UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA TOXICOLÓGICA

Juliana Sorraila de Oliveira

EFEITO NEUROPROTETOR DA BERBERINA NA NEUROTOXICIDADE INDUZIDA POR ESTREPTOZOTOCINA E LIPOLISSACARÍDEO EM RATOS

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Cinthia Melazzo de Andrade

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Aprovado em 29 outubro de 2019:

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Santa Maria,RS
2019

DEDICATÓRIA

A minha família, meus pais Julio e Iracema e minha irmã Bruna, meus maiores alicerces dessa vida. Só nós sabemos o que essa etapa representa em nossas vidas.

AGRADECIMENTOS

Agradeço primeiramente a Deus, o autor da vida, meu refúgio e fortaleza. Gratidão à Ele por tudo que faz em minha vida. Quando tudo se torna difícil são os teus cuidados que me fazem vencer. O teu amor me surpreende.

Agradeço aos meus pais Júlio e Iracema e minha irmã Bruna por representarem simplesmente TUDO em minha vida. Sou privilegiada por ter dentro de casa os maiores incentivadores que alguém poderia ter. Obrigada por estarem sempre ao meu lado e me amarem tanto. Obrigada por não medirem esforços para me ajudarem a alcançar todos meus sonhos. Tudo o que faço na minha vida é pensando em vocês. Amo muito vocês.

À minha orientadora Cinthia, pela oportunidade de realizar o estágio final do curso de graduação, o mestrado e, posteriormente, o doutorado. Obrigada por tornar a minha trajetória acadêmica mais leve durante esses quase 6 anos juntas. Sou imensamente grata à Deus por ter te colocado em meu caminho. Obrigada pelas palavras de incentivo e conforto sempre que precisei e por sempre me receber com um sorriso alegre e animado. És um exemplo para mim além do profissional, mas como ser humana. Obrigada por tudo!

O meu namorado Luiz Fernando, que sempre esteve ao meu lado desde o início da minha trajetória acadêmica. Obrigada por me encorajar a nunca desistir e por estar presente em todas as etapas me incentivando e disponibilizando sua ajuda quando era preciso. Além de compreender minha ausência quando necessária. Amo você.

À minha amiga Fátima Abdalla, por ter-me depositado sua confiança e inicialmente ter sido a ponte que me ligou ao programa da pós-graduação. Obrigada por toda ajuda e por me "mostrar o caminho", literalmente, me ensinando os passos de cada etapa da pesquisa, laboratório, escritas e tudo mais. Obrigada pela paciência e dedicação e por sua amizade e carinho.

À minha amiga Indiara, colega de graduação e pós-graduação e companheira de apartamento, por ingressarmos juntas na realização desse sonho e dividirmos assim as alegrias e angustias desse caminho. Obrigada por "entrar no barco" junto comigo e por sempre se fazer presente quando eu precisei.

Aos meus colegas de laboratório, pela parceria na realização dos trabalhos e por contribuírem para a concretização das minhas pesquisas. Um agradecimento especial àqueles que foram além de colaboradores, amigos, Micheli, Guilherme, Jucimara, Taís, Jamile, Luana Lenz, Luana Magni, Camila, Ana, Bernardo, Cássia e John.

Agradeço à Universidade Federal de Santa Maria – UFSM, o Programa de Pós-Graduação em Bioquímica e a todos os demais que contribuíram para que este trabalho pudesse ser concluído.

EPÍGRAFE

"Prezado Cuidador,

Lembre-se de que sou uma pessoa consciente, portadora de uma doença que compromete minha memória, minha linguagem e meu raciocínio. Por isso, ajude-me a aceitar a demência (...). Não perca a paciência se eu pedir a mesma coisa por mais de uma vez. É a única maneira que tenho de dizer que eu não lembro o que falei antes.

Eu não sou deliberadamente teimoso, mau, ingrato ou desconfiado. A deterioração do meu cérebro faz com que eu me comporte diferente do que eu gostaria. Se eu tivesse um braço quebrado, você com certeza não ficaria irritado comigo por estar impossibilitado de fazer certas coisas, não é mesmo? Mas eu tenho um cérebro que está a cada dia se deteriorando. Então, não me culpe pelos efeitos que a doença de Alzheimer tem em minha habilidade de executar certas tarefas (...).

Eu não esqueço a finalidade de magoar, irritar, embaraçar ou confundir. A doença me faz confuso e desorientado. Nove de dez vezes você está certo em me lembrar de algo, vá em frente; por mais que eu demonstre constrangimento ou me aborreça. Eu sei que preciso que me lembrem de tudo.

Não tire todas as responsabilidades de mim. Eu estou vivo e quero estar incluído na sua vida (...). Não desista de mim. Me estimule sempre. Não solucione todos os meus obstáculos (...).

Não tenha vergonha de mim, não me esconda em casa. Leve-me para passear, ver o sol nascer, o jardim florido, as crianças na praça... eu posso até não entender o que estou fazendo nos lugares, mas com certeza SINTO (...). Transmita-me paz e serenidade. Não fale de mim como seu eu não estivesse ali (...).

Não zombe de mim quando eu fizer minhas confissões; quando eu confundir os nomes dos filhos, do cônjuge, dos netos, o local onde estou, quando eu me perder dentro de minha própria casa. Lembre-se que eu preciso de ajuda e compreensão. Por isso, conheça a doença para poder entender o que eu passo e sinto.

Você poderá se sentir sozinho quando a doença avançar, mas saiba que não foi minha escolha ter demência. Por isso, não me abandone. A natureza da minha doença me faz mudar de personalidade (...). Quando minhas pernas falharem para andar, dê-me sua mão terna para me apoiar (...), eu não escolhi ter Alzheimer. Não chore por mim, nem se deprima por ter que conviver com um demente. Não se sinta triste, ou impotente por me ver assim. Dê-me em seu coração, compreenda-me e me apoie (...)."

Autor desconhecido

RESUMO

EFEITO NEUROPROTETOR DA BERBERINA EM RATOS SUBMMETIDOS À NEUROTOXICIDADE INDUZIDA POR ESTREPTOZOTOCINA E À NEUROINFLAMAÇÃO INDUZIDA POR LIPOPOLISSACARIDEO

AUTORA: Juliana Sorraila de Oliveira
ORIENTADORA: Cinthia Melazzo de Andrade

A Doença de Alzheimer (DA) é considerada a maior causa de demência e caracterizada por apresentar alterações dos sistemas colinérgico e purinérgico, estresse oxidativo, processo inflamatório e mudanças metabólicas que, por sua vez, são responsáveis pela morte celular cerebral com consequente perda de memória e outros sintomas significativos. Por outro lado, a Berberina (BRB), um alcaloide muito utilizado na Medicina Tradicional Chinesa tem apresentado efeitos significativos em doenças neurodegenerativas, entre elas, a DA. Os benefícios descritos pelo uso da BRB dizem respeito, dentre outras, à sua capacidade antioxidante, anti-inflamatória e neuroprotetora. Sendo assim, o objetivo do presente estudo foi investigar os efeitos da BRB sobre a memória e relacionar estas mudanças com os sistemas colinérgico e purinérgico e estresse oxidativo através de dois modelos experimentais em ratos muito utilizados na investigação de compostos candidatos ao tratamento da DA. Para tanto, investigou-se primeiramente o efeito da BRB nas doses de 50 e 100 mg/kg sobre o modelo de demência induzido pela injeção intracerebroventricular de streptozotocina (ICV-STZ). Os ratos foram submetidos a ICV-STZ (3 mg/kg) ou solução salina e, 3 dias depois, iniciou-se o tratamento com BRB nas doses de 50 ou 100 mg/kg ou solução salina durante 21 dias. A avaliação comportamental da memória, bem como, parâmetros de estresse oxidativo e atividade de ectoenzimas em amostras de córtex cerebral e hipocampo foram investigados. Os resultados demonstraram que a BRB em ambas as doses foi eficaz na proteção contra o comprometimento da memória, o estresse oxidativo e a diminuição na atividade das enzimas NTPDase, 5'-Nucleotidase e adenosina desaminase (ADA). Posteriormente, investigou-se a ação da BRB em um modelo experimental de neuroinflamação induzido por lipopolissacarídeo (LPS). Os ratos foram submetidos por 8 dias consecutivos a uma injeção (ip) diária de LPS na dose de 250 ug/kg do peso corporal e a BRB na dose de 50 mg/kg administrada via oral 30 minutos após o LPS. A BRB demonstrou eficácia na proteção da memória de reconhecimento, no aumento da atividade da acetilcolinesterase, na proteção contra o estresse oxidativo e na diminuição da atividade das ectoenzimas NTPDase e 5'-nucleotidase em amostras de córtex cerebral e hipocampo dos ratos submetidos à injeção de LPS. Diante dos resultados apresentados, podemos sugerir que a BRB possui uma potente atividade antioxidante prevenindo a demência do tipo Alzheimer além de uma potencial atividade moduladora sobre as vias do sistema colinérgico e purinérgico. Nesse contexto, o uso da BRB pode ser apontado como uma nova estratégia terapêutica e multialvo a ser considerada no tratamento da DA.

Palavras-chave: *Coptis chinensis*. Memória. Demência. Sistema colinérgico. Sistema purinérgico. Estresse oxidativo.

ABSTRACT

EVALUATION OF THE BERBERINA NEUROPROTECTOR EFFECT ON STREPTOZOTOCIN-INDUCED NEUROTOXICITY AND LPS-INDUCED NEUROINFLAMMATION

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Alzheimer's disease (AD) is considered the major cause of dementia and is characterized by changes in the cholinergic and purinergic system, oxidative stress, inflammatory process and metabolic changes that, in turn, are responsible for brain cell death with consequent memory loss and other significant symptoms. On the other hand, Berberine (BRB), an alkaloid widely used in traditional Chinese medicine has had significant effects on neurodegenerative diseases, including AD. The benefits described by the use of BRB concern, among others, its antioxidant, anti-inflammatory and neuroprotective ability. Thus, the aim of the present study was to investigate the effects of BRB on memory and to relate these changes with the cholinergic system, purinergic system and oxidative stress through two experimental rat models widely used in the investigation of AD candidate compounds. Therefore, the first investigation was through the study of BRB at doses of 50 and 100 mg/kg on the model of dementia induced by intracerebroventricular injection of streptozotocin (ICV-STZ). The rats were submitted to ICV-STZ 3 mg/kg or saline solution and, 3 days later, BRB treatments at 50 or 100 mg/kg or saline for 21 days were started. Behavioral assessment of memory as well as oxidative stress parameters and activity of ectoenzymes in samples of cerebral cortex and hippocampus were investigated.. The results demonstrated that BRB at both doses was effective in protecting against memory impairment, oxidative stress and decreased activity of the enzymes NTPDase, 5'-Nucleotidase and adenosine deaminase. The second study concerns the analysis of BRB on the experimental model of lipopolysaccharide-induced neuroinflammation (LPS). The animals were submitted for 8 consecutive days to an injection (ip) per day of LPS at a dose of 250 µg / kg body weight and BRB at a dose of 50 mg/kg was administered orally 30 minutes after LPS for 8 days. BRB has been shown to be effective in protecting recognition memory, increased acetylcholinesterase activity, oxidative stress and decreased NTPDase and 5'-nucleotidase ectoenzyme activity in cerebral cortex and hippocampus samples from LPS animals. Taken together, we can suggest that BRB has a potent antioxidant activity on Alzheimer's dementia with the potential to modulate cholinergic and purinergic system pathways. In this context, the use of BRB can be pointed as a new therapeutic and multi-target strategy to be considered in the treatment of AD.

Keywords: *Coptis chinensis*. Memory. Dementia. Cholergic system. Purinergic system. Oxidative stress.

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ARTIGO I

LISTA DE ABREVIATURAS

ACh	Acetilcolina
AChE	Acetilcolinesterase
ADA	Adenosina desaminase
ADO	Adenosina
ADP	Adenosina difosfato
APOE	Apolipoproteína E
AMP	Adenosina monofosfato
ATP	Adenosina trifosfato
Aβ	β-amiloide
BRB	Berberina
Ca^{+2}	Cálcio
ChAT	Colina acetiltransferase
CTR	Controle
DA	Doença de Alzheimer
DNA	Ácido desoxirribonucleico
eNOS	Óxido nítrico sintetase endotelial
ERO	Espécies reativas de oxigênio
GLUT 2	*Glucose Transporter*
ICV	Intracerebroventricular
ICV-STZ	Intracerebroventricular de Estreptozotocina
IL-1β	Interleucina 1 beta
IL-6	Interleucina 6
JNK	*c-Jun N-terminal kinases*
K^{+}	Potássio
LPS	Lipopolissacarídeos
mAChR	Receptor muscarínicos
MCP-1	Proteína quimiotática para monócitos-1
nAChR	Receptor nicotínico
Na^{+}	Sódio
NFKB	Fator nuclear kappa B
NTPDase	*Nucleoside Triphosphate Diphosphohydrolases*
PGE2	Prostaglandina E2
PPA	Proteína precursora amiloide
P1	Purinoreceptores do tipo 1
P2	Purinoreceptores do tipo 2
P2X	Purinoreceptores do tipo 2X
P2Y	Purinoreceptores do tipo 2Y
SNC	Sistema Nervoso Central
STZ	Estreptozotocina
TGF-β	Fator de transformação do crescimento beta
TNF-α	Fatores de Necrose Tumoral Alfa
δ-ALA-D	Ácido δ -aminolevulínico desidratase

SUMÁRIO

1 INTRODUÇÃO

As doenças neurodegenerativas estão intimamente relacionadas ao processo de envelhecimento e levam entre outras desordens, à perda progressiva e incapacitante de determinadas funções do sistema nervoso central (SNC), com deficiência na neurotransmissão, neuroplasticidade e neurogênese, além de apoptose (YOU et al., 2016). A doença de Alzheimer (DA) caracteriza-se como uma desordem neurodegenerativa e é considerada a principal causa de demência e um problema crítico de saúde pública (SUN et al., 2017; WORTMANN, 2012).

A identificação da DA ocorreu no início do século 20 por Alois Alzheimer, tendo sido fundamental para a compreensão da demência senil e a descrição atual das características clínicas e patológicas observadas nesta doença (BERCHTOLD, COTMAN, 1998). Assim, tornou-se possível estimar a prevalência de pessoas acometidas pela DA. Entre os anos de 1990 e 2016 foi descrito um aumento de 117% no número de casos de demência, de 20,2 milhões em 1990 para 43,8 milhões em 2016. Nesse mesmo estudo, estimou-se que em 2016 no Brasil mais de 1,5 milhões de pessoas apresentavam Alzheimer e outros tipos de demência, com mortalidade de aproximadamente 80 mil pessoas. Globalmente nesse mesmo ano, a demência foi a quinta maior causa de morte, com 2,4 milhões de óbitos. Ainda, o Brasil foi o segundo país com maior prevalência padronizada por idade para demência (GBD 2016 Dementia Collaborators, 2019).

Segundo o World Alzheimer Report (ALZHEIMER'S DISEASE INTERNATIONAL, 2019), a demência acomete atualmente cerca de 50 milhões de pessoas e acredita-se que esse índice aumentará para 152 milhões de pessoas até o ano de 2050. Ainda, o relatório cita que, em pesquisa realizada com 70 mil pessoas de 155 países e territórios, 95% das pessoas acredita que desenvolverá demência em algum momento da vida e 86% das pessoas faria um teste genético para saber a propensão para desenvolvimento de demência, salientando a crescente preocupação do público em geral acerca dessa doença. Com relação ao custo anual com a demência, esse é estimado em um trilhão de dólares, podendo dobrar até o ano de 2030 (ALZHEIMER'S DISEASE INTERNATIONAL, 2019). No Brasil, são gastos pelo governo aproximadamente 13 milhões, 18 milhões e 19 milhões de dólares por ano com as formas leve, moderada e severa do DA, respectivamente (FERRETTI et al., 2015).

A DA é caracterizada por diversas alterações na anatomia, fisiologia e funções cerebrais. A apresentação clínica dessa doença ocorre inicialmente pelo declínio gradual da memória de curto prazo e da cognição, com incapacidade de reter a informação recentemente adquirida. Com a progressão da doença, sintomas como perda da memória de longo prazo, deficiência no

processo cognitivo afetando a linguagem, raciocínio abstrato e função executiva, confusão, delírios, alterações de humor e perda de funções corporais são observados (BEKRIS et al., 2010).

Duas formas são descritas para a DA, a DA de início tardio, também denominado de Doença de Alzheimer Esporádica (DAE) e de início precoce, denominada de Doença de Alzheimer Familiar (DAF). A DAF representa aproximadamente 1% dos casos e manifesta-se comumente antes dos 65 anos de idade e evolui rapidamente. Descreve-se para esta forma uma relação 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 (BLENNOW; DE LEON; ZETTERBERG, 2007). Por sua vez, a DAE, responsável pelo grande número de casos, é considerada a forma mais comum da doença e na maioria dos casos após os 60 anos de idade. Embora haja especulações a respeito da relação da DAE com fatores genéticos, a sua principal causa está correlacionada com o processo de envelhecimento. Contudo, vale ressaltar que em ambas as formas da DA observa-se as mesmas características patológicas (BEKRIS et al., 2010).

As características neuropatológicas mais relevantes da DA incluem 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). Essas desordens em conjunto são as grandes responsáveis pela atrofia cerebral com consequente decréscimo das funções cognitivas e de memória (GARCIA-AYLLON et al., 2011; JAHN, 2013). Além disso, disfunção do sistema colinérgico, com aumento da atividade da enzima acetilcolinesterase (AChE) responsável pela degradação do neurotransmissor acetilcolina (ACh) em torno das placas amiloides (GARCIA-AYLLON et al., 2011) e deficiência dos níveis desse neurotransmissor, é um dos fatores que favorece o declínio da aprendizagem e memória na doença, por acometer áreas importantes para esses processos como hipocampo e córtex cerebral (FERREIRA-VIEIRA et al., 2016; PICCIOTTO; HIGLEY E MINEUR, 2012).

