exploratory preference in the STM test performed 1.5 h after training (When 3 is a new object). C) Exploratory preference in LTM Test performed 24 h after training (when 4 is a new object). Data are expressed as mean ± S.E.M of percentage of time exploring using 8 animals per group. * p <0.05 when compared to old object into same group. #p <0.05 when compared to new object of CTR group. ω p <0.05 when compared to the new STZ group object.

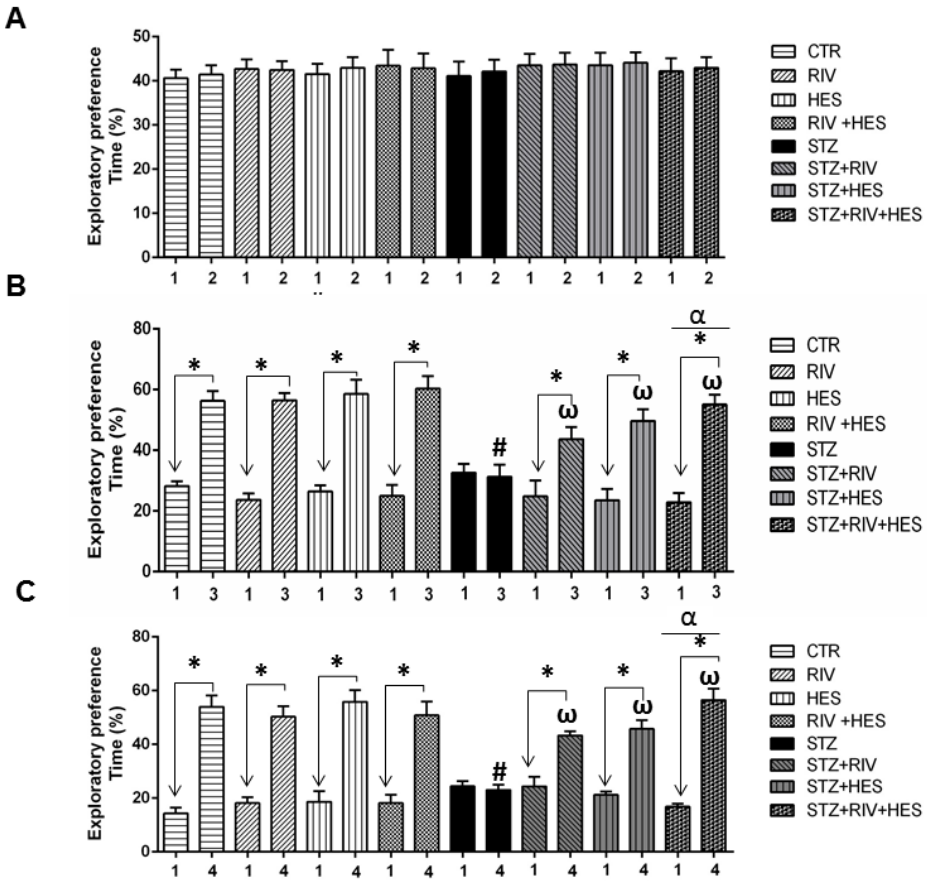

Figure 5: Effect of hesperidin (HES) and rivastigmine (RIV) on a new object recognition task in rats with STZ-induced memory impairment after 30 days of ICV-STZ. A) Exploration preference during training (two identical objects, 1 and 2). B) Exploratory preference in the STM test performed 1.5 h after training (When 3 is a new object). C) Exploratory preference in LTM Test performed 24 h after training (when 4 is a new object). Data are expressed as mean ± S.E.M of percentage of time exploring the objects. * p <0.05 when compared to old object into same group, #p <0.05 when compared to new object of CTR group, ω p <0.05 when compared to the new object of STZ group, α p <0.05 when compared to new object of STZ + RIV and STZ+HES.

