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Tese

Efeitos das antocianinas em modelos experimentais da doença de Alzheimer: estudos *in vitro* e *in vivo*

Simone Muniz Pacheco

Pelotas, 2018

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RESUMO

PACHECO, Simone Muniz. **Efeitos das antocianinas em modelos experimentais da doença de Alzheimer:** estudos *in vitro* e *in vivo*. 2018. 141f. Tese (Doutorado em Ciências – área de concentração Bioquímica e Bioprospecção) – Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2018.

A doença de Alzheimer (DA) é uma patologia neurodegenerativa caracterizada por um progressivo e irreversível declínio em várias funções intelectuais do indivíduo. As principais características neuropatológicas incluem deposição extracelular de placas formadas pelo peptídeo β-amiloide (Aβ) e o acúmulo intracelular de emaranhados neurofibrilares. Além disso, outros fatores também têm sido associados à patogênese da DA como estresse oxidativo, neuroinflamação, déficits na sinalização colinérgica, disfunção da Na^+,K^+-ATPase e a resistência à insulina no sistema nervoso central (SNC). Considerando que os fármacos disponíveis conseguem apenas aliviar os sintomas, a busca de novos tratamentos que possam prevenir, retardar ou tratar a doença são fundamentais. As antocianinas pertencem ao grupo dos flavonoides e tem sido descrito que essas possuem importantes atividades biológicas como ações antioxidantes, anti-inflamatórias e neuroprotetoras. Assim, o objetivo desse trabalho foi investigar o efeito do tratamento com extrato rico em antocianinas em modelos experimentais *in vitro* e *in vivo* da DA. Para o modelo *in vitro*, utilizou-se culturas de astrócitos corticais obtidas de ratos neonatos, as quais foram expostas ao lipopolissacarídeo (LPS), e tratadas com extrato rico em antocianinas (10, 30, 50 e 100 µg/mL) em dois tipos de protocolos: um de prevenção e o outro de reversão. Nesses protocolos foram avaliados parâmetros de viabilidade celular e de estresse oxidativo, e a atividade da enzima acetilcolinesterase (AChE). No primeiro protocolo, o extrato preveniu e, no segundo, o extrato reverteu as alterações promovidas pelo LPS como aumento dos níveis de espécies reativas de oxigênio (ERO) e de nitrito, aumento da atividade da AChE, diminuição da atividade da superóxido dismutase (SOD), do conteúdo tiólico e da viabilidade celular. Para o trabalho *in vivo*, realizou-se o modelo de demência esporádica do tipo Alzheimer através da injeção intracerebroventricular (ICV) de estreptozotocina (STZ) em ratos *Wistar*. Após 3 dias do procedimento, os animais receberam extrato rico em antocianinas (200 mg/kg; via oral) durante 25 dias. Nesses animais foram avaliados parâmetros comportamentais relativos à memória através do teste de reconhecimento de objetos e o do labirinto em Y. O córtex cerebral e o hipocampo foram utilizados para avaliar parâmetros de estresse oxidativo e atividades das enzimas AChE e Na^+K^+ATPase. O tratamento com antocianinas protegeu contra os déficits de memória e o aumento na atividade das enzimas AChE e Na^+K^+ATPase em córtex cerebral e hipocampo induzido pelo STZ. Quanto aos parâmetros de estresse oxidativo, o STZ induziu um aumento na peroxidação lipídica e diminuição da atividade das enzimas SOD, catalase (CAT) e glutationa peroxidase (GPx) em córtex cerebral. No hipocampo, o STZ acarretou um aumento nos níveis EROs, nitrito, peroxidação lipídica, e da atividade da SOD e uma diminuição da atividade da CAT e GPx. O tratamento com antocianinas foi capaz de reverter o dano oxidativo

induzido pelo STZ. Os achados do presente trabalho demonstraram que as antocianinas promoveram efeito glioprotetor, contribuindo para a manutenção da homeostase dos astrócitos, provavelmente, pelo seu efeito antioxidante e pela modulação da AChE, podendo, com isso, ter favorecido a proteção observada quanto à diminuição no déficit de memória, reforçando o potencial terapêutico promissor das antocianinas.

Palavras-chave: antocianinas; doença de Alzheimer; estreptozotocina; astrócitos; neuroproteção

ABSTRACT

PACHECO, Simone Muniz. **Effects of anthocyanins in experimental models of Alzheimer's disease:** *in vitro* and *in vivo* studies. 2018. 141f. Thesis (Doctorate em Ciências – área de concentração Bioquímica e Bioprospecção) – Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2018.

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a progressive and irreversible decline in various intellectual functions of the individual. The main neuropathological features include extracellular plaque deposition formed by the β-amyloid peptide (Aβ) and the intracellular accumulation of neurofibrillary tangles. Moreover other factors have also been associated with the pathogenesis of AD such as oxidative stress, neuroinflammation, cholinergic signaling deficits, Na^+,K^+-ATPase dysfunction, and central nervous system (CNS) insulin resistance. Considering that the available drugs can only alleviate the symptoms the search for new treatments that can prevent, delay or treat the disease are fundamental. Anthocyanins belong to the group of flavonoids and have been described that have important biological activities as antioxidant, anti-inflammatory and neuroprotective actions. Thus, the objective of this study was to investigate the effect of treatment with anthocyanin-rich extract in *in vitro* and *in vivo* experimental models of AD. In the *in vitro* model, cultures of cortical astrocytes from neonatal rats exposed to lipopolysaccharide (LPS) and treated with anthocyanin rich extract (10, 30, 50 and 100 µg/mL) in two types of protocols: one of prevention and the other of reversal. In these protocols, parameters of cell viability and oxidative stress, and the activity of the acetylcholinesterase (AChE) were evaluated. The extract prevented and reversed the changes promoted by LPS as increased levels of reactive oxygen species (ROS) and nitrite, increased AChE activity, decreased superoxide dismutase (SOD) activity, thiol content and cell viability. In the *in vivo* study, the model of sporadic dementia of the Alzheimer type was performed by intracerebroventricular injection (ICV) of streptozotocin (STZ) in *Wistar* rats. The animals received anthocyanin-rich extract (200 mg/kg, oral) for 25 days. Behavioral memory parameters were evaluated through the object recognition test and the Y-maze test. Cerebral cortex and hippocampus were used to evaluate oxidative stress parameters and AChE and Na + K + ATPase activities. Anthocyanin treatment was able to prevent memory deficits and increase AChE and Na + K + ATPase activity in cerebral cortex and hippocampus in the STZ-induced damage. For oxidative stress parameters, STZ induced an increase in lipid peroxidation and a decrease in activity of the enzymes SOD, catalase (CAT) and glutathione peroxidase (GPx) in cerebral cortex. In the hippocampus, STZ induced an increase in ROS, nitrite, lipid peroxidation, and SOD activity and a decrease in CAT and GPx activity. Treatment with anthocyanins was able to reverse the oxidative damage induced by STZ. These findings demonstrated that anthocyanins promoted a glioprotective effect, contributing to the maintenance of astrocyte homeostasis, probably due to its antioxidant effect and the modulation of AChE, which could have favored the protection observed regarding the decrease in the deficit of memory, reinforcing the promising therapeutic potential of anthocyanins

Keywords: anthocyanins; Alzheimer disease; streptozotocin; astrocytes; neuroprotection

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LISTA DE ABREVIATURAS E SIGLAS

AA	Alzheimer's Association
Aβ	β-amiloide
ACh	Acetilcolina
AChE	Acetilcolinesterase
ADRDA	Alzheimer's Disease and Related Disorders Association
APP	Proteína precursora amiloide
AKT	Proteína cinase B
ATP	Trifosfato de adenosina
BDNF	Fator neurotrófico derivado do cérebro
BHE	Barreira hematoencefálica
CAT	Catalase
ChAT	Colina acetiltransferase
CHT1	Transportador de colina de alta afinidade
DA	Doença de Alzheimer
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
GCLM	Glutamato cisteína ligase – subunidade modulatória
GFAP	Proteína ácida fibrilar glial
GLUT 2	Transportador de glicose do tipo 2
GPx	Glutationa peroxidase
GS	Glutamina sintetase
GSH	Glutationa
HO-1	heme oxigenase 1
ICV	Intracerebroventricular
IL - 1β	Interleucina -1β
IL - 6	Interleucina - 6
iNOS	Óxido nítrico sintase induzível

IRS-1	Substrato-1 do receptor de insulina
LCR	Líquido cefalorraquidiano
LPS	Lipopolissacarídeo
MTT	Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
NAD^+	Nicotinamida adenina dinucleotídeo oxidada
NF-kB	Fator nuclear kappa B
NIA	National Institute of Aging
NINCDS	National Institute of Neurological and Communicative Disorders and Strokes
NMDA	N-metil-D-aspartato
nNOS	Óxido nítrico sintase neuronal
OGT	*O*-β-*N*-acetil-glicosaminiltransferase
PARP	Poli-ADP-ribose-polimerase
PET	Tomografia de emissão de pósitrons
PI3K	Fosfatidilinositol-3-cinase
SNC	Sistema nervoso central
SOD	Superóxido dismutase
S100B	Proteína ligante de cálcio fração beta
STZ	Estreptozotocina
TLR4	Receptor *toll-like* tipo 4
TNF - α	Fator de necrose tumoral α
VAChT	Transportador vesicular de acetilcolina

LISTA DE SÍMBOLOS

H_2O_2	Peróxido de hidrogênio
$O_2^{\bullet -}$	Ânion superóxido
$OH^{\bullet}$	Radical hidroxila
$ONOO^-$	Peroxinitrito
$NO^{\bullet}$	Óxido nítrico
K^+	Potássio
Na^+	Sódio
Ca^{2+}	Cálcio

SUMÁRIO

1 INTRODUÇÃO

A doença de Alzheimer (DA) é considerada a principal causa de demência entre a população no mundo, sendo a idade o principal fator de risco para o seu desenvolvimento (GRAHAN, BONITO-OLIVA e SAKMAR, 2017). A prevalência da doença aumenta significativamente após os 65 anos de idade, apresentando uma taxa de incidência de 6 a 8% em indivíduos acima de 85 anos (MAYEUX e STERN, 2012). Alguns autores consideram essa patologia neurodegenerativa uma pandemia em decorrência do crescimento acelerado no número de casos – aumento de 244% entre 1990 e 2010 (GRILL et al., 2015). Estima-se que, em 2030, 67,5 milhões de pessoas no mundo apresentarão DA, sendo que este número poderá atingir 115,4 milhões até 2050 (NOWRANGI, LYKETSOS e ROSENBERG, 2015).

É bem estabelecido na literatura que a DA causa declínio cognitivo e comprometimento funcional progressivos, afetando a memória e outras funções intelectuais, o que leva a uma completa dependência para a realização das atividades básicas diárias (MAYEUX e STERN, 2012; HEPPNER, RANSOHOFF e BECHER, 2015). Os critérios clínicos ainda apresentam papel central no diagnóstico da DA, entretanto a validação de biomarcadores e exames de imagem serão de fundamental importância para o diagnóstico precoce da doença no futuro (NOWRANGI, LYKETSOS e ROSENBERG, 2015).

As principais alterações que caracterizam a DA incluem a deposição extracelular de placas formadas pelo peptídeo β-amiloide (Aβ) e o acúmulo intracelular de emaranhados neurofibrilares compostos pela proteína tau hiperfosforilada, promovendo a perda de sinapses e a morte neuronal (RASKIN et al., 2015). Atualmente, associam-se outros fatores à patogênese da doença como o estresse oxidativo, a neuroinflamação, déficits na sinalização colinérgica, a disfunção da Na^+,K^+-ATPase e a resistência à insulina no cérebro, encontrados inclusive em estágios iniciais da doença (AGOSTINHO, CUNHA e OLIVEIRA, 2010; CORREIA et al., 2011; ZHANG et al., 2013; DE LA MONTE e TONG, 2014; GRIMM, FRIEDLAND e ECKERT, 2016; CALSOLARO e EDISON, 2016; CERVELLATI et al., 2016).

Hoje existem quatro medicamentos comercializados para o tratamento da DA, sendo três inibidores da acetilcolinesterase (AChE) (donepezil, rivastigmina e galantamina) e um antagonista de receptores *N*-metil-D-aspartato (NMDA) (memantina) (SCHELTENS et al., 2016; GRAHAM, BONITO-OLIVA e SAKMAR, 2017). Os primeiros são utilizados principalmente nos estágios iniciais da doença e a memantina, nos casos de DA moderada a severa (SCHELTENS et al., 2016; GRAHAM, BONITO-OLIVA e SAKMAR, 2017). Entretanto, esses medicamentos conseguem apenas proporcionar um alívio sintomático, retardando o declínio na qualidade de vida dos indivíduos (GRAHAM, BONITO-OLIVA e SAKMAR, 2017). Assim, novos tratamentos que possam prevenir, retardar ou tratar a doença são fundamentais (SCHELTENS et al., 2016).

A literatura tem sugerido o potencial terapêutico dos compostos naturais na prevenção e tratamento de doenças neurodegenerativas (CHOI et al., 2012). Dentre esses, destacam-se as antocianinas que são compostos fenólicos pertencentes ao grupo dos flavonoides encontrados em vários frutos, vegetais e flores (HE e GIUSTI, 2010). Além disso, vários trabalhos da literatura têm demonstrado importantes atividades biológicas desses compostos como ações antioxidantes, anti-inflamatórias e neuroprotetoras (HE e GIUSTI, 2010; TSUDA, 2012).

Dados obtidos do nosso grupo de pesquisa já relataram os efeitos benéficos das antocianinas contra a neuroinflamação induzida por lipopolissacarídeo (LPS) (CARVALHO et al., 2017), a desmielinização induzida pelo brometo de etídio (CARVALHO et al., 2015) e o déficit de memória induzido por escopolamina (GUTIERRES et al., 2014a). Também já foi demostrado que o pré-tratamento com antocianinas preveniu alterações bioquímicas e comportamentais causadas pela demência esporádica do tipo Alzheimer induzida por estreptozotocina (STZ) (GUTIERRES et al., 2014b).

Assim, em virtude do crescente número de estudos que evidenciam os benefícios das antocianinas para a saúde, incluindo propriedades neuroprotetoras, o presente trabalho investigou o efeito neuroprotetor das antocianinas em modelos experimentais *in vitro* e *in vivo* da DA. O modelo *in vitro* avaliou a capacidade das antocianinas de prevenirem e/ou reverterem alterações decorrentes da exposição de astrócitos corticais ao LPS. O modelo *in vivo* analisou os efeitos neuroprotetores das antocianinas em parâmetros comportamentais e bioquímicos em um modelo de

demência esporádica do tipo Alzheimer induzida pela injeção intracerebroventricular (ICV) de STZ em ratos adultos.

2 OBJETIVOS

Objetivo geral

Investigar o efeito neuroprotetor das antocianinas em modelos experimentais *in vitro* e *in vivo* da doença de Alzheimer.

Objetivos específicos

a) Avaliar o efeito do tratamento com extrato rico em antocianinas em cultura primária de astrócitos expostas ao LPS em relação aos seguintes parâmetros:

- Viabilidade e proliferação celular.
- Estresse oxidativo.
- Atividade da enzima AChE.

b) Avaliar o efeito do tratamento com extrato rico em antocianinas em um modelo animal de demência esporádica do tipo Alzheimer induzida por STZ em relação aos seguintes aspectos:

- Parâmetros comportamentais para avaliação da memória.
- Parâmetros de estresse oxidativo em córtex cerebral e hipocampo.
- Atividade da AChE e da Na^+, K^+-ATPase e isoformas (α1 e α2/α3) em córtex cerebral e hipocampo.

3 REVISÃO DA LITERATURA

Doença de Alzheimer

A DA foi descrita primeiramente pelo psiquiatra alemão Alois Alzheimer em 1906 durante a 37ª Reunião de Psiquiatras do Sudoeste Alemão (HIPPIUS e NEUNDÖRFER, 2003; SMALL e CAPPAI, 2006). Nessa ocasião, ele apresentou um estudo de caso, relatando a rápida perda de memória e desorientação no tempo e no espaço de uma paciente de 51 anos, além da presença de atrofia cerebral, emaranhados neurofibrilares e depósitos incomuns no córtex cerebral através da análise histológica *post mortem* (HIPPIUS e NEUNDÖRFER, 2003; SMALL e CAPPAI, 2006).

Após mais de um século, a etiologia da doença não está completamente elucidada, entretanto é provável que haja o envolvimento de fatores genéticos e ambientais (MAYEUX e STERN, 2012). Atualmente, a DA pode ser classificada em dois tipos: a do tipo familiar e a do tipo esporádica. A DA do tipo familiar, também chamada de DA de início precoce pela sua ocorrência antes dos 60 anos, representa 1 a 5% dos casos e apresenta um padrão de transmissão autossômica dominante que envolve mutações nos genes da proteína precursora amiloide (APP) e das presenilinas 1 e 2 (MAYEUX e STERN, 2012; GRIMM, FRIEDLAND e ECKERT, 2016). Além disso, possui uma progressão mais rápida, iniciando principalmente entre a quarta e a quinta década de vida (MAYEUX e STERN, 2012).

A DA do tipo esporádica ou de início tardio, por acometer os indivíduos após os 60 anos, compreende mais de 95% dos casos da doença, apresentando componentes genéticos como a presença do alelo ε4 do gene da apolipoproteína E, e não genéticos como idade avançada, sexo feminino, estilo de vida (dieta, atividade física, tabagismo), comorbidades (doença cerebrovascular, traumatismo craniano, diabetes tipo II, obesidade) e escolaridade (MCDOWELL, 2001; BEKRIS et al., 2010; MAYEUX e STERN, 2012; REITZ e MAYEUX, 2014; GRIMM, FRIEDLAND e ECKERT, 2016; SCHELTENS et al., 2016). A maior prevalência da DA no sexo feminino se deve, principalmente a fatores como maior expectativa de vida, depleção de estrógeno associada com a menopausa, menores níveis de testosterona e menor nível educacional (BIASIBETTI et al., 2017)

O sintoma inicial típico da doença é o comprometimento gradual da capacidade de lembrar de novas informações e, conforme a doença evolui, outros aspectos cognitivos são atingidos como a linguagem, o raciocínio abstrato, a tomada de decisão e a memória para eventos remotos, além de dificuldades motoras (ALZHEIMER'S ASSOCIATION, 2017; PIERCE, BULLAIN e KAWAS, 2017).
Sintomas psiquiátricos também são encontrados, podendo variar de leves (depressão, ansiedade, irritabilidade e apatia) a graves (agitação, agressividade, desinibição, delírios e alucinações) (NOWRANGI, LYKETSOS e ROSENBERG, 2015). Conforme a progressão da DA, estabelece-se um quadro de completa dependência para a realização das atividades cotidianas (ALZHEIMER'S ASSOCIATION, 2017). No estágio final da doença, ocorre restrição ao leito, mutismo, incontinências e desnutrição (ALZHEIMER'S ASSOCIATION, 2017; PIERCE, BULLAIN e KAWAS, 2017).

O diagnóstico da DA ainda é fundamentalmente clínico, baseando-se principalmente no histórico familiar, nas alterações cognitivas ou comportamentais, nos testes para avaliação física e neurológica, além dos exames sanguíneos e imagens cerebrais para excluir outras causas potenciais de demência (MCKHANN et al., 2011; ALZHEIMER'S ASSOCIATION, 2017). As primeiras diretrizes para o diagnóstico da DA foram estabelecidas em 1984 pelo *National Institute of Neurological and Comunicative Disorders and Strokes* (NINCDS) e pela *Alzheimer's Disease and Related Disorders Association* (ADRDA) (MCKHANN et al., 2011). Devido aos avanços nas pesquisas sobre a DA, surgiu a necessidade de revisão desses critérios, sendo que, em 2011, novas recomendações foram publicadas pelo *National Institute on Aging* em parceria com a *Alzheimer's Association* (NIA-AA) (MCKHANN et al., 2011).

Essas novas diretrizes adicionaram o uso de biomarcadores e exames de imagens, considerando-os como opcionais na prática clínica, uma vez que ainda são necessárias mais pesquisas para a validação dos mesmos no uso para diagnóstico, desempenhando papel proeminente no futuro (MCKHANN et al., 2011; HAMPEL et al., 2014; ALZHEIMER'S ASSOCIATION, 2017). Entretanto, a principal mudança nas recomendações envolve a sugestão de três estágios para a DA: o pré-clínico, o comprometimento cognitivo leve e a demência (CUMMINGS, 2012).

O estágio pré-clínico é caracterizado pela ausência de sintomas perceptíveis como a perda de memória, mas já apresenta evidências como a acumulação do peptídeo Aβ no cérebro identificado pela tomografia de emissão de pósitrons (PET) ou, ainda, a diminuição da concentração do peptídeo no líquido cefalorraquidiano (LCR) (SPERLING et al., 2011; DUBOIS et al., 2016; ALZHEIMER'S ASSOCIATION, 2017). Essas alterações indicam que os sinais da doença poderiam iniciar décadas antes dos sintomas surgirem (ALZHEIMER'S ASSOCIATION, 2017).

No estágio de comprometimento cognitivo leve, já ocorre um declínio cognitivo em determinada área, incluindo a memória, a função executiva, a atenção, a linguagem e as habilidades visuoespaciais (ALBERT et al., 2011). Porém, essa diminuição ainda não interfere de forma significativa nas atividades diárias do indivíduo (ALBERT et al., 2011). Além disso, também foi observado um aumento na razão proteína tau/Aβ no LCR e atrofia hipocampal nesses pacientes (HAMPEL et al., 2014; REITZ e MAYEUX, 2014). Estima-se que, após cinco anos do diagnóstico de comprometimento cognitivo leve, em torno de metade dos pacientes preencherão os requisitos para algum tipo de demência, especialmente a DA (CASELLI et al., 2017). A figura 1 mostra uma representação esquemática das alterações cognitivas, metabólicas, estruturais e moleculares em relação ao aparecimento dos sintomas da DA de acordo com o tempo estimado.

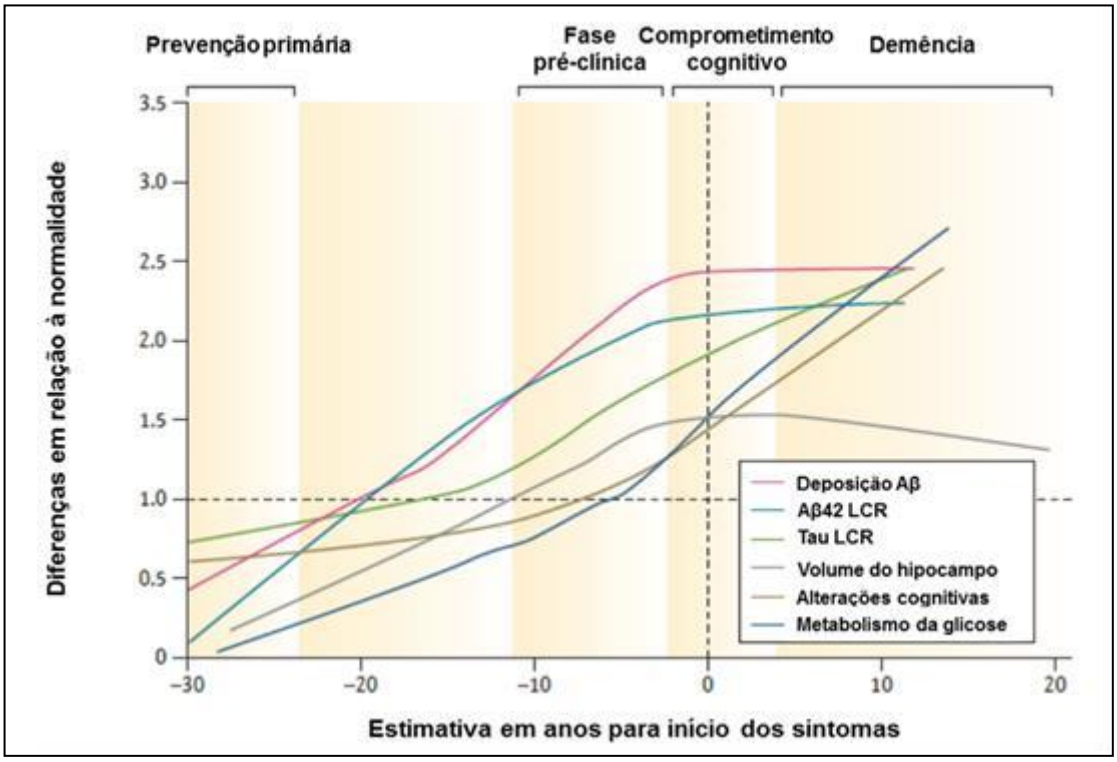

Figura 1- Representação esquemática das alterações cognitivas, metabólicas, estruturais e parâmetros moleculares.

Fonte: Adaptado de Masters et al., 2015.

Os mecanismos envolvidos no desenvolvimento e na progressão da DA ainda não foram totalmente esclarecidos. Sabe-se que a DA é uma patologia progressiva e que apresenta modificações em aspectos moleculares, fisiológicos, anatômicos e clínicos inter-relacionadaos (RASKIN et al., 2015). Em virtude de todas essas variáveis, seu estudo é bastante complexo. Há décadas a teoria dominante para a sua patogênese é a hipótese da cascata amiloide (SELKOE e HARDY, 2016).

As duas principais características histológicas da doença são a presença de placas senis extracelulares compostas pelo peptídeo Aβ e o acúmulo intraneuronal de emaranhados neurofibrilares formados pela proteína tau hiperfosforilada (ALZHEIMER'S ASSOCIATION, 2017; CASELLI et al., 2017). Essas lesões levam à perda de sinapses e à morte neuronal, afetando particularmente os neurônios colinérgicos o que contribui para o déficit de memória (AGOSTINHO, CUNHA e OLIVEIRA, 2010; FERREIRA-VIEIRA et al., 2016). Dentre as áreas atingidas,

encontram-se as áreas límbicas, as neocorticais e as subcorticais (CASELLI et al., 2017).

Recentemente, a NIA-AA as atualizou novamente com foco nos biomarcadores a fim de utilizá-las em pesquisas e não na prática clínica em geral. Assim, a DA foi descrita como uma associação de alterações neuropatológicas, sendo definida *in vivo* por biomarcadores e por exame *post mortem*, e não por sintomas clínicos (JACK JR et al., 2018). Sabe-se que entre 30 a 40% de idosos sem comprometimento cognitivo possuem alterações neuropatológicas da DA na autópsia e biomarcadores amiloides (JACK JR et al., 2018).

O sistema de biomarcadores sugerido envolve a utilização de três grupos: grupo A referente a marcadores da presença de placas de peptídeo Aβ; grupo T relacionado aos marcadores de emaranhados neurofibrilares de proteína tau hiperfosforilada; e grupo N referente à neurodegeneração e ao dano neuronal (JACK JR et al., 2018). Através da combinação desses marcadores, é possível gerar quatro perfis que indicam ou não a presença da doença como pode ser observado na tabela 1.

Tabela 1 - Perfis de biomarcadores e estágios cognitivos

	Estágios cognitivos		
Biomarcadores	**Sem alterações**	**Comprometimento leve**	**Demência**
A-/T-/N-	Biomarcadores normais; sem alteração cognitiva	Biomarcadores normais; com comprometimento cognitivo leve	Biomarcadores normais; com demência
A+/T-/N-	Alterações patológicas pré-clínicas	Alteração patológica para DA; com comprometimento cognitivo leve	Alteração patológica para DA; com demência
A+/T-/N+	Alteração patológica indicativa ou não de DA; sem alteração cognitiva	Alteração patológica indicativa ou não de DA; com comprometimento cognitivo leve	Alteração patológica indicativa ou não de DA; com demência
A+/T+/N- **A+/T+/N+**	DA pré-clínica	DA com comprometimento cognitivo leve	DA com demência

Fonte: Adaptado de Alzheimer's Association, 2018

Entretanto, há evidências da participação de outros componentes como o estresse oxidativo, a neuroinflamação, déficits na sinalização colinérgica, a disfunção da Na^+,K^+-ATPase e a resistência à insulina no cérebro, encontrados inclusive em estágios iniciais da doença (AGOSTINHO, CUNHA e OLIVEIRA, 2010; CORREIA et al., 2011; ZHANG et al., 2013; DE LA MONTE e TONG, 2014; GRIMM, FRIEDLAND e ECKERT, 2016; CALSOLARO e EDISON, 2016; CERVELLATI et al., 2016; ALZHEIMER'S ASSOCIATION, 2018).