A ACh é um neurotransmissor de ampla distribuição no SNC, apresenta múltiplas funções neuromoduladoras, promove o desenvolvimento neuronal, regula a descarga neuronal, afeta a transmissão sináptica, promove plasticidade sináptica e modula os circuitos neurais (RAMANATHAN et al., 2015). A ACh exerce suas funções através da interação com dois tipos de receptores, os receptores nicotínicos (nAChR) e os receptores muscarínicos (mAChR). Os nAChR distribuem-se nas regiões pré-, pós-, peri- e extrassinápticas do cérebro e estão

relacionados ao aprendizado e memória, desenvolvimento neuronal e sistema de recompensa (GOPALAKRISHNAN et al., 1997). Já, os mAChR estão envolvidos no controle da função extrapiramidal, vestibular, memória, aprendizado e atenção, respostas emocionais, modulação do estresse, do sono e da vigília (VENTURA et al., 2010).

Na fenda sináptica a ação da ACh cessa quando é hidrolisada pela AChE em colina e acetato (AMENTA; TAYEBATI, 2008). Essa enzima é encontrada nos neurônios colinérgicos, nas proximidades das sinapses colinérgicas e em concentrações elevadas na junção neuromuscular (MASSOULIÉ et al., 1993). A AChE está amplamente distribuída no SNC e também é encontrada em eritrócitos, linfócitos e plaquetas de mamíferos. A enzima circulante pode ter papel não-catalítico como sugerido que variantes estruturais da AChE estão amplamente distribuídas pelos tecidos. Tem-se correlacionado sua participação no crescimento e adesão celular (DARBOUX et al., 1996), neurogênese (LAYER, 1990), sinaptogênese (LAYER, 1991) e hematopoiese (LEV-LEHMAN et al., 1994).

Em adição, é atribuído a ACh um papel fundamental na regulação da inflamação através da via colinérgica anti-inflamatória, a qual representa um mecanismo de resposta do sistema nervoso central à presença de estímulos inflamatórios (GALLOWITSCH-PUERTA; PAVLOV, 2007). O processo inflamatório tem sido intimamente relacionado com múltiplas vias neurodegenerativas e contribui significativamente para a perda estrutural e funcional de neurônios, processo característico de doenças neurodegenerativas, incluindo a DA (CHEN et al., 2016; STEPHENSON et al., 2018). Inflamação sistêmica e neuroinflamação apresentam uma importante relação com a perda de memória e o déficit cognitivo, além de ser responsável por um desequilíbrio na sinalização redox (LESZEK et al., 2016; STEPHENSON et al., 2018).

Outra anormalidade que ocorre no cérebro de pacientes com DA é o distúrbio nos metabolismos de glicose e insulina. Sendo assim, a diabetes do tipo II pode ser considerada como um fator de risco para o desenvolvimento da DA (BIESSELS et al., 2005). Devido ao estado cerebral de resistência crônica da insulina com uma estreita relação com a neurodegeneração da DA, essa patologia tem sido também denominada de diabetes do tipo III (DE LA MONTE E WANDS, 2008). Esses distúrbios no metabolismo de glicose e insulina são também relacionados à disfunção mitocondrial (SIVITZ E YOREK, 2010). O metabolismo da glicose é o processo pelo qual o carboidrato é quebrado através de múltiplos caminhos enzimáticos com o objetivo de produção de energia. Nesse processo ocorre a geração de ATP embora o transporte de elétrons da cadeia respiratória ocorra em grande parte com eficiência, uma pequena porcentagem de elétrons é liberada prematuramente para o oxigênio, resultando

na formação de espécies reativas de oxigênio (ERO), incluindo radicais livres e peróxidos (MOSCONI, 2013).

O estresse oxidativo ocorre após o desequilíbrio entre a quantidade de ERO e capacidade das células de detoxificar essas moléculas através de suas defesas antioxidantes (HUANG, ZHANG E CHEN, 2016). Neste caso, as substâncias oxidantes geradas podem levar a oxidação proteica, lipídica, do DNA ou glicoxidação em diversos tecidos e órgãos, alterando suas estruturas e funções. O cérebro por ser composto, em grande parte, de lipídios facilmente oxidáveis, com uma alta taxa de consumo de oxigênio e baixos níveis de antioxidantes endógenos, torna-se altamente susceptível a ação deletéria das ERO. Ainda, subprodutos da peroxidação lipídica podem induzir neurodegeneração e morte celular pelas vias apoptótica e necrótica (BHAT et al., 2015).

A Ácido δ-aminolevulínico desidratase (δ-ALA-D) é uma enzima contendo grupamentos tiois que podem ser oxidados sob condições de estresse oxidativo. Por sua vez, a inibição da δ-ALA-D resulta em alta concentração de seu substrato (ácido 5-aminolevulínico), o qual possui um efeito pró-oxidativo. Diante disso, sugere-se que a inibição de δ-ALA-D possa ser usada como um índice de estresse oxidativo e, ainda, estar relacionada com o desenvolvimento da DA, podendo ser aplicada como um marcador da doença (GARLET et al., 2019).

Por esta razão, a ocorrência do estresse oxidativo se torna uma das principais vias patogênicas da DA (HUANG, ZHANG E CHEN, 2016). Evidências sugerem que o hipometabolismo da glicose e o estresse oxidativo possam ocorrer já na fase pré-clínica da doença e tornar-se mais proeminente nas regiões cerebrais que apresentam degeneração na DA, como no córtex cerebral e hipocampo (MOSCONI, 2013).

No que se refere à sinalização através do ATP, sabe-se que o sistema purinérgico também está intimamente relacionado com as doenças neurodegenerativas e com a DA (BURNSTOCK, 2016). A sinalização purinérgica é uma importante via moduladora de variados processos fisiológicos, estando envolvida em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo secreção exócrina e endócrina, respostas imunes, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK, 2006).

A liberação de ATP nos terminais pré e pós-sinápticos pode ocorrer como um mecanismo fisiológico ou em resposta a danos celulares, como hipóxia e injúrias (BURNSTOCK, 2006). Este nucleotídeo também pode ser armazenado em vesículas sinápticas, sendo liberado por exocitose como um co-transmissor juntamente com neurotransmissores

como a acetilcolina (ACh) e o glutamato (ZIMMERMANN, 1996). Além disso, o ATP pode ser liberado por exocitose nas células neuronais e nas células não neuronais através de transportadores que se ligam a esse nucleotídeo ou via canais acoplados à conexina ou panexina (SABIROV E OKADA, 2005).

O ATP e seus nucleotídeos exercem suas atividades através de purinoreceptores do tipo P1 e P2 (sendo ainda classificados em P2X e P2Y) (BURNSTOCK, 2012; BURNSTOCK E KENNEDY, 1985). Os receptores P1 são divididos em quatro subtipos de acordo com suas características, sendo todos acoplados a proteína G e exibindo sete domínios transmembrana formados por aminoácidos hidrofóbicos. A adenosina (ADO), um nucleosídeo com propriedades neuroprotetoras e neuromodulatórias, exerce seus efeitos através da ativação desses purinoreceptores (FREDHOLM et al., 2000; STEHLE et al., 1992).

A família P2X consiste de receptores ionotrópicos que quando ativados resultam na abertura de canais iônicos na membrana celular, que permitem a passagem de cátions Na^{+}, K^{+} e Ca^{+2}. Essa família de receptores está dividida em sete membros (P2X1-7), os quais podem ser encontrados em neurônios, células gliais e músculo liso (FIELDS E BURNSTOCK, 2006; MOLLER et al., 2000; NORTH, 2002; NORTH E VERKHRATSKY, 2006). Os receptores P2X7 estão relacionados ao processo neuroinflamatório e são capazes de transformar o fenótipo fagocítico da micróglia (neuroprotetor) em inflamatório (neurodegenerativo). Esses receptores, em especial, têm demonstrado forte relação com a via amiloidogênica da DA e por isso tem ganhado atenção especial no estudo dessa doença (BURNSTOCK, 2016; DIAZ-HERNANDEZ et al., 2012).

A família P2Y consiste em receptores metabotrópicos acoplados a uma proteína G e foram funcionalmente descritos oito membros, que apresentam uma ampla distribuição no tecido vascular, nervoso e cardíaco. Além disso, esses receptores também estão envolvidos no processo neuroinflamatório, pois desempenham papel importante na comunicação neurônio-glia. Lesões neuronais ativam receptores astrocitários P2Y levando à liberação de prostaglandina E2 (PGE2), causando gliose reativa (XIA e ZHU, 2011); ou à liberação de glutamato, mediando a modulação sináptica (DOMERCQ et al., 2006). Como resultado, os receptores P2Y influenciam a permeabilidade da barreira hematoencefálica através da indução de óxido nítrico sintetase endotelial (eNOS) e as células gliais ativadas induzem a expressão de quimiocinas, como a proteína quimiotática para monócitos-1 (MCP-1), levando ao recrutamento de monócitos ao SNC (KIM et al., 2011).

A sinalização induzida pelas moléculas de nucleotídeos de adenina (ATP, ADP, AMP e ADO) depende diretamente da atividade de enzimas ancoradas na superfície da membrana

celular, conhecidas como ectoenzimas. Entre elas, a NTPDase (E.C 3.6.1.5, CD39 ou apirase), que catalisa a hidrólise do ATP em ADP e ADP em AMP, controlando assim os níveis de ATP e ADP no meio extracelular (ZIMMERMANN, ZEBISCH E STRATER, 2012), a 5' - nucleotidase (E.C 3.1.3.5, CD73), responsável pela hidrolise de AMP em adenosina (COLGAN et al., 2006; ZIMMERMANN, 2001) e a adenosina desaminase (EC 3.5.4.4, ADA), responsável por regular as concentrações de adenosina, através da sua conversão em inosina (ZIMMERMANN, 2001).

Alterações na atividade ou expressão das enzimas do sistema purinérgicos estão associadas a processos neurodegenerativos, demência e déficit cognitivo. Em estudo de Fuchs (1991), observou-se que a atividade da 5'-nucleotidase aumenta em diferentes regiões do cérebro a medida em que o animal envelhece. Ainda, ratos desmielinizados (SPANEVELLO et al., 2006) e diabéticos (SCHMATZ et al., 2009) apresentaram aumento na atividade dessa enzima em sinaptossomas de córtex cerebral, o que pode levar ao aumento nos níveis de ADO.

Em adição, a diminuição na atividade da enzima NTPDase é prejudicial para neurotransmissão e atividade neural (VAILLEND et al., 2002), uma vez que essa enzima desempenha importante papel no aprendizado e memória em diferentes tarefas (ZHAN et al., 2004). Além disso, observou-se piora na memória de ratos tratados com inibidores da atividade da NTPDase (ZHAN et al., 2004). A inibição da atividade dessa enzima está relacionada ao desenvolvimento de doenças neurodegenerativas (KUMAR E KURUP, 2002), como na DA em que tanto sua atividade quanto sua expressão estão diminuídas (LIGURI et al., 1990).

Muitos dos processos patológicos observados na DA podem ser induzidos pela injeção intracerebroventricular de estreptozotocina (ICV-STZ), sendo este o modelo experimental animal para desenvolvimento de demência esporádica do tipo Alzheimer (KALAFATAKIS E ZARROS, 2014). De forma similar ao que acontece perifericamente, um dos alvos do STZ no cérebro é o transportador de glicose do tipo 2 (GLUT 2) (KNEZOVIC et al., 2017). Assim, animais submetidos a este modelo apresentam deficiência no metabolismo energético de glicose, estresse oxidativo, disfunção colinérgica, aumento da atividade da AChE, perda da memória (SALKOVIC-PETRISIC et al., 2013), perda da massa encefálica, declínio cognitivo, disfunção mitocondrial e apoptose (GRIEB, 2016). Além disso, a administração da ICV-STZ ocasiona hiperfosforilação da proteína tau e aumento da expressão do peptídeo β-amiloide (ELCIOGLU et al., 2016; LIU et al., 2014), sendo utilizada como um modelo capaz de acompanhar as características iniciais e tardias da DA(SALKOVIC-PETRISIC et al., 2013).

Evidências sugerem que a patogênese da DA envolve mecanismos imunológicos no cérebro, que estão relacionados com enrolamento incorreto e agregados de proteínas. Esse

processo contribui para o surgimento de placas senis e emaranhados neurofibrilares, bem como para a progressão e gravidade da doença (HENEKA et al., 2015). Em adição, autores sugerem que a neuroinflamação é um importante componente em diversas desordens do sistema nervoso central, incluindo a DA, uma vez que processo inflamatório é fator de risco para déficit cognitivo e demência (BETTCHER; KRAMER, 2014).

Um estudo realizado em animais transgênicos sugere que a neuroinflamação possui um importante papel no processo de deposição cerebral de amiloide (GUO et al., 2002). Foi demonstrado que citocinas inflamatórias, como IL-1β, IL-6, TNF-α ou fator de transformação do crescimento beta (TGF-β) podem aumentar a expressão da proteína precursora amiloide (PPA) (HIROSE et al., 1994) e a formação de βA (BLASKO et al., 1999). Além disso, lipopolissacarídeos (LPS) de bactérias gram-negativas podem ser responsáveis pela formação de proteínas amiloides extracelulares, observando-se uma relação diretamente proporcional, ou seja, quanto mais alto os níveis de LPS maior a formação de placas amiloides (HOLMES E COTTERELL, 2009;ZHAN et al.,2018).

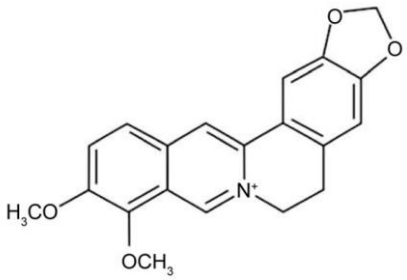
A administração de múltiplas injeções de LPS estimulam a microglia através da ativação da via de sinalização de NF-κB. Por sua vez, a inflamação sistêmica e, consequentemente, a neuroinflamação causam elevação dos níveis de proteína β-amiloide e morte celular neuronal, resultando em comprometimento cognitivo como o que ocorre na DA (ZHAO et al., 2019).Além disso, o modelo de indução de neuroinflamação por LPS é capaz de ocasionar aumento do estresse oxidativo (JANGRA et al., 2016), aumento na atividade da AChE (MING et al., 2015), ativação de astrócitos e aumento na liberação de mediadores pró-inflamatórios (WANG et al., 2014; ZHAO et al., 2011). Assim, um dos modelos animais mais importantes e amplamente utilizados de neuroinflamação e neurodegeneração induzida perifericamente é a indução por injeções de LPS (CATORCE E GEVORKIAN, 2016). Entre os modelos de neuroinflamação não manipulados geneticamente para DA, o modelo animal induzido por LPS é comumente utilizado (ZAKARIA et al., 2017) e tem sido útil na avaliação de medicamentos e produtos naturais no tratamento da DA (CATORCE E GEVORKIAN, 2016).

Como não existe cura para a DA, a estratégia terapêutica para essa doença baseia-se no uso de inibidores da AChE, visando retardar ou amenizar o déficit colinérgico e dessa forma atenuar os sintomas e as alterações comportamentais (VIEGAS, 2011). Entretanto, são relatadas algumas reações adversas ao uso destes medicamentos, como problemas gastrointestinais, neuropsiquiátricos, renais e cardiovasculares (KROGER et al., 2015). Por esta razão, a busca por intervenções terapêuticas eficazes para o tratamento da DA e com capacidade de abranger uma maior gama de vias patogênicas da doença se faz necessária. Neste sentido, alguns avanços

no tratamento da doença através do uso de compostos naturais, têm sido promissores (BERTÉ et al., 2018; FEITOSA, 2016; HOWES E PERRY, 2011; MONTEIRO et al., 2018; PENIDO et al., 2017).

A berberina (BRB – Figura 1), um alcaloide isoquinolina, isolado principalmente da erva chinesa *Coptis chinensis*, tem demonstrado segurança e eficácia na utilização tanto em humanos quanto em animais (JIA et al., 2012; KONG et al., 2004; MOGHADDAM et al., 2013). Dentre as múltiplas atividades farmacológicas descritas para esse fitoterápico, cita-se a ação antimicrobiana (HAN et al., 2011), a anti-inflamatória (MO et al., 2014) e a antioxidante (ABD EL-WAHAB et al., 2013), podendo também agir sobre o metabolismo de glicose e dos lipídios (CALICETI et al., 2016).

Figura 1 – Estrutura molecular da Berberina.



Fonte: JIANG et al (2015)

A BRB pode estar presente em raízes, rizomas, caule e casca de plantas do gênero *B. vulgaris*, assim como em muitas outras plantas. Diversos estudos clínicos realizados sugeriram uma ampla gama de aplicações terapêuticas para o uso da BRB principalmente com a finalidade de diminuir os lipídios e melhorar a resistência à insulina (propriedades mais estudadas). Porém, pode-se observar ensaios clínicos sobre doenças cardiovasculares, tratamentos oncológicos, doenças gastrointestinais e endócrinas e assim por diante. Demonstra-se ainda que a BRB possui toxicidade muito baixa em doses usuais e revela benefícios clínicos sem efeitos colaterais importantes. Apenas reações gastrointestinais leves podem ocorrer em alguns pacientes (IMENSHAHIDI; HOSSEINZADEH, 2019).

No que se refere à toxicidade da BRB, estudos tem demonstrado que uma dose de 20,8 g de BRB/kg de peso corporal é segura para administração por via oral em camundongos (KHEIR et al., 2010). Dessa maneira, levando em consideração a taxa metabólica por kg de peso corporal aproximadamente sete vezes maior em camundongos do que nos 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 confirmada em outros estudos, utilizando uma dose de 1000 mg/kg, por dia durante três meses (ZHANG et al., 2008) e em uma dose de 500 mg/kg, três vezes ao dia (YIN et al., 2008).

Devido a capacidade da BRB 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 E QIAN 2006), uma vez que dentre outras ações, esse composto é capaz de inibir a atividade da AChE e de outras importantes enzimas relacionadas à esta doença (JI; SHEN, 2012), diminuir os níveis de β-amiloide e modular a APP (ASAI et al., 2007).

O efeito neuroprotetor da BRB foi demonstrado contra danos isquêmicos (CHAI et al., 2013; PIRES et al., 2014) e neurodegeneração em doenças como a DA, Parkinson e Huntington (AHMED et al., 2015). O efeito de neuroproteção se deve à múltiplas ações desenvolvidas por esse alcaloide, entre elas, prevenção do aumento da AChE e da morte celular neuronal de regiões do hipocampo e córtex cerebral (DE OLIVEIRA et al., 2016), além de possuir capacidade de agir reduzindo o nível de TNF-α e a atividade da caspase-3, assim como inibir a apoptose, com redução da relação *Bax/Bcl-2* no tecido cerebral (ABDEL MONEIM, 2015). Além disso, a BRB está envolvida na via de sinalização de sobrevivência/apoptose Akt/GSK3β/ERK 1/2 e na inibição da atividade da JNK (SIMOES PIRES et al., 2014).