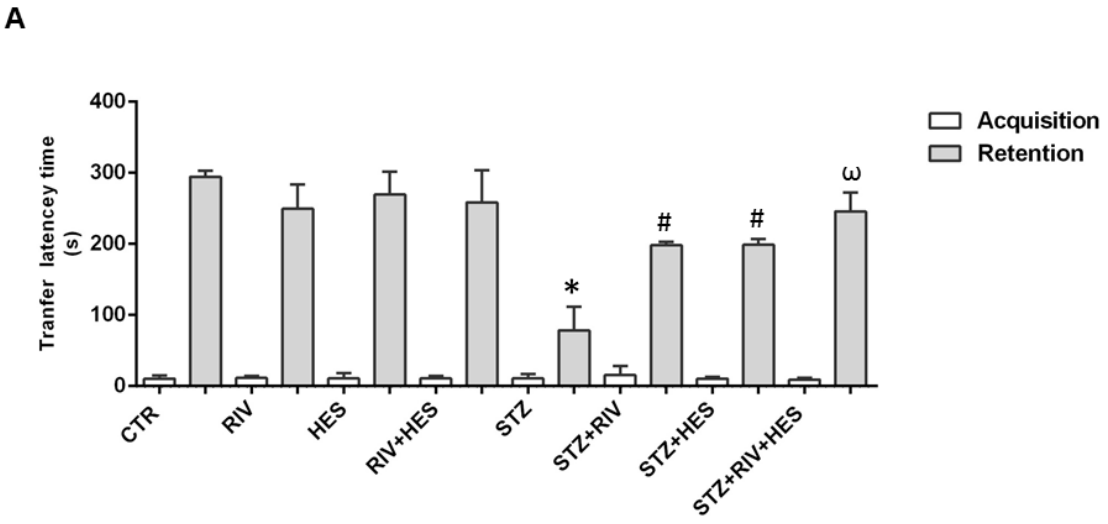

Figure 6: Effect of ICV-STZ, hesperidin (HES) and rivastigmine (RIV) on a passive evasion of detachment. Data values are expressed as mean ± SEM. * p <0.05. * P <0.05 compared to the CTR group, P <0.05 compared to the STZ group, ω <P <0.05 compared to the STZ + RIV and STZ + HES groups.

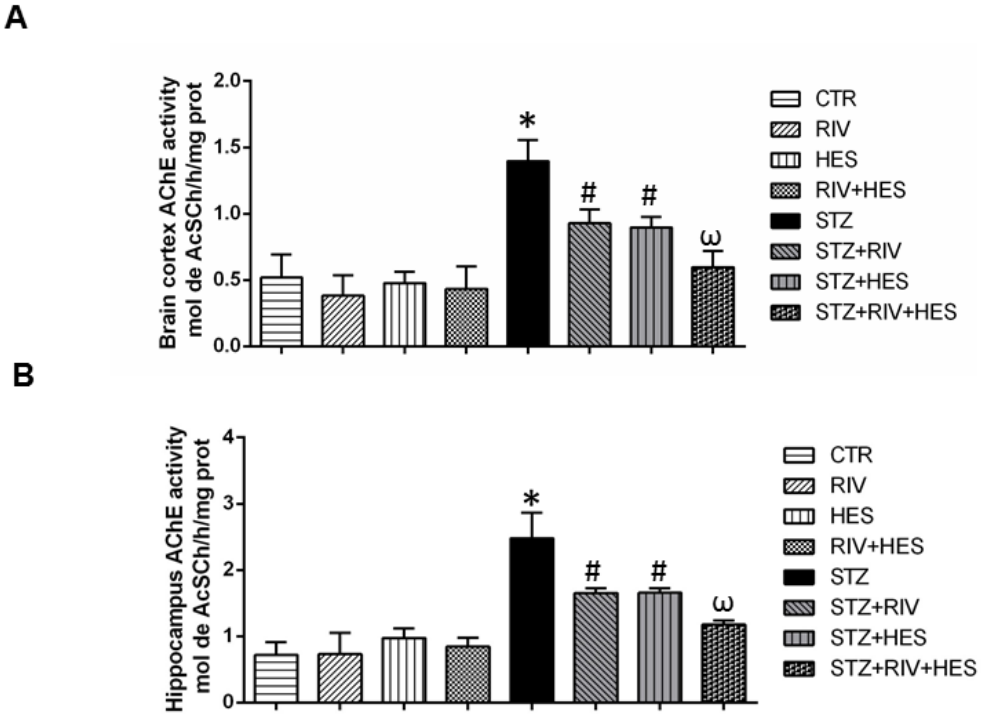

Figure 7: Effect of hesperidin (HES) and rivastigmine (RIV) treatment on AChE activity in cerebral cortex and hippocampal synaptosomes of ICV-STZ rats. Data values are expressed as mean ± SEM (n = 8). * P <0.05 compared to the CTR group, # P <0.05 as Compared to CTR and STZ, ω <P <0.05 compared to STZ + RIV and STZ + HES.

