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Juliana Sorraila de Oliveira

EFEITO DA BERBERINA SOBRE TESTES COMPORTAMENTAIS E ATIVIDADE DA ACETILCOLINESTERASE EM 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
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2016

DEDICATÓRIA

Dedico este trabalho aos meus pais Julio e Iracema e à minha irmã Bruna, meus alicerces da vida.

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RESUMO

EFEITO DA BERBERINA SOBRE TESTES COMPORTAMENTAIS E ATIVIDADE DA ACETILCOLINESTERASE EM RATOS SUBMETIDOS A UM MODELO DE DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER

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A Doença de Alzheimer (DA) é considerada a principal causa de demência no mundo. Assim, a busca por novas intervenções terapêuticas para seu tratamento é de extrema importância. A berberina (BRB), é um alcalóide isoquinolina que apresenta múltiplas ações farmacológicas, tais como antioxidante, anti-inflamatória e neuroprotetora. Estudos pré-clínicos vem sugerindo a sua utilização em patologias neurodegenerativas, entre elas a DA. Deste modo, o objetivo deste estudo foi investigar os efeitos da BRB sobre a aprendizagem e memória espacial, comportamento de ansiedade, atividade da acetilcolinesterase (AChE) e morte celular em um modelo de demência esporádica do tipo Alzheimer induzido por estreptozotocina (STZ) em ratos. Foram utilizados 60 ratos wistar machos, distribuídos em seis grupos (n= 10): Controle, BRB 50 mg/kg, BRB 100 mg/kg, STZ + salina, STZ + BRB 50 mg/kg, STZ + BRB 100 mg/kg. Os ratos STZ receberam uma injeção de STZ (3 mg/kg, 5 µL) intracerebroventricular (ICV) e foram tratados com BRB ou salina via oral. Os animais controle receberam ICV-salina (5 µL) e foram tratados com as mesmas soluções. O comportamento foi avaliado através dos testes de Water Maze e labirinto em cruz elevada. As análises de morte celular e a atividade da AChE foram realizadas em córtex cerebral e hipocampo. Os resultados revelaram que BRB foi capaz de prevenir a perda de memória, o comportamento ansiogênico, o aumento na atividade da AChE e a morte celular induzida por ICV-STZ, demonstrando um importante efeito neuroprotetor. Portanto, sugere-se que este composto pode ser um potencial candidato para o tratamento coadjuvante da DA.

PALAVRAS-CHAVE: Doença Neurodegenerativa. Sistema Colinérgico. Demência Esporádica. Morte Celular Neuronal.

ABSTRACT

EFFECT OF BERBERINE ON BEHAVIORAL TESTS AND ACETYLCHOLINESTERASE ACTIVITY IN RATS SUBMITTED SPORADIC DEMENTIA OF ALZHEIMER'S TYPE

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Alzheimer's Disease (AD) is considered one of the main causes of dementia. Thus, the research for new therapeutic interventions for treatment is of utmost importance. Berberine (BRB) is an isoquinoline alkaloid that has multiple pharmacological actions including antioxidant, anti-inflammatory and neuroprotective properties. Preclinical studies have suggested their use in neurodegenerative disorders, including AD. Thus, the aim of this study was designed to investigate the effects of BRB on behavioral tests, acetylcholinesterase (AChE) activity and cell death in rat model of streptozotocin (STZ)-induced experimental dementia of Alzheimer's type. Sixty Adult male Wistar rats were treated with BRB or saline orally once daily for 21 days and then bilaterally injected with a single intracerebroventricular-streptozotocin (ICV-STZ) (3 mg/kg, 5μL). The rats were divided into six groups (n = 10): Group I: control group; group II received BRB 50 mg/kg, group III received BRB 100 mg/kg, group IV received STZ, group V received STZ + BRB 50 mg/kg and group VI received STZ + BRB 100 mg/kg. The behavior was assessed through the Water Maze test and elevated plus maze. Behavioral parameters were assessed through Morris water maze and elevated plus maze tests. Cell death analysis and AChE activity were performed on cerebral cortex and hippocampus. The results revealed that BRB prevented memory loss, anxiogenic behavior, elevation in AChE activity and cell death induced by ICV-STZ, thereby demonstrating significant neurobehavioral and neuroprotective effects. In conclusion, our results suggest that BRB may be a potential candidate for adjuvant treatment of AD.

KEY WORDS: Neurodegenerative Disease. Cholinergic System. Sporadic dementia. Neuronal Cell Death.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

A	Acetato
ACh	Acetilcolina
AChE	Acetilcolinesterase
APOE	Spolipoproteína E
APP	*Amyloid-precursor protein*
Aβ	β-amilóide
BRB	Berberina
BuChE	Butirilcolinesterase
CoA	Coenzima A
CTR	Controle
DA	Doença de Alzheimer
DAE	Doença de Alzheimer Esporádica
DAF	Doença de Alzheimer Familiar
ICV	Intracerebroventricular
ICV-STZ	Intracerebroventricular de Estreptozotocina
mAChR	Receptor muscarínicos
nAChR	Receptor nicotínico
PRIMA	*Proline-rich membrane anchor*
SNC	Sistema Nervoso Central
SNP	Sistema Nervoso Periférico
STZ	Estreptozotocina

SUMÁRIO

1 INTRODUÇÃO

A doença de Alzheimer (DA) é uma doença neurodegenerativa caracterizada como a principal causa de demência (CARAMELLIA; BARBOSA, 2002), que leva à morte de neurônios envolvidos em funções cognitivas, de memória e motoras (BUCHMAN; BENNET, 2011; JAHN, 2013). Além disso, a DA também pode estar relacionada a outros sintomas neuropsiquiátricos, como ansiedade (RAMAKERS et al., 2013), delírios, apatia e depressão (BOUBLAY; SCHOTT; KROLAK-SALMON, 2016).

Um dos achados mais evidentes na DA é a perda sináptica e neuronal que leva à degeneração dos neurônios colinérgicos. Neste quadro, os níveis de acetilcolina (ACh), um neurotransmissor envolvido em processos de aprendizagem e memória, são reduzidos devido à sua rápida hidrólise pela enzima acetilcolinesterase (AChE), levando à (GARCÍA-AYLLÓN et al., 2011). Deste modo, é fato comumente aceito que a perda da transmissão colinérgica tem uma forte correlação com o comprometimento da cognição e da memória observados na DA, especialmente em áreas do encéfalo relacionadas com a aprendizagem, memória, comportamento e respostas emocionais que incluem o córtex cerebral e o hipocampo (EZEKIEL et al., 2004).

A injeção intracerebroventricular de estreptozotocina (ICV-STZ) em ratos tem sido utilizada como um modelo experimental de demência esporádica do tipo Alzheimer por ter capacidade de mimetizar muitos processos patológicos encontrados nesta doença (KALAFATAKIS; ZARROS, 2014). Entre eles, o prejuízo no metabolismo energético de glicose, estresse oxidativo, disfunção colinérgica, aumento da atividade da AChE (SALKOVIC-PETRISIC et al., 2013). Além disso a ICV-STZ também apresenta hiperfosforilação da proteína tau, aumento da expressão do peptídeo β-amilóide (ELCIOGLU et al., 2016; LIU et al., 2014), entre outros efeitos que levam a uma progressiva deterioração cognitiva caracterizada por déficits de aprendizado e memória (AWASTHI et al., 2010).

Dado o fato de não existir cura efetiva para a DA, a estratégia terapêutica utilizada no tratamento desta doença se baseia principalmente no uso de inibidores da AChE, visando retardar ou amenizar o déficit colinérgico e dessa forma atenuar os sintomas e alterações comportamentais observados na doença (VIEGAS, 2011). Todavia, reações adversas referentes ao uso destes medicamentos, como

problemas gastrointestinais, neuropsiquiátricos, renais e cardiovasculares têm sido relatados (KRÖGER et al., 2015).

Dessa maneira, a busca por intervenções terapêuticas eficazes para o tratamento da DA se faz necessária. Portanto, com base em avanços no tratamento da doença através do uso de compostos naturais, a fitoterapia parece promissora (HOWES; PERRY, 2011).

Destaca-se assim a berberina (BRB), um alcalóide isoquinolina, isolado principalmente da erva chinesa *Coptis chinensis*, que tem demonstrado segurança e eficácia em humanos e animais (KONG et al., 2004; MOGHADDAM et al., 2013). Dessa forma, dados da literatura revelam que a BRB apresenta múltiplas atividades farmacológicas, como antimicrobiana, anti-inflamatória (HAN; LIN; HUANG, 2011; MO, 2014) e antioxidante (EL-WAHAB et al., 2013). Além do mais, dada a sua capacidade de ultrapassar a barreira hematoencefálica e dessa forma desempenhar efeitos farmacológicos positivos no encéfalo (TAN et al., 2013; WANG et al., 2005a; WANG et al., 2005b) têm-se demonstrado evidências pré-clínicas da utilidade da BRB em várias doenças neurodegenerativas e neuropsiquiátricas, tais como a DA (AHMED, 2015; ZHU; QIAN 2006), uma vez que dentre outras ações, este composto é capaz de inibir a atividade da AChE e de outras importantes enzimas relacionadas à esta doença (JI; SHEN, 2012).

Diante do exposto, torna-se relevante elucidar os efeitos da BRB sobre os parâmetros de aprendizagem e memória espacial, bem como da ansiedade, da atividade da AChE e morte celular em 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 berberina sobre a aprendizagem e memória espacial, ansiedade, atividade da enzima acetilcolinesterase e sobre a morte celular neuronal de ratos submetidos a um modelo de demência esporádica do tipo Alzheimer.

2.2 OBJETIVOS ESPECÍFICOS

Avaliar os efeitos da BRB nas doses de 50 e 100mg/kg em ratos controles e em ratos submetidos a um modelo de DETA induzido por ICV-STZ e tratados ou não com BRB:

- A aprendizagem e memória espacial através do teste de Water Maze;
- O comportamento de ansiedade através do teste de Cruz Elevada;
- A atividade da enzima acetilcolinesterase em sinaptossomas do hipocampo e córtex cerebral;
- A morte celular neuronal, através de marcação com Iodeto de propídio em hipocampo e córtex cerebral.

3 REVISÃO DE LITERATURA

3.1CARACTERÍSTICAS NEUROPATOLÓGICAS DA DOENÇA DE ALZHEIMER

Atualmente, devido ao aumento da expectativa de vida da população, há um crescente interesse no estudo de doenças neurodegenerativas (BARBOSA et al., 2006), uma vez que o envelhecimento do encéfalo é geralmente associado com o aumento da vulnerabilidade neuronal (MATTSON; MAGNUS, 2006). Essas doenças são, portanto, intimamente relacionadas com o envelhecimento e se apresentam como patologias caracterizadas pela morte celular cerebral, o que leva à perda progressiva e incapacitante de determinadas funções do sistema nervoso central, com deficiência na neurotransmissão, neuroplasticidade e neurogênese, além do aumento da apoptose (YOU et al., 2016).