Em concordância com as propriedades relatadas para o uso da BRB, nossos estudos prévios demonstraram que esse composto foi capaz de exercer um efeito neuroprotetor contra o aumento da atividade da AChE em sinaptossomas de córtex cerebral e hipocampo de animais submetidos ao modelo ICV-STZ, prevenindo a perda de memória espacial e morte celular cerebral desses animais (DE OLIVEIRA et al., 2016). Além disso, a neuroproteção conferida pela BRB tem sido reportada devido sua vasta gama de atividades farmacológicas adicionais que são úteis para tratar perturbações do SNC. Dessa forma, elucidar os mecanismos dessas ações benéficas da BRB em doenças neurodegenerativas, tais como a DA é de grande importância.

Diante do exposto, a BRB pode ser considerada uma substância coadjuvante promissora para o tratamento da DA, pois tem a capacidade de agir como um composto multialvo capaz de atuar em diversos dos mecanismos que desencadeiam os sinais clínicos dessa doença degenerativa. Por esta razão, elucidar os seus efeitos sobre os parâmetros comportamentais cognitivos, sobre o estresse oxidativo, sua ação no sistema colinérgico e purinérgico em modelo

animal experimentalmente controlado, torna-se relevante e poderá impactar de forma significativa a abordagem terapêutica da DA.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo do presente estudo foi investigar a ação da BRB sobre prejuízos de memória, do sistema colinérgico e do purinérgico, bem como sobre parâmetros de estresse oxidativo em dois modelos experimentais de demência em animais induzidos por estreptozotocina e por lipopolissacarídeo.

2.2 OBJETIVOS ESPECÍFICOS

EXPERIMENTO 1: Avaliar os efeitos de duas doses de BRB (50 ou 100 mg/kg) em ratos submetidos ou não a um modelo de demência induzido por injeção intracerebroventricular de estreptozotocina (ICV-STZ) sobre:

- A memória através do teste de reconhecimento de objetos.
- Parâmetros oxidativos tais como os níveis de espécies reativas, peroxidação lipídica e proteínas carbonil em amostras de córtex cerebral e hipocampo e a atividade da ácido δ -aminolevulínico desidratase no córtex cerebral dos ratos.
- Sistema antioxidante através da análise dos níveis de tiois totais, glutationa reduzida, bem como atividade da glutationa transferase em córtex cerebral e hipocampo dos ratos.
- Ectoenzimas do sistema purinérgico, tais como a NTPDase, 5′-nucleotidase e ADA em sinaptossomas do córtex cerebral e hipocampo dos ratos.

EXPERIMENTO 2: Determinar a ação da BRB (50 mg/kg) em ratos submetidos ou não a um modelo de demência induzido por lipopolissacarídeo (LPS) intraperitoneal sobre:

- Peso corporal.
- Atividade locomotora.
- Memória de reconhecimento através do teste de reconhecimento de objetos.
- Parâmetros oxidativos tais como os níveis de espécies reativas, peroxidação lipídica e proteínas carbonil em amostras de córtex cerebral e hipocampo dos ratos.

- Sistema antioxidante através da análise dos níveis de tiois totais e glutationa reduzida no córtex cerebral e hipocampo dos ratos.
- Ectoenzimas do sistema purinérgico, tais como a NTPDase e 5′-nucleotidase no córtex cerebral e hipocampo dos ratos.

3. CAPÍTULO I: ARTIGO I – NEUROPROTECTIVE EFFECTS OF BERBERINE ON RECOGNITION MEMORY IMPAIRMENT, OXIDATIVE STRESS, AND DAMAGE TO THE PURINERGIC SYSTEM IN RATS SUBMITTED TO INTRACEREBROVENTRICULAR INJECTION OF STREPTOZOTOCIN.

Artigo publicado em: *Psychopharmacology*

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ABSTRACT

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease. The present study investigated the effects of 50 and 100 mg/kg berberine (BRB) on recognition memory, oxidative stress, and purinergic neurotransmission, in a model of sporadic dementia of the Alzheimer's type induced by intracerebroventricular (ICV) injection of streptozotocin (STZ) in rats. Rats were submitted to ICV-STZ 3 mg/kg or saline, and three days later, were started on a treatment of BRB or saline for 21 days. The results demonstrated that BRB was effective in protecting against memory impairment, increased reactive oxygen species, and the subsequent increase in protein and lipid oxidation in the cerebral cortex and hippocampus, as well as δ-aminolevulinate dehydratase inhibition in the cerebral cortex. Moreover, the decrease in total thiols, and the reduced glutathione and glutathione S-transferase activity in the cerebral cortex and hippocampus of ICV-STZ rats, was prevented by BRB treatment. Besides an antioxidant effect, BRB treatment was capable of preventing decreases in ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), 5′-nucleotidase (EC-5'-Nt), and adenosine deaminase (ADA) activities in synaptosomes of the cerebral cortex and hippocampus. Thus, our data suggest that BRB exerts a neuroprotective effect on recognition memory, as well as on oxidative stress and oxidative stress-related damage, such as dysfunction of the purinergic system. This suggests that BRB may act as a promising multipotent agent for the treatment of AD.

KEYWORDS: Reactive species; Antioxidant; Ectoenzymes; Alzheimer.

1. INTRODUCTION

Alzheimer's disease (AD) is considered a neurodegenerative disease, characterized by extracellular accumulation of the β-amyloid protein (Cenini et al. 2016) and intracellular hyperphosphorylation of the tau protein (Guo et al. 2016), as well as mitochondrial damage, synaptic loss, and inflammation (Kuruva and Reddy 2016).These abnormalities occur in the cerebral cortex and hippocampus (Nelson et al. 2012), and lead to deficits in learning and memory abilities (Santos et al. 2016). It is estimated that 10% of the world's population aged over 60–65 years is affected by AD (Meraz-Rios et al. 2014).While the pathogenesis of AD is not completely understood, increasing age is a major risk factor for of AD; thus, mitochondrial dysfunction and oxidative damage may also play an important role in the pathogenesis of AD (Jiang et al. 2016). Furthermore, the accumulation of reactive oxygen species (ROS) is a key mechanism involved in the aging process, which can cause direct injury to the central nervous system (CNS) (Droge and Kinscherf 2008).

It is known that the neurons are at high risk of oxidative stress because of their large oxygen demand but relatively low levels of antioxidants. As such, oxidative stress occurs when there is a significant increase in the amount of oxidized components, and an impairment in the balance between pro-oxidants and antioxidants (Cho et al. 2016; Di Pietro et al. 2014; Feuerstein et al. 2016). To this end, the body has enzymatic and non-enzymatic antioxidant mechanisms to counteract oxidative damage; these antioxidants include antioxidant enzymes, e.g., glutathione-S-transferase (GST), and non-enzymatic antioxidant factors, e.g., thiols (T-SHs) and reduced glutathione (GSH). However, once ROS overwhelm the cellular antioxidant activity, oxidative stress occurs, leading to the accumulation of cytotoxic compounds that not only results in damaged proteins and enzymes, but also in lipid destruction (Lee et al. 2012).

Furthermore, importantly, purinergic signaling is related to neurodegenerative diseases and appears to play an important role in neurodegeneration, neuroprotection and neuroregeneration(Burnstock 2016). The levels of adenosine triphosphate (ATP) and its hydrolysis product, adenosine, in the synaptic cleft are controlled by cell surface-located enzymes, collectively known as ectonucleotidases, and adenosine deaminase (ADA). Ectonucleotidases are enzymes responsible for the hydrolysis of ATP as well as other nucleotides such as adenosine diphosphate (ADP) and adenosine monophosphate AMP; ADA is responsible for the hydrolysis of adenosine (Cardoso et al. 2015). Few data are available in the literature related to the role of these enzymes in the AD, however, it is known that the ATP released and not degraded by less efficient or dysfunctional ectoenzymes triggers excitotoxic damage and neuro-inflammation in the brain tissue (Roszek and Czarnecka 2015). Thus, ATP release that occurs during neuronal injury contributes to the chronic inflammation seen in AD. Furthermore, there is evidence for the involvement of both ATP and ADP receptors of this disease. It is suggested that the ADP receptors are implicated in the metabolism of β-amyloid protein and ATP receptors in the reactive oxygen species production (Burnstock 2016).

The intracerebroventricular injection of streptozotocin (ICV-STZ) model in rats is considered an appropriate model for the investigation of new compounds for the treatment of AD, since it is capable of mimicking many of the processes that occur in this disease (Grieb 2016). Several studies have used this model to demonstrate neuroprotective effects against the damage associated with AD. In previous work, our research group has demonstrated that cholinergic neurotransmission is impaired in ICV-rats, via alteration of acetylcholinesterase (AChE) activity, which consequently causes impaired memory (de Oliveira et al. 2016). In addition, the ICV-STZ model has been show to alter glucose metabolism, insulin signaling, synaptic dysfunction, tau hyperphosphorylation, Aβ deposition, and neuronal apoptosis (Kamat et al. 2016) without alteration of peripheral metabolism. Interestingly, this dysfunction in cognitive and memory abilities is able to be observed for up to 14 weeks (Mehla et al. 2013).

Anticholinesterase drugs used for the treatment of AD lead to a modest clinical improvement in disease symptoms; however, they cannot prevent or reverse disease pathology (Huang and Mucke 2012). Therefore, it is necessary for research to investigate new multi-target therapeutic agents that have the capacity to fully address the multifaceted nature of the disease. As such, some investigators have turned their attention to agents with antioxidant capabilities in the search for drugs to combat against AD (Jiang et al. 2016).

Berberis, the source of berberine (BRB), is a herb used in Chinese medicine which is becoming increasingly prevalent in clinical settings (Lan et al. 2015). Extensive literature has demonstrated that BRB has effects against various diseases, including tumors, diabetes, cardiovascular disease, hyperlipidemia, inflammation, bacterial and viral infections, cerebral ischemia trauma, and mental disease (Imenshahidi and Hosseinzadeh 2016). Further, the beneficial role of BRB against neurodegeneration and AD has been demonstrated by numerous studies (Ahmed et al. 2015; de Oliveira et al. 2016; Jiang et al. 2015).

BRB appears to have strong potential for inhibition and prevention of AD through its antioxidant capacities (Jung et al. 2009). The promising results of this compound to date provide a convincing and substantial basis to support further scientific exploration and development of the therapeutic potential of BRB against AD. Moreover, since BRB has shown promising effects in the model of Alzheimer's type dementia induced by STZ (de Oliveira et al. 2016), our research group seeks to elucidate the possible mechanisms of action involved in the effects of this flavonoid on this type of disease. Thus, the objective of this work was to evaluate the action of BRB on recognition memory and parameters of oxidative stress, such as ROS, lipid peroxidation, protein carbonylation, δ-aminolevulinate dehydratase (δ-ALA-D) activity, T-SHs, GSH levels, and GST activity. This study also aimed to examine the action of BRB on the purinergic system through investigation of the activity of ectonucleotidases (Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases), Ecto-5'-nucleotidase (EC-5'-Nt), and adenosine deaminase (ADA), in synaptosomes of the cerebral cortex and hippocampus.

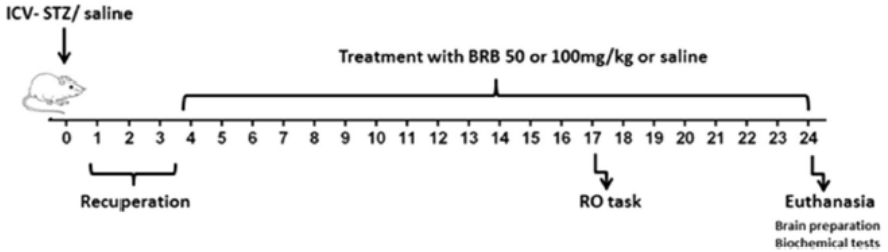

Figure 1. Experimental procedure. ICV-STZ: Intracerebroventricular injection of streptozotocin. BRB: Berberine. RO: Object recognition

2. MATERIALS AND METHODS

2.1 Chemicals

The substrates ATP, ADP, AMP, adenosine, as well as 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), Tris (hydroxymethyl) aminomethane, Coomassie Brilliant Blue G, Trizma base, STZ, and BRB were obtained from Sigma-Aldrich (St. Louis, MO, USA). All reagents used in the experiments were of analytical grade and of the highest purity.

2.2 Animals

All animal procedures were approved by the Animal Ethics Committee for the care and use of laboratory animals (protocol number: 109/2013) and followed the National Institutes of Health guide for the care and use of Laboratory animals. Relevant animal experiment guidelines were followed. Male Wistar rats, weighing 300–350 g, were obtained from the Central Animal House of the Federal University of Santa Maria (UFSM). The animals were maintained at a constant temperature (23 ± 1°C) under a 12 hour light/dark cycle, with *ad libitum* access to food and water.

2.3 Intracerebroventricular streptozotocin (ICV-STZ) administration

Animals were anesthetized with an intraperitoneal injection of thiopental (1 ml/kg) and maintained with isoflurane inhalation. The head was positioned in a stereotaxic apparatus and the skull was exposed. Two holes were drilled through the skull for the bilateral placement of a microinjector into the lateral cerebral ventricles, according to the following coordinates: 0.8 mm anterior-posterior to the bregma; 1.5 mm lateral to the sagittal suture; and 4.0 mm ventral to the brain surface (Watson 1996). Through a skull hole, a 28-gauge Hamilton® syringe of 10μL attached to stereotaxic apparatus and piston of the syringe was lowered manually. Rats in the STZ groups received an ICV injection of STZ dissolved in saline (Khan et al. 2012), and rats in the control groups received the same volume of saline. The injection of STZ was administered at concentration of 3 mg/kg body weight at volume of 5 ul/injection site in a speed to 1 μL/min into each lateral ventricle. The time total time of infusion was approximately 10 minutes.

2.4 Berberine administration

BRB was used at 50 or 100 mg/kg body weight at a dose of 1 ml/kg and was dissolved in saline and given daily by oral gavage. These submaximal doses were selected to investigate the biological potential against experimentally induced neurodegeneration based on reports in the literature on the safety of the compound (de Oliveira et al. 2016; Kheir et al. 2010). Furthermore, 50 and 100 mg/kg dosages have been demonstrated to produce protective action against various experimental disease conditions in rats, such as protecting or delaying oxidative stress and modulating AChE activity in rat cerebral cortex and hippocampus (Bhutada et al. 2011; de Oliveira et al. 2016; Kalalian-Moghaddam et al. 2013).

The rats were randomly divided into six groups, with ten animals per group: control (CTR), BRB 50 mg/kg (BRB 50), BRB 100 mg/kg (BRB 100), STZ plus saline (STZ), STZ plus BRB 50 mg/kg (STZ + BRB 50), and STZ plus BRB 100 mg/kg (STZ + BRB 100). The animals were allowed to recover from surgery for three days, with oral administration of BRB or saline beginning on day four, by gavage.

2.5 Novel object recognition task

On the 17th day of treatment, the animals were submitted to the novel object recognition task, as previously described by Gomes (Gomes et al. 2014), with modifications. The task was performed in a wooden box, 56 × 40 × 30 cm, where the animals were exposed to objects of different shapes and colors, but the same size. The box and objects were cleaned with 30% ethanol immediately before and at the end of each behavioral evaluation. The task consisted of habituation, training, and testing sessions, each lasting eight minutes. Firstly, the animals were individually habituated to the behavioral apparatus. Twenty-four hours later, the training

session performed, in which the animals were exposed to two of the same objects (object A), and the exploration time was recorded. The test session was carried out 24 hours after training. Rats were placed back in the behavioral chamber and one of the familiar objects (object A) was replaced by a novel object (object B). The times spent exploring the familiar and the novel object were recorded. The discrimination index was then calculated, taking into account the difference between time spent exploring the new and familiar objects, using the formula:

([(T novel – T familiar) / (T novel + T familiar)] x 100 (%))

The discrimination index was used as a memory parameter.

2.6 Brain tissue preparation

At the end of the behavioral test, on day 24, the animals were euthanized. The cranium was opened, the structures were gently removed, and the cerebral cortex and hippocampus were separated. The brain structures were homogenized in a glass potter in a solution of 10mM Tris – HCl (pH 7.4), on ice, at a proportion of 1:10 (w/v). The resulting homogenate was used to determine the oxidative stress parameters.

2.7 Preparation and isolation of synaptosomes

The cerebral cortex and hippocampus structures were dissected to isolate the synaptosomes, according to the method described by Nagy and Delgado-Escueta(Nagy and Delgado-Escueta 1984). The cerebral cortex and hippocampus were homogenized separately in medium I containing 320 mM sucrose, 0.1 mM Ethylenediaminetetraacetic acid (EDTA), and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, in a motor driven Teflon-glass. The synaptosomes were isolated using a discontinuous Percoll gradient. The pellet was suspended in an isoosmotic solution, and the final protein concentration was adjusted to 0.4-0.6 mg/mL. Synaptosomes were prepared fresh daily, maintained at 0-4°C throughout the procedure, and were used to measure NTPDase, 5′-nucleotidase, and ADA activities.

2.8 Lactate dehydrogenase

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

2.9 Measurement of intracellular reactive oxygen species (ROS) production

2′-7′-Dichlorofluorescein (DCF) levels were determined as an index of reactive species production by the cellular components (Myhre et al. 2003). Aliquots (50 µl) of brain supernatants were added to a medium containing Tris - HCl buffer (10 mM; pH 7.4) and 1 mM 2′-7′- dichlorofluorescein diacetate (DCFH-DA). After addition of DCFH-DA, the medium was incubated in the dark for 1 h until the fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm; both slit widths were at 1.5 nm). DCF levels were determined using a standard curve of DCF, and results were corrected by the protein content.

2.10 Thiobarbituric acid reactive substance (TBARS) measurement

TBARS levels were determined according to Ohkawa et al. (Ohkawa et al. 1979), by measuring the concentration of malondialdehyde (MDA), as an end product of the reaction lipid peroxidation with thiobarbituric acid (TBA). Briefly, the reaction mixture, containing 200 µl of brain supernatants or standard (0.03 mMMDA), 200 µl of 8.1% sodium dodecyl sulfate (SDS), 500 µl of 0.8% TBA, and 500 µl of acetic acid solution (2.5 M HCl, pH 3.4), was heated at 95°C for 120 min. The absorbance was measured at 532 nm. Levels of TBARS in tissues were expressed as nmol MDA/mg of protein.