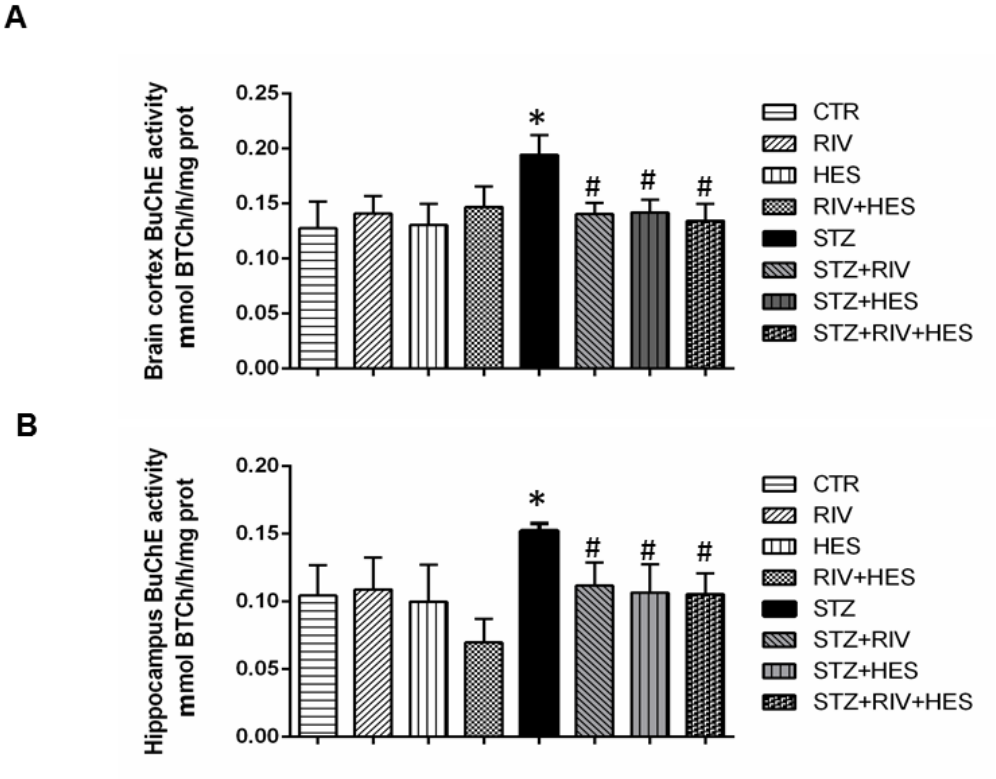

Figure 8: Effect of hesperidin (HES) and rivastigmine (RIV) treatment on BuChE activity in cerebral cortex and hippocampus of ICV-STZ rats. Data values are expressed as mean ± SEM (n = 8). * P <0.05 compared to the CTR group, # P <0.05 as Compared to STZ.