Além disso, algumas das doenças neurodegenerativas são hoje consideradas as maiores causas de demência no mundo. Elas podem ser distinguidas conforme o tipo de neurônios e regiões do encéfalo que são primeiramente afetados, bem como os diferentes sintomas apresentados em razão das funções iniciais perdidas (RICHARDS et al., 2016).

Citada pela primeira vez há mais de um século por Alois Alzheimer (ALZHEIMER, 1907), a DA representa atualmente a forma mais comum de demência em idosos (REITZET 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 em 2015 havia cerca de 46,8 milhões de pessoas no mundo vivendo com DA e 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 existem muitos dados a respeito da incidência da DA no Brasil, mas estimou-se que um milhão de pessoas fossem acometidas por esta doença em 2012 no país (FERRI, 2012).

Dados ainda mais preocupantes demonstraram que a expectativa de vida do paciente com a DA é curta, pois o falecimento do indivíduo costuma ocorrer em cerca de 3 a 9 anos após o diagnóstico (QUERFURTH; LAFERLA, 2010), sendo ocasionado geralmente por complicações da imobilidade, uma vez que em estágios mais avançados da doença os indivíduos possuem dificuldades de movimentação e

se tornam muito mais vulneráveis a infecções, tais como a pneumonia, que é muitas das vezes um dos principais fatores que contribuem para a morte de pessoas com DA (ALZHEIMER'S ASSOCIATION, 2014). Além disso, os outros sintomas neurológicos que podem ocorrer mais tarde no curso da doença incluem convulsões, hipertonia, mioclonia, incontinência, e a morte pode ainda estar associada a uma inanição geral e desnutrição (BIRD, 2008).

Pode-se observar que a progressão da DA se dá em primeiro momento por um declínio gradual da memória de curto prazo e da cognição, onde uma incapacidade de reter a informação recentemente adquirida é tipicamente a apresentação inicial. Adicionalmente, com a progressão da doença, os pacientes começam a exibir outros sintomas, incluindo 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 e enfim a morte (BEKRIS et al., 2010).

Sabe-se que a DA pode ser diferenciada de duas formas: a DA de início tardio, também denominado de Doença de Alzheimer Esporádica (DAE) e de início precoce, denominada de Doença de Alzheimer Familiar (DAF). Sendo que, a DAF geralmente se manifesta antes dos 65 anos de idade e evolui rapidamente, sendo bem relacionada com alterações genéticas que podem se manifestar em gerações sucessivas, por mutações autossômicas dominantes associadas a três genes alocados no cromossomo 21, identificados como presenilinas 1 e 2 e a apolipoproteína E (APOE) ε4, e representando em média 1% de todos os casos de DA (BLENNOW; DE LEON; ZETTERBERG, 2007). Já a DAE, que é, portanto a forma mais comum da doença, ocorre em grande parte após os 60 anos e embora alguns estudos tenham demonstrado alguma relação com fatores genéticos, a principal causa para a DAE ainda correlaciona-se principalmente com o envelhecimento (BEKRIS et al., 2010).

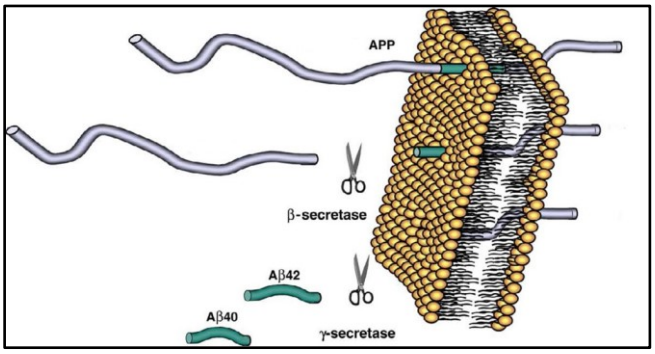
Ambas as formas da doença são definidas pelas mesmas características patológicas, principalmente o decréscimo das funções cognitivas, afetando, sobretudo, a memória, a linguagem, a capacidade de julgamento, a atenção e as funções executivas (BEKRIS et al., 2010). Dessa maneira, as características neuropatológicas mais relevantes da DA incluem a atrofia cerebral com perdas neuronais e sinápticas envolvendo vários sistemas de neurotransmissão. Além disso, apresenta placas senis extracelulares compostas de agregados filamentosos

da proteína β-amiloide (Aβ) e emaranhados neurofibrilares intracelulares, formados principalmente pela proteína tau hiperfosforilada (SERRANO-POZO et al., 2011).

A formação do peptídeo Aβ, formado por 40 a 42 resíduos de aminoácidos, se deriva de uma grande proteína transmembrana chamada de proteína precursora β-amilóide, ou APP (do inglês *amyloid-precursor protein*), abundantemente encontrada na maioria dos tecidos humanos. Quando constituindo a proteína maior, o peptídeo é composto de dois segmentos de α-hélices que atravessam a membrana. Após uma clivagem proteolítica anormal, o peptídeo Aβ remanescente, e relativamente instável, abandona a membrana e perde sua estrutura α-helicoidal e quando agregado na configuração de folha β pregueada se torna insolúvel e neurotóxico e constitui um dos principais eventos patogênicos descritos na DA (CHAMPE et al., 2006; NELSON; COX, 2011). Essa proteólise incorreta da APP (Figura 1) ocorre através de β e γ secretases (WEINER et al., 2012).

Apesar de grandes estudos terem se voltado para esta linha, é demonstrado que estes genes não correspondem unicamente à causa da DA, uma vez que estão ligados a apenas 10% do total de pacientes com esta doença (FRANK; GUPTA, 2005). Além disso, essas descobertas abrem caminho para outras investigações, uma vez que há evidências de que a perda de sinapses também ocorre em regiões onde não existem placas amiloides (SANTOS, 2009).

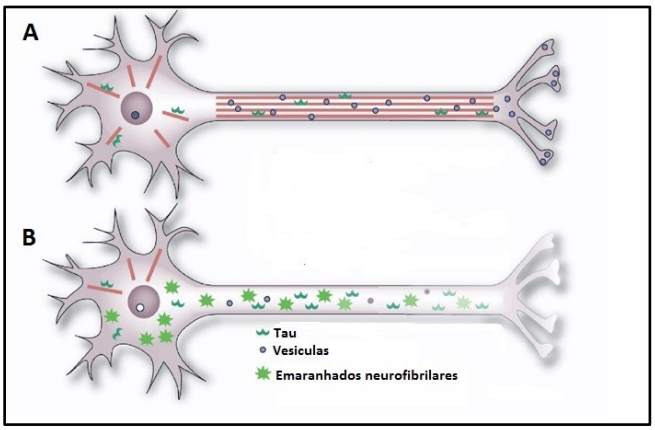
Figura 1 - A geração de fragmentos de β-amilóide a partir da APP.



Adaptado de WEINER et al., 2012

Em relação aos emaranhados neurofibrilares, é demonstrado que em um estado normal, a proteína tau é solúvel e promove a junção e estabilização dos microtúbulos. Entretanto, em estado patológico, a proteína tau exibe propriedades de solubilidades alteradas, com estrutura com forma filamentosa e está anormalmente hiperfosforilada em certos resíduos (FERLA; ODDO, 2005). Como demonstrado na figura 2, a hiperfosforilação da tau provoca a desestabilização dos microtúbulos axonais e prejudica assim o transporte axonal, comprometendo a função neuronal e sináptica (LQBAL et al., 2005).

Figura 2 - Diferenças esquemáticas entre um neurônio normal (A) em que a proteína tau normal mantém a rede de microtúbulos que suportam o transporte intraneuronal e um neurônio de um paciente com DA (B), em que a proteína tau hiperfosforilada forma emaranhados neurofibrilares.



Adaptado de TROJANOWSKI; LEE, 2005.

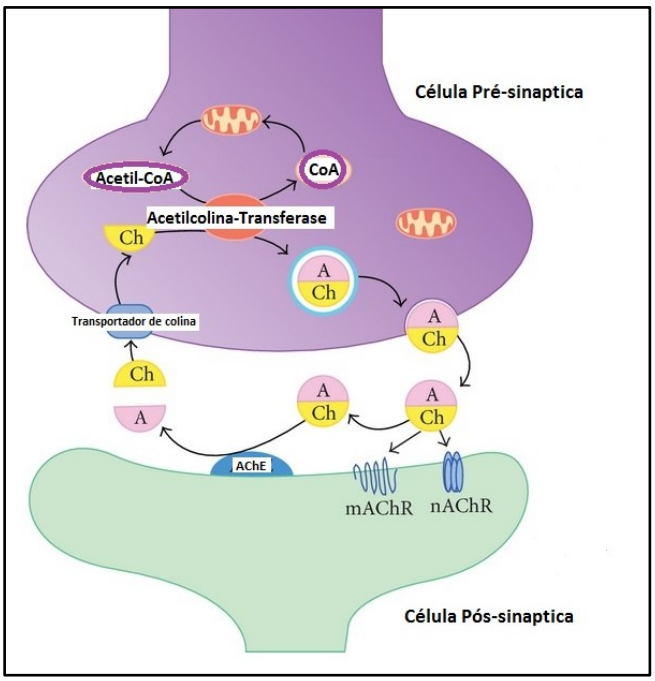
Além disso, a agregação do peptídeo Aβ e a hiperfosforilação da proteína tau podem estar relacionadas com a perda da neurotransmissão colinérgica central observada na DA (KAR et al., 2004). Dessa forma, uma linha crescente de estudos tem demonstrado que além das características neuropatológicas acima citadas, a DA se caracteriza por uma hipofunção colinérgica, com degeneração de neurônios colinérgicos, além de alterações na atividade de enzimas responsáveis pela síntese e degradação do neurotransmissor ACh, sendo esta hipofunção colinérgica associada com o declínio cognitivo observado nesta doença neurodegenerativa (DUMAS; NEWHOUSE, 2011;FERREIRA-VIEIRA et al., 2016; SCHLIEBS; ARENDT, 2011).

A hipótese do envolvimento da neurotransmissão colinérgica na DA é introduzida desde a década de 80 (BARTUS et al., 1982; COYLE et al., 1983), com relatos da sua importância nos processos de aprendizagem e memória já na década de 70 (DEUTSCH, 1971) e se mantém até os dias atuais como alvo de investigações nesta patologia (FERREIRA-VIEIRA et al., 2016). O neurotransmissor ACh, é sintetizado no terminal pré-sináptico dos neurônios a partir de colina e acetil-coenzima A (Figura 3) e utilizado em neurônios parassimpáticos pré e pós-ganglionares e em neurônios simpáticos pré-ganglionares, com importante papel nos sistemas nervosos periférico (SNP) e central (SNC) (FERREIRA-VIEIRA et al., 2016).