2.11 Protein carbonyl levels

The carbonylation of proteins was determined using the modified Levine method (Levine et al. 1990). Firstly, the brain supernatants were precipitated using 10% trichloroacetic acid (TCA) and centrifuged at 1800 g for 5 min, discarding the supernatant. Next, 0.5 ml of 10 mmol 2,4-dinitrophenylhydrazine (DNPH) in 2 mol HCl was added to this protein precipitate, and incubated at room temperature for 30 min. During incubation, the samples were mixed vigorously every 15 min. After incubation, 0.5 ml of 10% TCA was added to the protein precipitate and centrifuged at 1800 g for 5 min. After discarding the supernatant, the precipitate was washed twice with 1 ml of ethanol/ethyl acetate (1:1), and the supernatant was centrifuged out, in order to remove the free DNPH. The precipitate was dissolved in 1.5 ml of protein dissolving solution (2 g sodium dodecyl sulfate and 50 mg EDTA in 100 ml 80 mmol phosphate buffer, with pH 8.0) and incubated at 37°C for 10 min. The color intensity of the supernatant was measured using a spectrophotometer at 370 nm against 2 mol HCl. Carbonyl content was calculated using the molar extinction coefficient (21 x 10^3 1/mol cm), and results were expressed as nmol/mg of protein.

2.12 δ-Aminolevulinic acid dehydratase activity (δ-ALA-D)

Brain δ-ALA-D activity in the cerebral cortex was assayed according to the method of Sassa(Sassa 1982), by measuring the rate of formation of porphobilinogen (PBG). Since this technique requires a large amount of sample to evaluate the activity of this enzyme, it was not possible to perform this evaluation in the hippocampus structure. δ-ALA-D activity was expressed as nmol PBG/mg of protein/h.

2.13 Determination of total thiols (T-SHs)

T-SHs were assayed spectrophotometrically using the method of Boyne and Ellman (Boyne and Ellman 1972), with some modifications. An aliquot of 200 µl of brain supernatants (S1) in a final volume of 900 µL of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis DTNB (50 µL). A standard curve using cysteine was added to calculate the content of thiol groups in the samples; thiol group content was expressed as nmol T-SH/g tissue.

2.14 Measurement of reduced glutathione (GSH)

GSH was measured spectrophotometrically with Ellman's reagent (Ellman 1959). An aliquot of 200 µl of supernatants in a final volume of 900 µl of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM DTNB (50

μL). A standard curve using glutathione was added to calculate the content of GSH in samples; content was expressed as nmol GSH/g tissue.

2.15 Assay of glutathione S-transferase (GST)

The GST enzymatic assay was performed as previously described by Habig et al. (Habig et al. 1974), with a modification to the spectrophotometric method. GST activity was quantified in tissue homogenates in a reaction mixture containing 1-chloro-2,4-dinitrobenzene (CDNB) (1mM) and GSH (1mM) as substrates in 0.1 M K^+-phosphate buffer, with a pH of 7.5, at 37°C. The reaction was initiated by adding GSH substrate. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm, after 2 min of incubation. Enzyme activity was determined using the molar extinction coefficient 9.6 mM^{-1} cm^{-1}, and was expressed as μmol/CDNB/min/mg of protein.

2.16 NTPDase, 5′-nucleotidase, and ADA activities in synaptosomes from the hippocampus and cerebral cortex

NTPDase activity was determined according to Schetinger et al. (Schetinger et al. 2000), whereas 5′-nucleotidase activity was determined according to Heymann et al. (Heymann et al. 1984). The NTPDase enzymatic assay of the synaptosomes was carried out in a reaction medium containing 5 mM KCl, 1.5 mM CaCl2, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris - HCl buffer, pH 8.0, in a final volume of 200 μl, as described in a previous study from our laboratory (Schetinger et al. 2000). The 5′-nucleotidase activity was determined using the method of Heymann et al. (Heymann et al. 1984), in a reaction medium containing 10 mM MgSO4 and 100 mM Tris - HCl buffer, pH 7.5, in a final volume of 200 μl. Synaptosomes preparation 20 μL (8-12 mg of protein) was added to the reaction mixture and pre-incubated at 37°C for 10 min. The reaction was initiated by the addition of 20 μl ATP or ADP (10 mM), or AMP (20 mM), and incubated for 20 min. In all cases, the reaction was stopped with 200 μl of 10% trichloroacetic acid, and the release of inorganic phosphate was measured using the method of Chan et al. (Chan et al. 1986).

The ADA activity was assessed according to the colorimetric method described by Giusti and Galanti(Giusti and Gakis 1971). The amount of complexes of ammonia released per minute was quantified spectrophotometrically from the degradation of adenosine. Results were expressed in units per liter (U/L).

2.17 Statistical analysis

All data were analyzed by two-way ANOVA followed by Tukey's post hoc test, using GraphPad software. Data are presented as mean ± standard error of mean (SEM), and $p < 0.05$ was considered to be statistically significant. Pearson's correlation coefficient was used to investigate correlations.

3. RESULTS

3.1 ICV-STZ decreases and BRB increases the recognition index in the novel object recognition task

Memory loss is considered to be the earliest sign of AD (Jahn 2013). Because of this, cognitive measures are often used to evaluate the stages and efficacy of interventions for the

disease (Aschenbrenner et al. 2015). Figure 2 shows the effect of ICV-STZ administration and the treatment with BRB, at doses of 50 and 100 mg/kg, on the recognition index of the new object. The animals submitted to ICV-STZ displayed a lower recognition index in the object recognition task, when compared to animals in the CTR group. There were no differences in the BRB50 and BRB100 groups, when compared to the CTR group. However, the treatment with BRB at doses of 50 and 100 mg/kg prevented the memory impairment induced by ICV-STZ in the object recognition task.

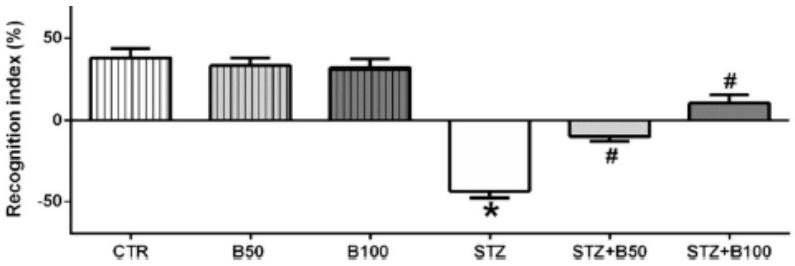

Figure 2. Effect of intracerebroventricular administration of streptozotocin (ICV-STZ) and berberine (BRB) on the novel object recognition task. Data are expressed as mean ± SEM. *p < 0.05 when compared to the control (CTR) group. #p < 0.05 when compared to the streptozotocin (STZ) group.

3.2 BRB prevents the increased ROS production induced by ICV-STZ

Our previous studies have demonstrated the neuroprotective action of BRB on some pathological pathways associated with AD (de Oliveira et al. 2016). However, evidence suggests that oxidative stress can also contribute to the etiopathology of AD (Mao et al. 2012; Wang et al. 2014). Thus, in an attempt to clarify the involvement of BRB in oxidative stress, we first evaluated the formation of ROS in animals submitted to the ICV-STZ model and treated with BRB at 50 and 100 mg/kg. The results presented in the Figure 3 reveal that there were significant increases in ROS production in the hippocampus and cerebral cortex of the STZ groups, when compared to the CTR groups. However, the treatment with BRB 50 and 100 mg/kg prevented this increase in both the cerebral cortex and hippocampus, compared to the STZ groups.

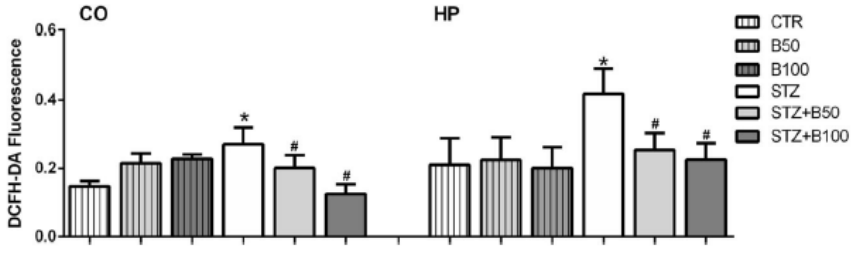

Figure 3. Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on reactive oxygen species (ROS) in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *p < 0.05 when compared to the control (CTR) group. #p < 0.05 when compared to the streptozotocin (STZ) group. DCFH-DA: 2′-7′- dichlorofluorescein diacetate.

3.3 BRB prevents the increased formation of TBARS induced by ICV-STZ

The next step was to investigate if BRB was capable of protecting against the damage caused by the increase in reactive molecules. To this end, we evaluated lipid damage through TBARS in the cerebral cortex and hippocampus. The results presented in Figure 4 reveal that

there was a significant increase in TBARS levels in the cerebral cortex and hippocampus of the STZ groups, when compared to the CTR groups. Nevertheless, the treatment with BRB 50 or 100 mg/kg in rats submitted to ICV-STZ significantly prevented the formation of TBARS, when compared to the STZ group only.

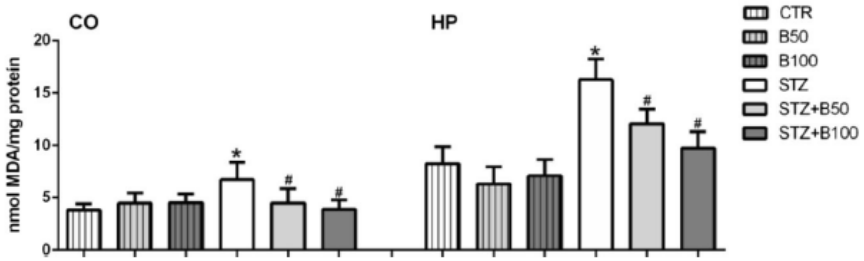

Figure 4. Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on thiobarbituric acid reactive substances (TBARS) in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *$p < 0.05$ when compared to the control (CTR) group. $^{\#}p < 0.05$ when compared to the streptozotocin (STZ) group. MDA: malondialdehyde.

3.4 BRB prevents the increase in protein carbonyl levels induced by ICV-STZ

In addition to increased membrane lipid damage, high ROS production is also capable of causing protein damage. In this study, we aimed to evaluate the potential antioxidant effect of BRB using the carbonylation of proteins test, the results of which are shown in Figure 5. It can be seen that there was a significant increase in the protein carbonyl levels in the cerebral cortex and hippocampus of the STZ groups, when compared to the CTR groups. However, in the ICV-STZ groups treated with BRB 50 or 100 mg/kg, a significant reduction in protein carbonyl levels was observed in both the cerebral cortex and hippocampus, when compared to the STZ groups.

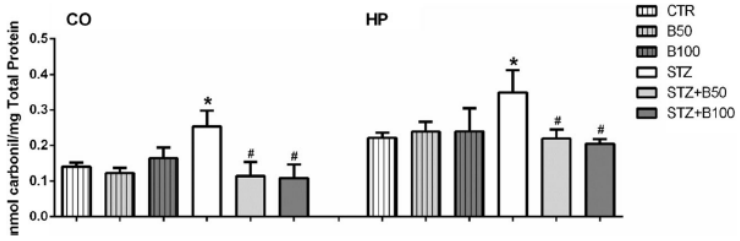

Figure 5. Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on protein carbonyl levels in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *$p < 0.05$ when compared to the control (CTR) group. $^{\#}p < 0.05$ when compared to the streptozotocin (STZ) group.

3.5 BRB prevents the inhibition of aminolevulinic acid dehydratase activity (δ-ALA-D) by ICV-STZ in the cerebral cortex

δ-ALA-D, an enzyme in the heme biosynthesis pathway, is essential for all aerobic organisms, and catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA) to form the monopyrrole porphobilinogen (PBG) (Fernandez-Cuartero et al. 1999). Malfunction of this enzyme can lead to heme deficiency, which is involved in the pathogenesis of AD, and also plays a critical role in increasing oxidative stress in the brain (Atamna and Frey 2004; Atamna and Frey 2007). In Figure 6, it can be seen that there was lower activity of δ-ALA-D in the cerebral cortex among the group that received the ICV-STZ and saline treatment, when compared to animals of the CTR group. Interestingly, treatment with BRB at doses of 50

and 100 mg/kg was capable of preventing the decrease in the activity of this enzyme, when compared to the STZ group.

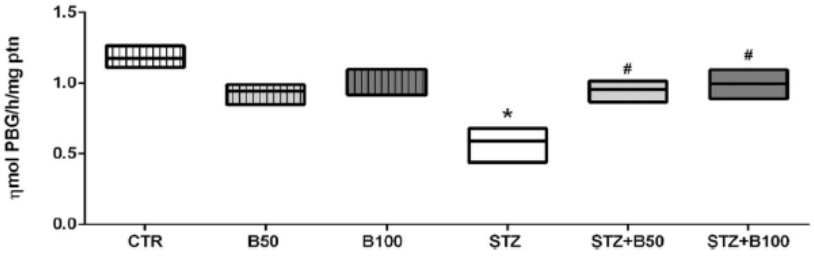

Figure 6. Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) onaminolevulinic acid dehydratase activity (δ-ALA-D) in the cerebral cortex. Data are expressed as mean ± SEM. *p < 0.05 when compared to the CTR group. #p < 0.05 when compared to the STZ group. PBG: Porphobilinogen.

3.6 Correlation between ROS and δ-ALA-D activity

Since we found that BRB was capable of preventing the increase in ROS production and the decrease in δ-ALA-D activity in the ICV-STZ model, we sought to evaluate if there was a correlation between these results. Figure 7 shows a statistically significant correlation between ROS production and δ-ALA-D activity in the cerebral cortex.

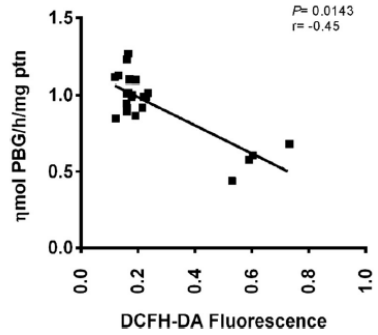

Figure 7. Correlation between reactive oxygen species (ROS) production and aminolevulinic acid dehydratase activity (δ-ALA-D) in the cerebral cortex. PBG: Porphobilinogen. DCFH-DA: 2′-7′- dichlorofluorescein diacetate.

3.7 BRB prevents the decrease in antioxidant compounds induced by ICV-STZ

Levels of T-SHs have been the focus of studies in AD, and are considered a novel oxidative stress marker in these patients (Gumusyayla et al. 2016). Among the T-SHs, GSH is considered the main non-protein thiol (Matamoros et al. 1999) and the most important radical scavenger which can directly act as a substrate for glutathione peroxidase (GPx) and GST (Lu 2013). In turn, GST catalyzes the conjugation of GSH to diverse electrophilic centers on lipophilic molecules, forming less active end products. As such, a decrease in GSH concentration and GST activity has been implicated in neurodegenerative diseases such as AD (Allen et al. 2012; Calabrese et al. 2006).

Thus, we sought to evaluate the levels of antioxidant compounds in the ICV-STZ model, as well as the action of BRB on these compounds. The results presented in Table 1 reveal a significant decrease in T-SHs and GSH levels, as well as in GST activity, of the cerebral cortex and hippocampus of animals submitted to ICV-STZ, when compared to control animals. However, treatment with BRB at doses of 50 and 100 mg/kg was capable of preventing the decrease in T-SHs and GSH levels, and the decreased activity of GST, in both brain structures, when compared to animals in the STZ groups.

	T-SHs nmol T-SH/g tissue Cerebral cortex	GSH nmol GSH/g tissue	GST µmol/CDNB/ min/mg of protein	T-SHs nmol T-SH/g tissue Hippocampus	GSH nmol GSH/g tissue	GST µmol/CDNB/ min/mg of protein
CTR	555.2 ± 82.7	36.4 ± 2.5	123.4 ± 5.8	580.1 ± 85.1	65.5 ± 4.6	140.1 ± 7.6
B50	509.1 ± 55.1	30.8 ± 2.1	122.6 ± 4.9	498.7 ± 79.6	60.4 ± 3.3	132.2 ± 6.5
B100	487.5 ± 38.4	30.2 ± 2.9	115.3 ± 4.9	495.4 ± 81.6	54.8 ± 5.3	122.5 ± 8.4
STZ	328.0 ± 40.4*	15.9 ± 3.5*	67.4 ± 6.6*	245.5 ± 46.2*	26.3 ± 3.5*	89.7 ± 8.1*
STZ+B50	455.9 ± 48.2#	22.3 ± 2.1#	81.7 ± 7.4#	427.2 ± 34.0#	36.5 ± 5.6#	105.5 ± 5.1#
STZ+B100	492.6 ± 67.3#	25.3 ± 3.2#	105.8 ± 4.5#	470.5 ± 48.1#	47.3 ± 2.6#	110.3 ± 8.5#

Table 1. Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on total thiols (T-SHs), glutathione (GSH) levels, and glutathione S-transferase (GST) activity, in the cerebral cortex and hippocampus. Data are expressed as mean ± SEM. *$p < 0.05$ when compared to the CTR group. #$p < 0.05$ when compared to the STZ group.

3.8 BRB prevents the decrease in ectoenzymes induced by ICV-STZ

Purinergic neurotransmission, through the action of ectoenzymes, may be of great relevance in the pathophysiology of brain disorders (Bonan 2012). Therefore, we investigated whether BRB administration could have a protective effect against a possible purinergic dysfunction caused by this experimental model. Figure 8 shows the effects of ICV-STZ injection and BRB treatment on NTPDase, 5′-nucleotidase, and ADA enzymes in synaptosomes of the cerebral cortex and hippocampus.

Figure 8A shows lower activity of NTPDase using ATP as a substrate in synaptosomes in both the cerebral cortex and hippocampus of animals that received ICV-STZ, when compared to the CTR groups. However, the treatment with BRB at 50 and 100 mg/kg prevented this decrease; in the animals that received ICV-STZ and were treated with BRB, higher activity of NTPDase in synaptosomes of the cerebral cortex and hippocampus was evident, in comparison to the STZ groups. Similar results were observed for activity of this enzyme when ADP was used as the substrate. Figure 8B demonstrates that treatment with BRB at both doses was capable of preventing the decrease in NTPDase activity induced by ICV-STZ, in both the cerebral cortex and hippocampus.