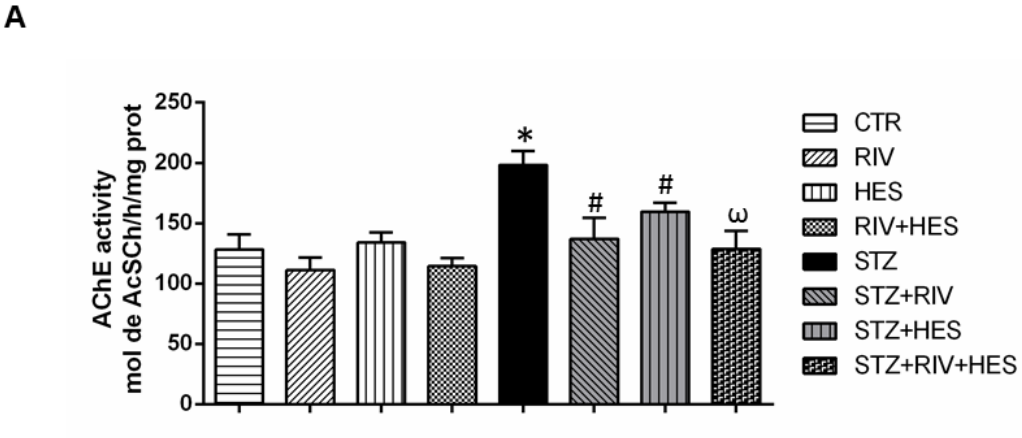

Figure 9: Effect of hesperidin (HES) and rivastigmine (RIV) treatment on AChE activity in the peripheral blood of ICV-STZ rats. Data values are expressed as mean ± SEM (n = 8). * P <0.05 compared to the CTR group, # P <0.05 compared to STZ, ω <P <0.05 compared to STZ + HES.

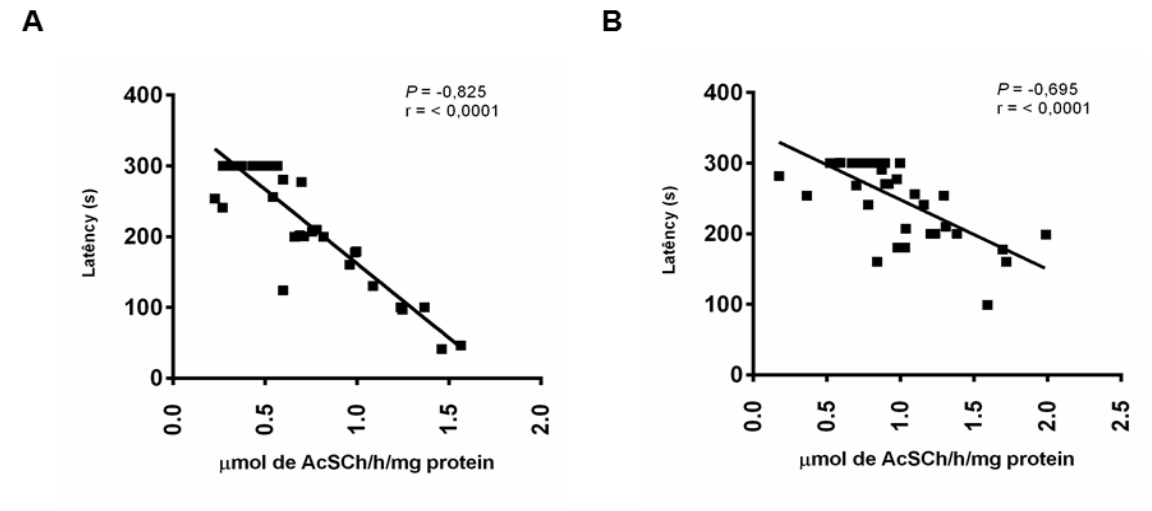

Figure 10: Correlation between acetylcholinesterase activity in the cerebral cortex (A) and hippocampus (B) with step-down passive avoidance task. Data values are expressed as mean ± SEM (n=8).