No SNP a ACh atua na sinalização do nervo para o músculo nas sinapses especializadas, designadas junções neuromusculares e pode ser responsável por algumas funções não-neuronais, como a proliferação, diferenciação, apoptose, locomoção, migração, funções imunes, organização do citoesqueleto, dentre outras (KAWASHIMAA, et al., 2015; KIRKPATRICK, 2008). Já no SNC, dada a ampla distribuição de neurônios colinérgicos em quase todas as regiões do encéfalo, a neurotransmissão da ACh é responsável em modular funções neurais importantes, dentre elas, a atenção, aprendizagem e a memória, em áreas importantes para esses processos como hipocampo e córtex cerebral (PICCIOTTO; HIGLEY; MINEUR, 2012).

Dessa maneira, quando os neurônios colinérgicos são despolarizados, a ACh é liberada das vesículas sinápticas na fenda sináptica, onde pode ativar seus receptores e uma vez presente na fenda sináptica ser rapidamente hidrolisada pela enzima acetilcolinesterase (AChE), liberando os produtos colina e acetato. Assim, duas classes de receptores são sensíveis a ACh, (figura 3) os receptores muscarínicos, principalmente associados ao SNP e ao músculo liso e cardíaco, e os receptores nicotínicos, que encontram-se no SNC e na placa motora terminal, que são as sinapses entre os nervos e músculos esqueléticos (FERREIRA-VIEIRA et al., 2016).

Figura 3 – Síntese e liberação de ACh



Adaptado de TOLEDO-IBARRA; ROJAS-MAYORQUÍN; GIRÓN-PÉREZ, 2013.

A= Acetato; AChE= Acetilcolinesterase; Ch= colina; CoA: Coenzima A; mAChR: receptor muscarínico; nAChR: receptor nicotínico.

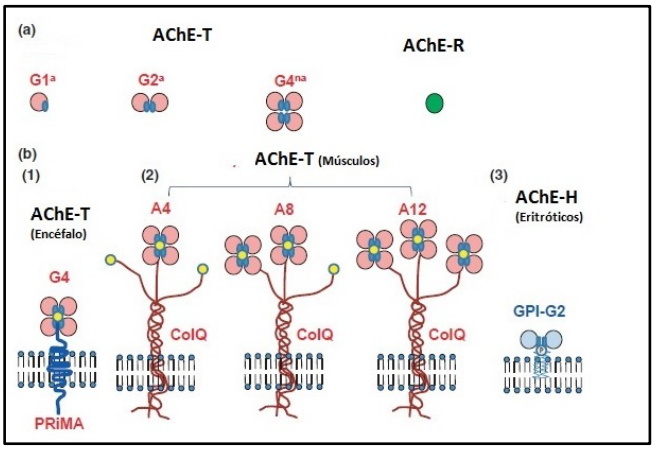
É demonstrado que a AChE é codificada a partir de um único gene, mas existe uma variedade de formas moleculares que varia de tecido para tecido. Estas formas são geradas por *splicing* alternativo do exão C terminal seguidos por modificação pós-transducional (SHEN et al., 2002; SILMAN; SUSSMAN, 2005).

Mamíferos expressam três tipos de mRNA para a enzima AChE diferindo em suas extremidades 3', a AChE-T, -H e -R, que codificam para subunidades AChE com diferentes sequências C-terminal, ou seja, a Ache T ou sináptica (AChET ou AChES), subunidades hidrofóbicas ou eritrocitárias (AChEH ou AChEE) e *read-through* (AChE-R), caracterizada como a forma solúvel e que liga-se à membrana por associação com diferentes tipos de âncoras de membrana (Figura 4). Além disso, as subunidades AChET ocorrem como monómeros, dímeros e tetrâmeros de subunidades catalíticas (G1, G2 e G4), sendo que tetrâmeros de AChET formam as unidades funcionais em sinapses colinérgicas, e estes tetrâmeros são geralmente ligados à subunidade estrutural prima, e também como formas assimétricas (A4, A8,

A12), contendo uma subunidade ColQ, que serve como uma âncora de membrana para AChE nas junções neuromusculares (MASSOULIÉ et al., 2008; MESHORER; SOREQ, 2006).

Nesse contexto, é demonstrado que a principal forma da AChE no SNC, a AChE Tetramérica (G4 designado) se encontra ligada à âncora de proteína rica em prolina (PRIMA, do inglês *Proline-rich membrane anchor*) ligada a membrana (NAVARATNAM et al., 2000;. PERRIER et al., 2002).

Figura 4 - Visão atual de formas alternativas de proteínas da AChE e sua ancoragem à membrana. (A) As formas solúveis da AChE são representadas pelas formas mono -, di e tetramética da AChE-T (G1, G2,G4), e a forma monomérica read-through (AChE-R). (B) as formas ligadas à membrana da AChE são representadas pela forma não anfifilica globular G4 da AChE-T, ancorada na membrana da célula neuronal por meio da âncora de membrana rica em prolina (PRIMA) (1), ou na junção neuromuscular via subunidade Q semelhante a colagénio (ColQ) (2). Em eritrócitos, uma forma hidrofóbica G2 dimérica da AChE (AChE-H) está ligado à membrana celular por meio de uma âncora de GPI (3).



Adaptado de HICKS et al., 2011.

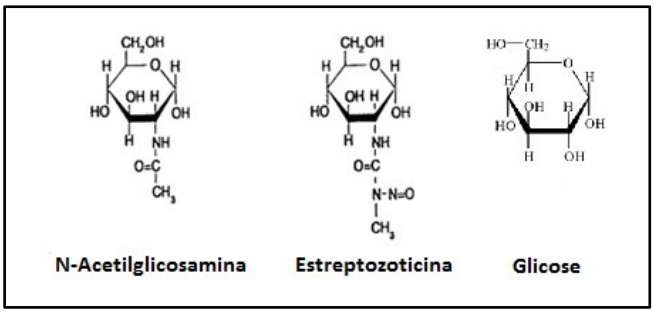
Dessa maneira, a literatura demonstra que déficits na transmissão colinérgica podem potencialmente influenciar todos os aspectos da cognição e comportamento, incluindo o processamento de informações em regiões do córtex cerebral e hipocampo (BENTLEY; DRIVER; DOLAN, 2011). Além das alterações cognitivas, sintomas neuropsiquiátricos são também frequentemente observados em pacientes com DA, estes sintomas não cognitivos incluem ansiedade, agressividade, delírio, excitação ou apatia, desinibição ou depressão (PINTON et al., 2011).

3.2 MODELO EXPERIMENTAL UTILIZANDO A INJEÇÃO INTRACEREBROVENTRICULAR DE ESTREPTOZOTOCINA (ICV-STZ)

Achados moleculares e comportamentais que seguem a perturbação do metabolismo de glicose e sinalização de insulina parecem mimetizar a DA. Nesse contexto, a administração ICV-STZ vem sendo associada em animais, a mudanças morfológicas, moleculares e comportamentais comparáveis à DA (SANTOS et al., 2012; SHOHAM et al., 2006).

A estreptozotocina (STZ) é um derivado de glucosamina-nitrosouréia, com a fórmula química de $C_8H_{15}N_3O_7$ obtido a partir de *Streptomyces achromogenes* (LEWIS; BARBIERS, 1959) utilizada como uma ferramenta experimental em modelos animais para estudar a DAE. Uma vez que, a STZ tem uma estrutura semelhante à glicose e N-acetilglicosamina (GlcNAc) é capaz de inibir a *O*-GlcNAc-seletiva *N*-acetil-β-D-glicosaminidase (*O*-GlcNAcase) (Figura 5). Para muitas proteínas celulares, a adição e remoção de O-GlcNAc é prejudicial para a função biológica, com evidência de essa glicosilação ser particularmente crítica na célula β pancreática (HANOVER, 2001; ZACHARA; HART, 2004a; ZACHARA; HART, 2004b).

Figura 5 – Estrutura química da N-acetilglicosamina e Estreptozotocina



Adaptado de ROBERT et al., 2001

Portanto, estudos têm sugerido que a administração ICV-STZ pode prejudicar constantemente a captação de glicose cerebral e produzir características moleculares e patológicas semelhantes a DAE (LABAK et al., 2010), além de levar a

um estado de resistência da insulina cerebral, perda da massa encefálica, declínio cognitivo, estresse oxidativo, disfunção mitocondrial e apoptose (GRIEB, 2016).

Evidências clínicas sugerem que as alterações nos níveis de insulina, assim como da cascata de sinalização no encéfalo de pessoas com DA têm uma influência sobre o metabolismo da acumulação do peptídeo Aβ (STANLEY; MACAULEY; HOLTZMAN, 2016). Interessantemente, a injeção ICV-STZ por provocar uma disfunção da insulina no encéfalo de roedores, pode levar a uma hiperfosforilação da proteína Tau, que induz a formação de emaranhados neurofibrilares (GRUNBLATT et al., 2007) e a um aumento da expressão do peptídeo Aβ (LESTER-COLL et al., 2006). Sugerindo dessa maneira que uma desordem neuro-endócrina está associada ao desenvolvimento da AD (LESTER-COLL et al., 2006).

Trabalhos demonstraram através de análises imuno-histoquímicas o aumento da expressão da proteína Tau e do peptídeo Aβ em ambas regiões de córtex cerebral e hipocampo em 3 semanas após a ICV-STZ (CHU; QIAN, 2005; LESTER-COLL et al., 2006). Entretanto o comprometimento da memória de roedores foi relatado já em torno de 14 dias (AGRAWAL et al., 2009), sendo ainda observado aos 21 dias (DESHMUKH et al., 2009), 40 dias (SHOHAM et al., 2003) e até em 14 semanas após a ICV-STZ (MEHLA; PAHUJA; GUPTA, 2009).

Dessa maneira, no que se refere ao uso deste modelo para a DAE, dados consistentes são apresentados a cerca dos déficits cognitivos, de aprendizagem e de memória de curto e longo prazo avaliados após a administração ICV-STZ em testes utilizados para avaliação de comportamentos de animais, como o labirinto aquático de Morris, esquiva passiva e reconhecimento de objetos (ISHRAT et al., 2006; SANTOS et al., 2012; SHOHAM et al., 2003, 2007). Esses prejuízos cognitivos da ICV-STZ são geralmente associados a uma disfunção colinérgica, que ocorre principalmente pelo aumento da atividade da AChE (AGRAWAL et al., 2009), ou diminuição da acetil-transferase (ChAT), que poderia levar a uma diminuição dos níveis de ACh (ISHRAT et al., 2006).

Há poucos dados na literatura a respeito dos comportamentos neuropsiquiátricos relacionados à injeção ICV-STZ em roedores. Mas sabe-se, por exemplo, que um comportamento do tipo ansiogênico é observado após a administração ICV-STZ quando avaliado através do teste de labirinto em cruz elevada (PINTON et al., 2011). Esta ação ansiogênica parece ainda estar envolvida em um ou mais de outros eventos, como: (I) anormalidades em vias metabólicas que

se encontram sob o controle da insulina por dessensibilização da cascata de sinalização neuronal; (II) redução do metabolismo da energia cerebral; (III) e deficiência na neurotransmissão colinérgica (PINTON et al., 2011). De fato, outros trabalhos tem também relacionado o papel do sistema colinérgico central na modulação do comportamento ansiogênico (KLINKENBERG; BLOKLAND, 2010).