When we analyzed the activity of 5'-nucleotidase using AMP as the substrate, lower activity of this enzyme in synaptosomes of the cerebral cortex and hippocampus of STZ groups was observed (Figure 8C), as compared to the CTR groups. Interestingly, treatment with BRB prevented this decrease; both the STZ+BRB 50 and STZ+BRB 100 groups presented with higher activity of 5'-nucleotidase in synaptosomes of the cerebral cortex and hippocampus, as compared to the STZ groups.

Figure 8D shows the ADA activity in synaptosomes of the cerebral cortex and hippocampus. We observed lower activity of ADA in both the cerebral cortex and hippocampus of animals that received ICV-STZ, as compared to the CTR groups. However, animals treated with BRB at 50 and 100 mg/kg presented with higher ADA activity in both structures when compared to STZ groups.

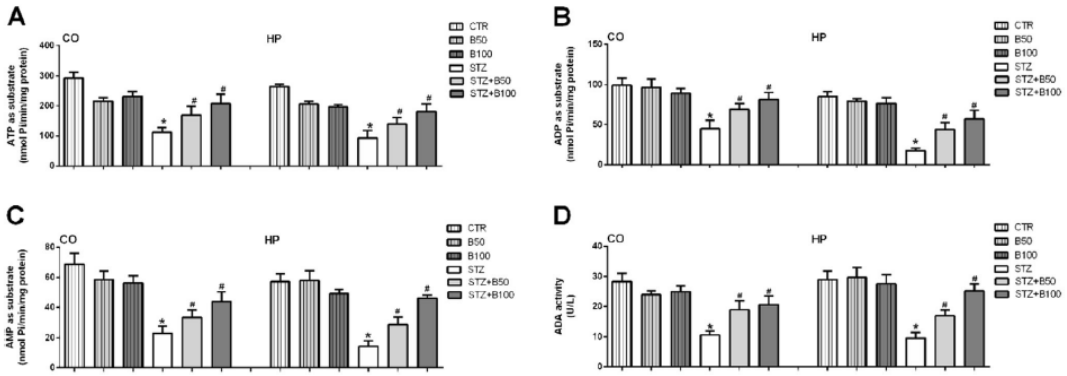

Figure 8. Effect of intracerebroventricular streptozotocin (ICV-STZ) and berberine (BRB) on NTPDase, 5′-nucleotidase, and ADA activities. Data are expressed as mean ± SEM. *p < 0.05 when compared to the CTR group. #p < 0.05 when compared to the STZ group.

3.9 Correlation between ROS production, TBARS levels, and ectoenzymes activities

Since we observed a protective effect of BRB on oxidative stress and the decrease in activity of ectoenzymes induced by ICV-STZ, we sought to investigate if there was a correlation between these results. Table 2 shows that there was a significant correlation between ROS production and both NTPDase and ADA activities, as well as a significant correlation between TBARS levels and NTPDase, 5′-nucleotidase, and ADA activities in the cerebral cortex. No significant correlation was observed between ROS production and 5'-nucleotidase activity in the cerebral cortex.

Similar results were observed in the hippocampus, with a significant correlation between ROS production and NTPDase, 5′-nucleotidase, and ADA activities, and between TBARS levels and NTPDase, 5′-nucleotidase, and ADA activities.

	ROS	**T-BARS**	**ROS**	**T-BARS**
	Cerebral cortex		Hippocampus	
NTPDase (ATP as substrate)	$p = 0.016$	$p = 0.001$	$p = 0.001$	$p = 0.001$
	$r = -0.65$	$r = -0.80$	$r = -0.75$	$r = -0.81$
NTPDase (ADP as substrate)	$p = 0.002$	$p = 0.001$	$p = 0.001$	$p = 0.001$
	$r = -0.49$	$r = -0.84$	$r = -0.79$	$r = -0.09$
5′-nucleotidase	n.s.	$p = 0.004$	$p = 0.001$	$p = 0.001$
		$r = -0.71$	$r = -0.80$	$r = -0.08$
Adenosine deaminase	$p = 0.017$	$p = 0.001$	$p = 0.001$	$p = 0.001$
	$r = -0.52$	$r = -0.76$	$r = -0.83$	$r = -0.0$

Table 2. Correlations between reactive oxygen species (ROS) production, Thiobarbituric acid reactive substance (TBARS) levels, 5′-nucleotidase, and adenosine deaminase (ADA) activities. n.s. = non-significant.

4. DISCUSSION

Our previous research has demonstrated that BRB has neuroprotective effects on loss of spatial memory, anxious behavior, modulation of the cholinergic system, and cell death (de Oliveira et al. 2016). Nevertheless, due to the still unknown cause of AD and the multiple pathological pathways attributed to the disease, a multipotent drug is required. Thus, we sought to further evaluate the action of BRB on recognition memory, parameters of oxidative stress, and ectoenzymes of the purinergic system in animals submitted to the dementia model induced by STZ.

It is well established that recollection is impaired early on in the course of AD (Simon et al. 2016). For this reason, with regard to treatment of AD, it is of particular interest to investigate the action of compounds on memory damage. Interestingly, the behavioral test of object recognition revealed a neuroprotective effect of BRB at both doses (50 and 100 mg/kg) in ICV-STZ-treated animals. BRB's ability to improve memory deficits is in agreement with a number of studies of memory impairment (de Oliveira et al. 2016; Haghani et al. 2015; Huang et al. 2017; Patil et al. 2015). In agreement with this results, our previous study demonstrated the BRB's capacity to improves also the spatial memory deficit (de Oliveira et al. 2016). The literature demonstrates that the neuroprotective mechanism of BRB could result from BRB's action on the inhibition of β-amyloid production (Huang et al. 2017) or the inhibition of AChE and cell death (de Oliveira et al. 2016). Besides, BRB has shown great therapeutic potential against neurodegenerative diseases due to its small size and its ability to effectively cross the blood-brain barrier allowing to act on a number of molecular targets (Jiang et al. 2015).

In addition to memory damage, AD is associated with abnormalities such as mitochondrial dysfunction, increased oxidative stress, failure of energy metabolism, and disorders in several neurotransmission systems (Ferrer 2012). In the process of oxidative stress, excessive ROS are produced, mainly by mitochondria (Alberici et al. 2011). In this study, we assessed several markers of oxidative stress and found that ICV-STZ in rats is capable of increasing ROS production, TBARS, and carbonyl protein levels, as well as inhibiting the δ-ALA-D enzyme. The increase in ROS production, and consequent increase in protein and lipid oxidation evaluated by TBARS and carbonyl proteins, is already established in AD, and suggests that oxidative stress is involved in disease-related synaptic loss (Ansari and Scheff 2010). The findings from the present study are also consistent with other studies using this model of dementia in rats. Other authors have also demonstrated an increase in ROS levels (Saxena et al. 2011) as well as ROS-induced damage to biomolecules of lipids and proteins (Khan et al. 2012).

ROS production can be exacerbated by δ-ALA-D inhibition; this enzyme is considered a marker of oxidative stress because its active sulfhydryl group renders it highly sensitive to pro-oxidant elements which impair its function (Baierle et al. 2014). Inhibition of δ-ALA-D mainly affects heme biosynthesis, resulting in the accumulation of the substrate ALA, which contributes to overproduction of ROS, and consequently, to the process of oxidative stress (Jacques-Silva et al. 2001); this may be an additional factor involved in the cognitive decline in AD (Baierle et al. 2014). δ-ALA-D inhibition is a well described marker of metal intoxication (Abdalla et al. 2014; do Nascimento et al. 2015), and some studies using animal models have shown a decrease in the activity of this enzyme in metabolic disorders (Brito et al. 2007; Folmer et al. 2003; Folmer et al. 2002) and streptozotocin-induced diabetes (Schmatz et al. 2012).

Furthermore, experimental evidence increasingly suggests that functional heme deficiency is an important factor contributing to the pathogenesis of AD (Atamna and Frey 2004; Ghosh et al. 2015). However, to our knowledge, this is the first work to demonstrate δ-ALA-D activity in the ICV-STZ model, and the action of BRB on this enzyme, correlating this activity with oxidative stress. Interestingly, we demonstrated that treatment with BRB was effective in protecting against increased ROS production and low δ-ALA-D activity, and observed a significant correlation between these results, suggesting a possible mechanism behind the reduction in oxidative stress with BRB administration.

In an attempt to reverse an imbalance caused by increases in oxidizing agents, an organism produces endogenous antioxidants. Generally, most of these oxidative compounds are conjugates with GSH and turn into detoxification products during the reaction catalyzed by GSTs. Thus, the evaluation of antioxidant compounds is of particular interest in neurodegenerative diseases, especially in AD, given that it has been demonstrated that the concentration of antioxidant compounds is decreased in AD brains (Ansari and Scheff 2010;

Calabrese et al. 2006; Gumusyayla et al. 2016). In this regard, in our work we found a decrease in levels of antioxidant compounds, such as T-SHs and GSH, as well as low activity of GST. Similar results were observed in other studies using the ICV-STZ model (Khan et al. 2012; Naghizadeh and Mansouri 2015). In fact, these data are indicative of increased oxidative stress, which is known to be an early event in the development of AD, and has an important role in the fast progression of neurodegenerative diseases (Manoharan et al. 2016; Meraz-Rios et al. 2014); oxidative stress is known to accelerate the Abeta- or tau-induced neurotoxicity, and is implicated in neuronal apoptosis and deterioration of cognitive function (Zhao and Zhao 2013).

In the present study, treatment with BRB at 50 and 100 mg/kg for 21 days protected against increased ROS production, TBARS levels, carbonyl proteins levels, and δ-ALA-D inhibition, and decreased antioxidants compounds such as total thiols, GSH, and GST, of animals submitted to ICV-STZ. The antioxidant activity of BRB has already been well described (Liang et al. 2014; Mojarad and Roghani 2014), although its mechanisms are not fully elucidated. The mechanisms behind BRB's ability to reduce oxidative stress seem to be related to multiple cellular pathways, including the downregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is a major source of ROS production in cells (Liang et al. 2014), and modulation of nitric oxide production (Mojarad and Roghani 2014). Several studies have shown that BRB has the ability to increase levels of antioxidant compounds, both directly through its defense against ROS, and indirectly as a cofactor of antioxidant enzymes (Dkhil 2014; Liang et al. 2014), thereby reducing levels of carbonyl protein and lipid peroxidation (Mojarad and Roghani 2014).

Thus, we suggest that pretreatment with BRB could attenuate oxidative stress in ICV-STZ animals due to its ability to increase antioxidant compounds, as demonstrated by increased levels of TSHs, GSH, and GST activity, thereby reducing ROS and ROS-related damage. Furthermore, we suggest a decrease in the formation of ROS results from the increase in the activity of δ-ALA-D. Since this enzyme is a metalloenzyme, and thiol (–SH) groups are required for its normal activity (Grotto et al. 2010; Valentini et al. 2007), the increase in the activity of this enzyme by BRB may contribute to the reduction in oxidative stress observed in this model.

In addition, oxidative stress may be closely related to damage of the purinergic system. Low cytosolic GSH is critical for maintaining plasma membrane integrity and ATP levels in synaptosomes (Martinez et al. 1995). Further, the increased oxidative damage to lipids and proteins, and the decline of antioxidant enzyme activities, is more localized to synapses (Ansari and Scheff 2010), and the enzymes that control ATP and adenosine levels are also located at the synaptic cleft (Bonan 2012). Thus, we sought to evaluate the activity of the enzymes that participate in the degradation of ATP and adenosine in synaptosomes of the cerebral cortex and hippocampus.

Given that it has higher metabolic rates and energy demands, the brain depends a lot on mitochondrial function. In this regard, ATP, upon its release, can be metabolized by the action of ecto-enzymes that convert ATP to inosine (Zimmermann et al. 2012). ATP is an important neurotransmitter in purinergic synapses, and is involved in the processes of synaptogenesis, neuritic growth, and control of cerebral blood flow (Molteni et al. 2002). On the other hand, adenosine is considered an important neuroprotective compound (Bonan 2012). Moreover, proper regulation of the purinergic signaling by ectonucleotidases and ADA may be crucial in pathological conditions of the central nervous system, such as AD, because these enzymes are involved in the mechanisms of acquisition and modulation of memory processing (Bonan 2012).

Our results revealed that ICV-STZ in rats also could contribute to the impairment of energy metabolism in the hippocampus and cerebral cortex by reducing the NTPDase enzyme, which degrades ATP in ADP and ADP in AMP, as well as reducing 5'-nucleotidase, which is

responsible for the degradation of AMP in adenosine, and ADA, which leads to the hydrolysis of adenosine to inosine. As such, changes in the ATP/adenosine balance may dramatically influence memory (Burnstock et al. 2011), and damage to purine metabolism observed in the present study may affect the complex equilibrium between intracellular ATP and its related metabolites and products.

It is known that low activity of NTPDase, and the consequent increase in extracellular ATP in the synaptic cleft, can impair purinergic signaling and stimulate an increase in intracellular calcium levels, which leads to neuronal damage (Pubill et al. 2001). In addition, low activity of 5'-nucleotidase can result in a decrease in adenosine levels, which is of particular interest in AD given its neuromodulating capability and its positive experimental effects in neurodegenerative diseases (Rahman 2009). It also plays an important role in glucose homeostasis, through insulin secretion, glucose release and clearance, glycogenolysis, and glycogenesis (Koupenova and Ravid 2013). Recently, inosine has also been shown to have an important role in neuronal damage (Bhattacharyya et al. 2016; Junqueira et al. 2016; Kovacs et al. 2011; Moore et al. 2016; Parkinson Study Group et al. 2014); thus, a decreased adenosine/inosine ratio may also be an important factor in AD.

Furthermore, since ICV-STZ is described by changing glucose metabolism and insulin signaling in the brain (Grieb 2016), some of the other neuroprotective mechanisms described to BRB are the capacity to directly act through insulin-dependent and -independent mechanisms thereby altering glucose homeostasis in the brain (M and C 2017). In this way, BRB could act facilitating the uptake of glucose in the brain by increase in the levels of glucose transporters (Chen et al. 2017) or by increase insulin receptor expression (Zhang et al. 2010).

Based on our data, we believe that the increase in oxidative stress, and consequent membrane damage, can lead to decreased viability of neurons and activity of the ectoenzymes, since they are bound to the cell membrane. Importantly, our hypothesis is supported by the significant correlations observed between ROS production, TBARS levels, and ectoenzymes activities. In addition, our preliminary evaluation is consistent with these results, since an increase in cell death was observed in the cerebral cortex and hippocampus of animals submitted to the ICV-STZ model (de Oliveira et al. 2016). Considering that there are no reports in the literature describing the mechanism of action of BRB involved in the increase in activity of ectoenzymes, we believe that BRB prevents reduction in the activity of these enzymes in synaptosomes of the cerebral cortex and hippocampus of animals submitted to the ICV-STZ model through its antioxidant capacity. We consider that BRB can prevent oxidative stress and damage to cell membranes, as observed in our results, thereby protecting against cell death, conserving the activity of membrane bound enzymes, preventing damage to the cholinergic and purinergic neurotransmission systems, and consequently, improving memory.

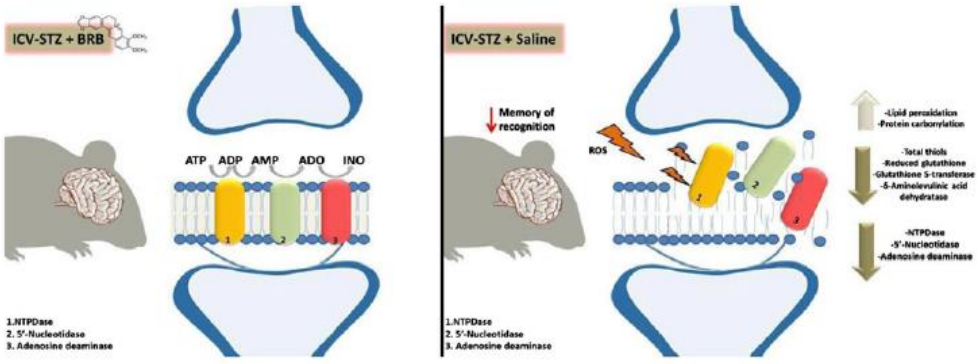

Figure 9. Graphical Abstract. ICV-STZ+BRB= intracerebroventricular of streptozotocin + oral treatment with berberine. ICV-STZ+saline= intracerebroventricular of streptozotocin + oral treatment with saline. ATP=

Adenosine triphosphate. ADP= Adenosinadifosfato. AMP= Adenosine monophosphate. ADO= adenosine. INO= Inosine.

5. CONCLUSIONS

Oral administration of 50 and 100 mg/kg BRB for 21 days was effective at preventing damage to recognition memory, preventing oxidative stress, and preserving purinergic neurotransmission. The strengths of this study include the objective assessment of the effects of BRB on oxidative stress and δ-ALA-D activity, and that these results were also related to the activity of ectoenzymes of synaptosomes in the cerebral cortex and hippocampus of animals with STZ-induced dementia. Moreover, the results provide a better understanding of the damage caused by ICV-STZ, since the activity of the purinergic system and δ-ALA-D in this model of dementia has not been fully elucidated in the literature. In summary, the combined effects of BRB lead to its ability to cause a cascade of pathological events, including memory loss, oxidative stress, and altered ATP neurotransmission in ICV-STZ model rats. Therefore, this compound could be considered a promising multi-target drug for the prevention and protection against AD.

Conflict of interest statement

Declarations of interest: none. The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior on number 88882.182124/2018-01, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and the Federal University of Santa Maria, RS, Brazil, andthe FINEP research grant ‘‘Rede Instituto Brasileiro de Neurociências (IBNet)’’, Instituto Nacional de Ciências Tecnológicas (INCT). All the experiments comply with the current laws of Brazil.