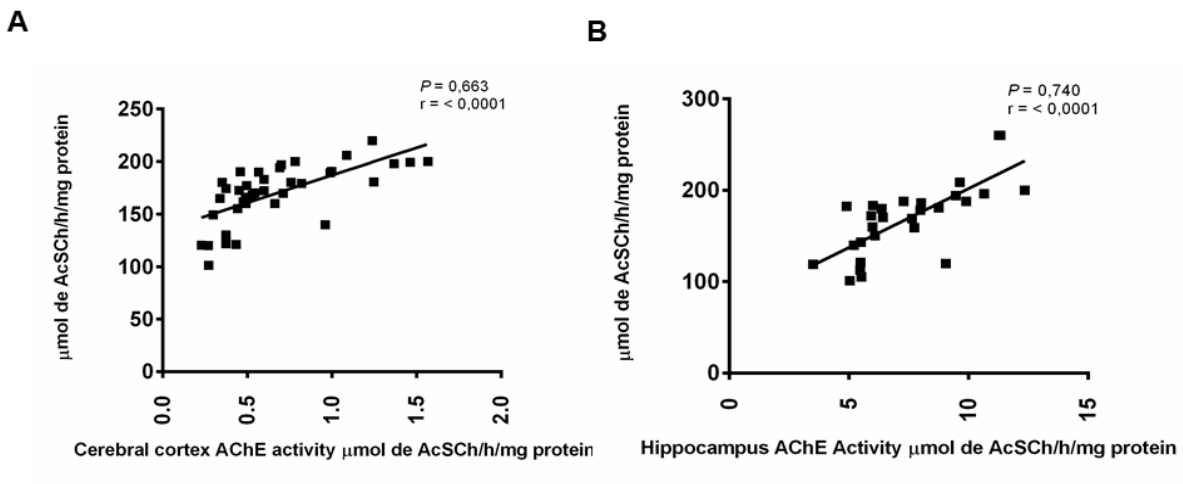

Figure 11: Correlation between acetylcholinesterase activity in the cerebral cortex (A) and hippocampus (B) with activity of peripheral acetylcholinesterase. Data values are expressed as mean ± SEM (n=8).

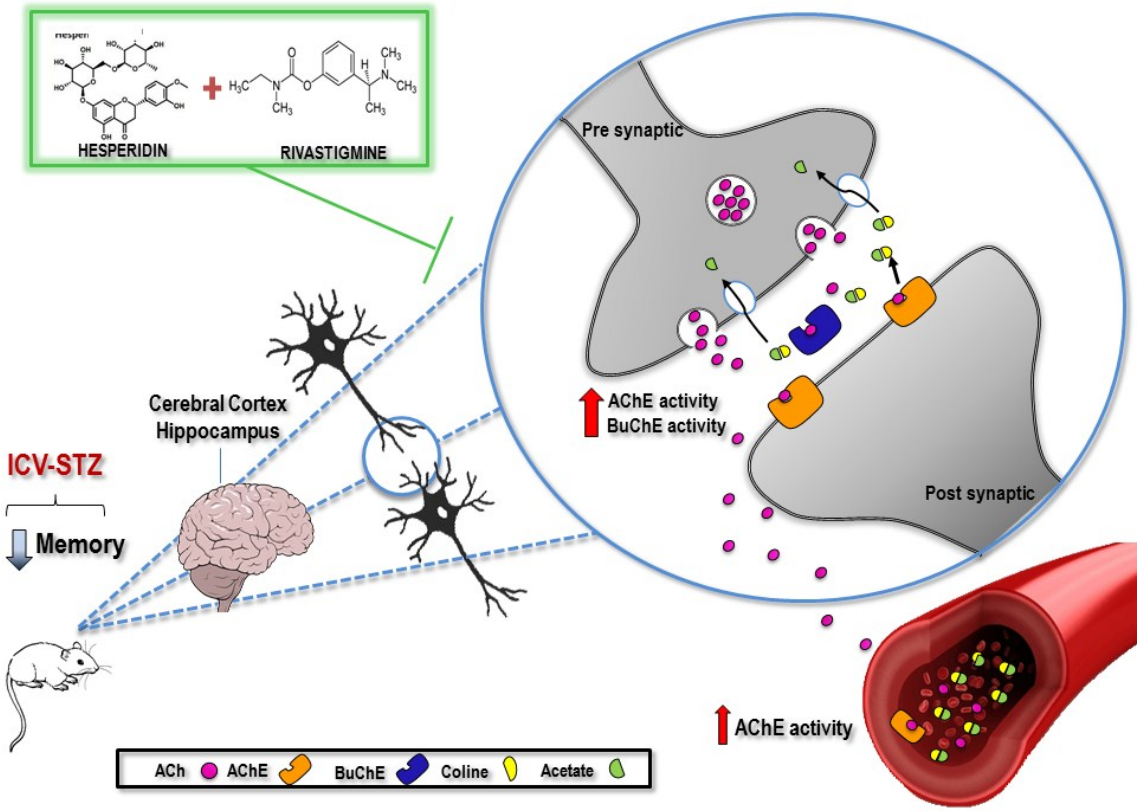

Figure 12: The HES and more interestingly its association with RIV was capable to decrease the activity of AChE and BuChE. Consequently, the increase of ACh neurotransmitter available was capable to improve the learning and memory of rats submitted to the model of ICV-STZ.