Além do mais, células colinérgicas parecem ter uma maior demanda para produção de energia e, portanto, serem mais sensíveis a prejuízos na energia relacionados com o envelhecimento (SZUTOWICZ et al., 2006), o que tem reforçado a pesquisa em modelos animais que mimetizam certas características neurológicas típicas da DA, tais como o modelo da ICV-STZ (LESTER-COLL et al., 2006). Dessa forma, estudos anteriores demonstraram que a insulina e seus receptores modulam a memória e a aprendizagem e podem estar envolvidos em distúrbios neuropsiquiátricos, tal qual a ansiedade (AKANMU et al., 2009; MARKS et al., 2009; ZHAO et al., 2004).

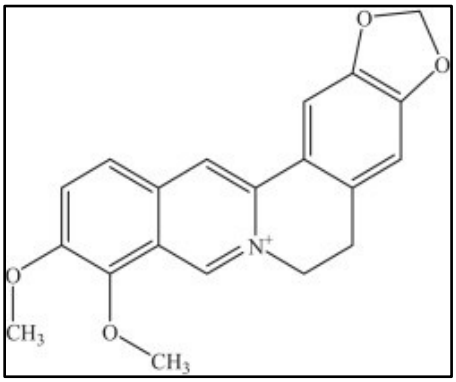
3.3 PROPRIEDADES FARMACOLÓGICAS DA BERBERINA (BRB)

A BRB é um sal de amônio quaternário dentre o grupo dos alcalóides de isoquinolina (Figura 6) e o principal componente bioativo em plantas do gênero *Berberis*, pertencente à família *Berberidaceae* e que compreende aproximadamente 450-500 espécies que são nativas das regiões da América do Norte e do Sul, Europa, África e Ásia (IMANSHAHIDI; HOSSEINZADEH, 2008; SINGH et al., 2010). Como grande utilidade na medicina tradicional chinesa, as prescrições chinesas contêm grandes concentrações de BRB (LIU et al., 2016).

A BRB é largamente encontrada em folhas, galhos, raízes, rizomas, caule e cascas (Figura 7) de diferentes espécies de plantas medicinais, como, *Argemone mexicana*, *Berberis aristata*, *Berberis aquifolium*, *Berberis heterophylla*, *Berberis beaniana*, *Coptis chinensis*, *Coptis japonica*, *Coptis rhizome*, *Hydrastis canadensis*, *Phellodendron amurense*, *Phellodendron chínense Schneid*, *Tinosporacordifolia*, *Xanthorhiza simplicíssima* (BOSE et al., 1963; INBARAJ et al., 2001; JINGZHU et al., 1994; KNAPP et al., 1967; LIU et al., 2006; SATO; YAMADA, 1984; SRINIVASAN et al., 2008; VUDDANDA et al., 2010). No

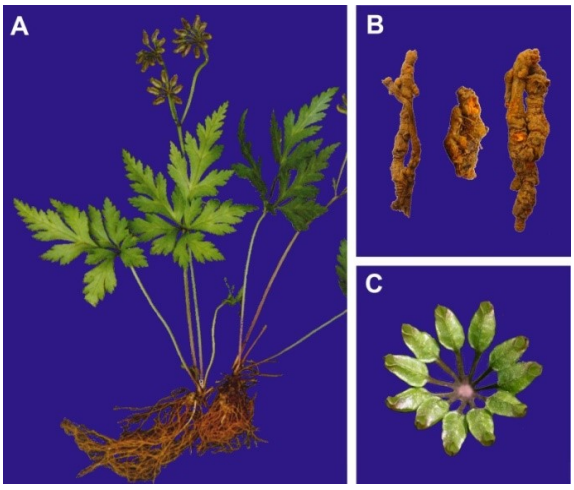
entanto, as raízes e cascas são mais ricas em BRB em comparação com outras partes (ANDOLA et al., 2010).

Figura 6 – Estrutura química da BRB



Adaptado de AHMED et al., 2015

Figura 7 – A: *Coptis chinensis* (planta inteira); B: rizoma seco ; C: carpelos.



Adaptado de XIANG et al., 2016

No que se refere à toxicidade da BRB, estudos tem demonstrado que uma dose de 20,8g de BRB/kg de peso corporal é segura para administração por via oral em camundongos (KHEIR et al., 2010). Dessa maneira, levando ainda em consideração taxa metabólica por kg de peso corporal de aproximadamente sete

vezes maior em camundongos do que os seres humanos, uma dose segura de BRB para seres humanos seria de aproximadamente 2,97 g/kg (KHEIR et al., 2010). A segurança e eficácia do uso da BRB em seres humanos foi ainda confirmada em outros estudos, utilizando uma dose 1000mg/kg por dia durante 3 meses (Zhang et al., 2008) e em uma dose de 500 mg/kg, três vezes ao dia (Yin et al., 2008).

Apesar da sua ampla distribuição em várias ervas e com uma vasta gama de efeitos medicinais a BRB é fracamente absorvida pelo trato gastrointestinal. Neste sentido, trabalhos têm utilizado de outras substâncias como, por exemplo, o d-α-tocoferol e P-glicoproteína a fim de aumentar a sua absorção intestinal (PAN et al., 2002; SHEN et al., 2016). Evidências científicas mostraram ainda que a BRB é metabolizada através de desmetilação, glicuronidação e/ou sulfatação e que pode ultrapassar a barreira hematoencefálica, podendo ser detectada no líquor, seguida por eliminação lenta, o que justificaria a ação da BRB em tecidos cerebrais (AHMED et al., 2015).

Em relação às características cinéticas da molécula de BRB, é demonstrado que ratos que receberam uma dose 100 mg/kg de BRB por via oral, demonstraram uma biodisponibilidade de aproximadamente 0,68% (CHEN et al., 2011). Outro estudo revela que a concentração máxima aos 15 minutos após a administração oral de 25 mg/kg de BRB em ratos foi 16,74 ng/mL, e a concentração no plasma diminuiu rapidamente dentro de 12 horas, porém uma concentração muito baixa de BRB foi mantida no plasma durante 36 horas (GONG et al., 2014). Já outro trabalho realizado por Tan et al. (2013) demonstrou uma absorção de aproximadamente 47.86±6.4 ng/mL no cérebro de ratos, após uma dose oral de 200mg/kg de BRB.

Diversos efeitos farmacológicos têm sido descritos ao uso da BRB, como antifúngica (SILVA et al., 2016) antibiótica (MANOSALVA et al., 2016), anti-inflamatório e antioxidante (JAVAD-MOUSAVI et al., 2016), anti-neoplásico (NAVEEN; GAIKWAD; AGRAWAL-RAJPUT, 2016), e imunossupressor (MAHMOUDI et al., 2016), além de agir sobre o metabolismo de glicose e lipídios (CALICETI et al., 2016), e ter efeitos positivos sobre disfunções gastrointestinais (CHEN et al., 2015; WANG et al., 2015).

No que se refere à ação a da BRB sobre a morte celular, esta se demonstra como duplo caráter em relação aos efeitos apoptóticos, uma vez que tem capacidade de agir como antioxidante em células normais e um pró-oxidante em células cancerígenas. Dessa maneira a BRB pode incitar o estresse oxidativo e

favorecer o efeito apoptótico (HSU et al., 2007) ou ter um efeito anti-apoptótico sobre células normais (HU et al., 2012).

Por conseguinte, além dos efeitos acima citados, estudos tem sido voltados para a aplicação da BRB na pesquisa para tratamento de doenças neurodegenerativas e neuropsiquiátricas. Dessa maneira, a propriedade neuroprotetora da BRB tem sido descrita (FRIEDEMANN et al., 2015), contra danos isquêmicos (CHAI et al., 2013; PIRES et al., 2014), hipóxia (ZHANG et al., 2012) danos neuronais induzidos por peróxido de hidrogênio (HSU et al., 2012), desordens neurológicas que exigem a modulação do glutamato, neurodegeneração induzida por alumínio, doença de Parkinson, entre outras (AHMED et al., 2015).

Além disso, enfatiza-se que os efeitos da BRB sobre o SNC, no que se referem em especial modelos de DA, demonstraram melhora sobre a memória espacial em ratos após duas semanas de tratamento com BRB na dose de 50 mg/kg (ZHU; QIAN, 2006) e sob a disfunção de memória induzida por escopolamina em ratos, com melhora sob esse parâmetro e estimulo da atividade colinérgica após 14 dias de tratamento de BRB na dose 20 mg/kg, i.p. (LEE et al., 2012). Esses efeitos positivos da BRB sobre a DA podem ocorrer pela ação deste composto sobre a inibição de importantes enzimas relacionadas a esta doença como a AChE e a butirilcolinesterase (BuChE) (JI; SHEN, 2012) ou ainda sobre a diminuição dos níveis de β-amilóide e modulação da APP (ASAI et al., 2007).

Em outros modelos que levam ao prejuízo da memória é possível observar efeitos positivos da BRB sobre este parâmetro e sobre a atividade da AChE em trabalhos que utilizaram doses de 25 a 100 mg/kg em ratos (GAO et al., 2014; PATIL et al., 2015). Em virtude de ter a capacidade de modular também o sistema de neurotransmissores, bem como receptores no encéfalo, pesquisas farmacológicas tem voltado atenção para o uso da BRB em várias disfunções do SNC, como depressão, ansiedade, entre outras (KULKARNI; DHIR, 2010).

Com base nas propriedades relatadas para o uso da BRB, é observado forte efeito deste composto sobre neurônios em regiões diversas do encéfalo, tais como córtex cerebral e hipocampo. Assim, o efeito neuroprotetor da BRB é reportado por uma vasta gama de atividades farmacológicas adicionais que são úteis para tratar perturbações do SNC, como mostrado. Nesse contexto, a BRB se torna um potencial candidato para o tratamento de doenças neurodegenerativas.

4 MANUSCRITO CIENTÍFICO

A metodologia, os resultados, discussão e referências desta dissertação apresentam-se sob a forma de um manuscrito científico e representa a íntegra deste estudo. O manuscrito encontra-se nas normas da revista Neurotoxicology.