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4 CAPÍTULO II: ARTIGO II BERBERINE ATTENUATES COGNITIVE IMPAIRMENT, ALTERATIONS IN THE CHOLINERGIC AND PURINERGIC SYSTEM AND OXIDATIVE STRESS IN LIPOPOLYSACCHARIDE -INDUCED RATS NEUROINFLAMMATION MODEL

Artigo submetido para: *NeuroToxicology*

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ABSTRACT

Neuroinflammation is known as a risk factor for Alzheimer Disease (AD). This process contributes with increased neuropathological changes as the formation of senile plaques and neurofibrillar tangles and consequently to disease progression and severity. Berberine (BRB) is an isoquinoline alkaloid isolated from traditional Chinese medicinal herbs with potential to act on neurodegenerative diseases due your action anti-inflammatory, neuroprotective, and antioxidant. In this study, the protective effect of BRB against lipopolysaccharide (LPS)-induced neuroinflammation was investigated in the cerebral cortex and hippocampus of rats. The animals were submitted 8 consecutive days, one injection (PI) per day of LPS at a dose of 250 μg/kg body weight and BRB was administered p.o. at dose of 50 mg/kg/day, 30 min after LPS, for 8 days. Treatment of LPS injected rats with BRB improved recognition memory and prevented the increased acetylcholinesterase activity. BRB was also effective in protecting against increased levels of reactive oxygen species and related damage such as lipid peroxidation and carbonyl protein formation. Furthermore, the treatment with BRB 50 mg/kg improved antioxidant defensive system comprising total thiols and glutathione (GSH) in addition to increasing ectoenzymes activity such NTPDase and Ecto-5'-nucleotidase of LPS-injected group. Taken together, BRB administration could mitigate LPS induced memory deficits via attenuation of oxidative stress and modulation of enzymes like acetylcholinesterase and NTPDase and Ecto-5'-nucleotidase.

KEYWORDS: Dementia; Cholinergic Signaling; Purinergic Signaling; *Coptis Chinensis.*

1. INTRODUCTION

Neurodegenerative diseases are currently considered the leading cause of morbidity and disability and disability besides displaying great social and economic concern (1). Systemic inflammation and consequent neuroinflammation represent a close relationship with memory loss and cognitive deficits besides being responsible for an imbalance in redox signaling (1, 2).

The inflammatory process as being closely linked with multiple neurodegenerative pathways and contributes significantly to the loss of neuronal structure and function, characteristic of neurodegenerative diseases including Alzheimer's disease (AD) (1, 3). Evidence suggests that AD pathogenesis strongly interacts with immunological mechanisms in the brain which is related misfolded and aggregated proteins. It this process contribute to by emerging senile plaques and neurofibrillar tangles as well as to disease progression and severity (4).

One of the most important and widely-used animal models of peripherally induced neuroinflammation and neurodegeneration is through injections of lipopolysaccharide (LPS) (5). Among the non-genetically manipulated neuroinflammation models for AD, LPS-induced animal model is commonly used (6) and has been helpful in assessing of drugs and natural products (5).

Berberine (BRB), an isoquinoline alkaloid isolated from traditional Chinese medicinal herbs, has shown promising pharmacological activities, including anti-inflammatory, memory enhancement and antioxidant effects besides may act as a promising anti-neurodegenerative agent with therapeutic potential to combat AD (7, 8). Therefore, this study was designed to evaluate the efficacy of BRB against LPS-induced neuroinflammation in rats. Thus, the objective of this work was to evaluate the action of BRB on recognition memory and parameters of oxidative stress, such as ROS, lipid peroxidation, protein carbonylation and antioxidant levels (such as total thiol (T-SHs) and reduced glutathione (GSH)). Furthermore, the study also aimed to examine the action of BRB on the cholinergic system by analyzing acetylcholinesterase activity and on the purinergic system through investigation of the activity of ectonucleotidases (Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and Ecto-5'-nucleotidase (EC-5'-Nt)) of the cerebral cortex and hippocampus.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL DESIGN

This project was approved by the Ethics Committee of the Federal University of Santa Maria, (Nº 5580160118). Male Wistar rats weighing between 200-250 g were divided into 4 different groups (n = 8): control (CTR), Berberine (BRB), LPS, LPS + BRB. LPS groups received, on 8 consecutive days, one injection (PI) per day of LPS at a dose of 250 μg/kg body weight, dissolved in 0.9% saline while CTR and BRB groups received only 0.9% saline vehicle on the same volume and time period. Thirty minutes after the application (PI), animals from group BRB and LPS + BRB were treated with BRB at a dose of 50mg/kg orally and CTR and LPS groups received 0.9% saline by the same route. The animals were treated during eight days of experiments. Animal weight was monitored daily at approximately 8 hours after treatment. Behavioral tests were performed on the seventh and eighth day. After this period, on the 9th day, euthanasia was performed followed by separation and homogenization of brain structures to perform analysis.

2.2. OPEN FIELD TEST

This test was performed to identify changes in locomotor and exploratory capacity of animals as previously described by Zanin and Takahashi (1994) (9). The apparatus consists of a wooden box lined with waterproof material with dimensions 70 x 70 x 30 cm. The floor was divided into 16 squares measuring 12 x 12 cm each for open field evaluation. The session lasted five minutes and was recorded for further processing by an automated activity monitoring system (AnyMaze, Stoelting, USA). The animals were initially submitted to a training session. The tests were performed 2h and 24 after training. Locomotor activity was defined by the total number of areas crossed by the animal's four legs.

2.3 OBJECT RECOGNITION TEST

The object recognition task was used to study recognition memory in rats (10). The animals were trained and individually placed in the open field containing 2 similar objects (A1 and A2) and allowed to explore them freely for 5 minutes. The animals were then removed and 24 hours after the training session the retention test was performed. In these 5-minute test

sessions, the rats were individually reintroduced into the open field where one of the objects presented during training was randomly replaced by a new object (A1 and B). This task consists of spontaneous and differential exploration of familiar and new objects and recognition performance is derived from the time spent exploring both stimuli. The test was repeated after the end of the trial period to assess treatment efficacy. For this, new objects (C1, C2 and D) were used.

2.4 BRAIN TISSUE PREPARATION

After euthanasia the cranium was opened and the structures were gently removed and separated into the cerebral cortex and hippocampus. The brain structures were homogenized in a glass potter in a solution of 10 mMTris–HCl, with pH 7.4, on ice, at a proportion of 1:10 (w/v). The resulting homogenate was used to determine the oxidative stress parameters, acetylcholinesterase activity and activity of ectoenzymes.

2.5 DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY IN THE BRAIN

The AChE enzymatic activity was determined by the Ellman et al method, (11) as modified by Rocha et al (12) 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.

2.6 MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

Reactive oxygen species were evaluated by quantifying of 2′-7′-Dichlorofluorescein (DCF) (13). Aliquots (50 μl) of cerebral cortex and hippocampus supernatants were added to a medium containing Tris - HCl buffer (10 mM; pH 7.4) and 1 mM 2′-7′- dichlorofluorescein diacetate (DCFH-DA). After addition of DCFH-DA, the medium was incubated in the dark for

1 h until the fluorescence measurement procedure (488 excitation and 525 emission). Results were expressed as DCFH-DA Fluorescence.

2.7 THIOBARBITURIC ACID REACTIVE SUBSTANCE (TBARS) MEASUREMENT

Lipid peroxidation was determined according to Ohkawa et al. (14). Briefly, the reaction mixture, containing 200 μl of cerebral cortex and hippocampus supernatants or standard (0.03 mMMDA), 200 μl of 8.1% sodium dodecyl sulfate (SDS), 500 μl of 0.8% TBA, and 500 μl of acetic acid solution (2.5 M HCl, pH 3.4), was heated at 95°C for 120 min. The absorbance was measured at 532 nm. Levels of TBARS in tissues were expressed as nmol MDA/mg of protein.

2.8 DETERMINATION OF PROTEIN CARBONYL LEVELS

The carbonylation of proteins was determined through the method of Levine (15). Cerebral cortex and hippocampus supernatants were used in a mixture containing 0.5 ml of 10 mmol 2,4-dinitrophenylhydrazine (DNPH) in 2 mol HCl and reaction was incubated at room temperature for 30 min. After incubation, 0.5 ml of 10% TCA was added to the protein precipitate and centrifuged at 1800 g for 5 min. After discarding the supernatant, the precipitate was washed twice with 1 ml of ethanol/ethyl acetate (1:1), and the supernatant was centrifuged out, in order to remove the free DNPH. The precipitate was dissolved in 1.5 ml of protein dissolving solution (2 g sodium dodecyl sulfate and 50 mg EDTA in 100 ml 80 mmol phosphate buffer, with pH 8.0) and incubated at 37°C for 10 min. The color intensity of the supernatant was measured using a spectrophotometer at 370 nm against 2 mol HCl. Carbonyl content was calculated using the molar extinction coefficient (21 x 10^3 1/mol cm), and results were expressed as nmol/mg of protein.

2.9 DETERMINATION OF TOTAL THIOLS (T-SHS)

T-SHs were evaluated according with Boyne and Ellman (16), with some modifications. For the reaction an aliquot of 200 μl of cerebral cortex and hippocampus supernatants in a final volume of 900 μL of solution with phosphate buffer was used. The reaction product was measured at 412 nm after the addition of 50 μL of 10 mM 5-5-dithio-bis DTNB. Results were calculated using curve of cysteine. Thiol group content was expressed as nmol T-SH/g tissue.

2.10 MEASUREMENT OF REDUCED GLUTATHIONE (GSH)

For the quantification of GSH levels the method described by Ellman (17) was used. The cerebral cortex and hippocampus samples were mixed (1: 1) with 10% trichloroacetic acid (TCA) and centrifuged at 4000 × g for 10 min. After centrifugation, protein pellet was discarded and free NPSH groups were determined in the clear supernatant. An aliquot of 200 µl of samples in a final volume of 900 µl of solution with phosphate buffer was used. The reaction product was measured at 412 nm after the addition of 10 mM DTNB (50 µL). A standard curve using glutathione was added to calculate the content of GSH in samples. The results were expressed as nmol GSH/g tissue.

2.11 EVALUATION OF NTPDASE, 5′-NUCLEOTIDASE ACTIVITIES

NTPDase activity was determined according to Schetinger et al. (18), whereas the 5′-nucleotidase activity was determined according to Heymann et al. (19). First, samples of the cerebral cortex and hippocampus were homogenized in 10mM phosphate buffer and the supernatant was used for analysis of the activity of ectoenzymes. The NTPDase enzymatic assay was carried through a reaction containing 5 mM KCl, 1.5 mM CaCl2, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 µl. The 5′-nucleotidase activity was determined essentially by the method of Heymann et al. (19) in a reaction medium containing 10 mM MgSO4 and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 µl. The samples of cerebral cortex and hippocampus was added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of to 20 µl ATP or ADP (1 mM) or AMP (2 mM) and incubation time was 20 min. In all cases the reaction was stopped with 200 µl of 10 % trichloroacetic acid and the release of inorganic phosphate was measured by the method of Chan et al.(20). The results were expressed by nmol Pi/min/mg of protein.

2.12 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.

3 RESULTS

3.1 ANIMALS BODY WEIGHT MONITORING

The systemic effect of LPS or BRB was evaluated in order to know if the LPS could affect body weight of animals, and the results were presented in Fig. 1. There were no significant differences in the body weight of rats treated with LPS or BRB. Although the researchers observed a slight decline in the body weight of the animals in the LPS and LPS + BRB group on the third day of evaluation, this observation showed no significant difference in relation to another groups. The trend of body weight gain was gradual during the 8 days of experimentation in all groups.

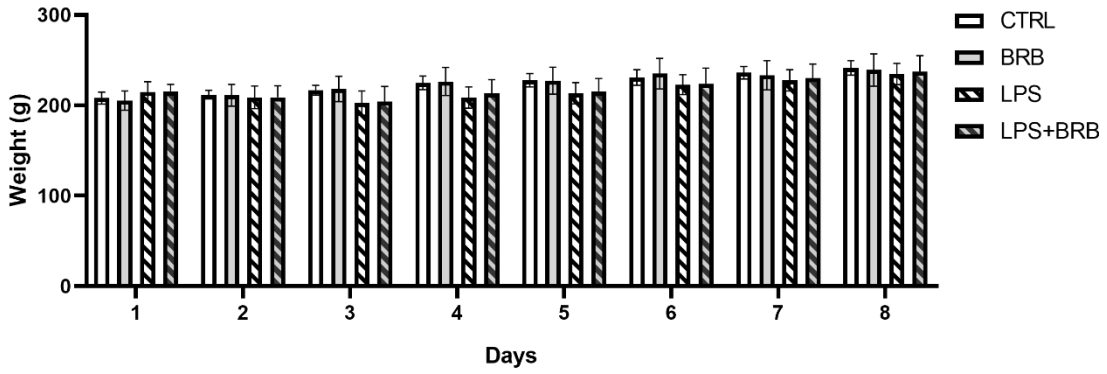

Figure 1: Effect of lipopolysaccharide (LPS) injection and berberine (BRB) on body weight. Data values are expressed as mean body weight in g ± SEM.

3.2 EVALUATION OF LOCOMOTOR ACTIVITY THROUGH THE OPEN FIELD TEST

In the current study, we determined the effects of injections repeated of LPS or BRB treatment on locomotor behavior. The open field test revealed that there were no significant differences for as distance travelled, mobile and immobile well as numbers of entries, exits or time in the corner, wall and center zone. In other words, open field locomotor activity monitoring revealed that the rats did not exhibit locomotor alteration.

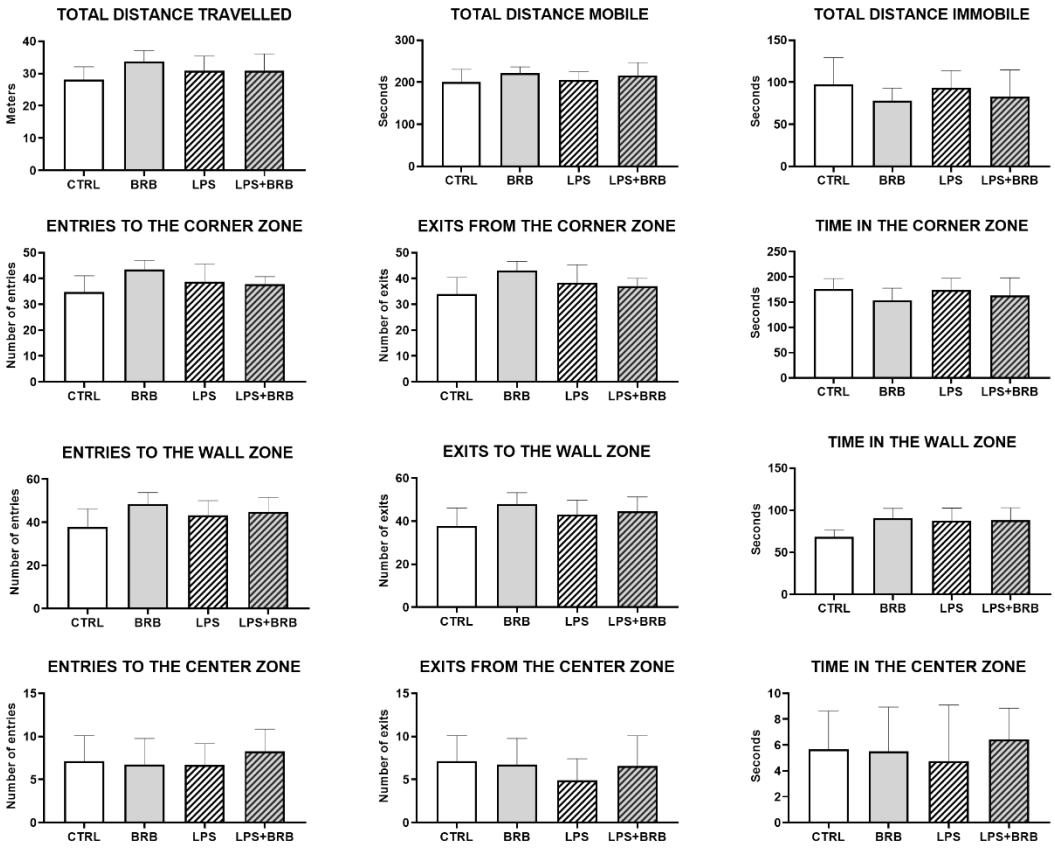

Figure 2. Effects of lipopolysaccharide (LPS) injection and berberine (BRB) treatment on locomotor activity. The behavioral tests were performed 2 h after the (i.p) LPS injection at a dose of 250 ug/kg or saline (SAL) or treatment with BRB 50 mg/kg. Either LPS or saline injection or BRB treatment did not modify the rat locomotor activity in the open field test. Data values are expressed as mean ± SEM.

3.3 BERBERINE (BRB) PREVENTS LIPOPOLYSCCHARIDE (LPS)-INDUCED RECOGNITION MEMORY DEFICIT

The release of inflammatory mediators through neuroinflammation contribute to AD progression and severity (4). For this reason, we sought to evaluate whether BRB could exert neuroprotective action on LPS-induced neuroinflammation. Figure 3 shows the effect of LPS and the treatment with BRB at dose of 50 mg/kg on the recognition index of the new object in of time 2h (short memory) and 24h (long memory). LPS animals without BRB treatment showed a significant reduction in recognition index in the short and long memory evaluation. However, the treatment with BRB 50 mg/kg did protect against memory impairment induced by LPS in both short and long memory evaluation.

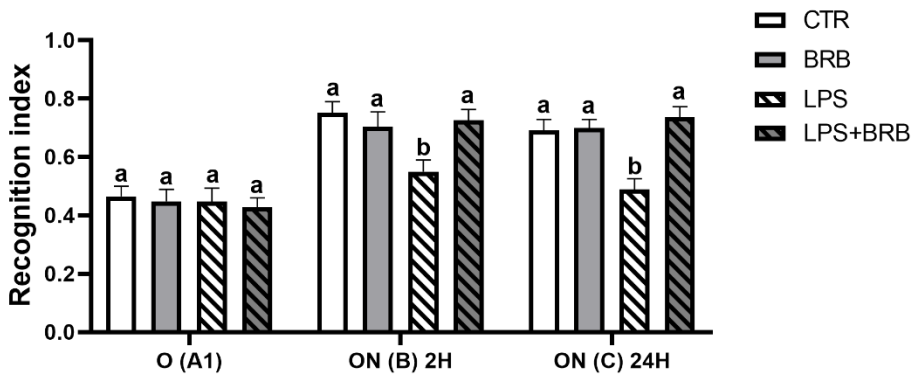

Figure 3. Effects of lipopolysaccharide (LPS) injection and berberine (BRB) treatment on the novel object recognition task. Data are expressed as mean ± SEM. Different letters indicate significant differences between groups ($p<0.05$). O = object. ON = new object.