5 CONCLUSÕES

Com os resultados apresentados nesta dissertação, pode-se concluir:

- A HES e o seu uso concomitante com RIV atenua o aumento da atividade das colinesterases no SNC, bem como no sangue periférico.

- O tratamento com HES e associado à RIV melhora o comprometimento da aprendizagem e da memória nos testes de reconhecimento de objetos e de memória não-espacial. Assim, este composto fornece neuroproteção em ratos submetidos a um modelo de Demência Esporádica do Tipo Alzheimer, além de potencializar o efeito da terapia convencional com RIV na DA.

- Desta maneira, inferimos que a HES tem capacidade de melhorar o déficit de memória no modelo de Demência Esporádica do Tipo Alzheimer e representar um potencial candidato adjuvante ao tratamento já estabelecido para pacientes com DA.

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ANEXO A – CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que o Projeto intitulado "EFEITO DA HESPERIDINA SOBRE COMPORTAMENTO, ESTRESSE OXIDATIVO, ATIVIDADE DAS ECTOENZIMAS E COLINESTERASES EM UM MODELO DE ALZHEIMER", protocolado sob o CEUA nº 1786040216, sob a responsabilidade de **Cinthia Melazzo de Andrade** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) em reunião de 14/04/2016.

We certify that the proposal "EFFECT OF THE BEHAVIOR HESPERIDIN , OXIDATIVE STRESS , AND ACTIVITY ECTOENZYMES CHOLINESTERASE IN AN ALZHEIMER'S MODEL", utilizing 72 Heterogenics rats (72 males), protocol number CEUA 1786040216, under the responsibility of **Cinthia Melazzo de Andrade** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 04/14/2016.

Vigência da Proposta: de 03/2016 a 12/2017 Área: Clínica de Pequenos Animais

Procedência:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	3 a 3 meses	N:	72
Linhagem:	Wistar			Peso:	300 a 350 g		

Resumo: A Doença de Alzheimer (DA) é considerada a principal causa de demência no mundo. Esses dados destacam a urgência de desenvolver intervenções terapêuticas eficazes para seu tratamento. Estudos pré-clínicos sugerem a utilização da hesperidina (HES), um flavonóide da subclasse flavona com ações antioxidantes, antiinflamatórias e neuroprotetoras, estudada em várias patologias neurodegenerativas, tais como a DA. Deste modo, este projeto visa investigar os efeitos da HES e rivastigmina (RIV) uma droga padrão para o tratamento de DA sobre o comportamento, atividade de enzimas do sistema colinérgico e purinérgico e parâmetros de estresse oxidativo em um modelo de demência esporádica do tipo Alzheimer induzido em ratos. Serão utilizados 72 ratos wistar machos, distribuídos em nove grupos (n= 8): controle (CTR), HES 50mg/kg, HES 100 mg/kg, RIV, STZ, STZ + HES 50 mg/kg, STZ + HES 100 mg/kg, STZ + RIV + HES 50 mg/kg, STZ + RIV + HES 100 mg/kg. Os ratos STZ receberão uma injeção de STZ (3mg/kg) intracerebroventicular (icv) e serão tratados com HES, RIV ou salina, já os animais controle receberão salina icv e serão tratados com as mesmas soluções. O tratamento será por via oral no volume de 1 mL/kg. Os testes comportamentais iniciarão no 28º dia após a cirurgia e logo após os animais serão submetidos à eutanásia para separação das amostras de hipocampo e córtex cerebral, assim como soro e plasma para a realização das análises. Espera-se com este estudo avaliar o possível efeito protetor da hesperidina no envolvimento da DA neste modelo experimental, através de análise comportamental, além das avaliações de estresse oxidativo e de enzimas do sistema colinérgico e purinérgico.

Santa Maria, 18 de abril de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
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