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

BERBERINE PROTECTS THE IMPAIRMENT OF MEMORY AND ANXIOGENIC-LIKE BEHAVIOR IN RATS SUBMITTED SPORADIC DEMENTIA OF ALZHEIMER'S TYPE: INVOLVEMENT OF THE ACETYLCHOLINESTERASE AND CELL DEATH

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ABSTRACT

The present study aimed to investigate the effects of BRB on spatial and learning memory, anxiety, acetylcholinesterase activity and cell dealth in intracerebroventricular streptozotocin (ICV-STZ) induced experimental sporadic dementia of Alzheimer type. Sixty male Wistar rats were randomly divided into six different groups: control (CTRL), berberine 50 mg/kg (BRB 50), berberine 100 mg/kg (BRB 100), streptozotocin (STZ), streptozotocin plus berberine 50 mg/kg (STZ + BRB 50) and streptozotocin plus berberine 100 mg/kg (STZ + BRB 100). The rats were injected ICV-STZ (3 mg/kg) and on the fourth day, BRB treatment was done daily for 21 days. On the seventeenth day, the behavioral tests were carried out and the rats were submitted to euthanasia on the 24th day. The cell death analysis and AChE activities were performed on the cerebral cortex and hippocampus of the brain. The results revealed that BRB was able to prevent memory loss, anxiogenic behavior, increased AChE activity and cell death induced by ICV-STZ. This may explain in part, the possible protective mechanisms of BRB in ameliorating neurodegenerative diseases including AD and give better comprehension of BRB's effects in the brain. Thus, we conclude that BRB may serve as a potential neuroprotective agent.

KEYWORDS: neuroprotection; Alzheimer`s disease; streptozotocin; cholinergic system; berberine; behavior tests.

1. INTRODUCTION

Alzheimer's disease (AD) is one of the neurological disorders of the central nervous system that involves the deposition of neurotic plaques and neurofibrillary tangles[1, 2]. In 2015, it was estimated that 46.8 million people worldwide were affected by the disease and this number will almost double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050[3]. AD results in loss of neuronal function and synaptic damage, besides the cholinergic neurotransmission dysfunction, reduction in acetylcholine (ACh) levels in the synaptic process as well as a decrease in the number of nicotinic and muscarinic receptors, increase in oxidative stress and massive neuronal loss with subsequent impairment of memory, motor skills and reasoning, and loss of cognitive ability and dementia[4-6]. It is well known that the cholinergic system can be negatively affected by the accumulation of β-amyloid peptides (Aβ)[7].

Neuropsychiatric symptoms such as anxiety, impairment in learning and memory abilities that may result from the dystrophy of nerve cells in the terminal areas of the cortex and hippocampus, are strong evidences that correlate cholinergic dysfunction with progressive neurodegeneration[8, 9]. Therefore, it is believed that the cognitive decline is accompanied by impaired performance of daily activities, behavior, speech and visual-spatial perception [10]. However, possible inhibition of acetylcholinesterase (AChE) could help to maintain and increase the levels of ACh in the neuronal synapses, leading to a positive effect in AD patients[11]. Another important fact is that drastic abnormalities have been found to occur in cerebral glucose and energy metabolism in sporadic AD (sAD). It distorts neuronal insulin, disturbs insulin receptor signal transduction and contributes to the incidence of dementia[12].

Streptozotocin (STZ) is a glucosamine–nitrosourea compound ($C_8H_{15}N_3O_7$) derived from soil bacteria. Intracerebroventricular injection of STZ (ICV-STZ) in rats has been well accepted as a relevant model for sAD-like[13,14]. In addition, ICV-STZ can cause neuropathological changes including hyperphosphorylation of tau at multiple sites with reduction in its ability to bind to microtubules[15], the increase in oxidative stress and AChE activity[16], β-amyloid peptides accumulation[14], apoptosis[17] as well as progressive deterioration of memory, brain insulin resistance and behavioral disturbances[18,19]. Besides, this model can cause drastic abnormalities in

cerebral glucose characterized by decreased glucose utilization and energy metabolism in the brain[20].

It has been well documented in the literature that natural product therapy with antioxidant and anti-inflammatory properties, may play an important role in neuroprotection by facilitating cholinergic neurotransmission and consequently exert various beneficial effects in AD[21, 22]. Berberine (BRB) is a pure phenanthrene alkaloid isolated from the roots and bark plants from the genus Berberis and family Berberidaceae. It comprises approximately 450 - 500 species and has long-term use in Oriental medicine. The BRB has been reported for its wide range of therapeutic effects including anti-inflammatory and antioxidant[23], antiarrhythmic[24], cardioprotective[25], anticancer [26], hypolipidemic[27] and neuroprotective properties [28, 29]. Earlier study by JI and Shen (2009) has revealed the anticholinesterase effect of BRB[30]; however memory and anxiogenic-like behavior in sporadic Alzheimer's disease have not been fully studied. Considering the intense investigation of natural compounds for the treatment of AD, it is therefore important to evaluate the effect of BRB on anxiogenic-like and memory behavior, cell death and AChE activity in ICV-STZ injected rats.

2. MATERIAL AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), Tris-(hydroxymethyl)- aminomethane, ouabainoctahydrate, Coomassie Brilliant Blue G, Trizma Base, streptozotocin (STZ) and berberine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2 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: 109/2013).

2.3 Drugs administration

2.3.1 Intracerebroventricular streptozotocin (ICV-STZ) administration

The animals were anesthetized using thiopental (1 mL/kg) administered intraperitoneally. The head was positioned in a stereotaxic apparatus and skull exposed. Two holes were drilled through the skull for bilateral placement of a microinjector into the lateral cerebral ventricles using the following coordinates: 0.8 mm anterio-posterior to bregma; 1.5 mm lateral to the sagittal suture; and 4.0 mm ventral from the surface of the brain[31]. The STZ (3 mg/kg, body weight) was dissolved in saline[32]. Rats in the control group received ICV injection of the same volume of saline as in STZ treated group. The animals were allowed to recuperate from the surgery for three days. On the fourth day, oral administration of BRB was performed.

2.3.2 Berberine administration

Rats were treated by gavage with BRB (50 or 100 mg/kg bodyweight and 1 ml/kg) or saline daily for 21 days. In this study, the rats were randomly divided into six different groups containing ten animals per group: control (CTRL), BRB 50 mg/kg (BRB 50), BRB 100 mg/kg (BRB 100), streptozotocin plus saline (STZ), streptozotocin plus BRB 50 mg/kg (STZ + BRB 50) and streptozotocin plus BRB 100 mg/kg (STZ + BRB 100). The body weights were monitored throughout the study period. The BRB was dissolved in saline and dose selection was based on literature report on the safety of the compound. Previous studies have indicated that treatment with the same concentration may protect or delay oxidative stress and modulate AChE activity in rat hippocampus and cerebral cortex[33,34]. The CRT and STZ groups received by gavage only vehicle (saline). Behavioral investigations were carried out 17 days after the injection of STZ or vehicle

2.4 Behavioral procedure

2.4.1 Open Field Test

This test was performed to identify locomotory and exploratory disabilities that can influence the other test such as the morris water maze and elevated plus maze task,as previously described by Zanin and Takahashi (1994) with modifications[35]. In the seventeenth day of treatment, animals were transferred to a box of 56 × 40 × 30 cm, with the floor divided into 12 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 was made to record the number and duration of animal crossings from each quadrant, evaluate exploratory and locomotory activities. The passage is thus defined as the total number of areas crossed by four legs and total number of investigations of rats.

2.4.2 Morris water maze

The morris water maze test was carried out to evaluate the effects of BRB on spatial learning and memory in rats ICV-STZ, according to the method described by Morris (1984)[36]. The water maze was a circular black tank (150 cm diameter, 60 cm height, 30 cm depth) with an automatic heater to maintain a water temperature of 28 ± 1°C. This was placed in a room with several extra-maze visual illuminated by a sparse light. Around, submerged platform, painted black, is placed inside this pool, 2 cm below the surface of water which remained during all days of the test. Rats climb this platform to escape the necessity of swimming. Region within 15 cm from border was regarded as border zone while the inner region was called central zone that was further divided into four quadrants. Acquisition trials were performed keeping the platform in different quadrants. The animals underwent 24-hour training and four trials a day for four consecutive days. After the animals find the platform, they remained on it for 40 seconds after each test. When the animal failed to reach the escape platform within 1 min period, it was 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.

2.4.3 Elevated plus maze task

The anxiety-like behavior was evaluated using the task of the elevated plus maze as previously described by Pellow *et al* [37]. The apparatus consists of a wooden structure raised 50 cm from the floor in a room separated from the investigator. This apparatus is composed of two opposite facing open arms, whereas the other two arms are enclosed within the walls and of the same size (walls 40 cm). Initially, the animals were placed on the central platform of the maze in front of an open arm. The animals had 5 min to explore the apparatus; the time spent and the numbers of entries in the open- and closed-arms were subsequently recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

2.5 Brain tissue preparation and isolation of synaptosomes

After the behavioral tests, the animals were euthanized by decapitation. The brain was excised; the hippocampus and cerebral cortex were dissected to isolate the synaptosomes according to the method described by Dunkley *et al.*[38]. The hippocampus and cerebral cortex were separately homogenized in a 0.25M sucrose medium containing 10 mM HEPES (pH 7.4). The homogenate was centrifuged for 3 min at 2000x g at 4 °C and the supernatant was centrifuged again at 9500x g for 13 min. Then, the pellets were resuspended in 2 ml 0.25 M sucrose, 10 mM HEPES (pH 7.4) and were placed in 3 ml Percoll gradients containing 0.32M sucrose, 1 mM EDTA, 0 .25 mM dithiothreitol and 3, 10, or 23% Percoll, pH 7.4. The gradients were centrifuged at 25, 000 x g for 11 min at 4 ° C. The synaptosomes were collected between bands of 10 and 23% and diluted with 15 ml of HEPES (140 mM), NaCl (5 mM), KCl (5 mM), $NaHCO_3$ (1.2 mM), NaH_2PO_4 (1 mM $MgCl_2$), glucose (10 mM) and HEPES (10 mM) pH 7.4. After centrifugation at 22, 000 x g for 11 min at 4 ° C, the pellet was collected as the synaptosomes.

2.6 Determination of AChE activity in brain

The AChE enzymatic assay was determined by the modified spectrophotometric method described by Rocha *et al*[39] and previously described by Ellman *et al*[40]. The method is based on the formation of the yellow anion, 5,5′-dithio-

bis-acid-nitrobenzoic, measured by 412 nm at 25°C for 2 min. The reaction mixture contained 100 mM K^+-phosphate buffer (pH 7.5), 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and the enzyme (40 - 50 µg of protein) pre-incubated for 2 min. Then, the reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). The experiment was carried out in triplicate and the enzyme activity was expressed in µmol AcSCh/h/mg of protein.