3.4 BERBERINE (BRB) PREVENTS THE INCREASED ACETYLCHOLINESTERASE (AChE) ACTIVITY INDUCED BY LIPOPOLYSCCHARIDE (LPS)

AChE is able to accelerate the Aβ peptide aggregates in the AD and increasing its neurotoxicity (21). Besides, neuroinflammation and cholinergic dysfunction are related to memory impairment which are hallmarks of AD (22). Our evaluation of AChE activity in cerebral cortex (CO) and hippocampus (HP) samples demonstrated increased activity of this enzyme in both brain structures of LPS group animals. However, the BRB treatment showed a elevation inhibitionof AChE activity when compared to the untreated LPS group.

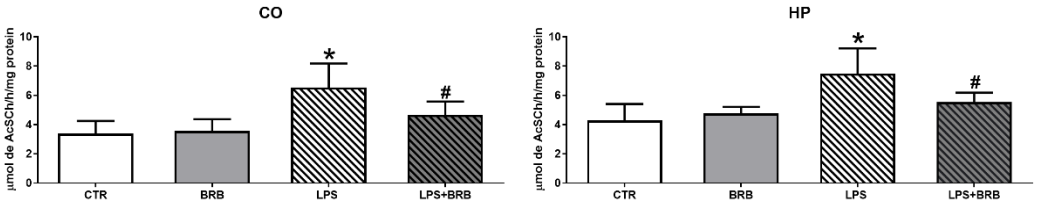

Figure 4. Effect of lipopolysaccharide (LPS) and berberine (BRB) 50 mg/kg on AChE acitivity in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *$p < 0.05$ when compared to the control (CTR) group. $^{\#}p < 0.05$ when compared to the LPS group.

3.5 BERBERINE (BRB) PREVENTS THE INCREASED ROS PRODUCTION AND ITS RELATED DAMAGE INDUCED BY LIPOPOLYSACCHARIDE (LPS)

In the neuroinflammation of AD, in addition to memory impairment, a several reactive oxygen species (ROS) are generated, establishing a status of oxidative stress (23). Our previous studies have demonstrated the BRB's ability to act as an antioxidant molecule in oxidative stress present in a model of sporadic dementia of the Alzheimer's type induced by intracerebroventricular (ICV) injection of streptozotocin (STZ) in rats (24). Now we seek to emphasize also the action antioxidant of BRB on oxidative stress generated in the inflammation process. The results presented in the figure 5 reveal that there were significant increases in ROS production in the cerebral cortex (fig. 5A) and hippocampus (fig. 5B) of the LPS group when compared to the CTR group. As a result of ROS elevated it was also possible to observe an increase in lipid peroxidation, evidenced by the increase in thiobarbituric acid reactive substances (TBARS) (fig. 5 C and D) and protein damage, as demonstrated by the increase in carbonyl proteins (fig. 5 E and F). The same effect was observed in both cerebral cortex and hippocampus of LPS group animals. Nevertheless, the treatment with BRB 50 mg/kg prevented the generation of ROS and, consequently, damage to lipids and proteins in both cerebral cortex and hippocampus of LPS + BRB group animals when compared to LPS group.

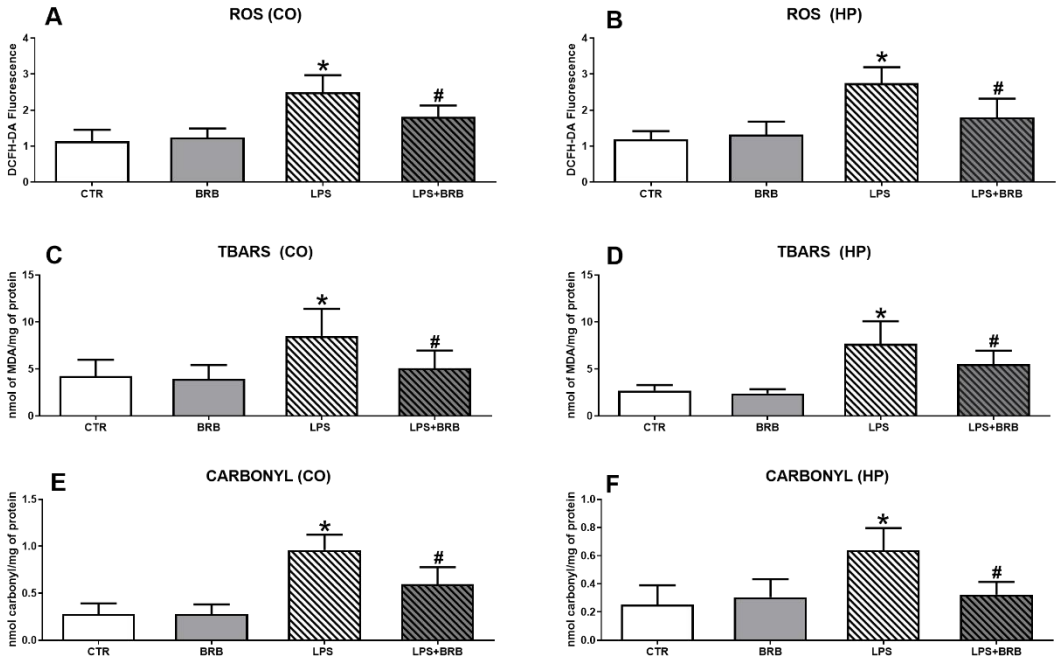

Figure 5. Effect of lipopolysaccharide (LPS) and berberine (BRB) on ROS, TBARS and Carbonyl levels in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *$p < 0.05$ when compared to the control (CTR) group. $^{\#}p < 0.05$ when compared to the LPS group.

3.6 BERBERINE (BRB) PREVENTS THE DECREASE OF TOTAL AND NON-PROTEIN THIOL (GSH) INDUCED BY LIPOPOLYSACCHARIDE (LPS)

Oxidative stress may induce an alteration in the antioxidant systems by modifying proteins that participate in these systems and/or depleting cellular stores of antioxidants (25). Furthermore, antioxidant system plays an important role to regulate the inflammatory (25) as well as cholinergic (26). Since we observed an increase in reactive species and related damage, we sought to evaluate the levels of antioxidant compounds as well as the action of BRB on these compounds on LPS-induced neurodegeneration. Thus, it can be seen from Figure 6 that the untreated LPS group showed decreased levels of both total (T-SH) (fig. 6 A and B) and non-protein thiols, represented by GSH (fig. 6 C and D), in structures of the cerebral cortex and hippocampus. However, the group of animals that received LPS injections and were treated with BRB 50mg/kg had higher total and non-protein thiol levels in both the cerebral cortex and hippocampus when compared to the LPS group.

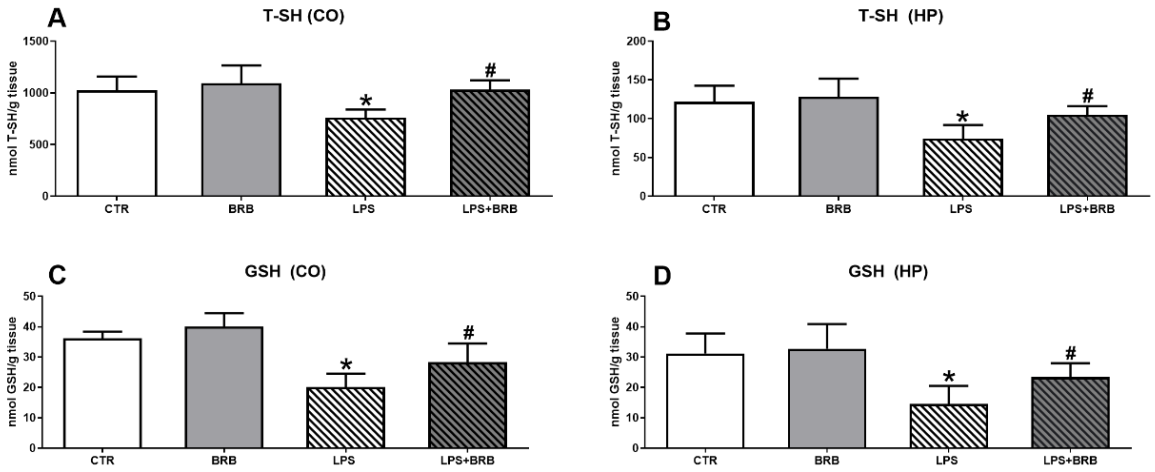

Figure 6. Effect of lipopolysaccharide (LPS) and berberine (BRB) on total thiois (T-SH) and non-protein thiols (GSH) levels in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *$p < 0.05$ when compared to the control (CTR) group. $^{\#}p < 0.05$ when compared to the LPS group.

3.7 BERBERINE (BRB) PREVENTS THE DECREASE IN ECTOENZYMES INDUCED BY LIPOPOLYSACCHARIDE (LPS)

Purinergic signaling is an important regulatory mechanism in a wide range of inflammatory diseases (27). Besides, neuroinflammation initiates the neurodegenerative processes, including AD, by activation of purinergic signaling (28). In view of this, we sought to evaluate the effect of LPS and BRB on the activity of ectoenzymes. Figure 7 shows lower activity of NTPDase using ATP as a substrate (fig. 7 A and B) and using AMP as a substrate (fig. 7 C and D) in both the cerebral cortex and hippocampus of animals of LPS group when compared to the CTR group. However, the group of animals that received the treatment with BRB 50 mg/kg (LPS+BRB group) showed higher activity of NTPDase compared to untreated animals (LPS group) in the structures of the cerebral cortex and hippocampus.

Similarly, the results demonstrated that 5'-nucleotidase showed activity resemblant to the enzyme NTPDase. That is, in the figure 7 (E and F) it is possible to observe that the activity of 5′-nucleotidase using AMP as the substrate was lower in the cerebral cortex and hippocampus of LPS group when compared to the CTR group. In its turn, the LPS+BRB group presented with higher activity of 5′-nucleotidase in both cerebral cortex and hippocampus, as compared to the LPS group.

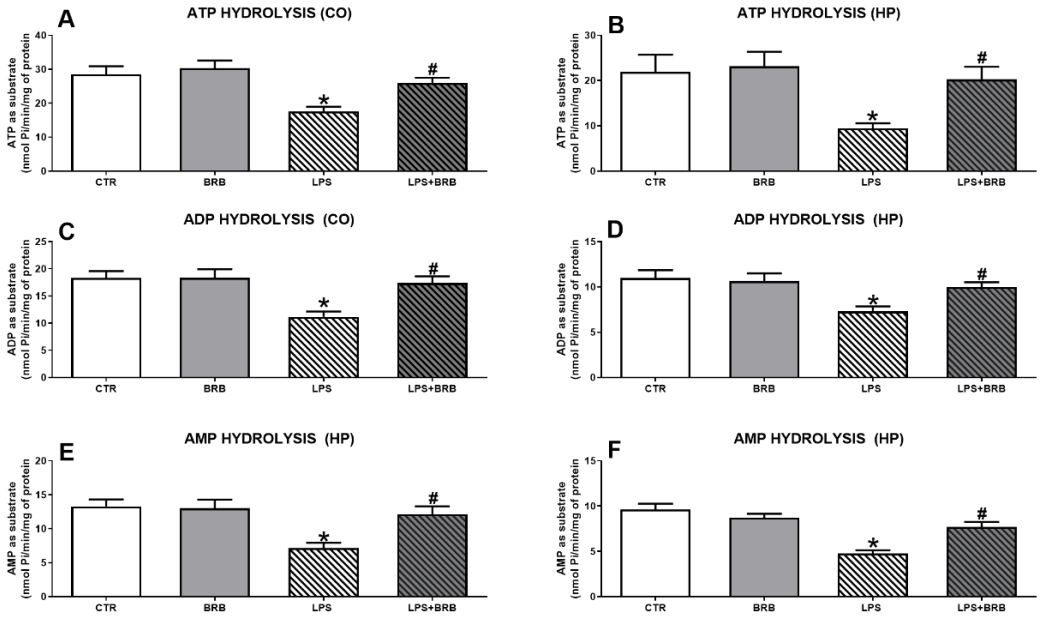

Figure 7. Effect of lipopolysaccharide (LPS) and berberine (BRB) on ectoenzymes activity (NTPDase and 5'-Nucleotidase) in the cerebral cortex (CO) and hippocampus (HP). Data are expressed as mean ± SEM. *p<0.05 when compared to the control (CTR) group. $^{\#}p < 0.05$ when compared to the LPS group.

4 DISCUSSION

Our previous research has demonstrated the neuroprotective action of BRB on memory deficits and alterations of the cholinergic and purinergic system, as well as on oxidative stress induced by intracerebroventricular injection of streptozotocin (24, 29). In turn, due to the benefits previously found for the use of BRB, our focus in this study was to evaluate the action of this alkaloid on another pathway also discussed in the pathogenesis of AD, that is neuroinflammation.

Peripheral infections followed by inflammatory process are considered important factors for the development of sporadic AD. Inflammation accelerates neurodegeneration of AD and activating microglial cells. In addition, toxic manifestations of gram-negative bacterial infections such as LPS may be responsible for the formation of extracellular amyloid proteins. This relationship is directly proportional, ie, the higher the levels of LPS, the higher the levels of amyloid plaques (30, 31).

The literature shows that peripherally-injected LPS induces a variety of central effects, this because this proinflammatory molecule is capable of stimulating from periphery the synthesis of pro-inflammatory cytokines in the brain released mainly from microglia. Besides, it is believed that high doses as well as multiple administration of LPS is able to increase the expression of pro-inflammatory cytokines in the brain of the animals (5).

Therefore, screen synthetic drugs and natural products targeting peripheral inflammation could be a promising additional treatment/prevention approach for neuro-degenerative diseases, like AD. In this sense, it was proposed to study the action of BRB on LPS-induced neuroinflammation. Berberis genus has antioxidant and anti-inflammatory property and protective effects in neurodegenerative disorders such as AD, Parkinson's disease (PD), and trauma-induced neurodegeneration (32).

In the current study we have shown the effects of BRB on LPS-induced neuroinflammation through the evaluations of related to memory, evaluated by object recognition test and acetylcholinesterase activity, in the redox profile, including ROS generation, lipid and protein injury, as well as, non-enzymatic antioxidant levels, besides the purinergic system, including ectoenzymes. Firstly we observed that the LPS did not affect neither spontaneous locomotor activity nor the body weight across a during the 8 days of evaluations. In addition, although we observed a slight decline in body weight of animals undergoing LPS injection on the third day of evaluation, but this difference was not significant. In its turn we did not had mortality during our experimental procedure and there was a complete

recovery of the rats until the end of the evaluations. This result excludes any interference of the BRB treatment and/or LPS injection on object recognition test.

The data of this research demonstrated that BRB prevents LPS-induced learning and memory dysfunctions as evidenced by better performance of animals in the object recognition test and by lower acetylcholinesterase activity that degrades less acetylcholine and makes this neurotransmitter more available for actuation. Previously, our research group has demonstrated a neuroprotective action of BRB on AD model streptozotocin-induced (24, 29). In addition, recent work has shown that this alkaloid is able to exert important action neuroprotective on Parkinson's disease (33), diffuse axonal injury (34), neurotoxicity (35), vascular dementia (36) traumatic brain injury (37) and neuroinflammation (38). A recent review shows that BRB may act as a promising anti-neurodegenerative agent by inhibiting the activity of the most important pathogenic enzymes, ameliorating intracellular oxidative stress, attenuating neuroinflammation, triggering autophagy and protecting neurons against apoptotic cell death (7).

Inflammatory pathways, reactive oxygen species and stress are also known to increase acetylcholinesterase (AChE) activity (39). Since berberine is able to inhibit acetylcholinesterase activity, this effect becomes interesting as it is reported that acetylcholinesterase inhibitors may be able to reduce neuroinflammation (40). Similar to our study, Sadraie et al. 2019 (38) demonstrated that 1 mg / kg LPS injections for 7 days are responsible for cognitive and memory deficits as well as an increase in oxidative stress in the hippocampus of animals. Also, using 50mg/mg BRB, the authors observed a prevention of the impact caused by LPS-induced neuroinflammation. The effects were attributed via partial suppression of apoptotic cascade, neuroinflammation, oxido-nitrosative stress, AChE, MAPK, and restoration of sirtuin 1.

In the present study, treatment with BRB at 50 mg/kg for 8 days protected against increased ROS production, damage to lipids and proteins and decreased antioxidant compounds such as total thiols and GSH of animals submitted to LPS-injection. These results together with the high levels in ROS generation indicate the presence of oxidative stress in LPS-induced neuroinflammation, which is in agreement with other authors (38). The literature shows that tissue level of GSH and antioxidant enzymes significantly decrease in brain tissue from LPS-exposed animals (41) and that an imbalance in the redox state is observed in this model (38).

The antioxidant BRB activity was revealed by changes in oxidative stress markers as well as antioxidant enzymes. In its turn, mechanisms of the antioxidant and anti-inflammatory activities of BBR were complex, which involved multiple cellular kinases and signaling pathways, such as AMP-activated protein kinase (AMPK), mitogen-activated protein kinases

(MAPKs), nuclear factor erythroid-2-related factor-2 (Nrf2) pathway, and nuclear factor- κB (NF- κ B) pathway (42). Still, the three main enzymes that generate higher levels of ERO are lipoxygenase, xanthine oxidase and cyclooxygenase-2 (COX2). Reduced activation of these enzymes in turn leads to reduced levels of ERO. Interestingly, the BRB is effective in reducing the activity of xanthine oxidase and COX2, which also decreases ERO levels and related damage (42).

In addition to memory damage by neuronal loss, oxidative stress has been recognized as a contributing factor in aging and in the progression of AD (43). Furthermore, the chronic inflammation is considered a risk factor to AD. Curiously, microglia activated in the brain during an inflammatory response is recognized as another source of ROS production directly mediated by Aβ and involves to the deposition of extracellular amyloid plaques. This increases of levels of Aβ could accelerate a production of ROS by directly binding to mitochondrial membranes, altering mitochondrial dynamics and function and abnormal energy metabolism with consequent loss of synaptic function (43).

Cellular respiration in the mitochondria converts nutrition-derived energy into adenosine 5′-triphosphate (ATP). ATP concentrations are regulated by ecto nucleoside triphosphate diphosphohydrolases (E-NTPDases) which catalyze the sequential dephosphorylation of nucleoside triphosphates to nucleoside monophosphates (ATP → ADP → AMP) and Ecto-5'-nucleotidase (AMP → Adenosine) (44). During inflammation, ATP is released from inflammatory cells and provides qualitative and quantitative information about pericellular injury to inflammatory cells via purinergic signaling (45). Therapeutic modulation of this signaling pathway influences disease progression in AD (28). Thus, we sought to evaluate the activity of the enzymes that participate in the degradation of extracellular nucleotides (e.g., ATP, ADP and AMP) in the cerebral cortex and hippocampus of animals submitted to neuroinflammation and treated with BRB.