Alterações envolvidas na Doença de Alzheimer

Existem vários mecanismos celulares e moleculares intrincados entre si na DA, não sendo possível ainda definir o que é a causa ou consequência no processo. Dentre as alterações envolvidas na doença, destaca-se a relação entre estresse oxidativo e neuroinflamação acionando os demais eventos encontrados na patologia (RASKIN et al., 2015).

Estresse oxidativo

O estresse oxidativo pode ser definido como uma condição que envolve um desequilíbrio entre a produção de várias espécies reativas e as defesas antioxidantes (HALLIWELL, 2006). Esse processo acaba por resultar em um acúmulo excessivo dessas espécies reativas que são representadas principalmente pelas espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) (KIM et al., 2015; CERVELLATI et al., 2016).

As espécies reativas incluem não apenas os radicais livres, mas também alguns não radicais como o peróxido de hidrogênio (H_2O_2) (HALLIWELL, 2006; KIM et al., 2015). Um radical livre possui como principal característica a presença de um ou mais elétrons não pareados como, por exemplo, o ânion superóxido ($O_2^{\bullet -}$), o radical hidroxila ($OH^{\bullet}$), o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) (HALLIWELL, 2006).

O cérebro é especialmente vulnerável ao estresse oxidativo devido a fatores como o elevado consumo de oxigênio (em torno de 20% em adultos). Isso ocorre em virtude da alta produção de trifosfato de adenosina (ATP) para a manutenção da homeostase iônica intraneuronal necessária para a propagação do potencial de ação e neurosecreção (HALLIWELL, 2006). Além disso, possui uma membrana lipídica

rica em ácidos graxos poli-insaturados, baixos níveis de defesas antioxidantes endógenas e elevado conteúdo de metais como ferro e cobre (HALLIWELL, 2006).

As ERO e ERN, em condições fisiológicas, apresentam importante função biológica como moléculas sinalizadoras, estando envolvidas em processos da resposta imune, da inflamação, da plasticidade sináptica, do aprendizado e da memória (GRIMM, FRIEDLAND e ECKERT, 2015; CERVELLATI et al., 2016). Entretanto, um desequilíbrio entre a formação e a remoção dessas moléculas, poderá levar a um dano oxidativo a macromoléculas como lipídios, proteínas e DNA, comprometendo o funcionamento celular e consequentemente a integridade dos neurônios (DARVESH et al., 2010; ZHAO e ZHAO, 2013; DE LA MONTE e TONG, 2014; PERSSON, POPESCU e CEDAZO-MINGUEZ, 2014; KIM et al., 2015).

A mitocôndria é considerada a principal fonte endógena de ERO (PERSSON, POPESCU e CEDAZO-MINGUEZ, 2014). No processo de transferência de elétrons para a produção energética, há uma pequena perda desses elétrons (menos de 5%) nos complexos I e III da cadeia transportadora de elétrons, sendo que esses acabam por se ligar ao oxigênio o que leva a formação do $O_2^{\bullet-}$ (HALLIWELL, 2006). Um aumento na produção do $O_2^{\bullet-}$ reflete em uma disfunção mitocondrial (CERVELLATI et al., 2016).

O $O_2^{\bullet-}$ formado pode ser convertido a H_2O_2 através da sua dismutação pela enzima superóxido dismutase (SOD) (SANTOS et al., 2013). Este é um passo importante, uma vez que esse H_2O_2 pode ser removido pela ação de outras enzimas antioxidantes como a catalase (CAT) ou a glutationa peroxidase (GPx) ou, ainda, pode ser convertido a $OH^{\bullet}$ por meio da reação de Fenton ou de Haber-Weiss (SANTOS et al., 2013). Além disso, o $O_2^{\bullet-}$ ainda pode reagir com o $NO^{\bullet}$, formando $ONOO^-$ que por sua vez é capaz de induzir a oxidação de lipídios e proteínas e a fragmentação do DNA (CERVELLATI et al., 2016).

Evidências sugerem que o estresse oxidativo está envolvido na patogênese da DA, sendo considerado, inclusive, uma característica precoce da doença (LIN e BEAL, 2006; PERSSON, POPESCU e CEDAZO-MINGUEZ, 2014; CERVELLATI et al., 2016). Além disso, também é relatado que esse poderia contribuir ou até mesmo ativar os mecanismos de processamento que levam à formação do peptídeo Aβ e da proteína tau hiperfosforilada (LIN e BEAL, 2006; LEUNER, MÜLLER e REICHERT,

2012; SELFRIDGE et al., 2013; DE LA MONTE e TONG, 2014; KIM et al., 2015; ABOLHASSANI et al., 2017).

O exato mecanismo implicado na origem do aumento da produção de ERO e ERN com consequente estabelecimento do estresse oxidativo na DA ainda não foi elucidado (KIM et al., 2015). Entretanto, esse excesso de ERO/ERN pode ser resultado da disfunção mitocondrial, acumulação anormal de metais de transição (ex: cobre, ferro e zinco), neuroinflamação, alteração no metabolismo da glicose, além da acumulação anormal do peptídeo Aβ e da proteína tau (QUERFURTH e LAFERLA, 2010; ZHAO e ZHAO, 2013; KIM et al., 2015; ORTIZ et al., 2017; ZILBERTER e ZILBERTER, 2017).

Estudos *post mortem* demonstraram o aumento da presença de produtos da peroxidação lipídica como o malondialdeído, 4-hidroxinonenal e F2-isoprostanos em várias regiões cerebrais e no LCR de pacientes com DA e comprometimento cognitivo leve (HALLIWELL, 2006; DARVESH et al., 2010; ZHAO e ZHAO, 2013). Também já foi constatado em portadores da DA a elevação nos níveis de carbonilas e de 3-nitrotirosina em hipocampo e neocórtex, indicando oxidação proteica, e aumento dos níveis de 8-hidroxideoxiguanosina, marcador de dano oxidativo ao DNA (nuclear e mitocondrial) (HALLIWELL, 2006; DARVESH et al., 2010; ZHAO e ZHAO, 2013; CERVELLATI et al., 2016). Entretanto, alterações desses marcadores a nível periférico (soro, plasma, urina) em indivíduos com DA são ainda inconclusivos (PERSSON, POPESCU e CEDAZO-MINGUEZ, 2014; CERVELLATI et al., 2016).

Níveis elevados de peroxidação lipídica, de proteínas e ácidos nucleicos oxidados e produtos de glicação avançada foram identificados em regiões cerebrais relacionadas com a DA, principalmente no hipocampo e no córtex entorrinal (AGOSTINHO, CUNHA e OLIVEIRA, 2010; MANOHARAN et al., 2016). Assim, propõe-se que o aumento do estresse oxidativo comprometeria a atividade sináptica e a integridade neuronal (AGOSTINHO, CUNHA e OLIVEIRA, 2010; DE LA MONTE e TONG, 2014; SCHEFF, ANSARI e MUFSON, 2016). Além disso, foi relatado que os níveis de estresse oxidativo estariam estritamente associados com alterações na cognição (SCHEFF, ANSARI e MUFSON, 2016).

Neuroinflamação

A neuroinflamação é considerada um processo importante na neurodegeneração causada pela DA, sendo sugerido que sua ocorrência poderia ser prévia e independente da deposição do peptídeo Aβ (CALSOLARO e EDISON, 2016). De um modo geral, neuroinflamação é um termo utilizado para definir uma resposta inflamatória originada no sistema nervoso central (SNC) após um dano e que leva a uma ativação de células da glia (MORALES et al., 2014). Uma resposta aguda é considerada benéfica, uma vez que contribui para o reparo tecidual, minimizando danos adicionais (WILKINS et al., 2014). Entretanto, quando essa resposta persiste, isto é, torna-se crônica, pode desencadear alterações no funcionamento cerebral, contribuindo para a disfunção e morte neuronal (AGOSTINHO, CUNHA e OLIVEIRA, 2010; CALSOLARO e EDISON, 2016).

Os principais tipos celulares envolvidos no processo neuroinflamatório são os astrócitos e a microglia (WILKINS et al., 2014). Durante o processo, essas células sofrem uma gradual ativação, levando a mudanças morfológicas e secreção de mediadores inflamatórios como as citocinas fator de necrose tumoral α (TNF-α) e interleucinas (IL-1β; IL-6); as prostaglandinas; as ERO e ERN; e as proteínas dos sistema complemento (AGOSTINHO, CUNHA e OLIVEIRA, 2010; MORALES et al., 2014). A ativação crônica da glia pode, através da contínua liberação dos mediadores inflamatórios, provocar disfunção neuronal e perda de sinapses, eventos relacionados com o declínio da memória, e preceder a morte neuronal que está associada a estágios mais tardios da DA (AZIZI e MIRSHAFIEY, 2012; RUBIO-PEREZ e MORILLAS-RUIZ, 2012; HENEKA, KUMMER e LATZ, 2014).

Os astrócitos são especialmente importantes, pois, além de serem as células gliais mais abundantes no SNC, possuem função essencial na transmissão sináptica e no processamento da informação, na regulação da plasticidade sináptica e no fornecimento do suporte neurotrófico e metabólico para as redes neuronais (AVILA-MUÑOZ e ARIAS, 2014; MORALES et al., 2014; MINTER, TAYLOR e CRACK, 2016). Ademais, os astrócitos atuam na homeostase do glutamato, evitando a excitotoxicidade; na manutenção da concentração de potássio (K^+) extracelular, controlando o processo de despolarização do neurônio; e na formação e manutenção da barreira hematoencefálica (BHE) (AGOSTINHO, CUNHA e OLIVEIRA, 2010; MORALES et al., 2014; CALSOLARO e EDISON, 2016; ACOSTA,

ANDERSON e ANDERSON, 2017). Enfim, quaisquer alterações na relação entre astrócitos e neurônios podem aumentar a neurotoxicidade e vulnerabilidade dos neurônios, iniciando uma cascata de dano neuronal (BAGYINSZKY et al., 2017; GONZÁLEZ-REYES et al., 2017).

A astrogliose reativa, que é caracterizada pela hipertrofia celular e pela elevada expressão da proteína ácida fibrilar glial (GFAP) e da proteína ligante de cálcio fração beta (S100B), já foi identificada em cérebro de portadores da DA, sendo associada à neurodegeneração (VERKHRATSKY et al., 2010; AVILA-MUÑOZ e ARIAS, 2014). Além disso, o grau de astrogliose foi correlacionado com a severidade do comprometimento cognitivo (VERKHRATSKY et al., 2010).

Disfunções no sistema colinérgico

A degeneração do sistema colinérgico é um importante componente da patofisiologia da DA reconhecida há mais de 30 anos (SABBAGH e CUMMINGS, 2011). Esse sistema está envolvido em importantes funções neuronais como atenção, aprendizagem, memória, resposta ao estresse e no ciclo sono-vigília (FERREIRA-VIEIRA et al., 2016).

O sistema colinérgico é aquele que utiliza a acetilcolina (ACh) como neurotransmissor (MESULAM, 2004). A ACh é sintetizada no citosol dos neurônios colinérgicos pré-sinápticos pela enzima colina acetiltransferase (ChAT) a partir dos substratos colina e acetil-CoA (FERREIRA-VIEIRA et al., 2016). Uma vez sintetizada, é transportada e armazenada em vesículas sinápticas através do transportador vesicular de acetilcolina (VAChT) (TATA et al., 2014). A sua liberação na fenda sináptica ocorre por exocitose, podendo interagir com os receptores muscarínicos e nicotínicos (TATA et al., 2014). Na fenda sináptica, a ACh é rapidamente hidrolisada pela AChE em acetato e colina que, por conseguinte, é recaptada pelo transportador de colina de alta afinidade (CHT1) para o neurônio pré-sináptico a fim de ser utilizada para síntese de novas moléculas de ACh (FERREIRA-VIEIRA et al., 2016).

Ao longo da progressão da DA, observam-se perdas importantes de neurônios colinérgicos como os do núcleo basal de Meynert e os do complexo banda diagonal septal que são, respectivamente, a principal fonte de inervação colinérgica do córtex cerebral e do hipocampo (MUFSON et al., 2008; FERREIRA-VIEIRA et al.,

2016). Os neurônios localizados no núcleo basal de Meynert são os principais afetados na DA, sendo que seu grau de perda está correlacionado com a severidade dos sintomas da doença (YAN e FENG, 2004; FERREIRA-VIEIRA et al., 2016).

Os neurônios colinérgicos diferem de outros neurônios, pois utilizam o acetil-CoA não apenas para a produção energética, mas também para a síntese de ACh, tornando-os mais vulneráveis à privação energética (SCHLIEBS e ARENDT, 2006). Além disso, compostos neurotóxicos como alumínio, $NO^{\bullet}$ e ERO demonstraram contribuir para a perda desses neurônios, uma vez que podem afetar o metabolismo energético (SCHLIEBS e ARENDT, 2006).

Na DA, as principais alterações encontradas no sistema colinérgico incluem a progressiva diminuição da atividade das enzimas ChAT e AChE (RASKIN et al., 2015). Entretanto, foi encontrado um aumento da atividade da AChE em regiões ao redor das placas formadas por peptídeo Aβ em vários estágios da doença (GARCÍA-AYLLÓN et al., 2011). Além disso, foi sugerido que a AChE poderia interagir diretamente com o peptídeo Aβ, aumentando a sua deposição, além de estar envolvida na modulação da formação do mesmo (GARCÍA-AYLLÓN et al., 2011; CAMPANARI et al., 2013).

Em relação à atividade da ChAT, um estudo demonstrou que a sua atividade estava preservada no neocórtex de indivíduos com comprometimento cognitivo leve (DAVIS et al., 1999). Também foi observado aumento da mesma em hipocampo e córtex frontal, indicando que alguns componentes da inervação colinérgica no hipocampo e no córtex são capazes de uma resposta compensatória e/ou de neuroplasticidade durante os estágios iniciais da DA (DEKOSKY et al., 2002; MUFSON et al., 2008).

Assim, sugere-se que, em estágios avançados da doença, exista uma severa perda de inervações colinérgicas corticais, enquanto que, em estágios iniciais ou no comprometimento cognitivo leve, não ocorre aparentemente nenhuma degeneração, mas sim uma perda da função colinérgica (SCHLIEBS e ARENDT, 2011). Dentre essas estão a redução da liberação e recaptação da ACh e alterações na expressão dos receptores (muscarínicos e nicotínicos) de ACh (SCHLIEBS e ARENDT, 2011).

Atualmente, a principal classe de medicamentos disponível para o tratamento da DA são os inibidores da AChE (GARCÍA-AYLLÓN et al., 2011). Esses agem evitando a degradação da ACh, com consequente aumento do seu conteúdo na

fenda sináptica, prolongando seu efeito (SCHLIEBS e ARENDT, 2006). Com isso, há uma melhora na transmissão colinérgica, reduzindo temporariamente o déficit cognitivo dos pacientes sintomáticos, principalmente, durante o primeiro ano de tratamento (DARREH-SHORI et al., 2004; SCHELTENS et al., 2016).

Alterações na Na^+,K^+-ATPase

A Na^+,K^+-ATPase é uma enzima ligada à membrana essencial para a manutenção do equilíbrio iônico dos neurônios, regulando a entrada de K^+ com a saída de sódio (Na^+) das células (ARNAIZ e ORDIERES, 2014). Uma mudança nesse equilíbrio leva à despolarização neuronal com a entrada de cálcio (Ca^{2+}), promovendo liberação de neurotransmissores e desequilíbrio osmótico com consequente prejuízo funcional (ZHANG et al., 2013; ARNAIZ e ORDIERES, 2014). A modulação da atividade da Na^+,K^+-ATPase afeta diretamente a transmissão sináptica, a plasticidade sináptica e o processo de aprendizado e memória (MOSELEY et al., 2007; ZHANG et al., 2013; PETRUSHANKO et al., 2016).

A Na^+,K^+-ATPase possui três subunidades: α, β e a γ (ARNAIZ e ORDIERES, 2014). A subunidade α é a catalítica, sendo a responsável pela troca de íons Na^+ e K^+ e é aonde se encontra o sítio de ligação para o ATP (ARNAIZ e ORDIERES, 2014). A subunidade β é a responsável por regular a atividade e a estabilidade conformacional da subunidade α e a subunidade γ provavelmente desempenha uma função reguladora de forma tecido-específica (ARNAIZ e ORDIERES, 2014).

A subunidade α apresenta quatro diferentes isoformas: α1, α2, α3 e α4 (MOSELEY et al., 2007). Dessas, três isoformas são expressas no cérebro: a α1 é encontrada em vários tipos celulares do SNC; a α2 é predominantemente expressa em astrócitos; e a α3 é expressa apenas em neurônios (MOSELEY et al., 2007).

Em análises *post mortem* de portadores da DA, a atividade da Na^+,K^+-ATPase se encontrava diminuída (ARNAIZ e ORDIERES, 2014). Foi observado que essa diminuição da atividade poderia ser em parte atribuída aos efeitos citotóxicos do peptídeo Aβ (ZHANG et al., 2013; PETRUSHANKO et al., 2016). Outro estudo demonstrou que o peptídeo Aβ na forma oligomérica foi capaz de interagir com a subunidade α3, resultando em aumento da concentração de Ca^{2+} intracelular e consequente morte neuronal (OHNISHI et al., 2015). Além disso, a Na^+,K^+-ATPase

também poderia ser um alvo do estresse oxidativo, promovendo a diminuição da sua atividade (ZHANG et al., 2013).

Modelos experimentais da Doença de Alzheimer

Estudos em modelos animais, os quais mimetizam sintomas e características neuroquímicas e fisiológicas envolvidas no DA, têm sido de extrema importância para a compreensão dos eventos biológicos envolvidos, bem como na busca de novos fármacos com ações mais específicas. Dessa forma, existem diferentes modelos animais para o estudo da DA, sendo que esses podem ser divididos em transgênicos e não transgênicos (NEHA et al., 2014). Dentre os não transgênicos, encontram-se os seguintes: infusão ICV do peptídeo Aβ; infusão ICV do ácido ocadaico; e injeção ICV de STZ (NEHA et al., 2014).

O STZ é uma substância que foi isolada, no final da década de 1950, de uma bactéria presente no solo denominada *Streptomyces achromogenes* (GRIEB, 2016). Essa substância é formada por uma estrutura N-metil-N-nitrosureia ligada ao carbono 2 de uma hexose (AL-AWAR et al., 2016). Foi primeiramente utilizada como antibiótico e posteriormente como um agente antitumoral (GRIEB, 2016). Entretanto, atualmente, o seu maior uso é para a indução do modelo experimental de diabetes melito do tipo I e do tipo II em roedores (GRIEB, 2016).

A ação diabetogênica do STZ se deve à citotoxicidade seletiva para as células β-pancreáticas, uma vez que essa substância é capaz de entrar na célula através do transportador de glicose do tipo 2 (GLUT 2), sendo que as mesmas apresentam altos níveis desse transportador (KAMAT et al., 2016). Após cruzar a membrana das células e ser metabolizado, o STZ gera alquilação do DNA através da molécula metilnitrosureia, causando sua fragmentação, e consequente ativação da enzima poli-ADP-ribose-polimerase (PARP) a fim de promover seu reparo (LENZEN, 2008; AL-AWAR et al., 2016). A excessiva ativação dessa enzima causa diminuição dos níveis de nicotinamida adenina dinucleotídeo oxidada (NAD^+) e, consequentemente, diminuição da formação de ATP, desencadeando a morte celular (SZKUDELSKI, 2001; LENZEN, 2008; GRIEB, 2016). Além disso, o STZ é capaz de provocar a metilação de proteínas, contribuindo para as alterações funcionais nas células (LENZEN, 2008).

Outros mecanismos envolvidos na toxicidade do STZ incluem a capacidade de liberar $NO^{\bullet}$ intracelularmente e a de gerar EROs (SZKUDELSKI, 2001). Há a formação do $O_2^{\bullet-}$ pela sua ação na mitocôndria e pelo aumento da atividade da enzima xantina oxidase, levando à produção de H_2O_2 e do $OH^{\bullet}$ (SZKUDELSKI, 2001; DE LA MONTE e TONG, 2009). Assim, tanto o $NO^{\bullet}$ quanto as EROs acabam por contribuir para a fragmentação do DNA, além de desencadear outros efeitos deletérios às células como inibição do ciclo de Krebs, diminuição do consumo de oxigênio pela mitocôndria e restrição da geração de ATP pela mesma (SZKUDELSKI, 2001; LENZEN, 2008).

A administração ICV de doses subdiabetogênicas (1 – 3 mg/kg) de STZ é capaz de promover alterações cerebrais similares às encontradas na DA do tipo esporádica em humanos como alteração no metabolismo da glicose, estresse oxidativo, redução na transmissão colinérgica, neuroinflamação, degeneração neuronal e comprometimento na aprendizagem e memória (SALKOVIC-PETRISIC e HOYER, 2007; AGRAWAL et al., 2009; SHARMA et al., 2012; RAI et al., 2013; KAMAT et al., 2016). Atualmente, é um modelo bastante utilizado, uma vez que permite acompanhar tanto as características iniciais como as tardias da doença (SALKOVIC-PETRISIC et al., 2013).

O mecanismo exato pelo qual o STZ exerce seus efeitos tóxicos no SNC ainda não foi completamente elucidado (DEHGHAN-SHASALTANEH et al., 2016; KNEZOVIC et al., 2017). Entretanto, parece que, de forma similar ao que acontece perifericamente, o alvo do STZ no cérebro é o GLUT 2, sendo seu efeito dependente da dose e do tempo de exposição (KNEZOVIC et al., 2017). Além disso, foi sugerido que o receptor de insulina e sua via de sinalização poderiam ser afetados pelo STZ intracelularmente através de seus produtos de degradação ou do estresse oxidativo induzido por ele (KNEZOVIC et al., 2017).

Um estudo recente investigou alterações em hipocampo de ratos machos após a infusão de STZ (3 mg/kg) em diferentes tempos (2, 4 e 8 semanas) (BIASIBETTI et al., 2017). Foi encontrado diminuição dos níveis da enzima ChAT (2, 4 e 8 semanas); diminuição da captação de glicose (4 e 8 semanas); diminuição dos níveis de glutationa (GSH) (4 e 8 semanas); e aumento da GFAP (2, 4 e 8 semanas) e da S100B (8 semanas) (BIASIBETTI et al., 2017). Outro estudo recente demonstrou que o STZ (3 mg/kg) foi capaz de causar uma resposta inflamatória

aguda (7 dias) e persistente (30 dias) através da ativação da microglia e dos astrócitos em áreas periventriculares e no hipocampo dorsal de ratos (BASSANI et al., 2017).

Kraska et al. (2012) identificou alterações provocadas pelo STZ (3 mg/Kg) após 1 semana, constatando processo inflamatório e alterações neuronais no septum que se agravaram após 3 meses. O septum apresenta projeções colinérgicas para regiões como córtex entorrinal, córtex cingulado e hipocampo, sendo que essas conexões estão envolvidas na memória de curto prazo e na atenção (KRASKA et al., 2012).

Além disso, outro trabalho demonstrou que o déficit cognitivo promovido pelo STZ (3 mg/kg) foi dependente do tempo transcorrido após a administração ICV, sendo considerada uma resposta aguda até um 1 mês após a injeção; entre 1 e 3 meses uma fase de compensação; e entre 6 e 9 meses uma fase de descompensação com declínio progressivo crônico (KNEZOVIC et al., 2015). Nesse mesmo trabalho, foi observado acumulação intracelular de peptídeo $A\beta_{1-42}$ em neocórtex a partir do 3º mês e agregados extracelulares após 6 meses em neocórtex e hipocampo (KNEZOVIC et al., 2015). Além disso, alterações neurofibrilares iniciais foram detectadas a partir do 1º mês em áreas dispersas do córtex temporal, sendo detectadas após 3 meses em áreas do hipocampo (KNEZOVIC et al., 2015) (Figura 2).

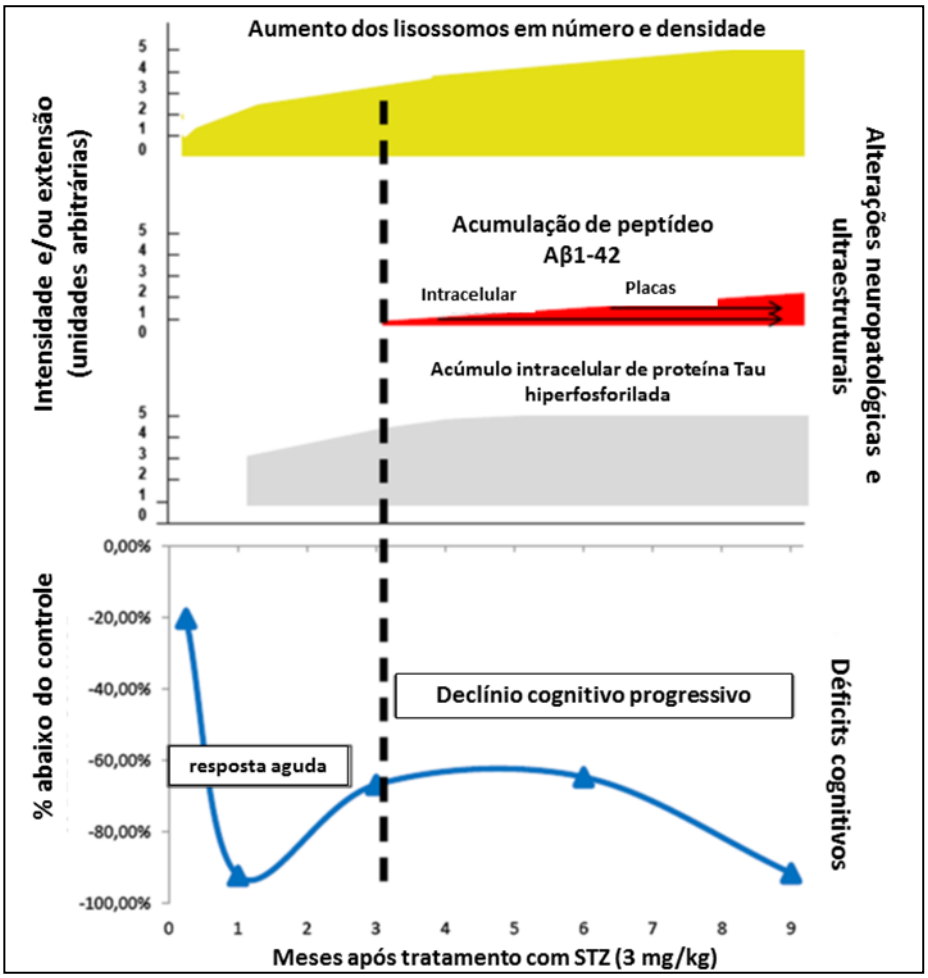

Figura 2 - Déficits cognitivos e alterações neuropatológicas e ultraestruturais em ratos submetidos à injeção intracerebroventricular de estreptozotocina durante 9 meses.
Fonte: Adaptado de Kzenovic et al., 2015.

Santos et al. (2018) avaliaram o efeito da administração ICV de STZ em relação aos níveis da enzima *O*-β-*N*-acetil-glicosaminiltransferase (OGT), enzima altamente expressa no tecido cerebral, à resistência à insulina e à atividade astroglial em hipocampo de ratos *Wistar* após 1 e 4 semanas. Foi observada diminuição dos níveis da OGT em ambos os períodos, destacando que *O*-glicosilação com *N*-acetilglicosamina parece estar diminuída na DA. Ainda, foi encontrado um aumento da fosforilação do substrato-1 do receptor de insulina (IRS-1) na serina 307 que está relacionado com a resistência à insulina, e redução da captação da glicose, ambos na semana 4, associando-se ao déficit cognitivo espacial. Observou-se também uma diminuição da glutamina sintetase (GS) e da

GSH em 1 e 4 semanas, sugerindo disfunção astroglial que, por conseguinte, pode estar envolvida com as alterações cognitivas e com a neurodegeneração nesse modelo.

Estudo com células Neuro-2a demonstrou que o mecanismo de ação do STZ envolve uma doação rápida e espontânea de $NO^{\bullet}$ e produção de ERO com prejuízo da atividade da fosfatidilinositol-3-cinase (PI3K), inativação da proteína cinase B (Akt) pela S-nitrosilação e morte celular por apoptose. Essas alterações acarretam na resistência à insulina, podendo assim explicar parcialmente as alterações cognitivas observadas (CRUNFLI et al., 2018).