2.7 Cell death assay with propidium iodide

Cell death was assessed by staining with propidium iodide (PI - Propidium iodide)[41]. Initially, the tissue (hippocampus or cerebral cortex) was incubated with trypsin for 10 min at 37 ° C and enzymatic digestion was stopped by addition of fetal calf serum (CULTILAB, Campinas Brazil). The cells were mechanically dissociated to obtain individual cells and filtered with filter pores (40 µM). The cells were resuspended at a density of 1 x 106 cells / ml in DMEM-F12 medium (Life Technology) supplemented with 2% B-27 (Life Technology), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). After 4 hours of culture under ideal conditions (37.5% CO_2 and 95% humidity), PI was added to obtain a final concentration of 2.5 µg/ml. The cells were then incubated for 5 minutes and evaluated in the immunofluorescence microscope Axiovert 200 (Zeiss). It is important to stress that only the plasma membrane of dead cells is permeable to PI. The obtained images were analyzed with ImageJ software (National Institutes of Health). The experiments were performed in triplicate.

2.8 Statistical analysis

All data were analyzed with GraphPad software using two-way ANOVA, followed by Tukey's post hoc test. Data are presented as mean ± SEM and $p<0.05$ was considered to be significant. The escape latency parameter in the water maze task and the body weight were evaluated by repeated measures analysis of variance, assuming $p< 0.05$. Pearson's correlation coefficient was used to investigate some correlations.

3 RESULTS

3.1 Body weight

Firstly, we evaluated whether the experimental model (ICV-STZ) used or/and the treatment with BRB, could interfere with the gain or loss of body weight, as presented in figure 1. The result revealed that there was a decrease in body weights of rats in all the groups on the third day after the animals had undergone the ICV with saline or STZ injection. However, there was gradual rise in the body weight of control, BRB 50 or 100 mg/kg groups, as well as, in STZ treated with BRB 50 or 100 mg/kg groups from the fourth day till the last day of experiment. However, there was significant decrease in body weight in STZ group when compared to control (CTR) group. In addition, there was no significant difference in the body weight of rat groups treated with BRB when compared to STZ group.

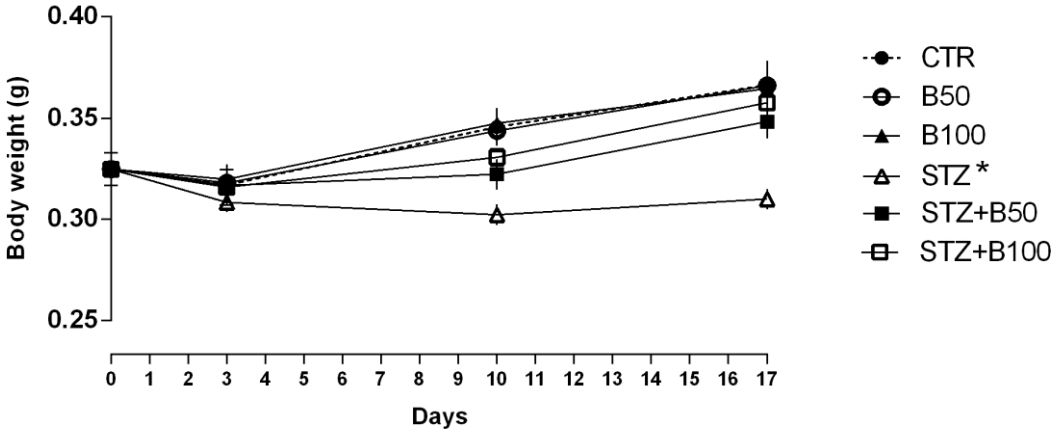

Figure 1: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on body weight. Data values are expressed as mean body weight in g ± SEM. *p<0.05, *STZ group compared to CRT group.

3.2 Open field

We considered if the possible impairment in the locomotory or exploratory activity resulting from the drugs used in this experiment could interfere with the evaluation of behavioral tests; the evaluation of these parameters was assessed using the open field test. The results obtained are presented in figure 2. The open

field test revealed that there was no significant difference in both exploratory (A) and spontaneous locomotory (B) activities between the control, BRB 50 mg/kg, BRB 100 mg/kg, streptozotocin, streptozotocin plus BRB 50 mg/kg and streptozotocin plus BRB 100 mg/kg groups.

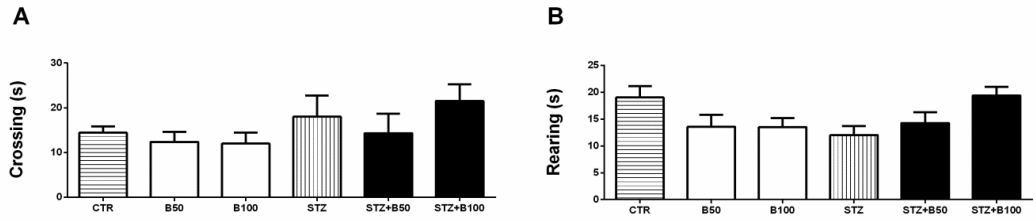

Figure 2: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on exploratory and locomotor activity. (A) Crossing, (B) Rearing. Data values are expressed as mean ± SEM.

3.3 Water maze

The water maze test may be used to assess the decrease in latency time in repeated trials and demonstrate intact learning and memory function, since AD results in memory deficits[42]. In this study, we sought to evaluate a possible neuroprotective effect of BRB using the Morris water maze model as shown in Figure 3. During the training phase, all rats learned the platform location, as evidenced by a decrease in the latency to locate the submerged platform. However, the ICV-STZ rats experienced delay in training as they traveled longer distance before they could find the platform than the control rats. In control rats treated with saline or with BRB at doses of 50 or 100 mg/kg, escape latency per day decreased gradually during the 4-day training. Furthermore, there was no significant difference in escape latency between the control rats treated with saline and that treated with 50 or 100 mg/kg BRB alone (Figure 3A). The escape latency in ICV-STZ rats was significantly higher than that of the control rats (Figure 3B). However, treatment with BRB (50 or 100 mg/kg) significantly reduced the escape latency in ICV-STZ rats (Figure 3C).

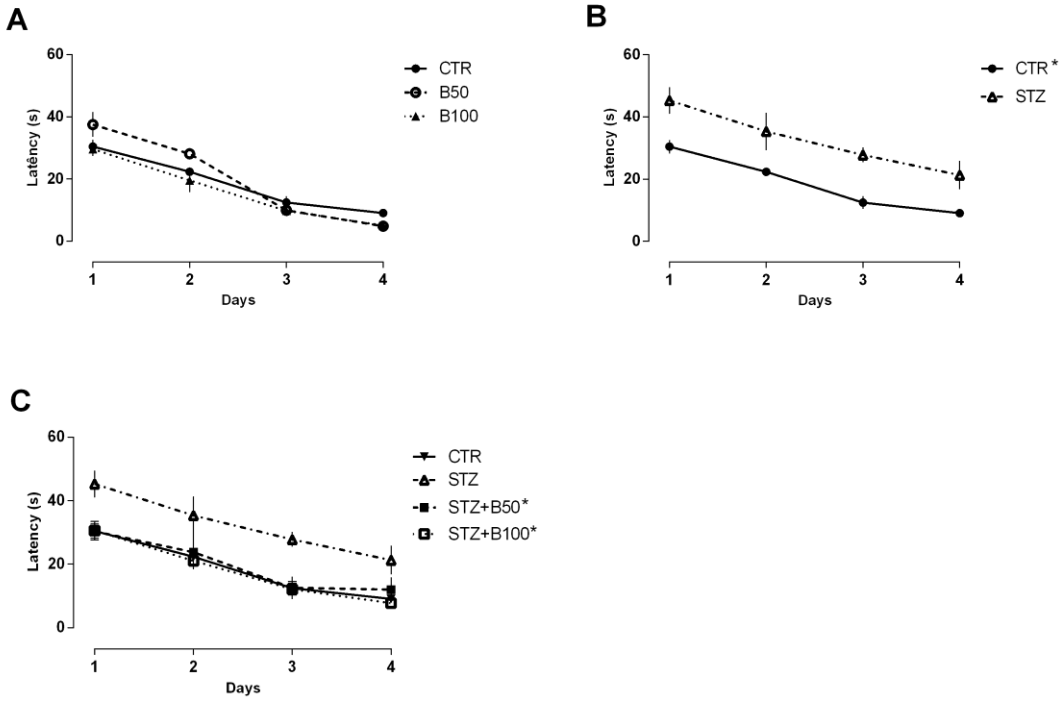

Figure 3: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on water maze test. Data values are expressed as mean ± SEM. *p<0.05, *compared to STZ.

3.4 Elevated plus maze task

Besides the loss of memory and cognition already demonstrated, other behavioral changes were also observed in AD[43]. Thus, we verified whether the BRB could improve the anxiogenic behavior observed in this disease. Anxiety-like behavior was evaluated in an elevated plus maze (figure 4). The number of entries in open (figure 4A) and closed arms (figure 4B) alongside with the time spent in the open arms (figure 4C) and close arms (figure 4D) were investigated in the maze. There were no significant differences in the number of entries into the open or closed arms in all the groups. However, it was observed that rats injected with ICV-STZ showed a significant reduction in the time spent in the open arms and increase in the time spent in the closed arms when compared to the control group. On the other hand, the groups that received ICV-STZ plus BRB treatment (50 or 100 mg/kg) increased the time spent in open arms and decreased time spent in the closed arms when compared to the STZ group. Furthermore, there was no significant change in the number of entries into the open or closed arms in ICV-STZ rats.

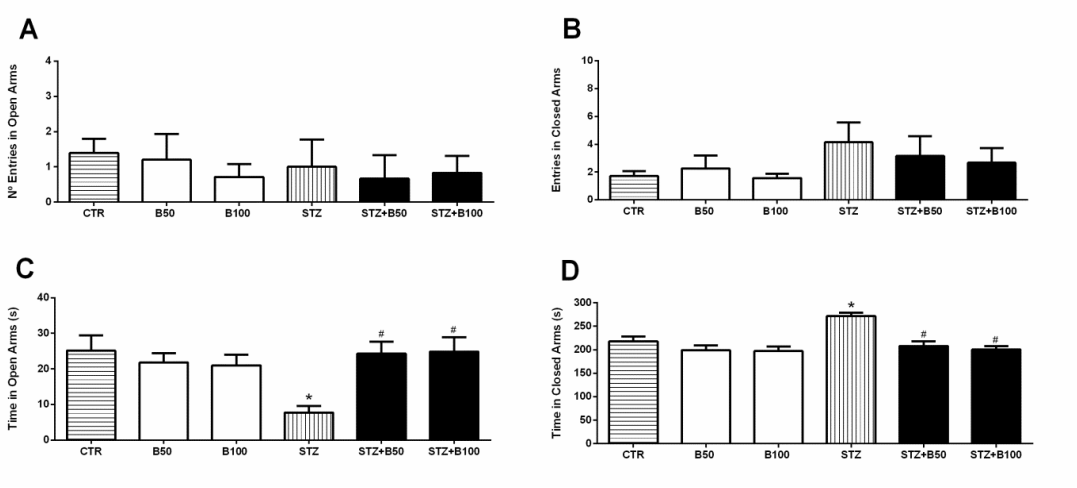

Figure 4: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on anxiety-like behavior in the elevated plus maze task. (A) Nº entries in open arms, (B) Nº entries in closed arms, (C) Time in open arms, (D) Time in closed arms. Data values are expressed as mean ± SEM. *p<0.05, compared to CRT. #p<0.05, compared to STZ.