Our results demonstrated a lower activity of E-NTPDase and Ecto-5'-nucleotidase in the cerebral cortex and hippocampus of animals submitted to multiple LPS injections. Similar to our findings the literature shows that macrophages stimulated with LPS present a decreased ATP and AMP hydrolysis in agreement with a decrease in NTPDase1, -3 and ecto-5′-nucleotidase expression and are more susceptible to ATP-induced cell death (46). In addition, microglia and astrocytes release ATP when exposed to Aβ and contribute to AD pathogenesis (28) in turn, it is known that abnormally high ATP levels may acerbate inflammatory responses (47).

The experimental findings of our work suggest that BRB was effective in protecting against decreased activity of the ectoenzymes NTPDase and Ecto-5'-nucleotidase. However, although there is not very data in the literature about the mechanisms underlying this action of BRB, we believe that its antioxidant capacity may contribute to prevent the decrease of ectoenzymes activity. Considering that the ectoenzymes are anchored to the membrane, preserving the cell membranes against the action of ERO consequently is possible to conserve the activity of these enzymes.

5 CONCLUSIONS

Oral administration of BRB 50 mg/kg was effective at preventing damage to recognition memory, acetylcholinesterase activity, oxidative stress and ectoenzymes activity in the neuroinflammation model induced by multiple injections of LPS. Neuroinflammation is an important mechanism involved in the pathogenesis and progression of AD. The evaluation of compounds that can act as an anti-inflammatory molecule and thus minimize the effects of the inflammatory process becomes interesting for the treatment of AD. Based on the results presented, we believe that berberine may be a potential active in the adjuvant AD treatment.

Compliance with ethical standards

Conflict of interest. The authors declare that they have no conflicts of interest.

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5. DISCUSSÃO

A DA é considerada a principal causa de demência e apresenta perspectivas de aumento do número de casos de acordo com o envelhecimento da população. A doença não apenas causa sofrimento para os pacientes, levando-os consequentemente a morte, como também afeta diretamente a qualidade de vida dos cuidadores, além de representar um grande custo para a sociedade. No que diz respeito a patogênese da DA sabe-se que esta é complexa e envolve o metabolismo anormal da proteína β-amiloide, hiperfosforilação da proteína tau, estresse oxidativo, neuroinflamação, dentre outros eventos patológicos. Por sua vez, os desafios atuais da DA incluem a falta de biomarcadores confiáveis para seu diagnóstico precoce, bem como a ausência de estratégias e tratamentos eficientes para impedir a progressão da doença (WANG et al., 2017). Diante disso, a busca pelo desenvolvimento de melhores métodos terapêuticos para a DA se faz necessária. Nesta tese, o objetivo inicial foi investigar a ação da BRB sobre a memória e relacioná-la com os sistemas colinérgico e purinérgico, assim como, avaliar o envolvimento do estresse oxidativo no processo de demência do tipo Alzheimer através de dois modelos experimentais em animais (ICV-STZ e LPS).

O emprego de compostos antioxidantes, como a BRB, torna-se cada vez mais popular no tratamento de transtornos relacionados ao dano oxidativo, processo inflamatório e alterações enzimáticas. Nas últimas duas décadas, uma maior atenção tem sido dada às atividades neuroprotetoras e antineurodegenerativas de compostos isolados de produtos naturais com alta eficácia e baixa toxicidade, como é o caso da BRB. Sendo, desta forma, considerados agentes terapêuticos promissores para doenças neurodegenerativas.

Evidências sugerem que a BRB, um alcaloide isoquinolina isolado de ervas medicinais chinesas tradicionais como a *Coptischinensis*, pode atuar como um agente neuroprotetor promissor, inibindo a atividade de importantes enzimas patogênicas da DA, impedindo o estresse oxidativo, atenuando a neuroinflamação, e, consequentemente, protegendo os neurônios contra a morte celular (FAN et al., 2019). Interessantemente, os metabólitos da BRB também são descritos por contribuírem para seus efeitos farmacológicos. Diante disso, metabolitos ativos como columbamina, berberrubina e desmetileno berberina também exibem efeitos farmacológicos semelhantes em comparação com a BRB, como efeitos antioxidantes, anti-inflamatórios, antitumorais, antimicrobianos, hepatoprotetores, neuroprotetores, hipolipidêmicos e hipoglicêmicos (WANG et al., 2017). Em conjunto, a BRB e seus metabólitos formam a base material da BRB *in vivo* que se torna interessante para seu uso em doenças neurodegenerativas.

Dentre os resultados observados nesta tese, destaca-se, primeiramente no artigo 1, que a BRB possui capacidade de atravessar a barreira hematoencefálica e exercer efeitos neuroprotetores importantes contra a perda cognitiva e demais alterações observadas nos experimentos deste estudo. Além disso, a BRB nas doses de 50 e 100 mg/kg impediu os prejuízos da memória de reconhecimento de objetos observados em animais experimentais induzidos a demência por streptozotocina, em nossa primeira avaliação (Artigo 1) e foi possível correlacionar a ação neuroprotetorada BRB com parâmetros de estresse oxidativo e atividade de ectoenzimas.

Curiosamente, neste estudo verificou-se que em ambas as doses a BRB foi eficaz na proteção contra comprometimento da memória, aumento das espécies reativas de oxigênio e subsequente aumento da oxidação de proteínas e lipídios no córtex cerebral e no hipocampo, bem como na inibição da ácido δ-ALA-D no córtex cerebral dos animais submetidos a demência pela ICV-STZ. Além disso em ambas as doses a BRB impediu a diminuição dos tióis totais, da glutationa reduzida e da atividade da glutationa S-transferase no córtex cerebral e no hipocampo dos animais. Por sua vez, observamos também a eficácia da BRB em impedir a diminuição da atividade das ectoenzimas NTPDase, 5'-nucleotidase e ADA em sinaptossomas do córtex cerebral e do hipocampo dos animais ICV-STZ. Assim, através do nosso primeiro experimento foi possível sugerir um efeito neuroprotetor da BRB 50 e 100mg/kg no reconhecimento da memória, sobre o estresse oxidativo e nos danos relacionados ao estresse oxidativo, assim como, na preservação da neurotransmissão purinérgica.

Os pontos fortes do nosso primeiro experimento incluíram a investigação dos efeitos da BRB no estresse oxidativo e na atividade da δ-ALA-D, bem como, a relação entre essas determinações com a atividade das ectoenzimas dos sinaptossomas do córtex cerebral e do hipocampo de ratos com demência induzida por streptozotocina. Além disso, os resultados proporcionaram uma melhor compreensão dos danos causados pela ICV-STZ, uma vez que a atividade de enzimas do sistema purinérgico e da δ-ALA-D nesse modelo de demência não foram completamente elucidadas na literatura. Em resumo, os efeitos combinados do BRB demonstraram na nossa primeira investigação a sua capacidade de prevenir uma cascata de eventos patológicos, incluindo perda de memória, estresse oxidativo e alterações na neurotransmissão de nucleotídeos. Diante disso, nós atribuímos a neuroproteção proporcionada pela BRB à sua capacidade antioxidante, uma vez que, ao diminuir os níveis de espécies reativas e aumentar as defesas antioxidantes torna-se também possível diminuir os danos as membranas celulares e, consequentemente, impedir alterações em enzimas ancoradas a membrana, como é o caso das ectoenzimas.

A demência induzida pela ICV-STZ em ratos é considerada um modelo eficiente de investigação de compostos promissores para o tratamento da DA, sendo muito utilizado em testes pré-clínicos de terapias farmacológicas da doença. Esse fato se deve a capacidade da ICV-STZ em diminuir cronicamente a captação de glicose cerebral e produzir vários outros efeitos que se assemelham as características moleculares, patológicas e comportamentais da doença (GRIEB, 2016).

Além disso, levando em consideração que o hipometabolismo da glicose é um sinal precoce e persistente da DA, e que o cérebro de pacientes portadores desta patologia apresentam sinalização de insulina prejudicada com danos sobre as células produtoras de insulina e/ou sensores de glicose no cérebro (GRIEB, 2016), a eficiência da BRB em atenuar os danos causados por esse modelo de investigação reforçam sua eficiência neuroprotetora. De fato, a suplementação de BRB tem sido eficiente em melhorar os níveis de receptores de insulina no cérebro e restaurar a expressão de GLUT 1 e GLUT 3. Ainda, a BRB pode atuar diferencialmente através de mecanismos dependentes e independentes de insulina, alterando a homeostase da glicose no cérebro e produzindo efeito benéficos sobre essa via patológica (SANDEEP; NANDINI, 2017).

Uma vez que um grande número de evidências sugere um papel neuroprotetor para o uso da BRB e que os primeiros objetivos desta tese foram alcançados, o segundo trabalho buscou avaliar se este alcaloide seria capaz de impedir também as alterações encontradas em um modelo de demência e neuroinflamação induzidos por LPS. A escolha desse modelo baseou-se no fato de que evidências crescentes sugerem que a patogênese da DA não se restringe ao compartimento neuronal, mas interage fortemente com os mecanismos imunológicos tanto perifericamente como em nível de SNC, observando-se ainda uma estreita relação entre proteínas b-amiloide e processo inflamatório, o qual contribui para a progressão e gravidade da doença (HENEKA et al., 2015).

Dado o fato de não observarmos inicialmente diferenças significativas em relação ao uso da BRB nas doses de 50 ou 100 mg/kg no nosso primeiro estudo, buscou-se avaliar no segundo experimento desta tese as ações benéficas para o uso da BRB utilizando-se apenas a menor dose (de 50 mg/kg). Além da avaliação de memória a fim de confirmar déficits cognitivos do modelo experimental, no segundo manuscrito, avaliamos também a atividade da AChE, parâmetros de estresse oxidativo e atividade de ectoenzimas.

Os nossos resultados demonstram que a BRB 50 mg/kg melhorou a memória de reconhecimento e impediu o aumento na atividade da AChE. Além disso, a BRB também foi eficaz na proteção contra níveis aumentados de espécies reativas de oxigênio e danos

relacionados, como a peroxidação lipídica e formação de proteínas carbonil. Por sua vez, o tratamento com BRB 50 mg/kg melhorou o sistema de defesa antioxidante, incluindo T-SH e GSH, além de aumentar a atividade das ectoenzimas, como NTPDase e Ecto-5'-nucleotidase no grupo de ratos induzidos à neuroinflamação por LPS e tratados com BRB. Concomitantemente, através dos resultados observamos em nosso segundo experimento que a administração de BRB pode atenuar os déficits de memória induzidos por LPS via redução do estresse oxidativo e modulação de enzimas como AChE e NTPDase e Ecto-5'-nucleotidase.

O modelo de neuroinflamação induzido por LPS é considerado eficiente em avaliações sobre a DA. O processo inflamatório é capaz de aumentar a formação de proteínas β-amiloide e favorecer a formação de placas senis com consequente lesão neuronal. Esses dados levam à hipótese de que o LPS atua nos receptores TLR4-CD14/TLR2 de leucócitos e micróglias e é responsável por produzir aumento dos níveis de citocinas mediado por NFkB. Por sua vez, o aumento de citocinas elevam os níveis de proteína β, danificam os oligodendrócitos e produzem lesão celular cerebral. Diante desses achados o modelo LPS pode servir como estudo para alvos de tratamento ou prevenção da DA esporádica (ZHAN; STAMOVA; SHARP, 2018). Interessantemente, nossas avaliações demonstraram importantes benefícios para o uso da BRB também sobre essa via patológica da DA.

Esses achados são importantes uma vez que evidências crescentes sugerem que a patogênese da DA não se restringe ao compartimento neuronal, mas inclui fortes interações com mecanismos imunológicos no cérebro. A formação de proteínas mal dobradas e agregados proteicos estão intimamente relacionados com células da glia como micróglia e astrócitos e desencadeiam uma resposta imune inata caracterizada pela liberação de mediadores inflamatórios, que, por sua vez, contribuem para a progressão da DA (HENEKA et al., 2015). Observa-se ainda que fatores externos como a inflamação sistêmica são capazes de interferirem nos processos imunológicos do cérebro e agravarem a doença. Por sua vez, compostos com capacidade de modular esse fator de risco e interferir em mecanismos imunológicos podem levar a futuras estratégias terapêuticas ou preventivas para a DA.

Através das avaliações realizadas tanto no modelo de demência induzido por ICV-STZ e por LPS a BRB demonstrou potente ação antioxidante. A literatura demonstra que a atividade antioxidante da BRB está relacionada à sua capacidade de impedir a geração mediada por NADPH oxidase de ânions superóxido e inibir a expressão da enzima óxido nítrico sintase induzível (iNOS). Além disso, a BRB pode bloquear a formação de demais radicais livres tais como o óxido nítrico, peroxinitrito e hidroxila e induzir defesas antioxidantes, aumentando os níveis de antioxidantes não enzimáticos e enzimáticos. Demonstra-se, por exemplo, que a BRB

inibe a redução de glutationa, vitamina C e vitamina E e aumenta a atividade da superóxido dismutase, catalase, glutationa peroxidase, bem como da glutationa S-transferase. Tais ações antioxidantes da BRB são capazes de diminuir o nível de peroxidação lipídica e prevenir alterações morfológicas, além de diminuir a apoptose celular (AHMED et al., 2015).

A capacidade da BRB em modular a atividade da AChE é um achado importante para nossa investigação. O sistema colinérgico desempenha funções consideráveis nos processos de aprendizagem e memória. Ainda, pesquisas a respeito da relevância desse sistema na DA demonstraram diversas características, como a diminuição na concentração da ChAT, enzima responsável pela síntese da ACh, no córtex e no hipocampo, bem como perda de neurônios colinérgicos. Ainda, relaciona-se as alterações do sistema colinérgico e depleções de acetilcolina com um maior grau de severidade dos déficits cognitivos. Diante disso, fármacos inibidores da AChE constituem-se as principais terapias medicamentosas utilizadas atualmente na DA e são empregadas com o intuito de tornar mais disponível os níveis de acetilcolina restante nos espaços sinápticos (FALCO et al., 2016).

Em relação aos nossos resultados a respeito das enzimas do sistema purinérgico, sabe-se que tais investigações são interessantes devido a relação deste sistema com a DA. Agregados proteicos de β-amiloide induzem a liberação de maiores níveis de ATP no espaço extracelular cerebral, que, por sua vez, estimulam os receptores purinérgicos P2X7. A ativação desses receptores resultam em um aumento da síntese e liberação de muitos mediadores pró-inflamatórios, como as citocinas e quimiocinas além de diminuir da atividade da α-secretase (CIEŚLAK; WOJTCZA, 2018). Por sua vez, nossas avaliações demonstraram um desbalanço na degradação do ATP por meio das alterações de ectoenzimas. Nós sugerimos que tais modificações nas atividades dessas enzimas podem tornar os níveis de ATP mais elevados e assim provocar prejuízos neuronais. Ainda, acreditamos que alterações no balanço de a degradação de ATP e a formação adenosina podem influenciar drasticamente a memória, uma vez que a adenosina é reconhecida por ter potente ação neuroprotetora, prevenindo danos neuronais e sendo considerada essencial na moderação de processos patológicos como a neurodegeneração (CIEŚLAK; WOJTCZA, 2018).

6. CONCLUSÕES

O tratamento diário por 21 dias com BRB nas doses de 50 e 100 mg/kg por via oral em ratos submetidos à demência induzida por injeção intracerebroventricular de estreptozotocina (ICV-STZ):

- Foi eficiente em proteger contra os déficits de memória de reconhecimento induzidos pela ICV-STZ.
- Demonstrou potente ação antioxidante uma vez que impediu a formação de maiores níveis de espécies reativas de oxigênio e, consequentemente, dos danos relacionados a estas espécies que incluíram a peroxidação lipídica e a formação de proteínas carbonil em amostras de córtex cerebral e hipocampo. Bem como, a atividade da ácido δ-aminolevulínico desidratase foi preservada no córtex cerebral dos animais tratados com BRB em ambas as doses.
- Agiu melhorando as defesas antioxidantes através dos tiois totais, glutationa reduzida e da atividade da glutationa transferase em córtex cerebral e hipocampo dos animais induzidos a demência pela ICV-STZ.
- Impediu a diminuição da atividade da NTPDase, 5′-nucleotidase e Adenosina desaminase em sinaptossomas do córtex cerebral e hipocampo dos animais.

O tratamento diário por 8 dias com BRB na dose de 50 mg/kg por via oral em ratos submetidos à demência induzida por injeção intraperitoneal de lipopolissacarídeo (LPS):

- Não alterou significativamente o peso corporal e a atividade locomotora dos animais. Por sua vez, BRB 50 mg/kg demonstrou ação neuroprotetora sobre a memória de reconhecimento dos animais submetidos a neuroinflamação por LPS.
- Demonstrou ação antioxidante nas avaliações dos córtex cerebral e hipocampo dos animais LPS ao atenuar os parâmetros oxidativos tais como os níveis de espécies reativas, peroxidação lipídica e proteínas carbonil além de impedir a diminuição das defesas antioxidantes como tiois totais e glutationa reduzida.

- Preservou a atividade de ectoenzimas do sistema purinérgico, tais como a NTPDase e 5′-nucleotidase no córtex cerebral e hipocampo dos animais induzidos a demência por LPS e tratados com BRB 50 mg/kg.

A BRB mostrou efeitos neuroprotetores em dois modelos que são reconhecidamente utilizados para desenvolvimento experimental da DA. Diversas vias patogênicas envolvidas no processo de desenvolvimento da demência e de falhas cognitivas foram bloqueadas quando se utilizou a BRB por via oral, sugerindo que o uso desse fitoterápico trará um benefício clínico importante na demência e em doenças neurodegenerativas. Além disso, devido a sua capacidade de modular enzimas relacionadas ao sistema colinérgico e purinérgico, bem como, de agir como uma potente molécula antioxidante, acreditamos que a BRB possa ser considerada uma estratégia terapêutica multialvo a ser utilizada no tratamento da DA. Contudo, estudos que revelem a influência de outras doses e esquemas de tratamento, como por exemplo o preventivo ou ainda em um estágio mais avançado de demência, são importantes para elucidar todos os benefícios da BRB no tratamento da DA.

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