Atualmente, esse modelo experimental tem seu uso bastante difundido para o estudo do potencial terapêutico de novos compostos tanto para prevenção quanto para o tratamento da DA (SALKOVIC-PETRESIC et al., 2013). Além disso, é especialmente utilizado para a investigação dos efeitos de compostos naturais como estratégia terapêutica na DA (KAMAT et al., 2016).

Entretanto, tem sido proposto que a neuroinflamação possui um papel importante no mecanismo das doenças neurodegenerativas, sendo considerada uma característica precoce encontrada na DA (NAZEM et al., 2015). Um modelo amplamente utilizado é o que envolve o uso do LPS tanto *in vitro* como *in vivo* (NAZEM et al., 2015). O LPS é um componente da membrana das bactérias gram-negativas e é considerado uma potente endotoxina (ZAKARIA et al., 2017). Essa molécula acaba por desencadear a liberação de citocinas pró-inflamatórias como TNF-α, lL-6 e IL-1β e a ativação do fator nuclear kappa B (NF-κB), a liberação de $NO^{\bullet}$ e o aumento da produção de ERO (BELLAVER et al., 2015). O LPS exerce seus efeitos por meio da sua ligação com a proteína CD14 presente na membrana da célula alvo, formando um complexo o qual interage com o receptor *toll-like* tipo 4 (TLR4), podendo ativar astrócitos e microglia no SNC (NAZEM et al., 2015).

Assim, podem ocorrer danos ao endotélio, depleção de GSH e disfunção mitocondrial, causando redução nos níveis de ATP. Com isso, o LPS influenciaria a consolidação da memória em roedores, induzindo déficits análogos aos observados na DA. Contudo, esses resultados poderiam ser afetados por vários fatores como a dose utilizada, o local de administração e a duração da exposição (ZAKARIA et al., 2017).

Compostos naturais x Doença de Alzheimer

Antocianinas

As antocianinas (do grego *anthos* = flores e *kianos* = azul) são definidas como pigmentos naturais hidrossolúveis pertencentes a uma classe de compostos mais ampla denominada flavonoides (HE e GIUSTI, 2010; FANG, 2015). Esses pigmentos são responsáveis pela grande diversidade de cores das flores e dos frutos, conferindo tonalidades de laranja, vermelho, violeta, roxo e azul (MIGUEL, 2011; HRIBAR e ULRIH, 2014). Todas essas cores auxiliam na atração de animais polinizadores e dispersores de sementes, facilitando a propagação das plantas (WU et al., 2006; HE e GIUSTI, 2010; KOVINICH et al., 2014). Além disso, as antocianinas desempenham ação fotoprotetora e sua síntese pode ser aumentada em situações de estresse biótico ou abiótico, participando do sistema de defesa vegetal (PETRONI e TONELLI, 2011; KOVINICH et al., 2014; KARPPINEN et al., 2016).

As antocianinas são compostas por uma estrutura básica chamada de antocianidina ou aglicona que é formada por dois anéis aromáticos (A e B) ligados a um anel heterocíclico com a presença de oxigênio (C) conforme pode ser observado na figura 3 (CASTAÑHEDA-OVANDO et al., 2009). Quando uma molécula de açúcar se liga a esta estrutura (forma glicosilada), as antocianidinas passam a ser chamadas de antocianinas (TSUDA, 2012; POJER et al., 2013). A forma aglicona raramente é encontrada na natureza, pois é muito instável (HRIBAR e ULRIH, 2014).

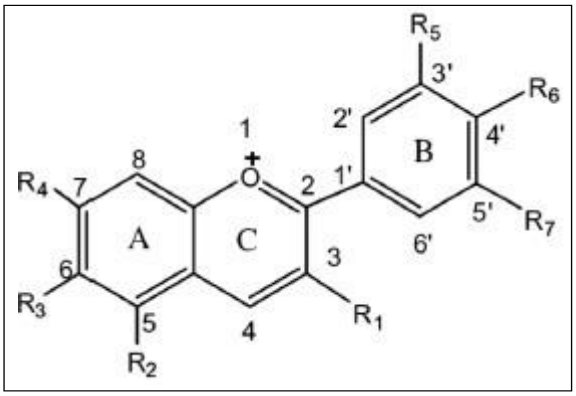

Figura 3 - Estrutura química básica das antocianinas.
Fonte: Pojer et al., 2013.

Atualmente, já foram isoladas 35 diferentes estruturas de antocianidinas e mais de 600 antocianinas foram identificadas (HRIBAR e ULRIH, 2014; SMERIGLIO et al., 2016). Entretanto, as antocianidinas mais prevalentes na natureza são a cianidina, a delfinidina, a pelargonidina, a malvidina, a peonidina e a petunidina, sendo que os glicosídeos de cianidina, delfinidina e pelargonidina são os mais comuns (KONG et al., 2003). As principais fontes alimentares de antocianinas incluem frutos como amora, framboesa, mirtilo, cereja, uva, romã, ameixa, e vegetais como rabanete, berinjela, repolho roxo e batata-doce roxa (HE e GIUSTI, 2010).

Estudos *in vivo* já demonstraram a capacidade das antocianinas de atravessar a BHE, sendo detectadas em regiões cerebrais relacionadas com o aprendizado e a memória como o córtex cerebral e o hipocampo (ANDRES-LACUEVA et al., 2005; KALT et al., 2008). Foram identificadas antocianinas não metabolizadas em cérebros de ratos alimentados com uma dieta suplementada com extrato de mirtilo (2%) durante 10 semanas (ANDRES-LACUEVA et al., 2005). Em outro trabalho, uma dieta enriquecida com antocianinas do extrato de amora (*Rubus fruticosus* L.) foi fornecida aos ratos durante 15 dias, encontrando-se no cérebro 0,25 ± 0,05 nmol/g de tecido de antocianinas (TALAVÉRA et al., 2005). Um estudo *in vitro* avaliou o transporte de antocianinas através de um modelo celular de BHE, sugerindo que esses compostos e seus metabólitos eram capazes de atravessar essa barreira influenciados pela lipofilicidade (FARIA et al., 2014).

As antocianinas têm demonstrado potencial efeito benéfico frente ao declínio cognitivo e à neurodegeneração relacionada ao envelhecimento (TSUDA et al., 2012; RENDEIRO, RHODES e SPENCER, 2015). Os mecanismos que podem estar envolvidos na sua capacidade neuroprotetora incluem atividade antioxidante, atividade anti-inflamatória, modulação da sinalização neuronal e da expressão gênica relacionadas com a plasticidade sináptica e aumento do fluxo sanguíneo cerebral (HE e GIUSTI, 2010; TSUDA, 2012; POJER et al., 2013; RENDEIRO, RHODES e SPENCER, 2015).

O efeito neuroprotetor das antocianinas tem sido sustentado por estudos *in vitro*, *in vivo* e em humanos. O pré-tratamento das células de neuroblastoma humano (SH-SY5Y) com cianidina 3-*O*-glicopiranosídeo ou cianidina foi capaz de inibir a formação de ERO a nível extracelular e extracelular/intracelular, respectivamente, sendo que a cianidina também evitou a indução de eventos

apoptóticos (TAROZZI et al., 2007). Em outro estudo, Tarozzi et al. (2010) demonstraram que a cianidina 3-*O*-glicosídeo inibiu a agregação do peptídeo $A\beta_{25-35}$ em oligômeros naquele mesmo tipo celular. Ainda o pré e co-tratamento com a cianidina 3-*O*-glicosídeo reduziu a morte celular tanto por apoptose como por necrose promovida pelos oligômeros do peptídeo $A\beta_{25-35}$ (TAROZZI et al., 2010). Células de neuroblastoma de camundongos (N2A) incubadas com os peptídeos $A\beta_{25-35}$ e $A\beta_{1-40}$ e, após tratadas com antocianinas, apresentaram diminuição da formação de ERO, preservação da homeostase do Ca^{2+} e inibição da ativação da enzima β-secretase (SHIH et al., 2011).

O tratamento com pelargonidina (10 mg/kg; oral) com início 3 dias antes da administração ICV do peptídeo $A\beta_{25-35}$ até o 22º dia após promoveu melhora do déficit cognitivo e atenuou o estresse oxidativo, o déficit colinérgico e a reatividade astrocitária em hipocampo de ratos adultos (SOHANAKI et al., 2016). Em um modelo animal de envelhecimento baseado na administração intraperitoneal de D-galactose, foi observado que a administração de antocianinas (100 mg/kg; intraperitoneal), durante 7 semanas, proporcionou melhora da memória de curta duração e espacial, além de reduzir os níveis de ERO, de peroxidação lipídica, de ativação dos astrócitos e supressão de vários marcadores inflamatórios como a óxido nítrico sintase induzível (iNOS), o TNF-α e o NF-κB em córtex cerebral e hipocampo (REHMAN et al., 2017).

Em ratos idosos (18 meses), a suplementação com antocianinas purificadas (179,0 µg/g de ração) durante 6 semanas promoveu aumento da memória espacial e incremento nos níveis do fator neurotrófico derivado do cérebro (BDNF) hipocampal e da expressão do mRNA do BDNF nas regiões CA1, CA3 e giro denteado do hipocampo (RENDEIRO et al., 2013). Estudo com camundongos transgênicos (APdE9) demonstrou que os animais alimentados com dieta rica em antocianinas iniciada aos 2,5 meses de idade, isto é, antes da deposição das placas Aβ, e continuada até os 13 meses, apresentaram melhora da hiperatividade e do déficit cognitivo, além de mostrar efeitos benéficos no metabolismo da APP e do peptídeo Aβ (VEPSÄLÄINEN et al., 2013).

Estudo também com camundongos transgênicos para a DA (APP/PS1) encontrou que a administração intraperitoneal de 12 mg/kg/dia de extrato de antocianinas de feijão preto coreano, durante 30 dias, foi capaz de melhorar a

memória espacial averiguada através dos testes de labirinto aquático de Morris e do labirinto em Y. Outro achado interessante do estudo foi a redução do estresse oxidativo nos camundongos tratados com o extrato através da ativação do fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2) e seus genes alvo como heme oxigenase 1 (HO-1) e glutamato cisteína ligase – subunidade modulatória (GCLM), podendo associar-se com a melhora da memória espacial nesses animais (ALI et al., 2017).

Khan et al. (2018) avaliaram o efeito anti-inflamatório e antioxidante das antocianinas em modelos *in vitro* e *in vivo* de neurotoxicidade induzido pelo LPS. No modelo *in vitro*, células de hipocampo de camundongo (HT22) foram tratadas com meio condicionado de células de microglia (BV2) expostas ao LPS (1µg/mL) e ao extrato de antocianinas de feijão preto coreano (100 µg/mL). Observou-se que as antocianinas foram capazes de inibir o aumento da expressão de TNF-α nessas células. No modelo *in vivo*, o tratamento de camundongos com antocianinas (24 mg/kg/dia intraperitonealmente) durante 2 semanas (com início 1 semana antes da aplicação do LPS intraperitonealmente) preveniu a produção de ERO, inibiu a neuroinflamação e a neurodegeneração e reduziu o dano à memória provocado pelo LPS.

Dados experimentais com idosos diagnosticados com demência do tipo Alzheimer de leve a moderada evidenciaram que o consumo diário de 200 mL de suco de cereja rico em antocianinas, durante 12 semanas, promoveu melhora na fluência verbal e da memória de curta e de longa duração (KENT et al., 2015). Também foi encontrado que o consumo de suco de uva Concord (6,3 a 7,8 mL/kg/dia – 46% de antocianinas) por indivíduos acima de 68 anos, portadores de comprometimento cognitivo leve, durante 16 semanas, promoveu aumento da ativação neuronal em áreas corticais e melhora da memória (KRIKORIAN et al., 2012).

É importante salientar que trabalhos prévios do nosso grupo de pesquisa já demonstraram que o pré-tratamento com antocianinas durante 7 dias foi capaz de prevenir déficit de memória e alterações nas atividades das enzimas AChE, Na^+,K^+-ATPase e Ca^{2+}-ATPase, e nos níveis de nitrato e nitrito em córtex cerebral e hipocampo de ratos submetidos a um modelo de demência esporádica do tipo Alzheimer induzida por STZ (GUTIERRES et al., 2014b).

Dessa forma, a fim de dar continuidade a esses estudos, o objetivo do presente trabalho foi avaliar se o tratamento com antocianinas é também capaz de reverter as alterações induzidas pelo STZ bem como avaliar o efeito desses compostos na modulação das respostas astrocitárias na prevenção e na reversão de insultos induzidos pelo LPS.

4 RESULTADOS

Os resultados que fazem parte dessa tese estão apresentados sob a forma de dois artigos. As seções materiais e métodos, resultados, discussão e referências encontram-se nos próprios artigos e representam a íntegra desse estudo.

Os itens discussão e conclusões que se encontram no final dessa tese apresentam interpretações e comentários gerais sobre os artigos contidos nesse trabalho.

As referências são referentes apenas às citações que aparecem nos itens introdução e discussão da tese.

Os artigos estão estruturados de acordo com as revistas as quais foram publicados: *Cellular and Molecular Neurobiology* (Artigo 1) e *The Journal of Nutritional Biochemistry* (Artigo 2).

Artigo 1

Glioprotective effects of lingonberry extract against altered cellular viability, acetylcholinesterase activity, and oxidative stress in lipopolysaccharide-treated astrocytes

Glioprotective effects of lingonberry extract against altered cellular viability, acetylcholinesterase activity, and oxidative stress in lipopolysaccharide-treated astrocytes

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Abstract

Altered astrocytic function is a contributing factor to the development of neurological diseases and neurodegeneration. Berry fruits exert neuroprotective effects by modulating pathways involved in inflammation, neurotransmission, and oxidative stress. The aim of this study was examined the effects of the lingonberry extract on cellular viability and oxidative stress in astrocytes exposed to lipopolysaccharide (LPS). In the reversal protocol, primary astrocytic cultures were first exposed to 1 µg/mL LPS for 3 h and subsequently treated with lingonberry extract (10, 30, 50, and 100 µg/mL) for 24 h and 48 h. In the prevention protocol, exposure to the lingonberry extract was performed before treatment with LPS. In both reversal and prevention protocols, the lingonberry extracts, from 10 to 100 µg/mL, attenuated LPS-induced increase in reactive oxygen species (around 55% and 45% respectively, $P<0.01$), nitrite levels (around 50% and 45% respectively, $P<0.05$), and acetylcholinesterase activity (around 45% and 60% respectively, $P<0.05$) in astrocytic cultures at 24 and 48 hours. Also, in both reversal and prevention protocols, the lingonberry extract also prevented and reversed the LPS-induced decreased cellular viability (around 45% and 90% respectively, $P<0.05$), thiol content ((around 55% and 70% respectively, $P<0.05$), and superoxide dismutase activity (around 50% and 145% respectively, $P<0.05$), in astrocytes at both 24 h and 48 h. Our findings suggested that the lingonberry extract exerted a glioprotective effect through an anti-oxidative mechanism against LPS-induced astrocytic damage.

Keywords: astrocyte, lingonberry extract, lipopolysaccharide, oxidative stress, acetylcholinesterase, cellular viability

1. Introduction

Polyphenols are compounds involved in the defense system of plants (Almeida et al. 2016). These substances are a central part of our diet since they are found in a variety of vegetables and fruits (Almeida et al. 2016). Berries are a very rich source of polyphenols, in particular, anthocyanins that are water-soluble pigments responsible for the red, purple, and blue colors found in these fruits (Del

Rio et al. 2010; Fang 2015; Shih et al. 2007). Lingonberry (*Vaccinium vitis-idaea* L.) is a small red berry of the Ericaceae family that grows in northern latitudes including Scandinavian countries, Canada, and China (Fan et al. 2012; Mane et al. 2011). The lingonberry extract consists primarily of a pool of 15 anthocyanins and cianidina-3-O-galactoside was to be the major (Mane et al. 2011). An increasing number of studies provided evidence for health benefits of anthocyanins including anti-oxidative, anti-inflammatory, and neuroprotective properties (Subash 2014; Zafra-Stone 2007). Previous studies showed that anthocyanins were able to cross the blood-brain barrier (BBB) of rodents and can be localize in several brain regions related to learning and memory (Faria et al. 2014; Andres-Lacueva et al. 2005; Youdim et al. 2003). In addition, our research group has already proven its benefit for the treatment of diseases that affect the central nervous system (CNS) suggesting that anthocyanins have potential to cross the BBB (Carvalho et al. 2017; Carvalho et al. 2015; Gutierres et al. 2014a; Gutierres et al. 2014b).

Neurodegenerative diseases, such as multiple sclerosis, Parkinson's disease (PD), and Alzheimer's disease (AD), are closely associated with inflammation and oxidative stress (Cunningham et al. 2009). Reactive oxygen species (ROS), excessive cyclooxygenase-2 (COX-2), nitric oxide synthases (NOS), and large release of pro-inflammatory cytokines have been associated with neurological damage (Bolaños and Heales 2010; Lin and Beal 2006; Wee Yong 2010).

Astrocytes are glial cells distributed throughout the CNS in an arrangement optimal for chemical and physical interaction with neuronal synapses and brain blood supply vessels, and can produce neurotrophic factors, cytokines, and neurotransmitters. In neurodegenerative diseases, astrocytes react to the damaging inflammatory microenvironment by increasing cell metabolism and engendering cell hypertrophy and proliferation. This condition of glial cell dysfunction is termed reactive astrocytosis and can be harmful for neurons (Hamby and Sofroniew 2010; Sofroniew and Vinters 2010). Reactive astrocytes stimulate the accumulation of extracellular glutamate, aggravate inflammation by the production of pro-inflammatory cytokines, and produce neurotoxic levels of ROS and nitric oxide (NO), thus promoting neuronal death and CNS injury (Alirezaei et al. 2011; Sofroniew and Vinters 2010).

Lipopolysaccharide (LPS) is a membrane component of gram-negative bacteria, which can stimulate glial cells and induce the expression of pro-inflammatory molecules that can damage the CNS (Dunn and Swiergiel 2005; Lau et al. 2007). Owing to its ability to set an inflammatory environment, LPS is used to model inflammation (Kipp et al. 2008). It has been demonstrated that LPS induced COX-2, inducible nitric oxide synthase (iNOS), pro-inflammatory cytokine expression (interleukin-1β (IL-1β), IL-6 and tumor necrosis factor alpha TNF-α), increased the number of glial fibrillary acidic protein (GFAP)-positive cells and neuronal apoptosis, reduced expression of brain-derived neurotrophic factor (BDNF) and caused memory decline (Catorce et al. 2016; Daulatzai et al. 2016). Thus, chronic neuroinflammation promote each other, leading to the establishment of a detrimental vicious cycle that is characteristic of neurodegenerative diseases. Consequently, targeting neuroinflammation represents a major therapeutic interest to modulate neuronal loss and is currently the subject of several studies.

Acetylcholine (ACh) is recognized as a neurotransmitter in the central and peripheral nervous systems. However, others studies also showed that ACh modulates the physiological function of cells and tissues, acting as a cell-to-cell signaling molecule via muscarinic and nicotinic ACh receptors (mAChRs and nAChRs) (Fujii et al. 2017). Evidence show potential anti-inflammatory of ACh in astrocytes may provide protection against degeneration of neurons and glioprotection against astrogliosis induced by LPS in vitro. More recently, it has been demonstrated that Ach in glial cells leads to blocking of the nuclear factor kappa B (NF-κB) pathway and a consequent reduction in neuroinflammation (Patel et al. 2017; Han et al. 2014; Liu et al. 2012; Shen et al. 2012). The effect induced by ACh is correlated with the essential role of acetylcholinesterase (AChE) activity that cleaves ACh into choline and acetate interrupted the cholinergic signaling (Ferreira-Vieira et al. 2016). For this reason, the investigation of inhibitors of this enzyme is important to control of neuroinflammation. The particular importance, previous studies showed that anthocyanins were able to inhibit AChE activity in the brain of rats submitted a model of memory deficit (Gutierres et al. 2014a; Gutierres et al. 2014b).

In this context, in the present study we evaluated the effects of lingonberry extract on cellular viability, AChE activity, and oxidative stress parameters in primary culture of rat astrocytes treated with LPS.

2. Materials and Methods

Chemicals

The LPS from *Escherichia coli* (055:B5), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), tetrabutylammonium salt malondialdehyde (MDA), and acetylthiocholine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The lingonberry extract (NutriPhy® Lingonberry) was obtained from Christian Hansen (SAS, Hoersholm, Denmark). The identification of polyphenols and anthocyanins in the lingonberry extract was described by Mane et al. (2011).

Animals

Wistar rats (aged 1–2 d) were maintained under a standard 12-h dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.) in a room at a controlled temperature (22 ± 2 ºC). All animal procedures were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, Brazil, under protocol number CEEA 0179/2015. The use of the animals is in accordance with the Brazilian Guidelines for the Care and Use of Animals in Scientific Research Activities (DBCA) and with the National Council of Control of Animal Experimentation (CONCEA).

Astrocytic Cultures

Primary astrocytic cultures were prepared as described by Gottfried et al. 1999. The cerebral cortices of newborn (1–2 d) Wistar rats were removed and dissociated in a calcium and magnesium free balanced salt solution (pH 7.4). After removing the meninges, the cerebral cortex was dissociated by sequential passages through a pipette. The cell suspension was settled through centrifugation for 10 min at 1,000 rpm. The sediment cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, pH 7.6). Subsequently, the cells were seeded in plates (6-well and 96-well) pretreated with poly-L-lysine. Four hours after seeding, the plates were washed with phosphate-buffered saline (PBS) and a new medium was added. Cultures were maintained at 5% CO_2, 37 ºC, and humidified atmosphere for 20 d until confluence for cell maturation and differentiation, and the fresh medium was replaced every 4 d.

Culture treatment with LPS and lingonberry extract

The lingonberry extract was dissolved in water and subsequently mixed with DMEM with 10% FBS to obtain solutions at final concentrations of 10, 30, 50, and 100 µg/mL. For the cytotoxicity test, astrocytes were exposed to lingonberry extract at the four different concentrations for 24 h and 48 h. To induce inflammation by LPS (Bellaver et al. 2015b) with the purpose of testing the potential of lingonberry extract in treating neuroinflammation, we used two different protocols, namely reversal and prevention protocols (Fig. 1). In the reversal protocol, the cells were first exposed to 1 µg/mL LPS for 3 h and were subsequently treated only with lingonberry extract for 24 h and 48 h. In the prevention protocol, to test the potential of the extract for preventing neuroinflammation, the cells were first exposed to different concentrations of lingonberry extract for 3 h and subsequently treated only with 1 µg/mL LPS for 24 h and 48 h. The control cells were maintained only in DMEM with 10% FBS.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined using the MTT assay, which consists of measuring the number of cells with metabolically active mitochondria based on the reduction of the tetrazolium salt MTT to formazan.

Briefly, cells were washed with PBS and 0.5 mg/mL MTT were subsequently added in each well. The cells were then incubated for 90 min at 37 °C and 5% CO_2. The MTT solution was removed and the precipitate was dissolved with dimethyl sulfoxide (DMSO). The absorbance was determined in a microplate reader at 492 nm, and was linearly proportional to the number of cells with active mitochondria.

The sulforhodamine B assay

The sulforhodamine B (SRB) assay was used for the determination of the cell proliferation based on the measurement of cell protein. Briefly, cultures were washed and fixed in 50% trichloroacetic acid for 45 min at 4 °C. After washing five times with distilled water, 0.4% SRB in acetic acid was added. After incubation for 30 min, the plates were washed five times with 1% acetic acid for the complete removal of unbound dye. Finally, the dye was eluded with 10 mM Tris solution and the absorbance was determined in a spectrophotometer at 530 nm.

Acetylcholinesterase (AChE) activity

The AChE activity in primary cortical astrocytes was determined as previously described (Ellman et al. 1961). The cell lysate was prepared in PBS and the cells were mechanically lysed with the aid of a scraper. First, the reaction system was composed of 10 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 100 mM phosphate buffer (pH 7.5), and 15 µL of lysate cell, and was incubated for 2 min at 27 °C in 96-well microplates. Subsequently, 8 mM acetylthiocholine was added and the absorbance was determined at 412 nm in a spectrophotometer for 2 min with 30-s intervals at 27 °C. All readings were performed in triplicate and the AChE activity was expressed as µmol AcSCh/h/mg of protein. Protein content was measured by using the Coomassie blue method (Bradford 1976).

Oxidative stress parameters

Reactive oxygen species (ROS) assay

Formation of ROS was determined according to Ali et al. (1992) with some modifications. The intracellular generation of ROS was determined by using the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, whereby DCFH-DA reacts with ROS and consequently emits fluorescence. Intact cells were incubated with 1 µM DCFH-DA in serum-free medium for 30 min at 37 °C. Subsequently, cells were washed with PBS and fluorescence was determined in a multi-well plate reader (485/520 nm). The ROS production was reported as the percentage of untreated cells (control).

Total thiol content (SH) assay

The total SH was determined in lysates of astrocytes using the DTNB method as described by Aksenov and Markesbery (2001). The reaction was based on the reduction of DTNB by thiols resulting in a yellow derivative (TNB) whose absorption is read at 412 nm. The results were expressed in nmol TNB/mg of protein.

Nitrite determination

The production of NO was determined by measuring the accumulated level of nitrite (indicator of NO) in the supernatant after 24 h or 48 h of LPS treatment with or

without different concentrations of lingonberry extract by using a colorimetric reaction with Griess reagent (Stuehr and Nathan 1989). For this reaction, 100 µL of sulfanilamide in 5% phosphoric acid were added to 100 µL cell-culture supernatants. After mixing the reagents, the samples were maintained at room temperature for 10 min for a correct reaction to take place. Subsequently, the samples were mixed with 100 µL of Griess reagent (0.1% N-[1-naphthyl] ethylenediamine dihydrochloride) and incubated for 10 min in the dark. The absorbance was measured at 540 nm using a 96-well microplate reader and the amount of nitrite in the supernatant was compared to a standard curve of known concentrations of sodium nitrate.

Superoxide dismutase (SOD) assay

The SOD activity was measured in the lysates according to Misra and Fridovich (1972). This method is based on the inhibition of superoxide dependent adrenaline auto-oxidation in a spectrophotometer adjusted at 480 nm. The specific activity of SOD was reported as units/mg of protein.

Catalase (CAT) assay

The CAT activity based on the decomposition of H_2O_2 monitored at 240 nm at ambient temperature was performed according to Aebi (1984). The CAT activity was reported as units/mg of protein.

Glutathione peroxidase (GPx) assay

The activity of GPx was performed using a commercial kit (Randox Laboratories Ltd, United Kingdom) according to the manufacturer's instructions. The GPx activity was reported as units of activity of the GPx/mg of protein.

Protein determination

Protein determination was performed according to the method of Lowry (1951) using bovine serum albumin as standard.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 and one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons.

Furthermore, all data were expressed as mean ± standard error and the differences between mean values were considered significant at $P < 0.05$.

3. Results

Lingonberry extract reverses and prevents LPS-induced alterations of astrocyte viability and proliferation

First, we determined the cytotoxic profile of the lingonberry extract by exposing astrocytic cultures to 10, 30, 50, and 100 µg/mL of the extract for 24 h and 48 h. Our results indicated no changes in cell viability when compared with non-exposed control cells, revealing that the lingonberry extract is not harmful for healthy CNS cells at these concentrations (Fig. 2a, b).

In the reversal protocol, exposure to LPS at a concentration of 1 µg/mL for 24h reduced the cell viability by approximately 40% when compared to control cells ($F_{5,20} = 32.39$, $P < 0.05$; Fig. 2c). This results may be due to the fact that LPS induces mitochondrial dysfunction in these cells through the increase of ROS and NO (Bellaver et al. 2015a). On the other hand, treatment with the lingonberry extract at 10, 30, 50, and 100 µg/mL for 24 h reversed the deleterious effect of LPS, and increased the cell viability rates by 35%, 30%, 30%, and 50%, respectively, indicating a glioprotective effect ($F_{5,20} = 32.39$, $P < 0.05$; Fig. 2c). Similar results were obtained after treatment for 48 h, where LPS also caused a 50% reduction in cell viability ($F_{5,20} = 23.58$, $P < 0.05$; Fig. 2d), and treatment with lingonberry extract effectively reversed this LPS effect ($F_{5,20} = 23.58$, $P < 0.05$; Fig. 2d).