3.5 Acetylcholinesterase (AChE) activity

One of the most common neurochemical changes in AD brain is a reduced concentration of ACh in hippocampus and cerebral cortex, and AChE increased is linked to its pathogenesis by increase cholinergic deficit. For this reason, we sought to evaluate the activity of this enzyme as well as the action of BRB on it. Figures 5A and 5B reveal the AChE activity in hippocampus and cerebral cortex respectively. AChE activity was significantly increased in both cerebral cortex and hippocampus of STZ group when compared to the control group. The treatment with BRB (50 or 100 mg/kg) significantly decreased AChE activity in both hippocampus and cerebral cortex of rats that received ICV-STZ when compared to STZ group. Furthermore, there was no significant difference in the AChE activities of the control and BRB (50 and 100 mg/kg) treated rats.

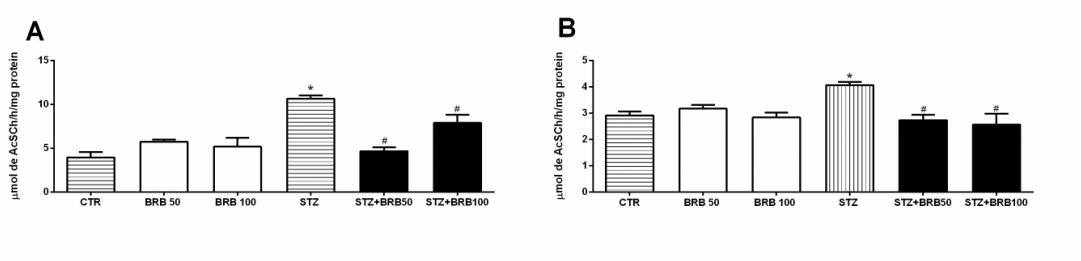

Figure 5: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on AChE activity. (A) Hippocampus, (B) Cerebral cortex. Data values are expressed as mean ± SEM. *p<0.05, compared to control group. #p<0.05, compared to STZ.

3.6 Cell death

It is well known that neuronal cell loss occur predominantly in AD[44]. However, it has been shown that cholinergic enhancement could be a neuroprotective mechanism that may prevent against cell death[45]. For this purpose, we investigated whether beyond the modulation of AChE, BRB could have protective effect on cell death. The effects of BRB treatment and ICV-STZ injection on the potential apoptosis in hippocampus and cerebral cortex are presented in Figure 6 and 7 respectively. There was no significant difference in the number of cell death in both hippocampus and cerebral of the BRB and control groups. Conversely, there were significant reductions in the number of dead cells in both hippocampus and cerebral cortex of STZ groups treated with BRB when compared to STZ group.

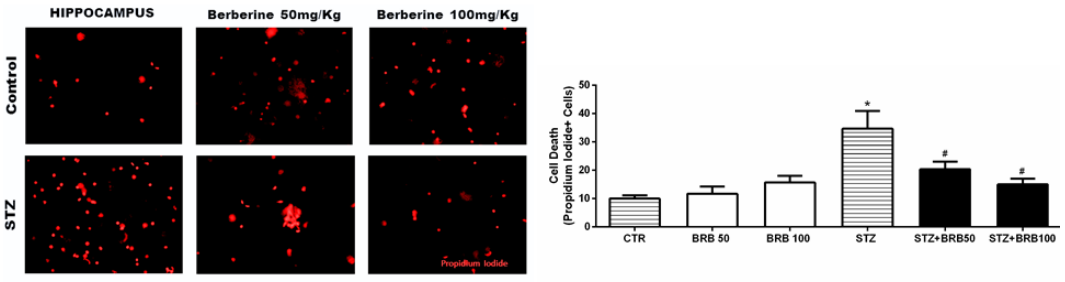

Figure 6: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on potential apoptotic in hippocampus. *p<0.05, compared to control group. #p<0.05, compared to STZ.

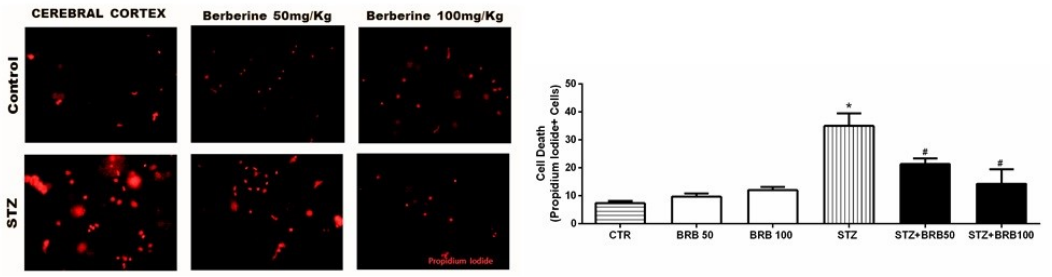

Figure 7: Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on potential apoptotic in cerebral cortex. *p<0.05, compared to control group. #p<0.05, compared to STZ.

3.7 Correlation between the AChE activity in brain regions and anxiety-like behavior or Water Maze tests

To verify the possible correlation of AChE activity with anxiety-like behavior or water maze tests, correlation of data was performed as shown in Figures 9 and 10. There was a negative correlation between AChE activity in hippocampus and time in open arms (figure 9A) while a positive correlation was observed between the AChE activity in hippocampus and time spent in enclosed arms (figure 9B). Conversely, no correlation was observed between the AChE activity in cerebral cortex and anxiety behavior (data not shown). Also, the correlation between AChE activity in the brain regions and spatial memory behavior was investigated (Figure 9A and B). There was a positive correlation between the AChE activity in both brain regions (hippocampus and cerebral cortex) and escape latency in water maze test.

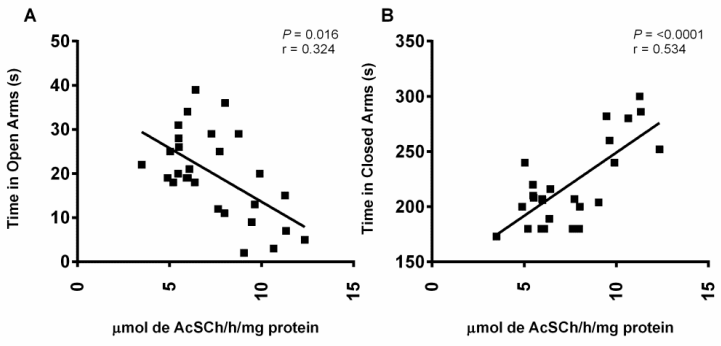

Figure 8: Correlation between the AChE activity in hippocampus and anxiety-like behavior in the elevated plus maze task. (A) Time in Open Arms, (B) Time in Closed Arms. Data values are expressed as mean ± SEM.

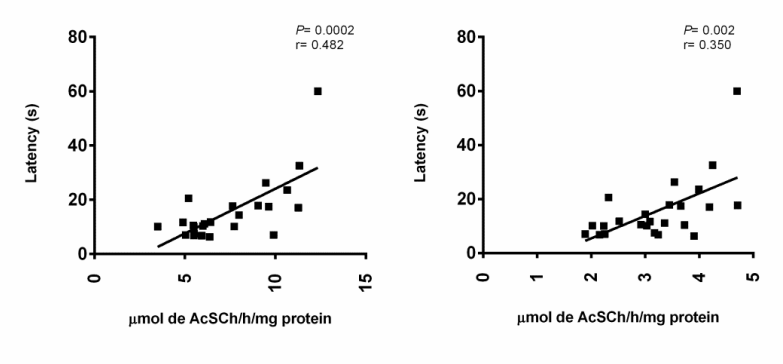

Figure 9: Correlation between the AChE activity in hippocampus or cerebral cortex in the water maze task. (A) Hippocampus, (B) Cerebral cortex. Data values are expressed as mean ± SEM

4. DISCUSSION

Experimental animal models of sAD induced by ICV-STZ injection may allow better understanding and provide insight into various treatment options[46]. Furthermore, it is well established in the literature that the small dose of STZ used in this study does not alter peripheral blood glucose levels and does not induce diabetes mellitus [47] but rather distort glucose metabolism in the brain [20].

Additionally, there was a large number of studies indicating the therapeutic potential of BRB in different neurodegenerative diseases including Brain Ischemia [25], Parkinson's disease[48] and Alzheimer's disease[49,50]. Besides, the inhibitory effects of BRB against the important key enzymes relevant to AD; acetylcholinesterase, butyrylcholinesterase and two isoforms of monoamine oxidase, have also been reported[30].

Thus, in view of structural and enzymatic changes that occur in sAD and mimicked by ICV-STZ injection in rat and previous reports on possible neuroprotective effect of BRB, we believed that administration of BRB may have promising effect and could be a potential candidate to attenuate neurodegeneration in sAD. We emphasize that to the best of our knowledge, this is the first work that describes the effect of BRB in doses of 50 and 100 mg/kg on apoptotic cells using propidium iodide (PI), AChE activity in synaptosomes of hippocampus and cerebral cortex, as well as the correlation of the AChE activity with anxiolytic-like behavior and memory impairment in rats ICV-STZ treated.

In this study, the weight of the animals was monitored immediately after the recovery from surgery until the end of the experiment. The observed decrease in body weight of rats that received ICV-STZ or saline injection and/or treated with saline or BRB in both doses may be attributed to alterations linked with impaired feeding resulting from hippocampal lesion, since the hippocampus is involved in the utilization of hunger state signals[51]. This agrees with reports from other studies where the same model was used in that there was loss in body weight of ICV-STZ treated rats and mice[20,52]. The prevention of loss of body weight by BRB may suggest that the BRB could exert some regulatory mechanism in the appetite control since BRB has capacity to cross the blood brain barrier and thus may have a direct or indirect action on the hippocampus.

In relation to spontaneous locomotor and exploratory activities assessed during the open field test, no significant difference was observed in both spontaneous locomotor and exploratory activities among the all groups. This rules out the interference by BRB treatment and/or STZ injection in performance of the tests of memory spatial and anxiogenic-like behavior.

Earlier report has shown that AD patients have spatial memory deficit[42]. Also, deficit in spatial memory have been reported in rat and rodent models of AD[53] using Morris Water Maze test. Thus, one of the objectives of this study was to evaluate the possible protective effect of BRB in sAD model induced by ICV-STZ in rats on the spatial memory. Accordingly, we found a significant decline in the latency time of the control rats when compared to ICV-STZ rats. This is in agreement with the report of Correia *et al* [47] that showed a decline in latency time was shown in control animal. Interestingly, treatment with 50 or 100mg/kg of BRB in rats that received ICV-STZ revealed shorter escape latency period of the animals in reaching the platform. Since the rats dislike swimming and their tendency to escape from the water was accomplished by finding an escape platform. Shorter escape latencies in STZ+BRB 50 and STZ+BRB 100 groups indicate effectively attenuated spatial memory deficit by BRB treatment in ICV-STZ rats. Therefore, we suggest that the BRB may have a positive effect on spatial memory deficits as evidenced by the Morris test. The result obtained from this study show similar trend with studies from other work where same doses of BRB were reported to ameliorate memory impairment in pilocarpine-induced epilepsy model in the treated rat model[54] and other Alzheimer's disease models[49, 55].