In the prevention protocol, our results showed that treatment with the lingonberry extract also prevented the LPS-induced alteration of cell viability at both 24 h and 48 h. After 24 h, LPS reduced the viability of astrocytes by 40% when compared with the control cells ($F_{5,20} = 12.06$, $P < 0.05$; Fig. 2e), while treatment with the extract at all concentrations significantly increased the cellular viability when compared with the LPS-alone group ($F_{5,20} = 12.06$, $P < 0.05$; Fig. 2e). Similar results were obtained after treatment with the extract for 48 h. While exposure to LPS resulted in a 50% decreased in astrocyte viability compared with the control group ($F_{5,20} = 12.06$, $P < 0.05$; Fig. 2f), treatment with lingonberry extract at 30, 50, and 100 µg/mL significantly increased the astrocyte viability rate when compared to LPS

alone to 50%, 100%, and 90%, respectively, of the LPS-alone level (Fig. 2f). Our findings revealed that the lingonberry extract exerts a protective effect against LPS-induced damage in astrocytes. It is well known that astrocytes react to the damaging inflammatory microenvironment by increasing the cell metabolism and engendering cell hypertrophy and proliferation (Hamby and Sofroniew 2010; Sofroniew and Vinters 2010). Exposure to LPS increased cell proliferation by 20% and 40% after 24 h and 48 h, respectively (Fig. 2g, h, i, j), and cell hypertrophy with increased cell body and higher cell density were observed (Fig. 3). In contrast, treatment with the lingonberry extract at 10, 30, and 50 µg/mL reversed these deleterious LPS effects by decreasing the proliferation rates of astrocytes to levels equal to controls, and by restoring homeostasis after 24 h of treatment ($F_{5,20}$ = 91,76, P < 0.001; Fig. 2g). The extract at all four concentrations decreased the proliferation and hypertrophy induced by LPS after 48 h of treatment, thus indicating a glioprotective effect ($F_{5,20}$ = 8,62, P < 0.0001; Fig. 2h). In addition, similar results were obtained after 48 h treatment in the prevention protocol, where the lingonberry extract at 10, 30, and 50 µg/mL prevented the deleterious LPS effects and restored homeostasis ($F_{5,20}$ = 69,62, P < 0.0001; Fig. 2j). Interestingly, morphologically we also observed prevention and reversion in the morphological changes induced by LPS observed in the astrocytes, in the groups exposed to anthocyanins, a morphology similar to that of the control group was observed with cell bodies of characteristic size to that of the astrocytes with absence of cells with morphology indicating cell death, plus one instead indicating the safety of the treated and glioprotective effect (Fig 3).

Lingonberry extract modulates the LPS-induced alterations of the AChE activity

In the reversal protocol, compared with the control group, we observed an increase in the AChE activity in astrocytes exposed to LPS for 24 h (65%, $F_{5,20}$ = 7.03, P < 0.05) and 48 h (75%, $F_{5,20}$ = 5.44, P < 0.05; Fig. 4). However treatment with lingonberry extract at 10, 30, 50, and 100 µg/mL decreased the AChE activity in astrocytes by approximately 40% after 24 h ($F_{5,20}$ = 7.03, P < 0.05) and 50% after 48 h ($F_{5,20}$ = 5.44, P < 0.05; Fig. 4). Similar results were obtained in the prevention protocol, where pretreatment with the lingonberry extract at all four concentrations prevented the LPS-induced increase in AChE activity at 24 h (40%) ($F_{5,18}$ = 4.48, P < 0.05) and 48 h (75%) ($F_{5,18}$ = 3.91, P < 0.05; Fig. 4).

Evaluation of oxidative damage markers in astrocytes exposed to LPS and lingonberry extract

In the reversal protocol, our results indicated an increase of 82% and 141% of ROS production in astrocytes exposed to LPS after 24 h ($F_{5,86} = 32.82$, $P < 0.05$) and 48 h ($F_{5,38} = 14.41$, $P < 0.05$), respectively. However, treatment with lingonberry extract at 10, 30, 50, and 100 µg/mL reversed this alteration by approximately 50–65% after 24 h ($F_{5,86} = 32.82$, $P < 0.05$) and 40–65% after 48 h ($F_{5,38} = 14.41$, $P < 0.05$; Table 1). In the prevention protocol, LPS also increased the ROS levels by approximately 60% after 24 h ($F_{5,85} = 23.70$, $P < 0.05$) and 84% after 48 h ($F_{5,87} = 6.88$, $P < 0.05$). The pretreatment with lingonberry extract at 10, 30, 50, and 100 µg/mL at both 24 h and 48 h prevented this increase by approximately 50% ($F_{5,85} = 23.70$, $P < 0.05$) and 40% ($F_{5,87} = 6.88$, $P < 0.05$), respectively (Table 1).

In both experimental protocols, LPS increased the NO levels both after 24 h and 48 h. In the reversal protocol, the increase was 96% at 24 h ($F_{5,18} = 5.06$, $P < 0.05$) and 32% at 48 h ($F_{5,18} = 40.08$, $P < 0.05$). Treatment with lingonberry extract at 10, 30, 50, and 100 µg/mL reversed this alteration by approximately 40% after 24 h ($F_{5,18} = 5.06$, $P < 0.05$) and 55% after 48 h ($F_{5,18} = 40.08$, $P < 0.05$; Table 1). In the prevention protocol, lingonberry extract at all concentrations prevented this LPS-induced NO production both at 24 h (11–55%) ($F_{5,18} = 82.18$, $P < 0.05$) and at 48 h (50–62%) ($F_{5,18} = 17.09$, $P < 0.05$; Table 1).

In the reversal protocol, LPS treatment also reduced the SH levels in astrocytes by approximately 30% ($F_{5,14} = 7.43$, $P < 0.05$) and 50% ($F_{5,14} = 7.17$, $P < 0.05$) at both 24 h and 48 h, respectively, compared with the control group. However, the lingonberry extract at only 100 µg/mL could reverse this LPS-induced damage by increasing the SH values by 40% after 24 h ($F_{5,14} = 7.43$, $P < 0.05$) and 70% after 48 h ($F_{5,14} = 7.17$, $P < 0.05$; Table 1). In the prevention protocol, LPS also decreased the SH content by approximately 30% at 24 h ($F_{5,14} = 11.28$, $P < 0.05$) and 50% at 48 h ($F_{5,17} = 11.46$, $P < 0.05$). In contrast, pretreatment with lingonberry extract at 50 and 100 µg/mL after 24 h prevented the reduction of SH content caused by LPS by approximately 45% and 70%, respectively ($F_{5,14} = 7.43$, $P < 0.05$). On the other hand, after 48 h, lingonberry extract at all four concentrations (10, 30, 50, and 100 µg/mL) prevented the SH reduction induced by LPS by 80%, 100%, 60%, and 60%, respectively ($F_{5,14} = 7.17$, $P < 0.05$; Table 1).

Evaluation of antioxidant enzymatic activities in astrocytes exposed to lingonberry extract and LPS

Figure 5 depicts the effect of LPS and lingonberry extract on the activity of anti-oxidative enzymes in astrocytes in the reversal protocol. Based on the results, LPS decreased the SOD activity at both 24 h ($F_{5,18}$ = 25.76, P < 0.05) and 48 h ($F_{5,18}$ = 75.39, P < 0.05) by approximately 50% and 40%, respectively, when compared with the control group. When astrocytes were exposed to LPS and treated subsequently with lingonberry extract (30, 50, and 100 µg/mL), an increase in the SOD activity was observed at both 24 h ($F_{5,18}$ = 25.76, P < 0.05) and 48 h ($F_{5,18}$ = 75.39, P < 0.05). No changes were observed in the CAT activity (P > 0.05; Fig. 5). Moreover, LPS significantly increased the GPx activity at 24 h (94%, $F_{5,18}$ = 10.63, P < 0.05). However, treatment with 10, 30, 50, and 100 µg/mL of lingonberry extract reversed this alteration by decreasing the GPx by 65%, 37%, 39%, and 64%, respectively ($F_{5,18}$ = 10.63, P < 0.05). After 48 h, a decrease in the GPx activity was observed only in astrocytes exposed to LPS and treated with 100 µg/mL lingonberry extract (P < 0.05; Fig. 5).

In the prevention protocol, the LPS treatment induced a decrease in the SOD activity at 24 h (50%) and 48 h (60%). However, pretreatment with lingonberry extract at 50 and 100 µg/mL prevented this SOD alteration at 24 h to 133% and 157%, respectively ($F_{5,11}$ = 13.64, P < 0.05). At 48 h, all concentrations of the lingonberry extract prevented the LPS-induced decrease in the SOD activity ($F_{5,14}$ = 15.33, P < 0.05; Fig. 6). In contrast, LPS treatment at both 24 h and 48 h resulted in no changes in the CAT and GPx activities in the prevention protocol (P > 0.05; Fig. 6).

4. Discussion

Neuroinflammation represents the cell response to tissue damage, and is characterized by the release of pro-inflammatory cytokines and free radicals. However, sustained, excessive, or inappropriate inflammation is the cause of astrocytic dysfunction and neuronal death (McGeer et al. 2003). Astrocytes play critical roles in physiological brain processes, including glutamate homeostasis, blood flow and excitability, metabolic support of neurons, and synaptic development and plasticity. In light of these roles, functional alterations in astrocytes are important

contributors to the progression of neurological diseases (Acosta et al. 2017). Therefore, the evaluation of compounds with anti-inflammatory and anti-oxidative actions targeting astrocytes is very important for the treatment or prevention of neuroinflammation.

In the present study, we demonstrated the ability of the lingonberry extract to protect against LPS-induced damage in astrocytes. Treatment with lingonberry extract both before and after exposure to LPS (i.e., prevention and reversal protocols, respectively) prevented or reversed the LPS-induced alterations in cell viability, hypertrophy, and proliferation in astrocytes. Our results also showed that lingonberry extract has the potential to enhance anti-oxidative defenses and decrease the production of ROS and reactive nitrogen species through the negative modulation of iNOS activity, thus leading to the regulation of the cell redox state and protecting against oxidative damage caused by LPS in astrocytes. In addition, LPS induced an increase in the astrocytic AChE activity, which was restored by treatment with the lingonberry extract. Similar results were found recently by our research group where it was possible to observe that anthocyanins control neuroinflammation in mice exposed to LPS. Carvalho et al. (2017) showed that anthocyanins extract prevents oxidative stress through carbonyl protein, No*x* (nitrite/nitrate) and lipid peroxidation reduction in cerebral cortex and hippocampus. Moreover it is capable to prevent the increase of proinflammatory interleukins as IL-1β, IL-6, TNF-α and interferon-γ (INF-γ) in hippocampus. They also showed that anthocyanins prevent the increase of GFAP levels in cerebral cortex and hippocampus of mice exposed to LPS suggesting that this extract modulate the astrocyte activation.

The Toll-like receptor 4 (TLR4), a LPS receptor, is a primary mediator of the innate immune response to pathogens (Park and Lee 2013). Activation of TLR4 by LPS triggers a response through signaling factors that induce the expression of genes related to inflammation (Pasare and Medzhitov 2005). Recent findings indicated that, after activating TLR4, astrocytes may cause changes in the BBB, promote inflammation, and modulate immune responses (Banks et al. 2015). Activation of TLR4 leads to astrocytic signaling via the NF-κB, mitogen-activated protein kinase (MAPK), and Janus kinase 1/signal transducer and activator of transcription 1 (JAK1/STAT1) pathways, which in turn regulate the expression of a wide range of pro-inflammatory molecules and downregulate the copper-zinc-

containing SOD (Gorina et al. 2011). Furthermore, TLR4 causes apoptotic death of astrocytes and autophagy primarily by glutamate excitotoxicity (Belkacemi and Ramassamy 2016; Sharma et al. 2016) upregulation of growth arrest specific 1 (Gas1) (Sun et al. 2016) and pro-inflammatory cytokine levels (Sun et al. 2016), and mitochondrial dysfunction with increased levels of oxygen/nitrogen species (Bellaver et al. 2015a). Based on these previous findings, chronically and abnormally activated astrocytes may affect the synaptic activity in neuronal networks contributing to development of neurological pathologies.

Lingonberry is a small red berry rich in functional compounds. The beneficial effects of berry fruits are related to phytochemicals such as anthocyanins, caffeic acid, quercetin, and ferulic acid. Flavanols are a major class of polyphenols in lingonberry extract (5.8% [w/w]), followed by flavonols (2.9%), phenolic acids (1.9%), and anthocyanins (1.5%) (Mane et al. 2011). Previous studies have demonstrated the actions of anthocyanins in several neurological conditions such as PD (Strathearn et al. 2014), neuroinflammation (Carvalho et al. 2017), demyelination (Carvalho et al. 2015), memory dysfunction (Gutierres et al. 2014a), and AD dementia (Gutierres et al. 2014b). The antioxidant potential of anthocyanins depends on the chemical structure of the molecule and this property is also influenced by number of hydroxyl groups, catechol moiety in the B ring, oxonium ion in the C ring, hydroxylation and methylation pattern, acylation and glycosylation (Pojer et al. 2013).

The precise mechanisms involved in the neuroprotective effects of anthocyanins are not completely understood. However, previous studies have suggested that the anti-inflammatory and anti-oxidative actions of these compounds may be due to the inhibition of the signaling pathway mediated by the transcription factor NF-κB (Morais et al. 2016), modulation of T-cell cytokine signaling, inhibition of IFN-γ signal transduction (Roth et al. 2016), upregulation of the anti-oxidative capacity by increased SOD activity, decreased NO contents and prostaglandin E2 (PGE2) (Wang et al. 2015), inhibition of COX-2 activity and ROS (Nair et al. 2015), and reduction of the levels of inflammatory cytokines (Sogo et al. 2015). In line with these previous findings, we showed that treatment with an extract rich in anthocyanins was capable of reversing and preventing alterations caused by LPS treatment in astrocytes through anti-oxidative mechanisms. This antioxidant protection of the lingonberry extract is associated with the increase in cell

mitochondria viability and the restoration of the SOD activity in astrocytes exposed to LPS.

In addition, the lingonberry extract increased the SH content in astrocytes exposed to LPS probably by preserving the thiol-containing glutathione (GSH) that is fundamental in redox status and protein function regulation (Moskaug et al. 2005; Poole 2015) because this biomolecule is highly susceptible to oxidative damage. In addition, lingonberry extract also reversed the alterations in GPx activity in astrocytes after 24 h of exposure to LPS, thus demonstrating that these compounds are capable of modulating the glutathione-related enzymatic system (Shih et al. 2007). Most importantly, previous studies have reported that the release of GSH by astrocytes is essential to maintain stable SH levels in the CNS (Wang and Cynader 2000) and that astrocytes protect neurons against NO toxicity through a GSH-dependent mechanism (Chen et al. 2001).

In neurological diseases, NO is produced due to increased expression of iNOS and can contribute to oxidative stress and neuronal death (Wakabayashi et al. 2010). Moreover, NO mediates the disruption of mitochondrial membrane, which contributes to cell death by bioenergetics failure, loss of intra-mitochondrial contents, increase of ROS production, and release of signaling molecules that regulate cellular apoptosis (Stewart and Heales 2003). Previous studies demonstrated that iNOS upregulation in glial cells caused neurodegeneration in PD (Liberatore et al. 1999) and brain immune cell activation in AD (Armato et al. 2013). In our study, we showed that lingonberry extract prevented and reversed the increase in the nitrite levels induced by exposure of astrocytes to LPS, demonstrating that the glioprotective effect of lingonberry extract is also associated with the regulation of NO levels.

In the present investigation, we also evaluated the effects of LPS and lingonberry extract on the activity of AChE, which is an important enzyme expressed in several cells including astrocytes (Thullbery et al. 2005) and is responsible for hydrolysis of the neurotransmitter ACh (Greenfield and Vaux 2002; Soreq and Seidman 2001). Alterations in AChE activity have been associated with several brain dysfunctions (Wee Yong 2010). Our results demonstrated that exposure of astrocytes to LPS increases the activity of AChE possibly by decreasing the levels of acetylcholine. It is important to consider that acetylcholine synthesis can be also inhibited by H_2O_2 and NO (Lüth et al. 2001). In line with this, lingonberry extract at all

time points and concentrations effectively reversed and prevented the increase of AChE induced in astrocytes by LPS, possibly due to the antioxidant action of the phytochemicals present in this extract.

Another important aspect to be discussed is that acetylcholine is a molecule with anti-inflammatory actions. Although the mechanisms involved in the control of the brain immune response remain unclear, studies have shown that an increase in AChE activity causes microglial activation and elevated levels of proinflammatory cytokines (Gnatek et al. 2012) and upregulation of iNOS activity (von Bernhardi et al. 2003). On the other hand, the increased acetylcholine levels inhibit the inflammatory effects of LPS through the inbuilt release of TNF-α by microglia through the nAChRs via MAPK (Shytle et al. 2004). These previous findings are in line with our results, which together suggested that compounds present in lingonberry fruits exert glioprotective action by modulating cholinergic signaling pathways.

5. Conclusion

The present study demonstrated that the lingonberry extract was capable of reversing and preventing the damage induced by LPS in primary astrocytic cultures. Our findings showed that lingonberry extract exerts a glioprotective effect through the increase of cellular viability, restoration of AChE activity, and anti-oxidative mechanism, thus contributing to the maintenance of astrocytic homeostasis (Fig. 7). Further studies are still necessary to evaluate the promising therapeutic potential of lingonberry extract against neuroinflammatory diseases.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Acosta C, Anderson HD., Anderson CM (2017) Astrocyte dysfunction in Alzheimer disease. J Neurosci Res. doi: 10.1002/jnr.24075

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126. doi: 10.1016/S0076-6879(84)05016-3

Aksenov MY, Markesbery WR (2001) Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302 (2-3):141–145. doi: 10.1016/S0304-3940(01)01636-6

Ali SF, LeBel CP, Bondy SC (1992) Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology 13(3):637-648

Alirezaei M, Kemball CC, Whitton JL (2011) Autophagy, inflammation and neurodegenerative disease. Eur J Neurosci 33(2):197-204. doi: 10.1111/j.1460-9568.2010.07500.x

Almeida S, Alves MG, Sousa M, Oliveira PF, Silva BM (2016) Are polyphenols strong dietary agents against neurotoxicity and neurodegeneration? Neurotox Res 30(3):345-366, 2016. doi: 10.1007/s12640-015-9590-4

Andres-Lacueva C, Shukitt-Hale B, Galli RL, Jauregui O, Lamuela-Raventos RM, Joseph JA (2005) Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory.
Nutr Neurosci 8(2):111–120. doi:10.1080/10284150500078117

Armato U, Chakravarthy B, Pacchiana R, Whitfield JF (2013) Alzheimer's disease: an update of the roles of receptors, astrocytes and primary cilia (review). Int J Mol Med 31(1):3-10. doi: 10.3892/ijmm.2012.1162

Banks WA, Gray AM, Erickson MA, Salameh TS, Damodarasamy M, Sheibani N, Meabon JS, Wing EE, Morofuji Y, Cook DG, Reed MJ (2015) Lipopolysaccharide-

induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. J Neuroinflammation 12:223. doi: 10.1186/s12974-015-0434-1

Belkacemi A, Ramassamy C (2016) Anthocyanins protect SK-N-SH cells against acrolein-induced toxicity by preserving the cellular redox state. J Alzheimers Dis 50(4):981-998. doi: 10.3233/JAD-150770

Bellaver B, Souza DG, Bobermin LD, Gonçalves CA, Souza DO, Quincozes-Santos A (2015a) Guanosine inhibits LPS-induced pro-inflammatory response and oxidative stress in hippocampal astrocytes through the heme oxygenase-1 pathway. Purinergic Signal 11(4):571–580. doi: 10.1007/s11302-015-9475-2

Bellaver B, Souza DG, Bobermin LD, Souza DO, Gonçalves CA, Quincozes-Santos A (2015b) Resveratrol protects hippocampal astrocytes against LPS-induced neurotoxicity through HO-1, p38, and ERK pathways. Neurochem Res 40(8):1600-1608. doi: 10.1007/s11064-015-1636-8

Bolaños JP, Heales SJR (2010) Persistent mitochondrial damage by nitric oxide and its derivatives: neuropathological implications. Front Neuroenergetics 2:1. doi: 10.3389/neuro.14.001.2010

Bradford M (1976) A rapid and sensitive method for the quantitation of microgramquantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72 (1-2):248-254. doi: 10.1016/0003-2697(76)90527-3

Carvalho FB, Gutierres JM, Bohnert C, Zago AM, Abdalla FH, Vieira JM, Palma HE, Oliveira SM, Spanevello RM, Duarte MM, Lopes ST, Aiello G, Amaral MG, Pippi NL, Andrade CM (2015) Anthocyanins suppress the secretion of proinflammatory mediators and oxidative stress, and restore ion pump activities in demyelination. J Nutr Biochem 26(4):378-390. doi: 10.1016/j.jnutbio.2014.11.006

Carvalho FB, Gutierres JM, Bueno A, Agostinho P, Zago AM, Vieira J, Frühauf P, Cechella JL, Nogueira CW, Oliveira SM, Rizzi C, Spanevello RM, Duarte MMF, Duarte T, Dellagostin OA, Andrade CM (2017) Anthocyanins control

neuroinflammation and consequent memory dysfunction in mice exposed to lipopolysaccharide. Mol Neurobiol 54(5):3350-3367. doi: 10.1007/s12035-016-9900-8

Catorce NM, Gevorkian G (2016) LPS-induced Murine Neuroinflammation Model: Main Features and Suitability for Pre-clinical Assessment of Nutraceuticals. Curr Neuropharmacol 14 (2): 155-164. doi: 10.2174/1570159X14666151204122017

Chen Y, Vartiainen NE, Ying W, Chan PH, Koistinaho J, Swanson RA (2001) Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. J Neurochem 77(6):1601-1610. doi: 10.1046/j.1471-4159.2001.00374.x

Cunningham C, Campion S, Lunnon K, Murray CL, Woods JFC, Deacon RMJ, Rawlins NP, Perry VH (2009) Systemic inflammation induces acute behavioral and cognitive changes and accelerates neurodegenerative disease. Biol Psychiatry 65:304–312. doi: 10.1016/j.biopsych.2008.07.024

Daulatzai AM (2016) Fundamental role of pan-inflammation and oxidative-nitrosative pathways in neuropathogenesis of Alzheimer's disease. Am J Neurodegener Dis 5:1-28.

Del-Rio D, Borges G, Crozier A (2010) Berry flavonoids and phenolics: bioavailability and evidence of protective effects. Br J Nutr 104(suppl3):S67-S90. doi: 10.1017/S0007114510003958

Dunn AJ, Swiergiel AH (2005) Effects of interleukin-1 and endotoxin in the forced swim and tail suspension tests in mice. Pharmacol Biochem Behav 81(3):688-693. doi: 10.1016/j.pbb.2005.04.019

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7(2):88-95. doi: 10.1016/0006-2952(61)90145-9

Fan ZL, Wang ZY, Zuo, LL, Tian SQ (2012) Protective effects of anthocyanins from lingonberry on radiation-induced damages. Int J Environ Res Public Health 9(12):4732-4743. doi: 10.3390/ijerph9124732

Fang J (2015) Classification of fruits based on anthocyanin types and relevance to their health effects. Nutrition 31:1301-1306. doi: 10.1016/j.nut.2015.04.015

Faria A, Meireles M, Fernandes I, Santos-Buelga C, Gonzalez-Manzano S, Duenas M, de Freitas V, Mateus N, Calhau C (2014) Flavonoid metabolites transport across a human BBB model. Food Chem 149:190–196. doi:10.1016/j.foodchem.2013.10.095

Ferreira-Vieira TH, Guimaraes IM, Silva FR, Ribeiro FM (2016) Alzheimer's Disease: targeting the cholinergic system. Curr Neuropharmacol 14(1):101-115. doi: 10.2174/1570159X13666150716165726

Fujii T, Mashimo M, Moriwaki Y, Misawa H, Ono S, Horiguchi K, Kawashima K (2017) Physiological functions of the cholinergic system in immune cells. J Pharmacol Sci 134(1):1-21. doi: 10.1016/j.jphs.2017.05.002

Gnatek Y, Zimmerman G, Goll Y, Najami N, Soreq H, Friedman A (2012) Acetylcholinesterase loosens the brain's cholinergic anti-inflammatory response and promotes epileptogenesis. Front Mol Neurosci 5:66. doi: 10.3389/fnmol.2012.00066

Gorina R, Font-Nieves M, Márquez-Kisinousky L, Santalucia T, Planas AM (2011) Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways. Glia 59(2):242-255. doi: 10.1002/glia.21094

Gottfried C, Valentim L, Salbego C, Karl J, Wofchuk ST, Rodnight R (1999) Regulation of protein phosphorylation in astrocyte cultures by external calcium ions: specific effects on the phosphorylation of glial fibrillary acidic protein (GFAP), vimentin and heat shock protein 27 (HSP27). Brain Res 833(2):142-149. doi: 10.1016/S0006-8993(99)01503-6

Greenfield S, Vaux DJ (2002) Parkinson’s disease, Alzheimer’s disease and motor neurone disease: identifying a common mechanism. Neuroscience 113(3):485-492. doi: 10.1016/S0306-4522(02)00194-X

Gutierres JM, Carvalho FB, Schetinger MR, Agostinho P, Marisco PC, Vieira JM, Rosa MM, Bohnert C, Rubin MA, Morsch VM, Spanevello R, Mazzanti CM (2014a) Neuroprotective effect of anthocyanins on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia in rats. Int J Dev Neurosci 33:88-97. doi: 10.1016/j.ijdevneu.2013.12.006

Gutierres JM, Carvalho FB, Schetinger MR, Marisco P, Agostinho P, Rodrigues M, Rubin MA, Schmatz R, da Silva CR, de P Cognato G, Farias JG, Signor C, Morsch VM, Mazzanti CM, Bogo M, Bonan CD, Spanevello R (2014b) Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type. Life Sci 96(1-2):7-17. doi: 10.1016/j.lfs.2013.11.014

Hamby ME, Sofroniew MV (2010) Reactive astrocytes as therapeutic targets for CNS disorders. Neurotherapeutics 7(4):494–506. doi: 10.1016/j.nurt.2010.07.003

Han Z, Li L, Wang L, Degos V, Maze M, Su H (2014) Alpha-7 nicotinic acetylcholine receptor agonist treatment reduces neuroinflammation, oxidative stress, and brain injury in mice with ischemic stroke and bone fracture. J Neurochem 131:498–508. doi: 10.1111/jnc.12817

Kipp M, Norkute A, Johann S, Lorenz L, Braun A, Hieble A, Gingele S, Pott F, Richter J, Beyer C (2008) Brain-region-specific astroglial responses in vitro after LPS exposure. J Mol Neurosci 35(2):235-243. doi: 10.1007/s12031-008-9057-7

Lau FC, Bielinski DF, Joseph JA (2007) Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. J Neurosci Res 85(5):1010-1017. doi: 10.1002/jnr.21205

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S (1999) Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med 5(12):1403-1409. doi: 10.1038/70978

Lin MT, Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443(7113):787-795. doi: 10.1038/nature05292

Liu Y, Hu J, Wu J, Zhu C, Hui Y, Han Y, Huang Z, Ellsworth K, Fan W (2012) α7 nicotinic acetylcholine receptor-mediated neuroprotection against dopaminergic neuron loss in an MPTP mouse model via inhibition of astrocyte activation. J Neuroinflammation 24: 9-98. doi: 10.1186/1742-2094-9-98

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275. doi: 10.1074/jbc.270.46.27489

Lüth HJ, Holzer M, Gärtner U, Staufenbiel M, Arendt T (2001) Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. Brain Res 913(1):57-67. doi: 10.1016/S0006-8993(01)02758-5

Mane C, Loonis M, Juhel C, Dufour C, Malien-Aubert C (2011) Food grade lingonberry extract: polyphenolic composition and in vivo protective effect against oxidative stress. J Agric Food Chem 59(7):3330-3339. doi: 10.1021/jf103965b

McGeer PL, Schwab C, Parent A, Doudet D (2003) Presence of reactive microglia in monkey substantia nigra years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration. Ann Neurol 54(5):599-604. doi: 10.1002/ana.10728

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247(10):3170–3175.

Morais CA, de Rosso VV, Estadella D, Pisani LP (2016) Anthocyanins as inflammatory modulators and the role of the gut microbiota. J Nutr Biochem 33:1-7. doi: 10.1016/j.jnutbio.2015.11.008

Moskaug JØ, Carlsen H, Myhrstad MC, Blomhoff R (2005). Polyphenols and glutathione synthesis regulation. Am J Clin Nutr 81(1suppl):277S-283S.

Nair V, Bang WY, Schreckinger E, Andarwulan N, Cisneros-Zevallos L (2015) Protective role of ternatin anthocyanins and quercetin glycosides from butterfly pea (Clitoria ternatea Leguminosae) blue flower petals against lipopolysaccharide (LPS)-induced inflammation in macrophage cells. J Agric Food Chem 63(28):6355-6365. doi: 10.1021/acs.jafc.5b00928

Park BS, Lee JO (2013) Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp Mol Med 45:e66. doi: 10.1038/emm.2013.97

Pasare C, Medzhitov R (2005) Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol 560:11-8. doi: 10.1007/0-387-24180-9_2

Patel H, McIntire J, Ryan S, Dunah A, Loring R (2017) Anti-inflammatory effects of astroglial α7 nicotinic acetylcholine receptors are mediated by inhibition of the NF-κB pathway and activation of the Nrf2 pathway. J Neuroinflammation 14(1):192. doi: 10.1186/s12974-017-0967-6

Pojer E, Mattivi F, Johnson D, Stockley CS (2013) The case for anthocyanin consumption to promote human health: a review. Compr Rev Food Sci Food Saf 12(5):483-508. doi: 10.1111/1541-4337.12024

Poole LB (2015) The basics of thiols and cysteines in redox biology and chemistry. Free Radic Biol Med 80:148-157. doi: 10.1016/j.freeradbiomed.2014.11.013

Roth S, Spalinger MR, Gottier C, Biedermann L, Zeitz J, Lang S, Weber A, Rogler G, Scharl M (2016) Bilberry-derived anthocyanins modulate cytokine expression in the intestine of patients with ulcerative colitis. PLoS One 11(5):e0154817. doi: 10.1371/journal.pone.0154817

Sharma A, Patro N, Patro IK (2016) Lipopolysaccharide-induced apoptosis of astrocytes: therapeutic intervention by minocycline. Cell Mol Neurobiol 36(4):577-592. doi: 10.1007/s10571-015-0238-y

Shen J-x, Yakel JL (2012) Functional α7 nicotinic ACh receptors on astrocytes in rat hippocampal CA1 slices. J Mol Neurosci 48:14–21. doi: 10.1007/s12031-012-9719-3

Shih PH, Yeh CT, Yen GC (2007) Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. J Agric Food Chem 55(23):9427-9435. doi: 10.1021/jf071933i

Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J, Ehrhart J, Silver AA, Sanberg PR, Tan J (2004) Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem 89(2):337-443. doi: 10.1046/j.1471-4159.2004.02347.x

Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathol 119(1):7-35. doi: 10.1007/s00401-009-0619-8

Sogo T, Terahara N, Hisanaga A, Kumamoto T, Yamashiro T, Wu S, Sakao K, Hou DX (2015) Anti-inflammatory activity and molecular mechanism of delphinidin 3-sambubioside, a Hibiscus anthocyanin. Biofactors 41(1):58-65. doi: 10.1002/biof.1201

Soreq H, Seidman S (2001) Acetylcholinesterase – new roles for an old actor. Nat Rev Neurosci 2(4):294–302. doi: 10.1038/35067589

Stewart VC, Heales SJ (2003) Nitric oxide-induced mitochondrial dysfunction: implications for neurodegeneration. Free Radic Biol Med 34(3):287-303. doi: 10.1016/S0891-5849(02)01327-8

Strathearn KE, Yousef GG, Grace MH, Roy SL, Tambe MA, Ferruzzi MG, Wu QL, Simon JE, Lila MA, Rochet JC (2014) Neuroprotective effects of anthocyanin- and proanthocyanidin-rich extracts in cellular models of Parkinsons disease. Brain Res 1555:60-77. doi: 10.1016/j.brainres.2014.01.047

Stuehr DJ, Nathan CF (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 169(5):1543-1555.

Subash S, Essa MM, Al-Adawi S, Memon MA, Manivasagam T, Akbar M (2014) Neuroprotective effects of berry fruits on neurodegenerative diseases. Neural Regen Res 9(16):1557-1566. doi: 10.4103/1673-5374.139483

Sun M, Yan H, Zou W, Wang Y, Li H, Wang X (2016) Lipopolysaccharide induces astrocyte activation and downregulates the expression of Kir4.1 channel. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 32(2):196-200.

Sun XL, Chen BY, Zhao HK, Cheng YY, Zheng MH, Duan L, Jiang W, Chen LW (2016) Gas1 up-regulation is inducible and contributes to cell apoptosis in reactive astrocytes in the substantia nigra of LPS and MPTP models. J Neuroinflammation 13:180. doi: 10.1186/s12974-016-0643-2

Thullbery MD, Cox HD, Schule T, Thompson CM, George KM (2005) Differential localization of acetylcholinesterase in neuronal and non-neuronal cells. J Cell Biochem 96(3):599-610. doi: 10.1002/jcb.20530

Von Bernhardi R, Ramírez G, De Ferrari GV, Inestrosa NC (2003) Acetylcholinesterase induces the expression of the beta-amyloid precursor protein in glia and activates glial cells in culture. Neurobiol Dis 14(3):447-57. doi: 10.1016/j.nbd.2003.08.014

Wakabayashi N, Slocum SL, Skoko JJ, Shin S, Kensler TW (2010) When NRF2 talks, who's listening? Antioxid Redox Signal 13(11):1649–1663. doi: 10.1089/ars.2010.3216

Wang J, Wang JA, Jiang YX, Zhou PP, Wang HH (2015) The mechanism of anthocyanin to anti-inflammatory and analgesic from Ligustrum fruit. Zhongguo Ying Yong Sheng Li Xue Za Zhi. 31(5):431-436.

Wang XF, Cynader MS (2000) Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem 74(4):1434-1442. doi: 10.1046/j.1471-4159.2000.0741434.x

Wee Yong V (2010) Inflammation in neurological disorders: a help or a hindrance? Neuroscientist 16(4):408–420. doi: 10.1177/1073858410371379

Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ, Rice-Evans C (2003) Interaction between flavonoids and the blood–brain barrier: in vitro studies. J Neurochem 85(1):180–192. doi**:** 10.1046/j.1471-4159.2003.01652.x

Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, Bagchi D (2007) Berry anthocyanins as novel antioxidants in human health and disease prevention. Mol Nutr Food Res 51(6):675-683. doi: 10.1002/mnfr.200700002

Legends of figures

Fig. 1 Experimental scheme of the two protocols used in the study. Astrocytes were obtained from primary cultures and maintained in standard conditions for 20 days. In the reversal protocol, astrocytes were exposed to lipopolysaccharide (LPS, 1 µg/mL) for 3 hours and subsequently treated with lingonberry extract. In the prevention protocol, cells were first treated with lingonberry extract for 3 hours and subsequently exposed to LPS

Fig. 2 Effects of lingonberry extract on cellular viability in primary astrocytic cultures after 24 hours (a) and 48 hours (b). Cellular viability of astrocytes treated with lipopolysaccharide (LPS) and lingonberry extract (reversal protocol) after treatment for 24 hours (c) and 48 hours (d). Cellular viability of astrocytes treated with lingonberry extract and LPS (prevention protocol) after treatment for 24 hours (e) and 48 hours (f). Cellular proliferation of astrocytes treated with LPS and lingonberry extract (reversal protocol) after treatment for 24 hours (g) and 48 hours (h). Cellular proliferation of astrocytes treated with LPS and lingonberry extract (prevention protocol) after treatment for 24 hours (i) and 48 hours (j). Values represent mean ± standard error of the mean. The experiments were performed in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey's *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, different from control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, different from LPS group

Fig 3 Representative phase-contrast microphotographs of astrocytes exposed to lipopolysaccharide (LPS) and treated with the lingonberry extract after 48 hours (images were taken using an Olympus inverted microscope; magnification 40×). Arrows indicate astrocytes evidencing the different morphologies found between the groups.

Fig. 4 Acetylcholinesterase (AChE) activity in primary astrocytic culture exposed to lipopolysaccharide (LPS) and lingonberry extract after 24 hours and 48 hours in both reversal and prevention protocols. Values represent the mean ± standard error from three independent experiments performed in triplicate. Data were analyzed by one-

way analysis of variance followed by Tukey's *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, different from control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, different from LPS group

Fig. 5 Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities in primary astrocytic culture exposed to lipopolysaccharide (LPS) and lingonberry extract after 24 hours and 48 hours in the reversal protocol. Values represent the mean ± standard error from three independent experiments performed in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey's *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, different from control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, different from LPS group

Fig. 6 Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities in primary astrocyte culture exposed to lipopolysaccharide (LPS) and lingonberry extract after 24 hours and 48 hours in the prevention protocol. Values represent the mean ± standard error from three independent experiments performed in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey's *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, different from control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, different from LPS group

Fig. 7 Mechanisms involved in the glioprotective actions of lingonberry extract in lipopolysaccharide (LPS)-induced damage in astrocytes. AChE, acetylcholinesterase; iNOS, inducible nitric oxide synthases; ROS, reactive oxygen species; SH, thiol content; SOD, superoxide dismutase.

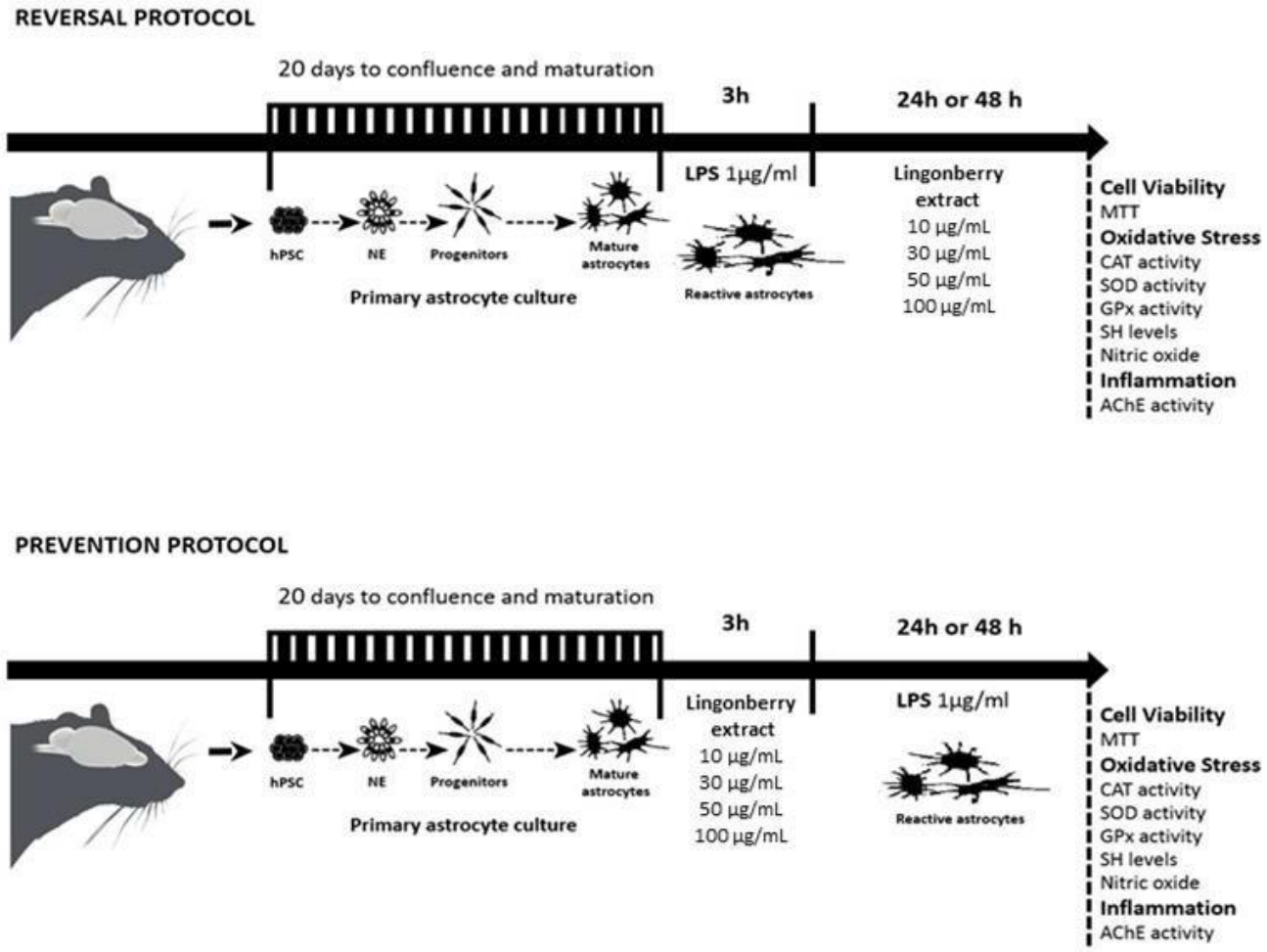
REVERSAL PROTOCOL
20 days to confluence and maturation
3h
24h or 48 h
LPS 1µg/ml
Lingonberry extract
10 µg/mL
30 µg/mL
50 µg/mL
100 µg/mL
hPSC
NE
Progenitors
Mature astrocytes
Primary astrocyte culture
Reactive astrocytes
Cell Viability
MTT
Oxidative Stress
CAT activity
SOD activity
GPx activity
SH levels
Nitric oxide
Inflammation
AChE activity
PREVENTION PROTOCOL
20 days to confluence and maturation
3h
24h or 48 h
Lingonberry extract
10 µg/mL
30 µg/mL
50 µg/mL
100 µg/mL
LPS 1µg/ml
hPSC
NE
Progenitors
Mature astrocytes
Primary astrocyte culture
Reactive astrocytes
Cell Viability
MTT
Oxidative Stress
CAT activity
SOD activity
GPx activity
SH levels
Nitric oxide
Inflammation
AChE activity

Fig. 1

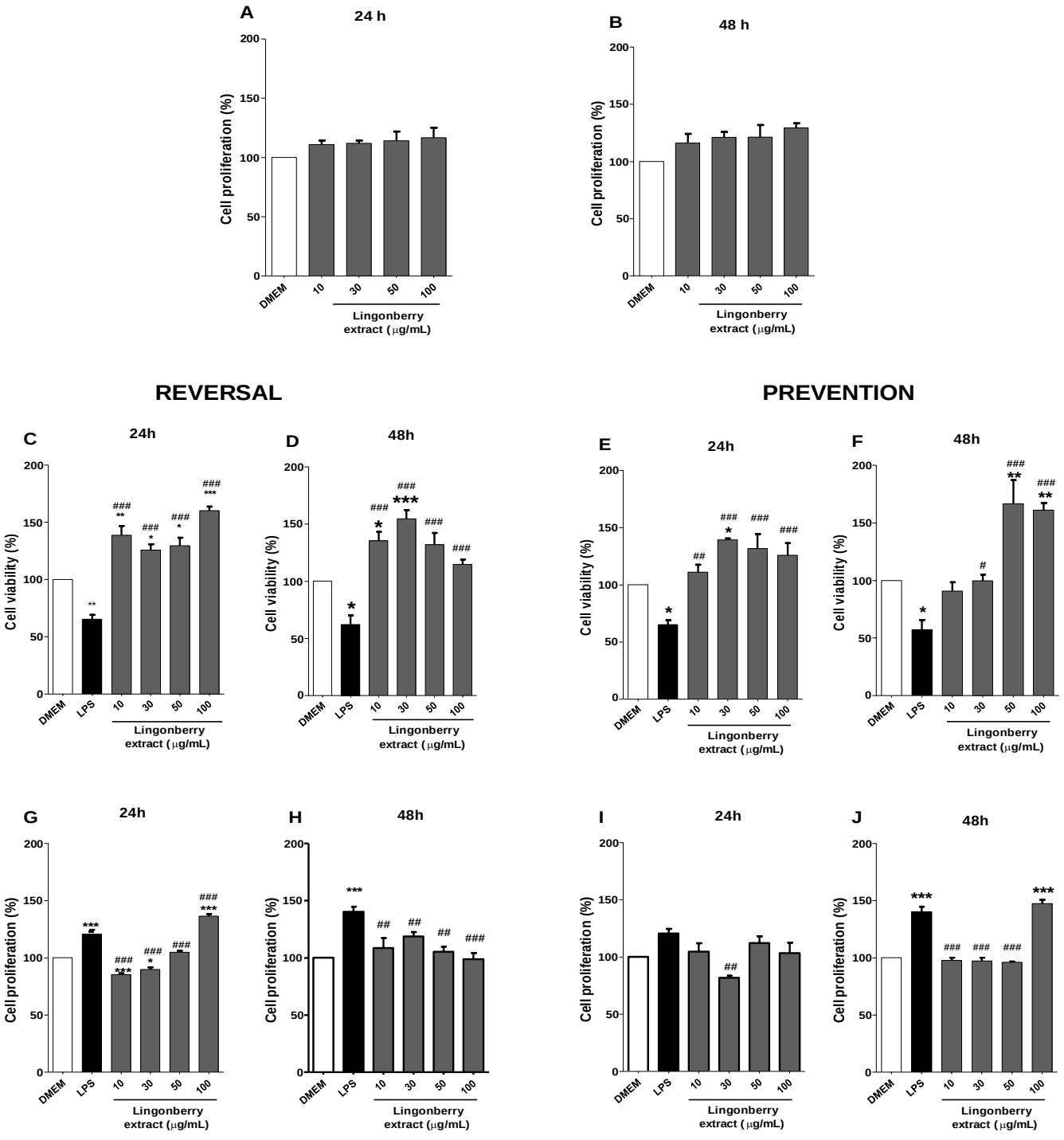
A
24 h
B
48 h
Cell proliferation (%)
DMEM
10
30
50
100
Lingonberry extract (μg/mL)
REVERSAL
PREVENTION
C
24h
D
48h
E
24h
F
48h
Cell viability (%)
LPS
G
24h
H
48h
I
24h
J
48h

Fig. 2

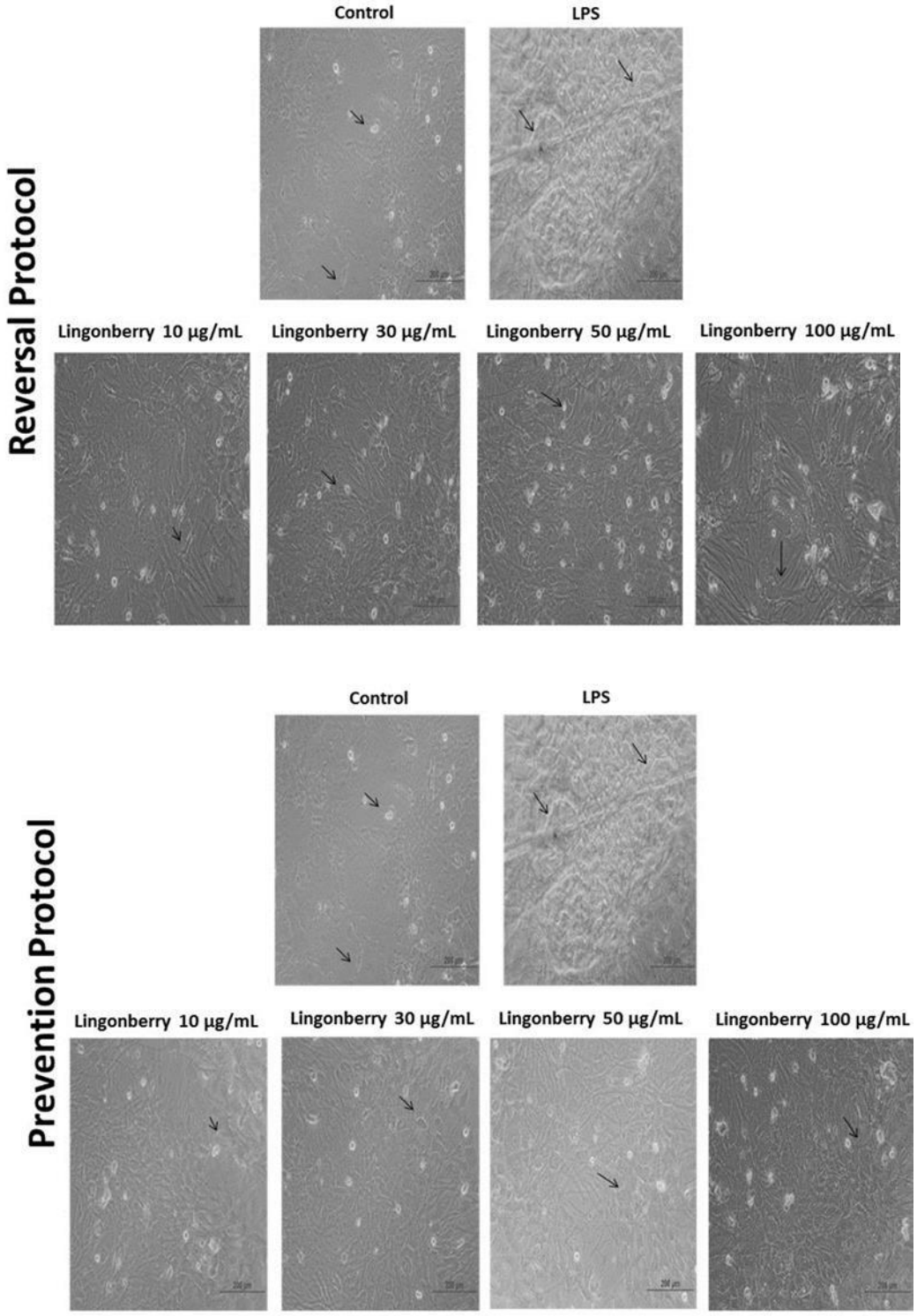
Reversal Protocol
Control
LPS
Lingonberry 10 µg/mL
Lingonberry 30 µg/mL
Lingonberry 50 µg/mL
Lingonberry 100 µg/mL
Prevention Protocol
Control
LPS
Lingonberry 10 µg/mL
Lingonberry 30 µg/mL
Lingonberry 50 µg/mL
Lingonberry 100 µg/mL

Fig. 3

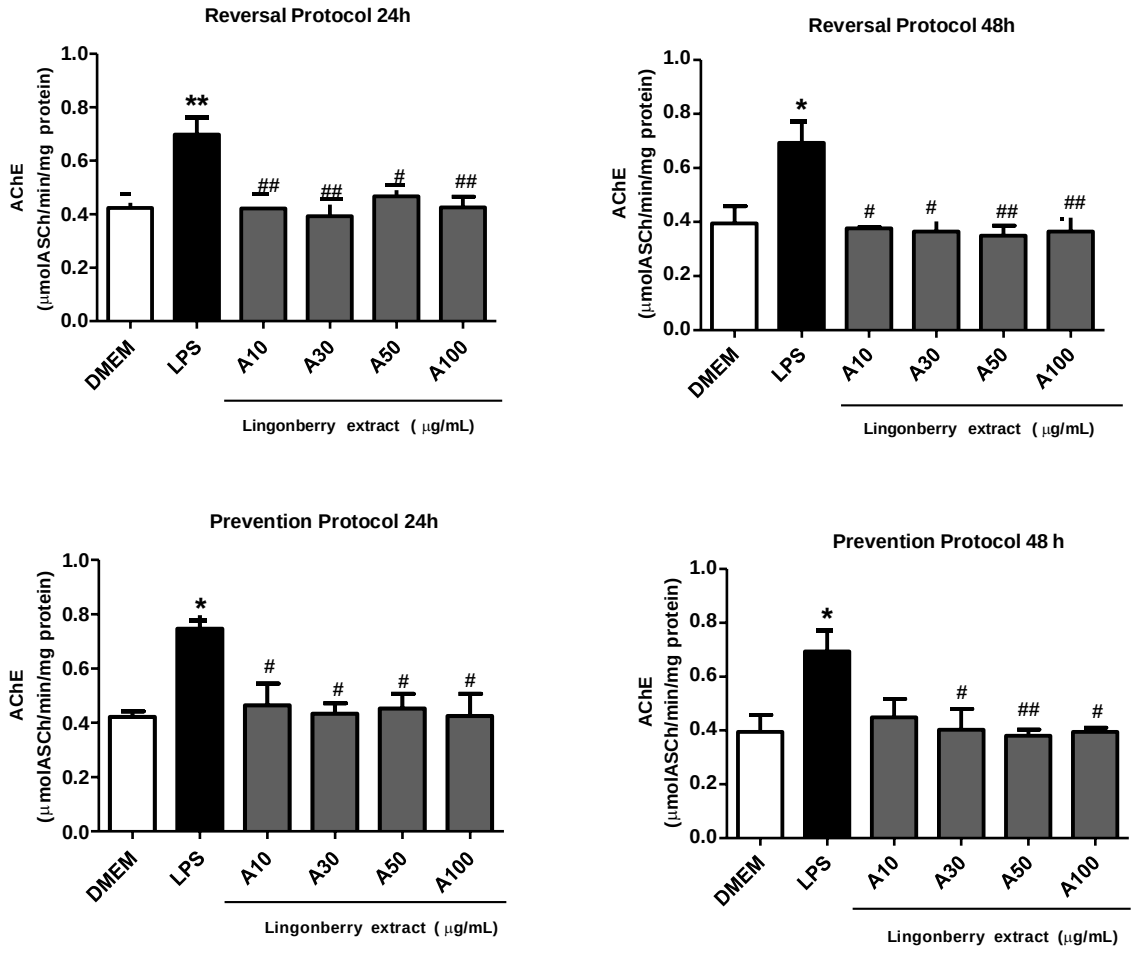
Reversal Protocol 24h
AChE
(μmolASCh/min/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
**
##
##
#
##
DMEM
LPS
A10
A30
A50
A100
Lingonberry extract (μg/mL)
Reversal Protocol 48h
AChE
(μmolASCh/min/mg protein)
*
#
#
##
##
DMEM
LPS
A10
A30
A50
A100
Lingonberry extract (μg/mL)
Prevention Protocol 24h
AChE
(μmolASCh/min/mg protein)
*
#
#
#
#
DMEM
LPS
A10
A30
A50
A100
Lingonberry extract (μg/mL)
Prevention Protocol 48 h
AChE
(μmolASCh/min/mg protein)
*
#
##
#
DMEM
LPS
A10
A30
A50
A100
Lingonberry extract (μg/mL)

Fig. 4

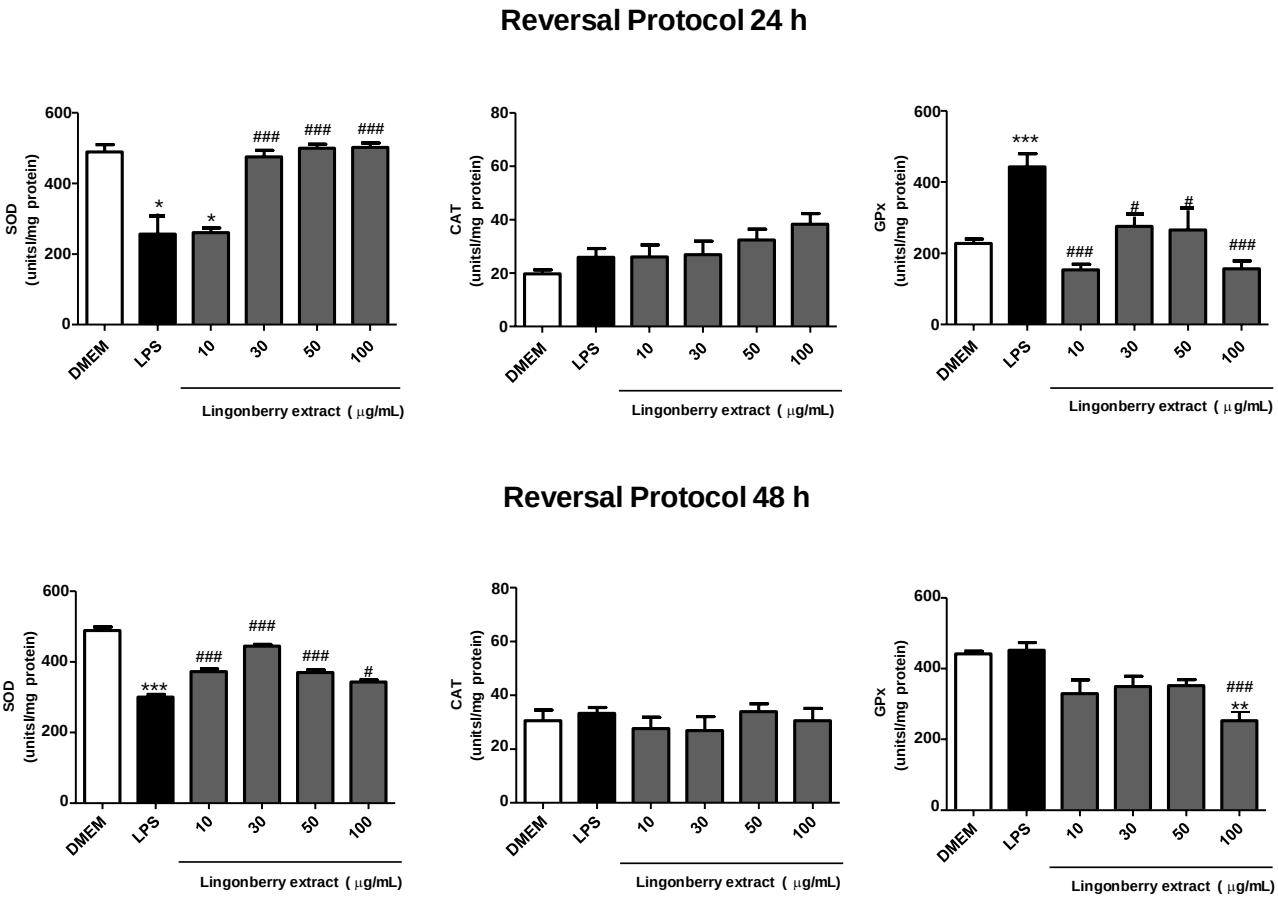
Reversal Protocol 24 h
SOD (units/mg protein)
CAT (units/mg protein)
GPx (units/mg protein)
DMEM
LPS
10
30
50
100
Lingonberry extract (μg/mL)
Reversal Protocol 48 h