One of the neuropsychiatric symptoms in the AD is anxiety[43]. This study revealed that ICV-STZ injection triggered an increase in anxiogenic-like behavior verified through the elevated plus-maze task. This result agrees with previous studies where anxiogenic behavior was increased in different models of dementia[56-58]. It is worth noting that anxiolytic drugs may increase the proportion of open arm exploration relative to total exploration while anxiogenic compounds may reduce open arm exploration [59]. However, treatment with 50 or 100 mg/kg of BRB reversed anxiogenic behavior induced by ICV-STZ suggesting the possibility that BRB may have anxiolytic effect.

Several mechanisms have been proposed to explain the anxiolytic effect of BRB. Peng *et al* [60] reported that the BRB could attenuate anxiety via the modulation of the serotonergic system. BRB may decrease the serotonergic system activity via activation of somatodendritic 5-HT1A autoreceptors and inhibition of postsynaptic 5-HT1A and 5-HT2 receptors. Lee *et al*[61] also reported that the anxiolytic action of BRB could be by possible modulation of central noradrenergic system. Thus, the anxiolytic mechanism of BRB may be attributed to the decreased the levels of norepinephrine and 5-hydroxytryptamine (5-HT)[60,61]. Furthermore, the modulation of anxiety-like behavior may be linked to the central cholinergic system[62] and this is closely related to pathogenesis of AD[11].

The AChE is an enzyme that hydrolyzes the neurotransmitter ACh in many tissues and is responsible for modulating the cholinergic system[63]. It is reported that there is a greater abundance of these enzyme at synapses than in extra-synaptic fractions and that the AChE found in nervous tissue is primarily of type G4 and is connected to the plasmatic membrane[64]. Besides, this isoform represents approximately 80% of the total brain AChE[65].

It is important to point out that cholinergic system also has a crucial function on the learning, memory, and cortical organization of movement[63]. Consequently, the AChE has been considered as an attractive target for the treatment of AD. In addition, cerebral cortex and hippocampus play an important role in the central nervous system and that AChE is primarily anchored to the plasmatic membrane. In this study we investigated the effects of BRB on the AChE activity in synaptosomes of cerebral cortex and hippocampus from ICV-STZ rats. As expect, we found a significant increase of AChE activity in the STZ group when compared to CRT group.

This result is in accordance with study by conducted Sachdeva et al., (2014) that have reported that ICV-STZ may increase the AChE activity[66].

In this way, considering or results we suggest that the increased AChE activity may cause a decrease in the levels of ACh in neuronal synapses and lead to the reduction of spatial memory. Interestingly, the administration of BRB was able to protect significantly the AChE activity increased in animals that received ICV-STZ injection and were treated with BRB50 or 100 mg/kg. Furthermore, the result from this study agrees with earlier studies where BRB was reported to inhibit AChE activity[33], having this manner, similar action to the reference drugs for the treatment of AD[11].

There are no published data that establish exact mechanism of relationship between the cholinergic system and anxiety-like behavior in ICV-STZ model. We correlated the AChE activity and the anxiolytic-like behavior and memory tests and found a positive relationship between AChE activity in hippocampus and anxiety test. This suggest that the loss of hippocampal cholinergic function may impair stimulus and be manifested in elevated anxiety-like behavior. Similarly, we observed a positive correlation between AChE activity in the hippocampus and cerebral cortex and memory behavior assessed by the water maze test. Results from our study follow the assumption that cholinergic dysfunction may be closely related to memory impairment and cognitive functions.

Another vital aspect of the study was the effect of ICV-STZ administration and BRB treatment on cell death. Post-fixation PI staining method has been known to be a fast, reliable, easily reproducible and valid method to examine the entirety of cytoarchitecture and cell morphology in neuronal tissues[67]. In this study, the observed increase in the number of dead cells in both hippocampus and cerebral cortex of STZ group when compared to CRT group may be attributed to AD conditions characterized by failure in information transfer from the synapses, decline in the number of neurons and eventual death of certain other brain regions. Although the exact cause of apoptosis in AD is not well known, it is speculated that alteration in expression of markers for apoptosis (p53 and Bcl-2), free radicals, insufficient levels of nerve growth factors, neurotoxic insoluble aggregates of Aβ and excessive levels of glutamate may be part of the possible mechanisms that could initiate a cascade of events leading to neuronal death[68]. The result obtained from this study agrees with some studies where markers of apoptosis were altered in the ICV-STZ

model[18, 69]. However, ICV-STZ rats treated with different doses of BRB (STZ+BRB50 and STZ+BRB100) for 21 days showed significant decrease in the number of dead cells and this may explain the anti-apoptotic effect of BRB. Reports from earlier studies have shown that BBR may resist apoptosis via inhibition of caspases[70, 71] and bolster B-cell lymphoma-2 (Bcl-2); an antagonist of apoptosis[72]. Pires *et al*[73] also reported that BRB may exert its positive effects on hippocampal organotypic culture and *in vitro* brain ischemia model by decreasing the incorporation of PI dye and modulating cellular signaling PI3K/Akt and JNK that could lead to decreased activation of caspase 3. The therapeutic effects of BRB may be attributed to its ability to cross the blood brain barrier. Thus, they may have protective action on several brain structures (including the cerebral cortex and hippocampus) and exert beneficial actions on the memory, anxiety, AChE and apoptosis. The multifaceted abilities of BRB may be attributed to its strong neuroprotective effects and conferred great therapeutic potential against neurodegenerative diseases such AD, due to its wide range of biological effects. Overall, BRB may protect the injuries induced by ICV-STZ, inhibit AChE activity, prevent spatial memory impairment and cell dealth, besides exert anxiolytic effect.

In summary, the findings of the present investigation suggest positive effects of BRB in the prevention of memory loss, anxiogenic behavior, increased AChE activity and cell death induced by ICV-STZ. Furthermore, the correction analyzes showed that the higher the activity of AChE, the greater is the loss of memory and higher the anxiogenic behavior in ICV-STZ rat model. BRB may therefore serve as a potential neuroprotective agent which could find clinical relevance in the treatment of sAD.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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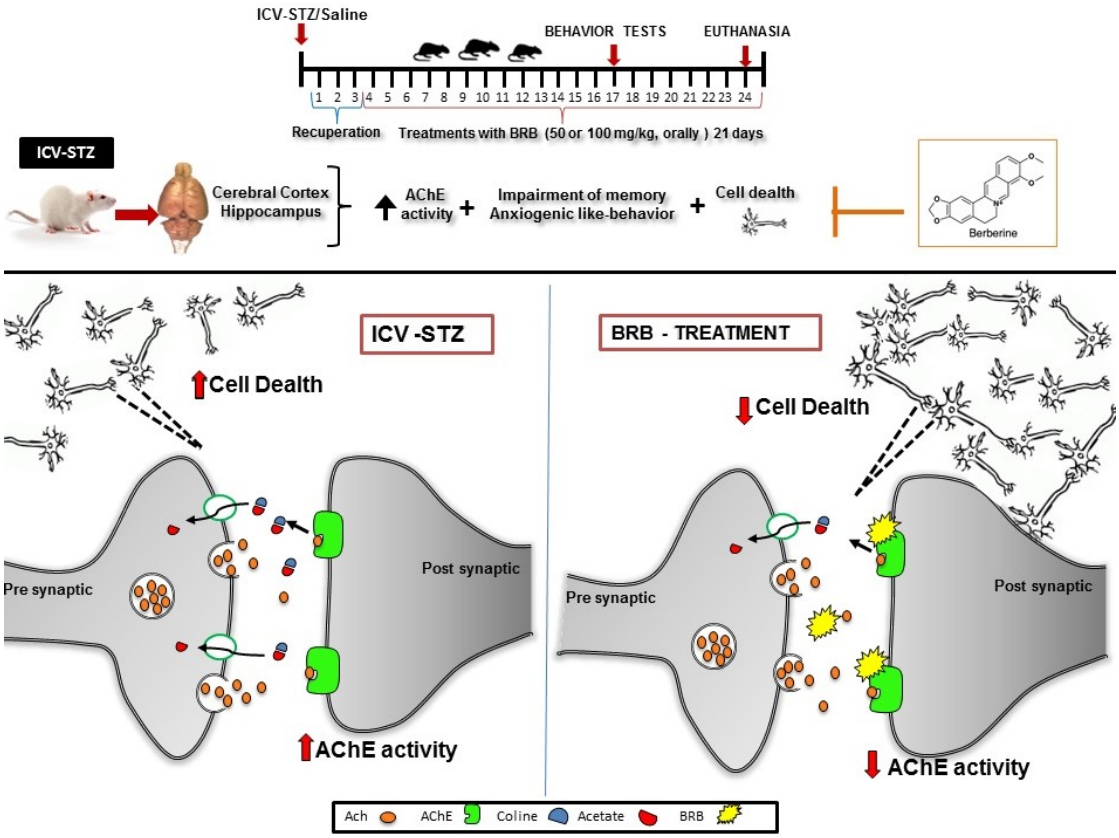
GRAPHICAL ABSTRACT



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5 CONCLUSÃO

Diante dos resultados obtidos pode-se observar que a BRB nas doses de 50 e 100 mg/kg foi capaz de prevenir os déficits de memória e aprendizagem, bem como o comportamento ansiogênico induzidos pela administração de ICV-STZ, assim como prevenir também o aumento da atividade da AChE e a morte celular neuronal em córtex cerebral e hipocampo. Dessa maneira, sugere-se que a BRB pode ser um potencial candidato para o tratamento coadjuvante na DA.

Os resultados encontrados são de grande importância, uma vez que juntamente com o envelhecimento da população é também crescente a prevalência de doenças neurodegenerativas, tais como a DA. Nesse contexto, torna-se de extrema relevância a busca por métodos eficazes não apenas para tratar a doença, mas também prevenir a neurodegeneração, uma vez que o diagnóstico da doença se dá em estágios mais avançados, já com a instalação do processo neurodegenerativo e a apresentação dos sinais clínicos.

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ANEXOS

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito da berberina sobre a memória, parâmetros de estresse oxidativo e atividade das ectonucleotidases e acetilcolinesterase em um modelo de demência esporádica do tipo Alzheimer induzida em ratos."

Número do Parecer: 109/2013

Pesquisador Responsável: Prof.ª Dr.ª Cinthia Melazzo de Andrade Mazzanti

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 16/01/2014.

Santa Maria, 16 de janeiro de 2014.

Prof. Dr. Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

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