Fig. 5

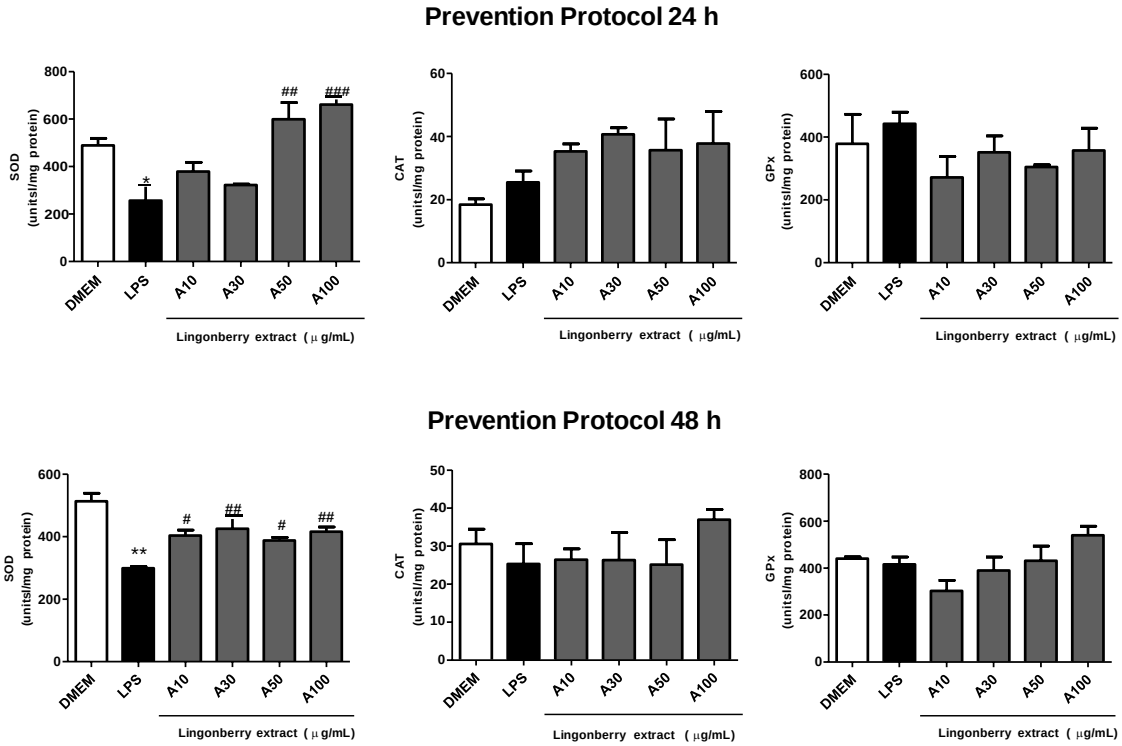
Prevention Protocol 24 h
SOD (units/mg protein)
CAT (units/mg protein)
GPx (units/mg protein)
DMEM
LPS
A10
A30
A50
A100
Lingonberry extract (μg/mL)
Prevention Protocol 48 h

Fig. 6

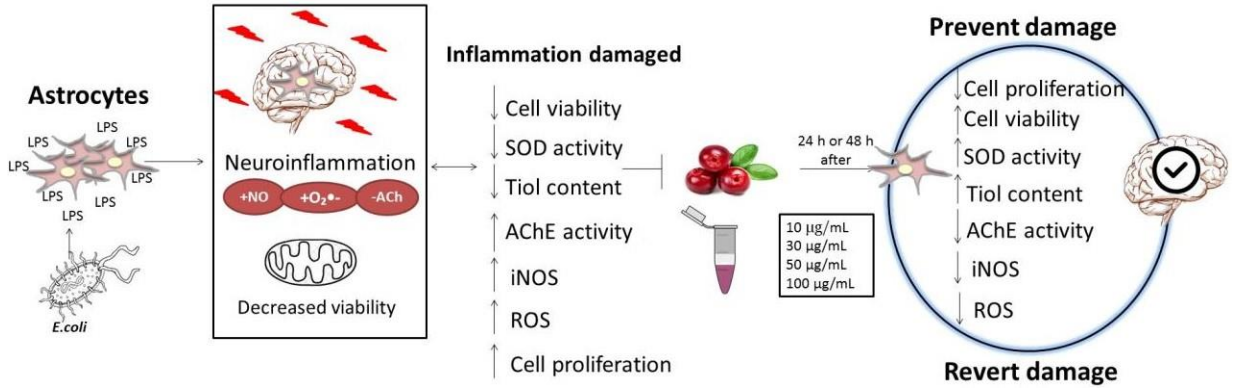
Astrocytes
LPS
E.coli
Neuroinflammation
+NO
+O2•-
-ACh
Decreased viability
Inflammation damaged
Cell viability
SOD activity
Tiol content
AChE activity
iNOS
ROS
Cell proliferation
24 h or 48 h after
10 µg/mL
30 µg/mL
50 µg/mL
100 µg/mL
Prevent damage
Cell proliferation
Cell viability
SOD activity
Tiol content
AChE activity
iNOS
ROS
Revert damage

Fig. 7

Table 1 -Reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) activity, and total thiol content (SH) in primary astrocytic culture exposed to lipopolysaccharide (LPS) and lingonberry extract (10-100 µg/mL) after 24 hours and 48 hours in both the reversal and prevention protocols.

	Reversal Protocol					
		24 h			**48 h**	
Groups	**ROS (%)**	**iNOS (nmol NO/mg of protein)**	**SH (nmol/mg of protein)**	**ROS (%)**	**iNOS (nmol NO/mg of protein)**	**SH (nmol/mg of protein)**
Control	100	79.4 ± 1.2	293±9.9	100	124.0±5.7	297.1±26.4
LPS	182.5±9.1***	156.1±9.9***	208.0±16.4**	241.9±24.5***	163.8±13.6**	156.8±7.8***
10 µg/mL	90.0±8.1$^{\#\#}$	78.3±7.4$^{\#\#\#}$	180.7±3.7***	147.2±11.6$^{\#\#}$	59.7±6.0$^{***\#\#\#}$	233.3±14.6
30 µg/mL	88.9±9.9$^{\#\#\#}$	106.3±15.8$^{\#}$	268.8±26.2	127.3±14.7$^{\#\#\#}$	72.3±0.6$^{***\#\#\#}$	241.9±12.4
50 µg/mL	73.3±7.2$^{\#\#\#}$	108.1±7.8$^{\#}$	237.9±8.2	123.5±17.5$^{\#\#\#}$	75.7±0.6$^{**\#\#\#}$	217.2±5.9
100 µg/mL	62.2±5.9$^{\#\#\#}$	100.0±10.4$^{\#\#}$	285.6±15.2$^{\#}$	85.1±12.6$^{\#\#\#}$	87.9±1.6$^{***\#\#\#}$	266.7±29.4$^{\#}$
	Prevention Protocol					
		24 h			**48 h**	
Groups	**ROS (%)**	**iNOS (nmol NO/mg of protein)**	**SH (nmol/mg of protein)**	**ROS (%)**	**iNOS (nmol NO/mg of protein)**	**SH (nmol/mg of protein)**
Control	100	79.4±1.2	301.5±7.16	100	121.0±6.3	297.1±26.4
LPS	160.2±9.8***	146.7±2.5***	208.4±16.42*	184.3 ±15.04***	173.3±13.7*	156.8±7.8***
10 µg/mL	73.92±4.86$^{\#\#\#}$	64.9±2.0$^{*\#\#\#}$	240.0±29.2	105.07±11.51$^{\#\#\#}$	76.8±6.5$^{*\#\#\#}$	285.7±9.1$^{\#\#\#}$
30 µg/mL	88.77±4.79$^{\#\#\#}$	113.2±3.6$^{***\#\#\#}$	198.0±16.3*	116.3±12.03$^{\#\#}$	85.6±18.4$^{\#\#\#}$	323.9±20.8$^{\#\#\#}$
50 µg/mL	68.37±9.12$^{\#\#\#}$	96.6±5.3$^{**\#\#\#}$	305.4±5.73$^{\#}$	109.7±14.71$^{\#\#}$	64.8±4.3$^{**\#\#\#}$	246.6±27.4$^{\#}$
100 µg/mL	74.48±8.87$^{\#\#\#}$	129.9±2.0$^{**\#}$	358.7±5.02$^{\#\#}$	105.3±14.46$^{\#\#\#}$	75.8±8.9$^{*\#\#\#}$	250.8 5.0$^{\#}$

Values represent the mean ± standard error from three independent experiments performed in triplicate. Data were analyzed by one-way analysis of variance followed by Tukey's *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, different from control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, different from LPS group.

Artigo 2

Anthocyanins as a potential pharmacological agent to manage memory deficit, oxidative stress and alterations in ion pump activity induced by experimental sporadic dementia of Alzheimer's type

Anthocyanins as a potential pharmacological agent to manage memory deficit, oxidative stress and alterations in ion pump activity induced by experimental sporadic dementia of Alzheimer's type

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Abstract

Anthocyanins (ANT) are polyphenolic flavonoids with antioxidant and neuroprotective properties. This study evaluated the effect of ANT treatment on cognitive performance and neurochemical parameters in an experimental model of sporadic dementia of Alzheimer's type (SDAT). Adult male rats were divided into four groups: control (1 mL/kg saline, once daily, by gavage), ANT (200 mg/kg, once daily, by gavage), streptozotocin (STZ, 3 mg/kg), and STZ *plus* ANT. STZ was administered via bilateral intracerebroventricular (ICV) injection (5 µL). ANT were administered after ICV injection for 25 days. Cognitive deficits (short-term memory and spatial memory), oxidative stress parameters, acetylcholinesterase (AChE) and Na^{+}-K^{+}-ATPase activity in the cerebral cortex and hippocampus were evaluated. ANT treatment protected against the worsening of memory in STZ-induced SDAT. STZ promoted an increase in AChE and Na^{+}-K^{+}-ATPase total and isoform activity in both structures; ANT restored this change. STZ administration induced an increase in lipid peroxidation and decrease in the level of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), in the cerebral cortex; ANT significantly attenuated these effects. In the hippocampus, an increase in reactive oxygen species (ROS), nitrite and lipid peroxidation levels, and SOD activity and a decrease in CAT and GPx activity were seen after STZ injection. ANT protected against the changes in ROS and antioxidant enzyme levels. In conclusion, the present study showed that treatment with ANT attenuated memory deficits, protect against oxidative damage in the brain, and restore AChE and ion pump activity in an STZ-induced SDAT in rats.

Keywords: anthocyanins; Alzheimer disease; oxidative stress; acetylcholinesterase; Na^{+}-K^{+}-ATPase; streptozotocin

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and reduced cognitive capacity [1]. It is the most common cause of dementia and its prevalence increases significantly with age [2,3]. It is estimated that 67.5 million people in the world will have AD by 2030 and this could reach 115.4 million by 2050 [4]. Sporadic AD is multifactorial and comprises the majority of cases of this disease [2,3,5,6].

The neuropathological changes underlying AD include extracellular senile plaques formed by β-amyloid peptide (Aβ) and intracellular accumulation of neurofibrillary tangles composed of hyperphosphorylated protein tau that promote synaptic dysfunction and neuronal death [7]. Furthermore, oxidative stress, neuroinflammation, altered ionic homeostasis, cholinergic dysfunction, and deficits in insulin signaling are also related with AD pathogenesis [8-10].

Oxidative stress has been associated with the initiation and progression of AD [11,12]. This is supported by the potential high vulnerability of neurons to reactive oxygen species (ROS), large amount of polyunsaturated fatty acids, high level of pro-oxidant metals, and low level of endogenous antioxidants in the brain [13,14]. Oxidative stress can induce membrane lipid damage, changes in enzymes critical to neuronal and glial function, and structural damage to DNA leading to tissue damage, synapse dysfunction, and cell death [15].

Furthermore, studies have also proposed a relationship between oxidative stress and dysfunction in acetylcholinesterase (AChE) and Na^+-K^+-ATPase activity in many diseases [16]. AChE is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) and this enzyme has been an important therapeutic target in AD since this pathology was first associated with the loss of cholinergic neurons and decrease in the level of ACh in the brain, leading to cognitive deficits [17,18]. In addition, Na^+-K^+-ATPase plays a role in intracellular ion and membrane potential homeostasis, regulating neuronal excitability. Alterations in Na^+-K^+-ATPase activity are associated with impaired synaptic responses, leading to alterations in the process of learning and memory [16].

Many studies in the literature have focused on the therapeutic potential of natural compounds aimed at the prevention of neurodegenerative diseases [19].

Anthocyanins (ANT), polyphenolic flavonoids found in various flowers, fruits, and vegetables, have demonstrated important biological activities, such as antioxidant and anti-inflammatory actions [20,21]. Our research group has reported the beneficial effects of ANT against lipopolysaccharide (LPS)-induced neuroinflammation [22], ethidium bromide-induced demyelination [23], and scopolamine-induced memory deficits [24]. Of particular importance in this study, we also demonstrated that pretreatment with ANT prevented the behavioral and neurochemical alterations caused by streptozotocin (STZ)-induced sporadic dementia of Alzheimer's type (SDAT) [25].

In this sense, considering the growing number of studies that provide evidence of the health benefits of ANT, the present study investigated the effect of ANT on protecting learning and memory ability in STZ-induced SDAT in rats. To delineate a possible mechanism of action for ANT, we also investigated the activity of key enzymes involved in cholinergic transmission, as well as oxidative and nitrosative stress markers, in the cerebral cortex and hippocampus of these animals.

2. Material and methods

Chemicals

Acetylthiocholine iodide (AcSCh), Coomassie Brilliant Blue G, ouabain, STZ, dichloro-dihydro-fluorescein diacetate (DCFH-DA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and adenosine triphosphate (ATP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). An anthocyanin-rich extract was obtained from grape skins (AC-12-R-WS-P/10120/Gin: 601412) and is commercially available from Christian Hansen A/S. All other reagents used in the detailed experiments were of analytical grade and the highest purity.

Animals

Forty adult male *Wistar* rats (2 months old) weighing 200–300 g were used in this study. The animals were kept in the Central Animal House of Federal University of Pelotas in colony cages under standard temperature (23 ± 1°C), relative humidity (45–55%), and lighting (12-h light/dark cycle) conditions for all trials. The rats had *ad libitum* access to an adequate commercial diet and water. This study was approved

by the Ethics Committee and Animal Experimentation of the Federal University of Pelotas under the protocol number CEEA 0179/2015.

Intracerebroventricular injection of STZ

For all surgical procedures, adult male rats were anesthetized with ketamine (100 mg/mL) and xylazine (20 mg/mL), administered intraperitoneally. The head of the animal was positioned in the stereotaxic apparatus and a midline sagittal incision was made in the scalp. The skull was drilled on both sides according to the stereotaxic coordinates used for the lateral ventricles. These coordinates were -0.8 mm anteroposterior and 1.5 mm mediolateral from the bregma and -4.0 mm dorsoventral to the dura with the bregma as the zero reference point [25,26]. The animals were divided into four groups: control (C), ANT, STZ, and STZ + ANT. The animals in the STZ and STZ + ANT groups received intracerebroventricular injection (ICV) of 3 mg/kg STZ dissolved in a citrate buffer (pH 4.5), delivered through the holes drilled in the skull [27]. The other groups received vehicle solution (citrate buffer) ICV. Both STZ and vehicle were injected using a 10 µL 28-gauge Hamilton® syringe and each ventricle received 5 µL of solution at a rate of 1 µL/min for five minutes.

Treatment with anthocyanin

Three days after the ICV injection of STZ or vehicle, the animals in the ANT and STZ + ANT groups were treated with 200 mg/kg ANT dissolved in saline daily by gavage for 25 days (see Figure 1). Animals in the Control and STZ groups received saline (1 mL/kg) also by gavage during the same time. The dose of ANT administered was selected according to findings from previous studies [25].

Behavioral tests

Open field test

After twenty-six days of STZ injection, the animals underwent an open field test. In this test, the animals were placed in the apparatus consisting of a square arena measuring 56 × 40 × 30 cm with the floor divided into twelve squares (12 × 12 cm each). Rats were placed individually in one of the four corners of the apparatus

and remained within the setup for five minutes. During this time, total crossing and rearing response were analyzed. The arena was cleaned with 40% ethanol after each individual session. This test allows quantification of locomotor activity and identification of motor disabilities and altered exploratory activity [25,28].

Object recognition test

Twenty-four hours after the open field test that was also used as habituation to the apparatus, the animals underwent an object recognition test to evaluate short-term memory. First, the animals were placed individually in the arena with two identical objects (object A and B) for five minutes and allowed to explore them freely (training session). After two hours, in the test session, the animals were placed again in the arena for five minutes but one of the previous objects (B) was replaced by a new object (object C). The time spent exploring the familiar and novel object was recorded. The objects used were pairs of plastic mounting blocks and were placed in a symmetrical position inside the arena. The arena and objects were cleaned between trials with 40% ethanol to remove residues and smells. Exploration was considered only when the rats were sniffing or touching the objects with their nose and/or forepaws. Results were analyzed using the recognition index for each animal calculated as follows, recognition index = TC/(TA+TC), where TA is the time taken to explore familiar object A and TC is the time taken to explore new object C [29].

Y-maze Test

Twenty-eight days after the STZ injection, the animals underwent a Y-maze test to evaluate spatial memory. This test used a Y-maze that had three arms, with each arm randomly designated as either the start arm (A), novel arm (B), or other arm (C). This test had a training session where the animal was placed in the apparatus on the start arm and was free to explore only the start arm and other arm for five minutes. The novel arm remained blocked throughout the training session. After two hours, the test session was performed with an open novel arm and the animal could freely explore all three arms over a five-minute period. The apparatus was cleaned with 40% ethanol after each session. The time spent in each arm was determined and the results were expressed as the percentage of time spent and number of entries on the new arm [30]. Twenty-four hours after the Y-maze test, the

animals were euthanized; and brain was removed and the hippocampus and cerebral cortex were dissected for evaluation using biochemical assays. Blood also was collected for analysis of glucose levels.

AChE activity determination

Samples of cerebral cortex and hippocampus were homogenized on ice in a glass potter with 10 mM Tris–HCl solution (pH 7.4). Aliquots of brain structure homogenates were stored at -80 °C until utilization. The protein content was determined using the Coomassie blue method, with bovine serum albumin as the standard solution [31].

The AChE enzymatic assay was determined using a spectrophotometric method [32]. This method is based on the formation of the yellow anion, 5,5'-dithio-bis-acid-nitrobenzoic, which was measured by absorbance at 412 nm, over a 2-min period at 25°C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM AcSCh. All samples were tested twice and the enzyme activity was expressed in µmol AcSCh/h/mg of protein.

Determination of total ATPase, Na^+-K^+-ATPase, and isoform activities

Na^+-K^+-ATPase activity was measured in the cerebral cortex and hippocampus supernatant as previously described [22]. Briefly, the assay medium consisted of 30 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, and 120 µg of protein in the presence or absence of ouabain (3 µM or 4 mM) to reach a final volume of 200 µL. The reaction was started by the addition of ATP to a final concentration of 3 mM. After 30 minutes at 37°C, the reaction was stopped by the addition of 50 µL of 50% (w/v) trichloroacetic acid (TCA). Saturating substrate concentrations were used, and the reaction rate was linear with respect to protein content and time. Appropriate controls were included in the assays for the non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described [33], using KH_2PO_4 as the reference standard. The absorbance at 630 nm was measured. The specific Na^+-K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and was expressed in nmol of Pi/mg of protein/min.

Different concentrations of ouabain were used to evaluate the activity of different Na^+-K^+-ATPase isoforms. A classical pharmacological approach based on the isoform-specific sensitivity to ouabain was used [34]. The experiments were performed as previously described [35]. To determine if treatments alter ouabain-sensitive ATPase activity, 3 µM or 4 mM ouabain was used (so as to only inhibit the Na^+-K^+-ATPase isoforms containing subunits α2/α3, or to every inhibit isoform, respectively).

Oxidative stress determination

2.8.1. Brain tissue preparation

The cerebral cortex and hippocampus were homogenized in sodium phosphate buffer (20 mM) containing KCl (140 mM), pH 7.4. The homogenates were centrifuged at 2500*g* for ten minutes at 4°C and the supernatant was separated to analyze oxidative stress parameters. The samples were stored at -80°C until utilization. Protein content was determined using the Lowry method [36], with bovine serum albumin as the standard solution.

Reactive oxygen species (ROS) determination

ROS formation was determined as previously described [37] with some modifications. The method was based on the oxidation of DCFH-DA to fluorescent dichlorofluorescein (DCF). DCF fluorescence was measured using excitation at 485 nm and emission at 520 nm 30 minutes after the addition of DCFH-DA to the sample. ROS levels were expressed as µmol DCF/mg of protein.

Nitrite levels quantification

Nitrite (NO_2^-) content was measured using the Griess reaction, as previously described [38]. Briefly, 50 µL of sample plus 50 µL of sulphanilamide in 5% phosphoric acid were incubated for 10 minutes at room temperature. Next, 50 µL of N-(1-naphthyl) ethylenediamine dihydrochloride was added and the mixture was incubated for 10 minutes at room temperature while protected from illumination. Absorbance at 540 nm was measured in a 96-well microplate reader. A sodium nitrite

solution was used as the reference standard and the results were expressed as µmol NO_2^- /mg of protein.

Thiobarbituric acid reactive substances (TBARS) level method

TBARS was used to determine lipid peroxidation through the malondialdehyde (MDA) levels according to a method described previously [39]. Briefly, 100 µL of tissue homogenate was mixed with 15% TCA and 0.67% thiobarbituric acid. This mixture was heated at 95°C for 30 minutes and cooled (4°C) after for 10 minutes. The absorbance was measured at 532 nm and the results were expressed as nmol TBARS/mg of protein.

Total thiol level assay

The total level of thiol groups was measured using the DTNB method according to a modified, previously described method [40]. Briefly, 10 µL of sample was mixed with 145 µL of PBS–EDTA (1 mM), pH 7.5. For the reaction, 10 µL of DTNB (10 mM) in PBS was added to the mixture. After one hour of incubation at room temperature, the absorbance was read at 412 nm and the results were expressed as nmol TNB/mg of protein.

Superoxide dismutase (SOD) activity assay

The activity of SOD was determined using a method that is based on measuring the inhibition of the autoxidation of adrenaline as described previously [41]. For this assay, the medium contained catalase (10 µM), 10 µL of sample, glycine buffer (50 mM, pH 10.2) and adrenaline (60 mM). SOD levels were assayed using the sample absorbance at 480 nm. The results were expressed as Units/mg of protein. One unit of SOD was defined as the amount of enzyme necessary to inhibit 50% of adrenaline autoxidation.

Catalase (CAT) activity assay

CAT activity was measured according to a previously described method [42]. For this analysis, 10 µL of sample was mixed with Triton X-100 (1:10 w/v) and potassium phosphate buffer (pH 7.0). The reaction was started with the addition of hydrogen peroxide (H_2O_2) in the reaction medium. This method is based on the ability

of the CAT to decompose H_2O_2 and is measured by the decrease in absorbance at 240 nm. One unit of CAT was defined as one µmol of H_2O_2 consumed per minute and the specific activity was expressed as Units/mg of protein.

Glutathione peroxidase (GPx) activity assay

The activity of GPx was analyzed using a commercial kit from Randox Laboratories Ltd. (United Kingdom) according to the manufacturer's instructions. This method is based on that previously described [43] in which GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is instantly converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The resultant decrease in absorbance at 340 nm was measured. One GPx unit was defined as 1 µmol of NADPH consumed per minute and the specific activity was recorded as Units/mg of protein.

Determination of glucose levels

Glucose levels were determined in serum using a commercial kit (Labtest® Diagnostica S.A. MG, Brazil), according with the manufacturer's instructions.

Statistical analysis

Statistical analysis of test results was carried out using one or two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* testing for multiple comparisons. $P < 0.05$ was considered to represent a significant difference in all experiments. All data were expressed as the mean ± SEM.

3. Results

Anthocyanins protect impairment of non-spatial memory induced by STZ administration

Figure 2 shows the effects of ANT treatment on the behavioral results from the open field, object recognition, and Y-maze tests. In the open field test, it was observed that STZ did not alter the number of total crossings or rearing responses,

suggesting that neither STZ nor ANT affected the locomotor or exploratory capacity of the animals (Fig. 2A and B).

Our results also demonstrated that STZ injection induced a memory impairment in the object recognition task and ANT treatment effectively attenuated the memory deficits [$F_{(1,23)} = 7.72$; $P < 0.05$; Fig. 2C] . In this study, we also evaluated the effects of the treatment with ANT and STZ on spatial memory in Y-maze apparatus. STZ decreased both the percentage of time spent [$F_{(1,32)} = 11.18$; $P < 0.01$; Fig. 2D] and number of entries [$F_{(1,36)} = 24.19$; $P < 0.001$; Fig. 2 E] into the novel arm. ANT treatment did not increase the time spent in the novel arm nor the number of entries into the novel arm.

Anthocyanins protect against alterations in AChE activity in both the cerebral cortex and hippocampus induced by STZ administration

Figure 3 shows the effect of ANT (200 mg/kg) and ICV-STZ (3 mg/kg) on AChE activity in the cerebral cortex and hippocampus of rats. STZ injection promoted a significant increase in AChE activity in both structures and ANT administration was able to protect against this increase in the cerebral cortex [$F_{(1,40)} = 7.73$; $P<0.01$; Fig. 3A] and hippocampus [$F_{(1,39)} = 12.67$; $P<0.01$; Fig. 3B].

Anthocyanins protect against alterations in Na^+-K^+-ATPase total and isoform activity in the cerebral cortex and hippocampus induced by STZ administration

Figures 4 and 5 shows the effect of ANT (200 mg/kg) and ICV-STZ (3 mg/kg) on the total ATPase, total Na^+-K^+-ATPase, and α2,3 and α1 isoforms activities in the cerebral cortex and hippocampus, respectively. STZ administration caused a significant increase in ATPase and Na^+-K^+-ATPase activity in the cerebral cortex and ANT treatment was able to protect against these changes [$F_{(1,20)} = 5.33$; $P<0.01$; Fig. 4A] [$F_{(1,20)} = 5.02$; $P<0.01$; Fig. 4B]. ANT treatment also protect against the increase induced by ICV-STZ administration in Na^+-K^+-ATPase α2,3 [$F_{(1,20)} = 14.06$; $P<0.001$; Fig. 4C] and α1 [$F_{(1,20)} = 14.70$; $P<0.001$; Fig. 4D] isoform activities.

Similar results were observed in the hippocampus. ICV-STZ also caused an increase in the total ATPase and Na^+-K^+-ATPase activities and ANT treatment was able to protect against these changes [$F_{(1,20)} = 5.24$; $P<0.05$; Fig. 5A] [$F_{(1,17)} = 4.97$; $P<0.05$; Fig. 5B]. ANT treatment also protect against the increase induced by ICV-

STZ administration in Na^+-K^+-ATPase α2,3 activity [$F_{(1,16)}$ = 5.71; P<0.05; Fig. 5C]. Regarding the Na^+-K^+-ATPase α1 isoform activity, no alterations were observed in any group evaluated in this study [$F_{(1,17)}$ = 0.1058; P=0.7490; Fig. 5D].

Anthocyanins protect against the changes in antioxidant responses in thecerebral cortex and hippocampus induced by STZ administration

Figure 6 shows the oxidative stress parameters in the cerebral cortex of rats that underwent STZ administration and were treated with ANT (200 mg/kg) for 25 days. First, it was observed that neither STZ nor ANT modified the ROS levels (Fig. 6A). However, STZ administration caused an increase in nitrite levels [$F_{(3,23)}$ = 5.39; P<0.05; Fig. 6B] that was not restore by ANT. STZ administration led to an increase in TBARS level, an effect attenuated by ANT treatment [$F_{(1,19)}$ = 6.21; P<0.05; Fig. 6C]. The sulfhydryl content did not significantly changed in this structure in any group (Fig. 6D). Regarding the antioxidant enzymes, it was observed that ICV-STZ injection promoted a reduction in their activity and ANT treatment was restored this effect on SOD [$F_{(1,15)}$ = 11.47; P<0.01; Fig. 6E] and CAT [$F_{(1,15)}$ = 7.29; P<0.05; Fig. 6F]. ANT also was able to protect against the decrease in GPx activity induced by STZ injection [$F_{(1,14)}$ = 8.89; P < 0.01; Fig. 6G]).

Figure 7 shows that ANT treatment was able to attenuated the increase in ROS levels ($F_{(1,15)}$ = 5.86; P < 0.05; Fig. 7A] in the hippocampus. Moreover ICV-STZ induced an increase in nitrite [$F_{(1,23)}$ = 8.27; P < 0.01; Fig. 7B] and TBARS levels [$F_{(1,20)}$ = 43.14; P < 0.001; 7C] however, ANT did not protect against this effect. No changes were observed in the total sulfhydryl content in any group (Fig. 7D). Regarding the antioxidant enzymes, it was observed that STZ injection increased SOD and decreased CAT and GPx activities. ANT administration protected against the changes in SOD [$F_{(1,15)}$ = 7.35; P<0.05; Fig. 7E], CAT [$F_{(1,15)}$ = 6.33; P<0.05; Fig. 7F], and GPx activity [$F_{(1,15)}$ = 7.50; P<0.05; Fig. 7G].

Treatment with anthocyanins and/or STZ did not alter the blood glucose levels

Figure 8 shows that the levels of blood glucose was not altered in any of the experimental groups evaluated in this study [$F_{(1,18)}$ = 1.47; P > 0.05].

4. Discussion

The present study investigated the neuroprotective potential of ANT in an STZ-induced model of SDAT in rats. This model promotes multiple alterations similar to those found in patients with AD, such as a decrease in brain glucose metabolism, oxidative stress, reduction in cholinergic signaling, neuroinflammation, neuronal loss, and impairment of learning and memory [44-47]. A recent study showed that pathological alterations in this model were dependent on the time since the STZ injection, with up to one month after injection considered an acute response, between one and three months a compensatory phase, and between six and nine months a decompensatory phase with a chronic progressive decline [48].

Our findings demonstrate that STZ impairs memory acquisition (short-term memory) and spatial memory. In addition, no difference between groups was observed in the open field test, excluding the possibility of STZ interfering with locomotor activity in the memory tests. These findings are in accordance with previous studies that used this rodent model [25,49-51-56].

The damage caused by ICV-STZ administration can reach septal and corpus callosum regions [57], and beyond, causing changes at the level of the fornix, anterior hippocampus, and periventricular structures [58]. These structures are involved in memory function, with the perirhinal cortex most strongly related to object recognition after short intervals and the hippocampus linked with spatial memory and long-term object recognition [59]. Moreover, the septum has cholinergic projections on to cortical regions and the hippocampus, which are also involved in memory acquisition [57,60], and it is suggested that ICV-STZ administration disrupts these connections, altering memory consolidation [57].

Treatment with ANT was able to protect against memory deficits induced by ICV-STZ only in the object recognition test. This finding is in accordance with previous studies that also showed that an ANT-rich diet could have learning and memory enhancing effects in mice with trimethyltin-induced neurotoxicity [61], improved short-term and working memory in old rats [62], and spatial learning and memory in D-galactose-treated rats [63] and transgenic AD murine model [64]. Furthermore, a recent study with humans showed that intake of an ANT-rich cherry juice for 12 weeks could have beneficial effects on cognitive performance in older adults with mild to moderate dementia [65].

In addition, our results also showed that ANT treatment restores AChE activity in the cerebral cortex and hippocampus in an experimental model of STZ-induced SDAT. It is well established in the literature that the cholinergic system plays a pivotal role in the regulation of learning and memory and shows changes during aging and AD progression [66,67]. In fact, cholinesterase inhibitors act by inhibiting the degradation of ACh, improving cholinergic transmission and reducing temporarily the cognitive deficit of symptomatic patients with AD [68]. The increase in AChE activity may lead to a decrease in ACh levels, contributing to memory deficits. This finding is in accordance with previous studies using STZ-induced SDAT [25,27,69,70].

Treatment with blueberries (*Vaccinium corymbosum* L.) for two months reduced AChE activity in D-galactose-treated rats [71]. In an in vitro assay, an ANT-rich grape skin extract inhibited AChE activity in a dose-dependent manner with an IC_{50} of 363.61 µg/mL [72]. Another study demonstrated that oral pretreatment with pelargonidin (10 mg/kg) attenuated the hippocampal increase in AChE activity in a model of AD induced by amyloid β_{25-35} peptide in rats [73]. Previous findings from our research group also demonstrated that ANT treatment protects against alterations in AChE activity and memory deficits caused by scopolamine administration [24].

The Na^+-K^+-ATPase enzyme plays a crucial role in maintaining the Na^+ and K^+ gradient across the plasma membrane. Three isoforms are expressed in the brain: the α1 isoform is found in many cell types, the α2 isoform is predominantly expressedin astrocytes, and the α3 isoform is exclusively expressed in neurons [74]. Data fromthe literature have demonstrated that dysfunctions in specific isoforms alter spatial learning and motor activity [74,75] and a decrease in Na^+-K^+-ATPase activity in the brain is associated with memory impairment [76,77].

In the present study, it was observed that STZ induced an increase in Na^+-K^+-ATPase activity, including the α1 and α2/α3 isoforms. A previous study reported a reduction in Na^+-K^+-ATPase activity 14 days after STZ injection, during the initial phase of the establishment of the SDAT model [25]. Other evidence has also showed that there is a reduction in the activity of this enzyme in different brain structures 21 days after STZ administration [78]. Studies have associated the reduction in Na^+-K^+-ATPase activity with markers of oxidative stress since this enzyme has -SH residues which makes it susceptible to oxidation. On the other hand, evidence shows that this enzyme has several amino acid residues, such as serine, tyrosine, and threonine,

that are targets of phosphorylation by cellular kinases. Glucose and insulin, for example, seem to play an important role in regulating Na^+-K^+-ATPase activity. It was described that 1, 2, and 3 hours after insulin administration there was a decrease in activity [79]. Moreover, the activation of insulin receptors results in reversible covalent modification of the catalytic subunits of intracellular signaling pathways that are utilized by insulin in controlling Na^+-K^+-ATPase activity, including phosphatidylinositol-3-kinase [80], AKT/ERK [81], PKA, and PKC [82,83]. Thus, is plausible suggest the metabolic dysfunctions caused by STZ administration mainly affect insulin receptor-dependent signaling pathways, impairing the regulation of Na^+-K^+-ATPase. However, our results showed an increase in Na^+-K^+-ATPase in brain after STZ administration demonstrating that other mechanisms besides insulin signaling may be involved in the alterations of this enzyme in this pathological condition [84,85].

Interestingly, it was demonstrated that pretreatment with ANT (200 mg/kg) for seven days prevented the decrease in the Na^+-K^+-ATPase in the cerebral cortex and hippocampus induced by scopolamine in rats [24]. It was also shown that ANT prevent the impairment of Na^+-K^+-ATPase in brain of experimentally demyelinated rats [22]. The beneficial effects of the ANT on brain function in learning and memory deficits and age-related neurodegeneration [21,86] include antioxidant and anti-inflammatory activities [20,87], modulation of neuronal signaling pathways and gene expression that are important to control synaptic plasticity [86], and improvement of cerebral blood flow [21]. Thus, considering that alterations in the Na^+-K^+-ATPase directly compromises axonal impulse transmission, our findings demonstrate that ANT also can contribute to the restoration of ATP levels in the brain.

Our results showed changes in oxidative stress parameters in the cerebral cortex and hippocampus after 28 days of ICV-STZ administration. It is well established that administration of ICV-STZ induces oxidative stress through ROS and reactive nitrogen species (RNS) generation [45]. ROS formation is involved with cellular injury in AD and is related in the initiation and development of memory impairments in rats [53,88]. Moreover, STZ treatment causes depletion of antioxidant systems, increase in the TBARS and protein carbonylation levels, decline in ATP level, and mitochondrial dysfunction [45,47].

Interestingly, the oxidative-nitrative damage caused by ICV-STZ was detected in the brain of rats 1 to 8 weeks after the injection [47]. The upregulation of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) protein and mRNA expression promoted by STZ injection may be an enzymatic source of the elevated nitrite level in the cortex and hippocampus in this model. Once expressed, iNOS produces a high level of nitric oxide (NO) constantly, having a toxic effect on neurons [86]. Moreover, the excess of superoxide ($O_2^{\bullet-}$) can react rapidly with NO, producing peroxynitrite ($ONOO^-$), which reacts with several biological molecules promoting damage and neuronal cell death [27,89-91].

Corroborating with our findings, other studies also showed a decrease in enzymatic antioxidant defense after ICV-STZ administration [27,92,93]. SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2, which can be neutralized through the action of other enzymes such as CAT and GPx [14]. It has been documented that ICV-STZ can reduce the GSH level. This is critical in eliminating H_2O_2 and organic peroxides by GPx since this enzyme depends on GSH as an electron donor for the reduction of peroxides [12,92,94]. The decrease in SOD, GPx, and CAT activity may impair $O_2^{\bullet-}$ and H_2O_2 removal and this can generate hydroxyl radicals and lipid peroxidation, triggering alterations in the biological properties of membranes [14,53,95]. In this study, lipid peroxidation was significantly increased in the ICV-STZ group as demonstrated by the TBARS content. Curiously, the higher SOD activity found in the hippocampus could be a compensatory mechanism in response to the increased $O_2^{\bullet-}$ accumulation. The hippocampus is especially vulnerable to the pathological alterations present in AD and the pyramidal neurons in the CA1 region is particularly susceptible to oxidative stress [96,97]. Furthermore, it has been shown that the neurons in this region contain higher levels of $O_2^{\bullet-}$ and ROS production [97]. Besides, there is a high demand for ROS/RNS as signaling molecules and this fact can be observed in CA1 neurons that require $O_2^{\bullet-}$ for long-term potentiation [97]. Evidences suggest that this scenario could lead a copper zinc superoxide dismutase (CuZnSOD) upregulation to facilitate the removal of the radicals and an increase in manganese superoxide dismutase (MnSOD) expression within the pyramidal neurons of the hippocampus [96,98] On the other hand, the overexpression of SOD may promote an increase in H_2O_2 formation that can induce a harmful oxidation of cell components [96,99].

In addition, ANT treatment was able to protect against the oxidative damage caused by ICV-STZ, suggesting that this effect may be associated with the improvement of dysfunctional memory. ANT have a direct free radical-scavenging activity due to the hydrogen donation capacity that is dependent on its chemical structure, especially the presence of hydroxyl groups in ring B [87,100]. The antioxidant action of ANT is further evidenced by indirect pathways, such as he modulation of antioxidant enzymes, reducing the formation of DNA oxidative abducts and endogenous ROS by inhibiting NADPH oxidase and xanthine oxidase, or by modifying mitochondrial respiration and arachidonic metabolism [87].

It is important to consider that ANTs are capable of crossing the blood brain barrier (BBB), and are found in several regions of the brain, including regions related to memory and learning such as the cortex and hippocampus [101-103]. In an in vitro study the transport of ANT across a BBB cell model was evaluated and suggested that these compounds and their metabolites were able to cross due to their lipophilicity [104].

In conclusion, the present study demonstrated that ANT treatment was able to protect against memory deficits in an experimental model of STZ-induced SDAT. These beneficial effects may be related with its antioxidant capacity and modulation of AChE and Na^+-K^+-ATPase activity in brain regions (Fig. 9).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

[1] Heppner FL, Ransohoff RM, Becher B. Immune attack: the role of inflammation in Alzheimer disease. Nat Rev Neurosci 2015;16(6):358-72.

[2] Bekris LM, Yu CE, Bird TD, Tsuang DW. Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol 2010;23(4):213-27.

[3] Mayeux R, Stern Y. Epidemiology of Alzheimer disease. Cold Spring Harb Perspect Med 2012;2(8):1-18.

[4] Nowrangi MA, Lyketsos CG, Rosenberg PB. Principles and management of neuropsychiatric symptoms in Alzheimer's dementia. Alzheimers Res Ther 2015;7:12.

[5] Mcdowell I. Alzheimer's disease: insights from epidemiology. Aging Clin Exp Res 2001;13(3):143-62.

[6] Scheltens P, Blennow K, Breteler MMB, Strooper B, Frisoni GB, Salloway S, et al. Alzheimer's disease. Lancet 2016;388(10043):505-17.

[7] Raskin J, Cummings J, Hardy J, Schuh K, Dean RA. Neurobiology of Alzheimer's disease: integrated molecular, physiological, anatomical, biomarker, and cognitive dimensions. Curr Alzheimer Res 2015;12(8):712-22.

[8] Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med 2010;362(4):329-44.

[9] Schliebs R, Arendt T. The cholinergic system in aging and neuronal degeneration. Behav Brain Res 2011;221(2):555-63.

[10] Awasthi M, Singh S, Pandey VP, Dwivedi UN. Alzheimer's disease: an overview of amyloid beta dependent pathogenesis and its therapeutic implications along with in silico approaches emphasizing the role of natural products. J Neurol Sci 2016;361:256-71.

[11] Fischer R, Maier O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. Oxid Med Cell Longev, 2015;2015:610813. http://dx.doi.org/10.1155/2015/610813

[12] Kim GH, Kim JE, Rhie SJ, Yoon S. The role of oxidative stress in neurodegenerative diseases. Exp Neurobiol 2015;24(4):325-40.

[13] Popa-Wagner A, Mitran S, Sivanesan S, Chang E, Buga AM. ROS and brain diseases: the good, the bad, and the ugly. Oxid Med Cell Longev 2013;2013:963520. http://dx.doi.org/10.1155/2013/963520

[14] Cervellati C, Wood PL, Romani A, Valacchi G, Squerzanti M, Sanz JM, et al. Oxidative challenge in Alzheimer's disease: state of knowledge and future needs. J Investig Med 2016;64(1):21-32.

[15] Huang WJ, Zhang X, Chen WW. Role of oxidative stress in Alzheimer's disease (review). Biomed Rep 2016;4(5):519-22.

[16] Zhang LN, Sun YJ, Pan S, Li JX, Qu YE, Li Y, et al. Na+-K+-ATPase, a potent neuroprotective modulator against Alzheimer disease. Fundam Clin Pharmacol 2013;27(1):96-103.

[17] Drever BD, Riedel G, Platt B. The cholinergic system and hippocampal plasticity. Behav Brain Res, 2011;221(2):505-14.

[18] Čolović MB, Krstić DZ, Lazarević-pašti TD, Bondžić AM, Vasić VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol 2013; 11(3):315-35.

[19] Choi DY, Lee YJ, Hong JT, Lee, HJ. Antioxidant properties of natural polyphenols and their therapeutic potentials for Alzheimer's disease. Brain Res Bull 2012;87(2-3):144-53.

[20] He J, Giusti MM. Anthocyanins: natural colorants with health-promoting properties. *Annu* Rev Food Sci Technol 2010;1:163-87.

[21] Tsuda T. Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Mol Nutr Food Res 2012;56(1):159-70.

[22] Carvalho FB, Gutierres JM, Bohnert C, Zago AM, Abdalla FH, Vieira JM, et al. Anthocyanins suppress the secretion of proinflammatory mediators and oxidative stress, and restore ion pump activities in demyelination. J Nutr Biochem 2015;26(4):378-90.

[23] Carvalho FB, Gutierres JM, Bueno A, Agostinho P, Zago AM, Vieira J, et al. Anthocyanins control neuroinflammation and consequent memory dysfunction in mice exposed to lipopolysaccharide. Mol Neurobiol 2017;54(5):3350-367.

[24] Gutierres JM, Carvalho FB, Schetinger MRC, Agostinho P, Marisco PC, Vieira JM, et al. Neuroprotective effect of anthocyanins on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia in rats. Int J Dev Neurosci 2014;33:88-97.

[25] Gutierres JM, Carvalho FB, Schetinger MR, Marisco P, Agostinho P, Rodrigues M, et al. Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type. Life Sci 2014:96(1-2):7-17.

[26] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 2nd ed San Diego: Academic Press; 1986.

[27] Tiwari V, Kuhad A, Bishnoi M, Chopra K. Chronic treatment with tocotrienol, an isoform of vitamin E, prevents intracerebroventricular streptozotocin-induced cognitive impairment and oxidative–nitrosative stress in rats. Pharmacol Biochem Behav 2009;93(2):183-9.

[28] Gutierres JM, Carvalho FB, Schetinger MR, Rodrigues MV, Schmatz R, Pimentel VC, et al. Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats. Life Sci 2012;91(23-24):1221-8.

[29] Da Silva AL, Piato AL, Ferreira JG, Martins BS, Nunes DS, Elisabetsky E. Promnesic effects of Ptychopetalumolacoides in aversive and non-aversive learning paradigms. J Ethnopharmacol 2007;109(3):449-57.

[30] Cognato GP, Agostinho PM, Hockemeyer J, Müller CE, Souza DO, Cunha RA. Caffeine and an adenosine A_{2A} receptor antagonist prevent memory impairment and synaptotoxicity in adult rats triggered by a convulsive episode in early life. J Neurochem 2010;112(2):453-62.

[31] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

[32] Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.

[33] Fiske CH, Subbarow Y. The nature of the “inorganic phosphate” in voluntary muscle. Science 1927;65(1686):401-3.

[34] Nishi A, Fisone G, Snyder GL, Dulubova I, Aperia A, Nairn AC, et al. Regulation of Na^+,K^+-ATPase isoforms in rat neostriatum by dopamine and protein kinase C. J Neurochem 1999;73(4):1492-501.

[35] Rambo LM, Ribeiro LR, Schramm VG, Berch AM, Stamm DN, Della-Pace ID, et al. Creatine increases hippocampal Na+,K+-ATPase activity via NMDA–calcineurin pathway. Brain Res Bull 2012;88(6):553-9.

[36] Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.

[37] Ali SF, LeBel CP, Bondy SC. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology 1992;13(3):637-48.

[38] Huang WC, Lin YS, Wang CY, Tsai CC, Tseng HC, Chen CL, et al. Glycogen synthase kinase-3 negatively regulates anti-inflammatory interleukin-10 for

lipopolysaccharide-induced iNOS/NO biosynthesis and RANTES production in microglial cells. Immunology 2009;128(1 suppl):e275-86.

[39] Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal**.** Methods Enzymol 1990;186(1):407-21.

[40] Aksenov MY, Markesbery WR. Change in thiol content and expression of glutathione redox system gene in the hippocampus and cerebellum in Alzheimer´s disease. Neurosci Lett 2001;302(2-3):141-5.

[41] Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247(10):3170-5.

[42] Aebi H. Catalase in vitro. Methods Enzymol 1984;105:121-6.

[43] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte gluthatione peroxidase. J Lab Clin Med 1967;70(1):158-69.

[44] Salkovic-Petrisic M, Hoyer S. Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach. J Neural Transm 2007;72:217-33.

[45] Kamat PK, Kalani A, Rai S, Tota SK, Kumar A, Ahmad AS. Streptozotocin intracerebroventricular-induced neurotoxicity and brain insulin resistence: a therapeutic intervention for treatment of sporadic Alzheimer's disease (sAD)-like pathology. Mol Neurobiol 2016;53(7):4548-62.

[46] Salkovic-Petrisic M, Osmanovic-Barilar J, Knezovic A, Hoyer S, Mosetter K, Reutter W. Long-term oral galactose treatment prevents cognitive deficits in male Wistar rats treated intracerebroventricularly with streptozotocin. Neuropharmacology 2014;77:68-80.

[47] Salkovic-Petrisic M, Knezovic A, Hoyer S, Riederer P. What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research. J Neural Transm 2013;120(1):233-52.

[48] Knezovic A, Osmanovic-Barilar J, Curlin M, Hof PR, Simic G, Riederer P, et al. Staging of cognitive deficits and neuropathological and ultrastructural changes in streptozotocin-induced rat model of Alzheimer's disease. J Neural Transm 2015;122(4):577-92.

[49] Saxena G, Patro IK, Nath C. ICV STZ induced impairment in memory and neuronal mitochondrial function: A protective role of nicotinic receptor. Behav Brain Res 2011;224(1):50-7.

[50] Deshmukh R, Kaundal M, Bansal V, Samardeep. Caffeic acid attenuates oxidative stress, learning and memory deficit in intra-cerebroventricular streptozotocin induced experimental dementia in rats. Biomed Pharmacother 2016;81:56-62.

[51] Ravelli KG, Rosário BD, Camarini R, Hernandes MS, Britto LR. Intracerebroventricular streptozotocin as a model of Alzheimer's disease: neurochemical and behavioral characterization in mice. Neurotox Res 2017;31(3):327-33.

[52] Shoham, S.; Bejar, C.; Kovalev, E.; Schorer-Apelbaum, D.; Weinstock, M. Ladostigil prevents gliosis, oxidative-nitrative stress and memory deficits induced by intracerebroventricular injection of streptozotocin in rats. Neuropharmacology 2007;52(3):836-43.

[53] Ishrat T, Hoda MN, Khan MB, Yousuf S, Ahmad M, Khan MM, et al. Amelioration of cognitive deficits and neurodegeneration by curcumin in rat model of sporadic dementia of Alzheimer's type (SDAT). Eur Neuropsychopharmacol 2009;19(9):636-47.

[54] Javed H, Khan MM, Ahmad A, Vaibhav K, Ahmad ME, Khan A, et al. Rutin prevents cognitive impairments by ameliorating oxidative stress and

neuroinflammation in rat model of sporadic dementia of Alzheimer type. Neuroscience 2012;210:340-52.

[55] Ejaz Ahmed M, Khan MM, Javed H, Vaibhav K, Khan A, Tabassum R, et al. Amelioration of cognitive impairment and neurodegeneration by catechin hydrate in rat model of streptozotocin-induced experimental dementia of Alzheimer's type. Neurochem Int 2013;62(4):492-501.

[56] Biasibetti R, Almeida Dos Santos JP, Rodrigues L, Wartchow KM, Suardi LZ, Nardin P, et al. Hippocampal changes in STZ-model of Alzheimer's disease are dependente on sex. Behav Brain Res 2017;316:205-14.

[57] Kraska A, Santin MD, Dorieux O, Joseph-Mathurin N, Bourrin E, Petit F, et al. In vivo cross-sectional characterization of cerebral alterations induced by intracerebroventricular administration of streptozotocin. PLoS One 2012;7(9):e46196.

[58] Shoham S, Bejar C, Kovalev E, Weinstock M. Intracerebroventricular injection of streptozotocin causes neurotoxicity to myelin that contributes to spatial memory deficits in rats. Exp Neurol 2003;184(2):1043-52.

[59] Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process, 2012;13(2):93-110.

[60] De Jaeger X, Cammarota M, Prado MA, Izquierdo I, Prado VF, Pereira GS. Decreased acetylcholine release delays the consolidation of object recognition memory. Behav Brain Res 2013;238:62-8.

[61] Jo YN, Jin DE, Jeong JH, Kim HJ, Kim D, Heo HJ. Effect of anthocyanins from Rabbit-eye blueberry (Vaccinium virgatum) on cognitive function in mice under trimethyltin-induced neurotoxicity. Food Sci Biotechnol 2015;24(3):1077-85.

[62] Ramirez MR, Izquierdo I, Do Carmo Bassols Raseira M, Zuanazzi JA, Barros D, Henriques AT. Effect of lyophilised *Vaccinium* berries on memory, anxiety and locomotion in adult rats. Pharmacol Res 2005;52(6):457-62.

[63] Rehman SU, Shah SA, Ali T, Chung JI, Kim MO. Anthocyanins reversed D-galactose-induced oxidative stress and neuroinflammation mediated cognitive impairment in adult rats. Mol Neurobiol 2017;54(1):255-71.

[64] Vepsäläinen S, Koivisto H, Pekkarinen E, Mäkinen P, Dobson G, McDougall GJ, et al. Anthocyanin-enriched bilberry and blackcurrant extracts modulate amyloid precursor protein processing and alleviate behavioral abnormalities in the APP/PS1 mouse model of Alzheimer's disease. J Nutr Biochem 2013;24(1):360-70.

[65] Kent K, Charlton K, Roodenrys S, Batterham M, Potter J, Traynor V, et al. Consumption of anthocyanin-rich cherry juice for 12 weeks improves memory and cognition in older adults with mild-to-moderate dementia. Eur J Nutr 2017;56(1):333-41.

[66] Anand P, Singh B. A review on cholinesterase inhibitors for Alzheimer’s disease. Arch Pharm Res 2013;36(4):375-99.

[67] Lagarde J, Sarazin M, Chauviré V, Stankoff B, Kas A, Lacomblez L, et al. Cholinergic changes in aging and Alzheimer disease an [^{18}F]-F-A-85380 exploratory PET study. Alzheimer Dis Assoc Disord 2017;31(1):8-12.

[68] Darreh-Shori T, Hellström-Lindahl E, Flores-Flores C, Guan ZZ, Soreq H, Nordberg A. Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer’s disease patients. J Neurochem 2004;88(5):1102-113.

[69] Saxena G, Singh SP, Agrawal R, Nath C. Effect of donepezil and tacrine on oxidative stress in intracerebral streptozotocin-induced model of dementia in mice. Eur J Pharmacol 2008;581(3):283-9.

[70] Sharma V, Bala A, Deshmukh R, Bedi KL, Sharma PL. Neuroprotective effect of RO-20-1724-a phosphodiesterase4 inhibitor against intracerebroventricular streptozotocin induced cognitive deficit and oxidative stress in rats. Pharmacol Biochem Behav 2012;101(2):239-45.

[71] Çoban J, Doğan-Ekici I, Aydın AF, Betül-Kalaz E, Doğru-Abbasoğlu S, Uysal M. Blueberry treatment decreased D-galactose-induced oxidative stress and brain damage in rats. Metab Brain Dis 2015;30(3):793-802.

[72] Pervin M, Hasnat MA, Lee YM, Kim DH, Jo JE, Lim BO. Antioxidant activity and acetylcholinesterase inhibition of grape skin anthocyanin (GSA). Molecules 2014;19(7):9403-18.

[73] Sohanaki H, Baluchnejadmojarad T, Nikbakht F, Roghani M. Pelargonidin improves memory deficit in amyloid β25-35 rat model of Alzheimer's disease by inhibition of glial activation, cholinesterase, and oxidative stress. Biomed Pharmacother 2016;83:85-91.

[74] Moseley AE, Williams MT, Schaefer TL, Bohanan CS, Neumann JC, Behbehani MM, et al. Deficiency in Na,K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. J Neurosci 2007;27(3):616-26.

[75] Lingrel JB, Williams MT, Vorhees CV, Moseley AE. Na,K-ATPase and the role of alpha isoforms in behavior. J Bioenerg Biomembr 2007;39(5-6):385-9.

[76] Lima FD, Souza MA, Furian AF, Rambo LM, Ribeiro LR, Martignoni FV, et al. Na+, K+-ATPase activity impairment after experimental traumatic brain injury: relationship to spatial learning deficits and oxidative stress. Behav Brain Res 2008;193(2):306-10.

[77] Da Silva FD, Pinz MP, De Oliveira RL, Rodrigues KC, Ianiski FR, Bassaco MM, et al. Organosulfur compound protects against memory decline induced by scopolamine through modulation of oxidative stress and Na+/K+ ATPase activity in mice. Metab Brain Dis 2017. http://dx.doi.org/10.1007/s11011-017-0067-4

[78] Ishrat T, Parveen K, Hoda MN, Khan MB, Yousuf S, Ansari MA, et al. Effects of pycnogenol and vitamin E on cognitive deficits and oxidative damage induced by intracerebroventricular streptozotocin in rats. Behav Pharmacol 2009;20(7):567-75.

[79] Kaur G, Arora SK. Acetylcholinesterase and Na+,K(+)-ATPase activities in different regions of rat brain during insulin-induced hypoglycemia. Mol Chem Neuropathol 1994;21(1):83-93.

[80] Sweeney G, Klip A. Regulation of the Na+,K+-ATPase by insulin: why and how? Mol Cell Biochem 1998;182(1-2):121-33.

[81] Al-Khalili L, Kotova O, Tsuchida H, Ehrén I, Féraille E, Krook A, et al. ERK1/2 mediates insulin stimulation of Na,K-ATPase by phosphorylation of the α-subunit in human skeletal muscle cells. J Biol Chem 2004;279(24):25211-8.

[82] Chibalin AV, Kovalenko MV, Ryder JW, Féraille E, Wallberg-Henriksson H, Zierath JR. Insulin- and glucose-induced phosphorylation of the Na(+),K(+)-adenosine triphosphatase alpha-subunits in rat skeletal muscle. Endocrinology 2001;142(8):3474-82.

[83] Rosić NK, Standaert ML, Pollet RJ. The mechanism of insulin stimulation of (Na+,K+)-ATPase transport activity in muscle. J Biol Chem 1985;260(10):6206-12.

[84] Lores GR, Bersier MG. Relationship between Na+, K+-ATPase and NMDA receptor at central synapses. Curr Protein Pept Sci 2014;15(8):761-77.

[85] Hernández-R J. Na+/K(+)-ATPase regulation by neurotransmitters. Neurochem Int 1992; 20(1):1-10

[86] Rendeiro C, Rhodes JS, Spencer JP. The mechanisms of action of flavonoids in the brain: Direct versus indirect effects. Neurochem Int 2015;89:126-39.

[87] Pojer E, Mattivi F, Johnson D, Stockley CS. The case for anthocyanin consumption to promote human health: a review. Compr Rev Food Sci Food Saf 2013;12(5):483-508.

[88] Rai S, Kamat PK, Nath C, Shukla R. A study on neuroinflammation and NMDA receptor function in STZ (ICV) induced memory impaired rats. J Neuroimmunol 2013;254(1-2):1-9.

[89] Halliwell, B. Oxidative stress and neurodegeneration: where are we now? J Neurochem 2006;97(6):1634-58.

[90] Darvesh AS, Carroll RT, Bishayee A, Geldenhuys WJ, Van der Schyf CJ. Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. Expert Rev Neurother 2010;10(5):729-45.

[91] Tota S, Awasthi H, Kamat PK, Nath C, Hanif K. Protective effect of quercetin against intracerebral streptozotocin induced reduction in cerebral blood flow and impairment of memory in mice. Behav Brain Res 2010;209(1):73-9.

[92] Khan MB, Khan MM, Khan A, Ahmed ME, Ishrat T, Tabassum R, et al. Naringenin ameliorates Alzheimer's disease (AD)-type neurodegeneration with cognitive impairment (AD-TNDCI) caused by the intracerebroventricular-streptozotocin in rat model. Neurochem Int 2012;61(7):1081-93.

[93] Mansouri MT, Naghizadeh B, Ghorbanzadeh B, Farbood Y, Sarkaki A, Bavarsad K. Gallic acid prevents memory deficits and oxidative stress induced by intracerebroventricular injection of streptozotocin in rats. Pharmacol Biochem Behav 2013;111:90-6.

[94] Correia SC, Santos RX, Santos MS, Casadesus G, Lamanna JC, Perry G, et al. Mitochondrial abnormalities in a streptozotocin-induced rat model of sporadic Alzheimer's disease. Curr Alzheimer Res 2013;10(4):406-19.

[95] Javed H, Vaibhav K, Ahmed ME, Khan A, Tabassum R, Islam F, et al. Effect of hesperidin on neurobehavioral, neuroinflammation, oxidative stress and lipid alteration in intracerebroventricular streptozotocin induced cognitive impairment in mice. J Neurol Sci 2015;348(1-2):51-9.

[96] Ceballos I, Javoy-Agid F, Delacourte A, Defossez A, Lafon M, Hirsch E, Nicole A, Sinet PM, Agid Y. Neuronal localization of copper-zinc superoxide dismutase protein and mRNA within the human hippocampus from control and Alzheimer's disease brains. Free Radic Res Commun 1991;12-13:571-80.

[97] Delacourte, A, Defossez A, Ceballos I, Nicole A, Sinet PM. Preferential localization of copper zinc superoxide dismutase in the vulnerable cortical neurons in Alzheimer's disease. Neurosci Lett 1988;92(3):247-53.

[98] DL, Strafaci JA, Freedman, ML. Differential neuronal expression of manganese superoxide dismutase in Alzheimer's disease. Med Sci Monit 2006;12(1)BR8-14.

[99] Wang X, Michaelis EK. Selective neuronal vulnerability to oxidative stress in the brain. Front Aging Neurosci 2010;2:12.

[100] Miguel MG. Anthocyanins: antioxidant and/or anti-inflammatory activities. J Appl Pharm Sci 2011;1(6):07-15.

[101] Andres-Lacueva C, Shukitt-Hale B, Galli RL, Jauregui O, Lamuela-Raventos RM, Joseph JA. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. Nutr Neurosci 2005;8(2):111-20.

[102] Talavéra S, Felgines C, Texier O, Besson C, Gil-Izquierdo A, Lamaison JL, et al. Anthocyanins metabolism in rats and their distribution to digestive area, kidney, and brain. J Agric Food Chem 2005;53(10):3902-8.

[103] Kalt W, Blumberg JB, McDonald JE, Vinqvist-Tymchuk MR, Fillmore SA, Graf BA, et al. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. J Agric Food Chem 2008;56(3):705-12.

[104] Faria A, Meireles M, Fernandes I, Santos-Buelga C, Gonzalez-Manzano S, Dueñas M, et al. Flavonoid metabolites transport across a human BBB model. Food Chem 2014;149:190-6.

Legends of figures

Figure 1 - Scheme for induction protocol of experimental model of sporadic dementia of Alzheimer's type and anthocyanins (200 mg/kg) treatment in rats.

Figure 2 - Effects of treatment with anthocyanins (200 mg/kg) on the results of behavioral tests of animals that were administered intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. The locomotor activity behavior was analyzed using the open field task on the number of total crossings (A) and rearing responses (B). The non-spatial memory and spatial memory was analyzed respectively using the object recognition task (C) and Y-maze apparatus: percentage of time spent (D) and number of entries (E) into the novel arm. Data are expressed as mean±S.E.M. *Denotes significant difference from the vehicle group for $P<0.05$; ***$P<0.001$. # Denotes significant difference from the STZ group for $P<0.05$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 3 - Acetylcholinesterase (AChE) activity in the cerebral cortex (A) and hippocampus (B) in animals treated with intracerebroventricular streptozotocin (STZ; 3 mg/kg) and/or anthocyanins (200 mg/kg). Data are expressed as mean ± S.E.M. **Denotes significant difference from the vehicle group for $P<0.01$. ##Denotes a significant difference compared with the STZ group for $P<0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 4 - Total ATPase (A), Na^+-K^+-ATPase (B), Na^+-K^+-ATPase α2,3 (C) and Na^+-K^+-ATPase α1 (D) isoform activity in the cerebral cortex in animals treated with intracerebroventricular streptozotocin (STZ; 3 mg/kg) and/or anthocyanins (200 mg/kg). Data are expressed as mean ± S.E.M. *Denotes significant difference from the vehicle group for $P<0.05$; ** $P<0.01$; ***$P<0.001$. # Denotes a significant difference compared with the STZ group for $P<0.05$; ##$P<0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 5 - Total ATPase (A), Na^+-K^+-ATPase (B), Na^+-K^+-ATPase α2,3 (C) and Na^+-K^+-ATPase α1 (D) isoform activity in the hippocampus in animals treated with

intracerebroventricular streptozotocin (STZ; 3 mg/kg) and/or anthocyanins (200 mg/kg). Data are expressed as mean ± S.E.M. *Denotes significant difference from the vehicle group for $P<0.05$; ** $P<0.01$. # Denotes a significant difference compared with the STZ group for $P<0.05$; ## $P<0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 6 - Effects of anthocyanins treatment (200 mg/kg) on reactive oxygen species level (A); nitrite level (B); TBARS level (C); sulfhydryl content (D); superoxide dismutase activity (E); catalase activity (F); and glutathione peroxidase activity (G) in the cerebral cortex of animals administered with intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. Data are reported as means ± S.E.M. *Denotes significant difference from the vehicle group for $P<0.05$; ** $P<0.01$; *** $P<0.001$. #Denotes significant difference from the STZ group for $P<0.05$; ## $P<0.01$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 7 - Effects of anthocyanins treatment (200 mg/kg) on reactive oxygen species level (A); nitrite level (B); TBARS level (C); sulfhydryl content (D); superoxide dismutase activity (E); catalase activity (F); and glutathione peroxidase activity (G) in the hippocampus of animals administered with intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. Data are reported as means ± S.E.M. *Denotes significant difference from the vehicle group for $P<0.05$; ** $P<0.01$. # Denotes significant difference from the STZ group for $P<0.05$ (Two or one-way analysis of variance followed by Bonferroni *post hoc* test).

Figure 8 - Effects of anthocyanins treatment (200 mg/kg) blood glucose levels in serum of animals administered with intracerebroventricular streptozotocin (STZ; 3 mg/kg) injection. Data are reported as means ± S.E.M.

Figure 9 - Neuroprotective potential of anthocyanins against damage caused by the administration of intracerebroventricular streptozotocin in an experimental model of sporadic dementia of Alzheimer's type.

Ethics Committee protocol number
CEEA 0179/2015

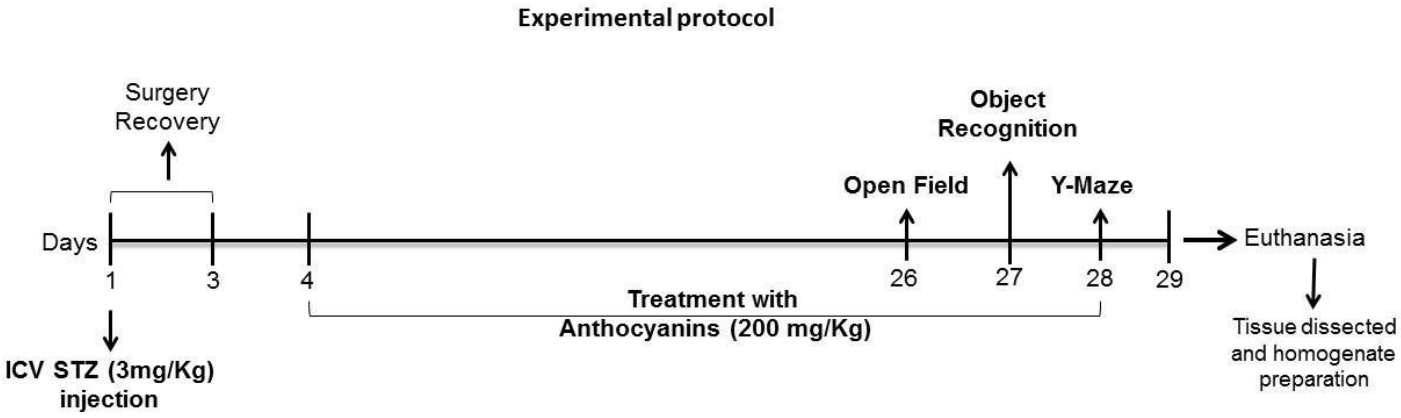

Figure 1

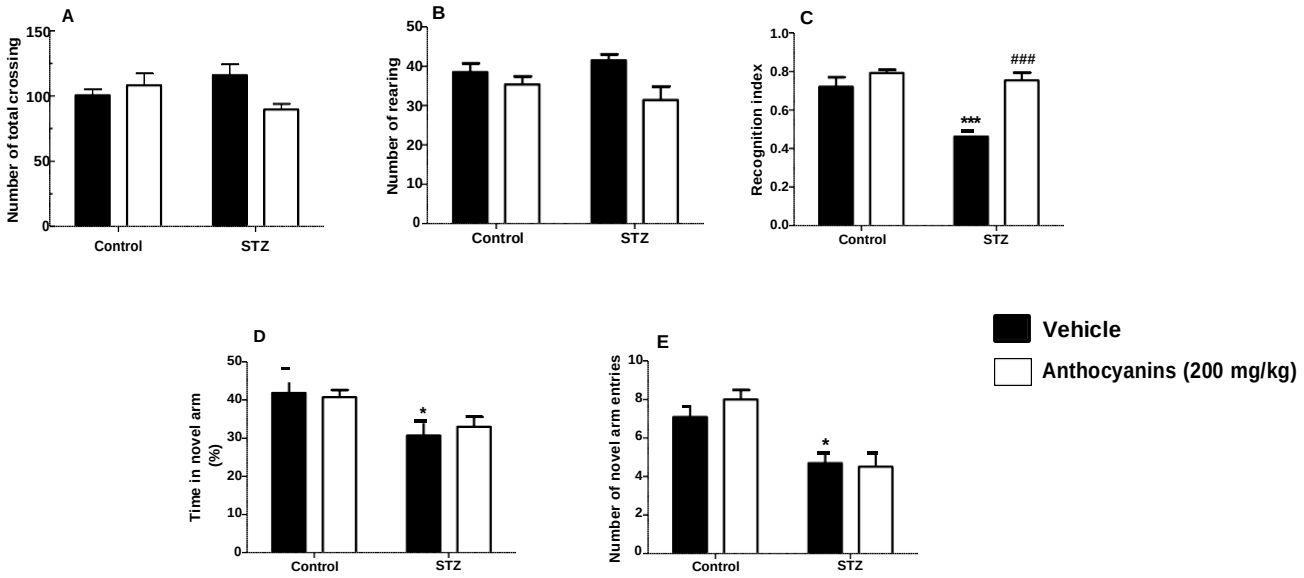
A
Number of total crossing
150
100
50
0
Control
STZ
B
Number of rearing
50
40
30
20
10
0
Control
STZ
C
Recognition index
1.0
0.8
0.6
0.4
0.2
0.0
***
###
Control
STZ
D
Time in novel arm (%)
50
40
30
20
10
0
*
Control
STZ
E
Number of novel arm entries
10
8
6
4
2
0
*
Control
STZ
Vehicle
Anthocyanins (200 mg/kg)

Figure 2

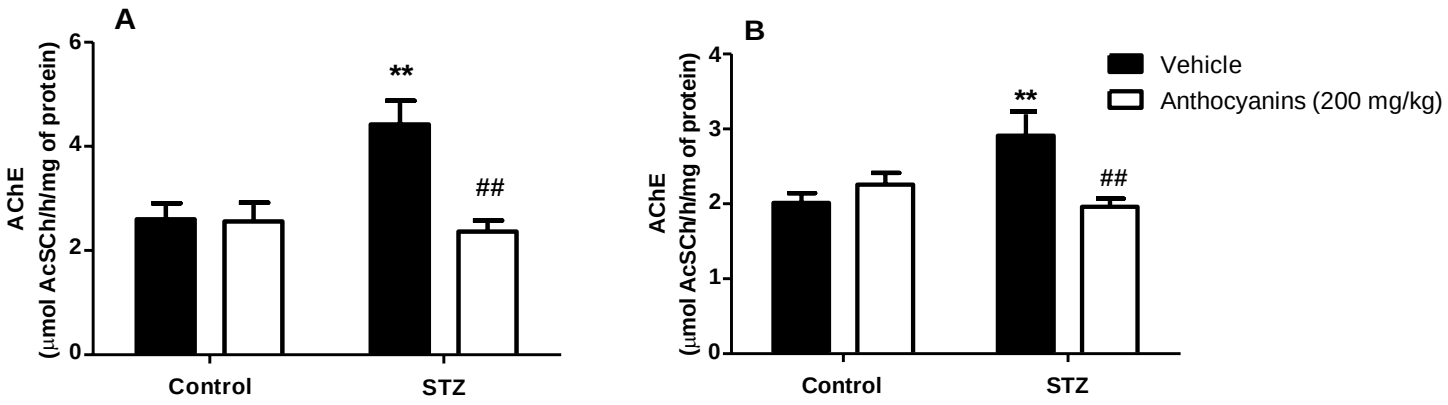
A
AChE
(μmol AcSCh/h/mg of protein)
6
4
2
0
**
##
Control
STZ
B
AChE
(μmol AcSCh/h/mg of protein)
4
3
2
1
0
**
##
Control
STZ
Vehicle
Anthocyanins (200 mg/kg)

Figure 3

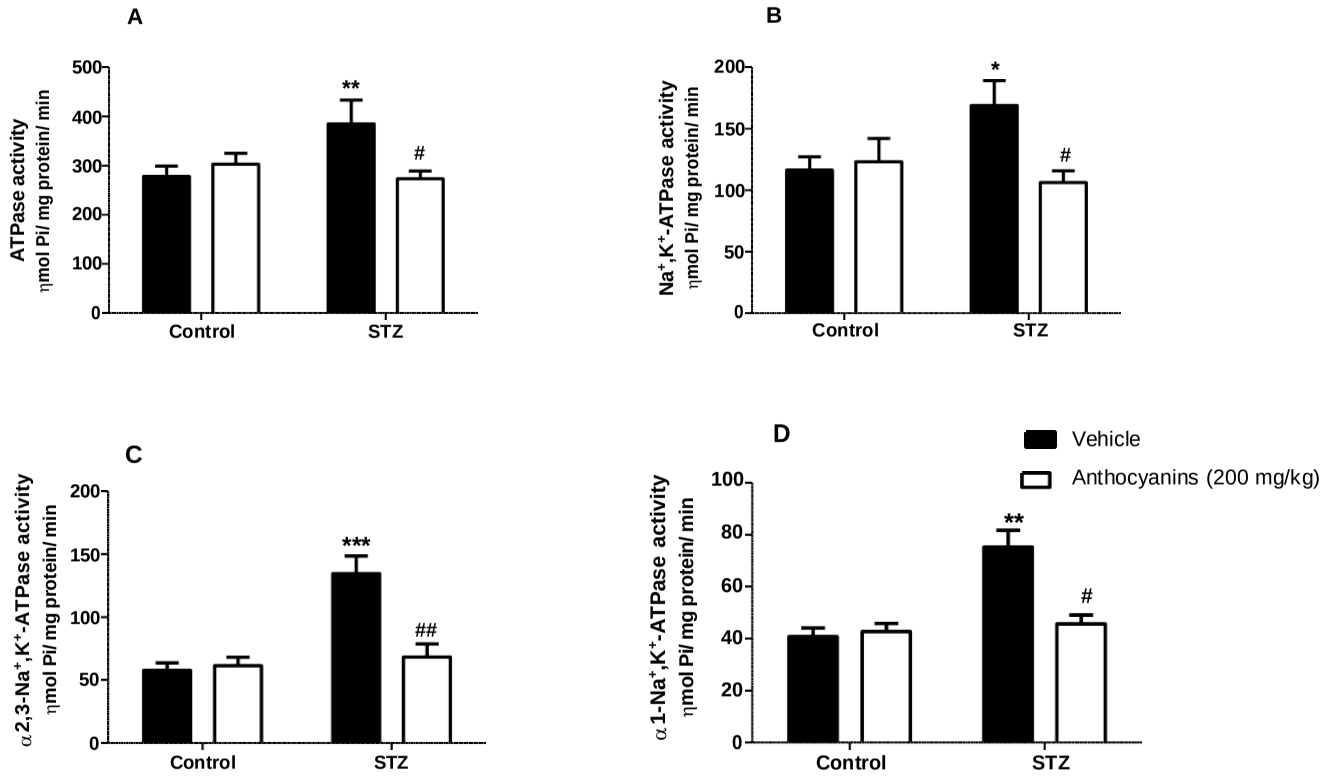
A
ATPase activity
ηmol Pi/ mg protein/ min
500
400
300
200
100
0
**
#
Control
STZ
B
Na+,K+-ATPase activity
ηmol Pi/ mg protein/ min
200
150
100
50
0
*
#
Control
STZ
C
α2,3-Na+,K+-ATPase activity
ηmol Pi/ mg protein/ min
200
150
100
50
0
***
##
Control
STZ
D
Vehicle
Anthocyanins (200 mg/kg)
α1-Na+,K+-ATPase activity
ηmol Pi/ mg protein/ min
100
80
60
40
20
0
**
#
Control
STZ

Figure 4

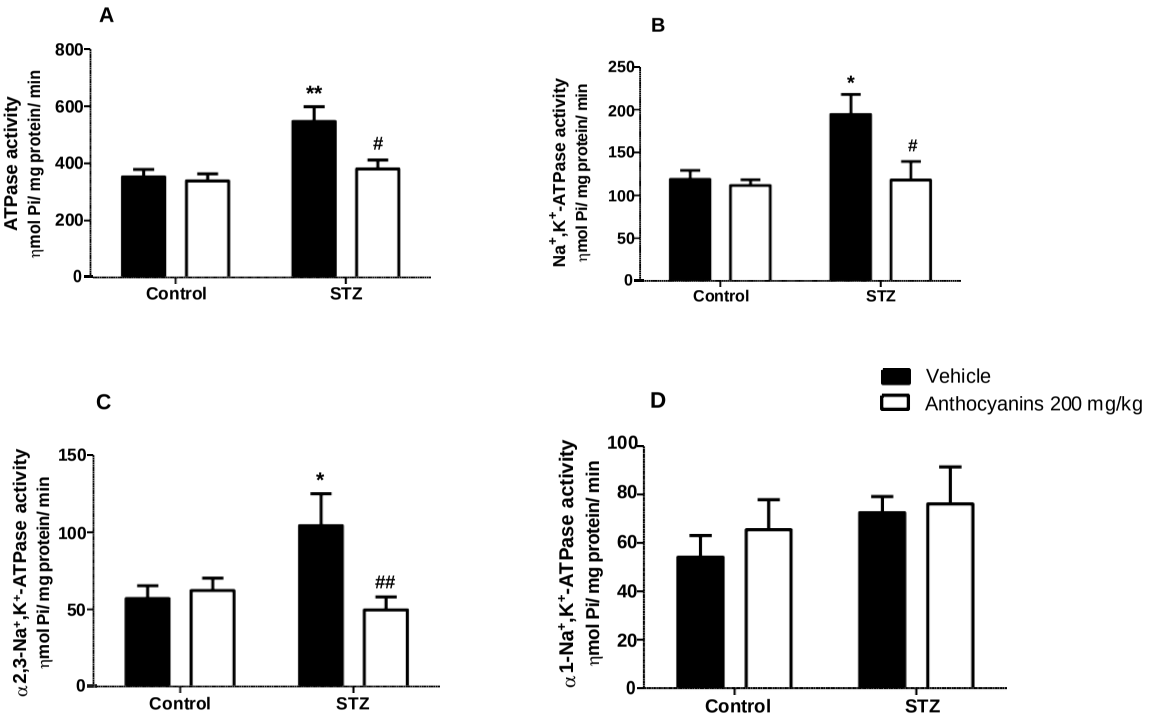
A
ATPase activity
ηmol Pi/ mg protein/ min
800
600
400
200
0
**
#
Control
STZ
B
Na+,K+-ATPase activity
ηmol Pi/ mg protein/ min
250
200
150
100
50
0
*
#
Control
STZ
C
α2,3-Na+,K+-ATPase activity
ηmol Pi/ mg protein/ min
150
100
50
0
*
##
Control
STZ
D
α1-Na+,K+-ATPase activity
ηmol Pi/ mg protein/ min
100
80
60
40
20
0
Control
STZ
Vehicle
Anthocyanins 200 mg/kg

Figure 5

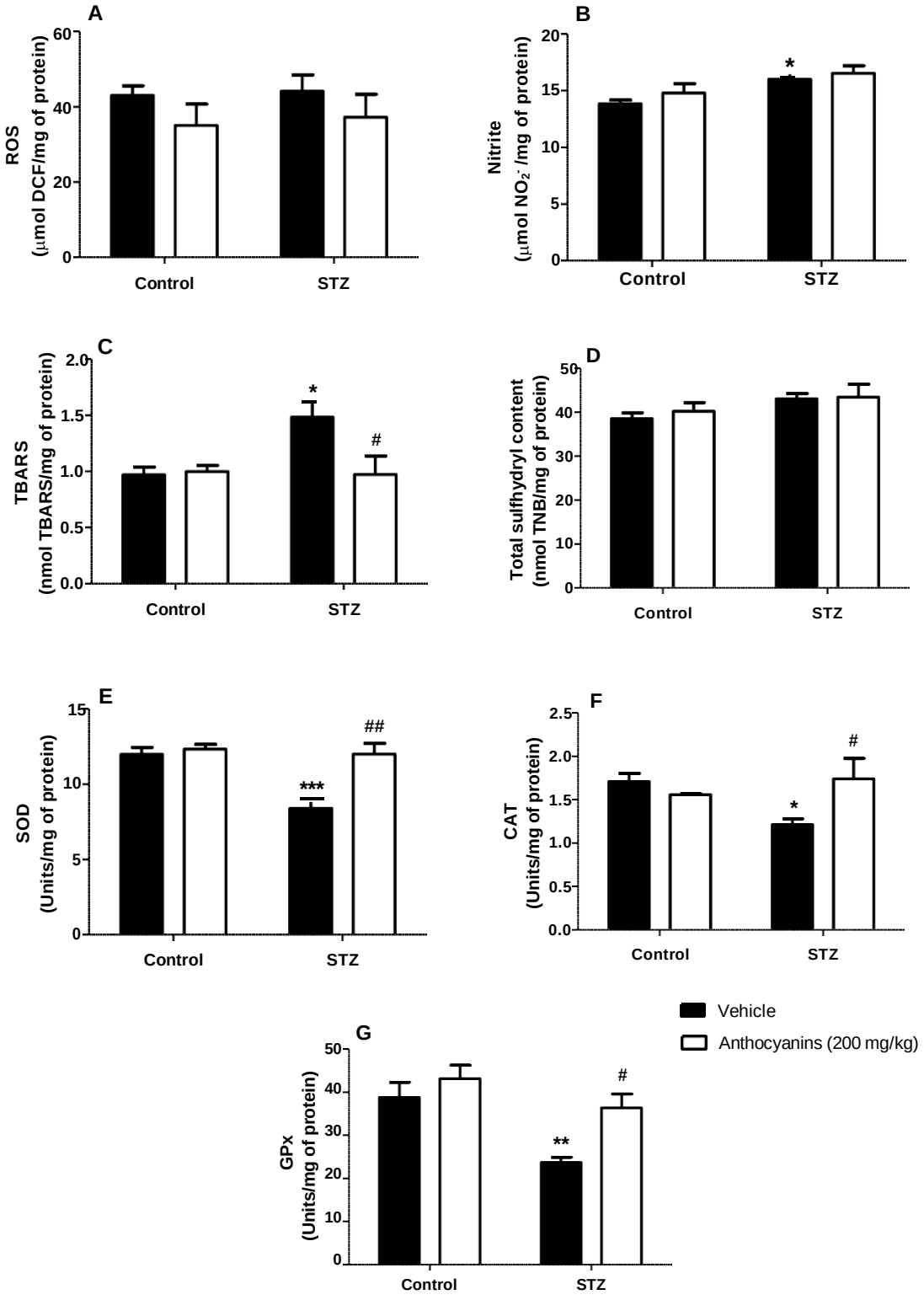
A
ROS
(µmol DCF/mg of protein)
60
40
20
0
Control
STZ
B
Nitrite
(µmol NO2- /mg of protein)
20
15
10
5
0
*
Control
STZ
C
TBARS
(nmol TBARS/mg of protein)
2.0
1.5
1.0
0.5
0.0
*
#
Control
STZ
D
Total sulfhydryl content
(nmol TNB/mg of protein)
50
40
30
20
10
0
Control
STZ
E
SOD
(Units/mg of protein)
15
10
5
0
***
##
Control
STZ
F
CAT
(Units/mg of protein)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Control
STZ
Vehicle
Anthocyanins (200 mg/kg)
G
GPx
(Units/mg of protein)
50
40
30
20
10
0
**
#
Control
STZ

Figure 6

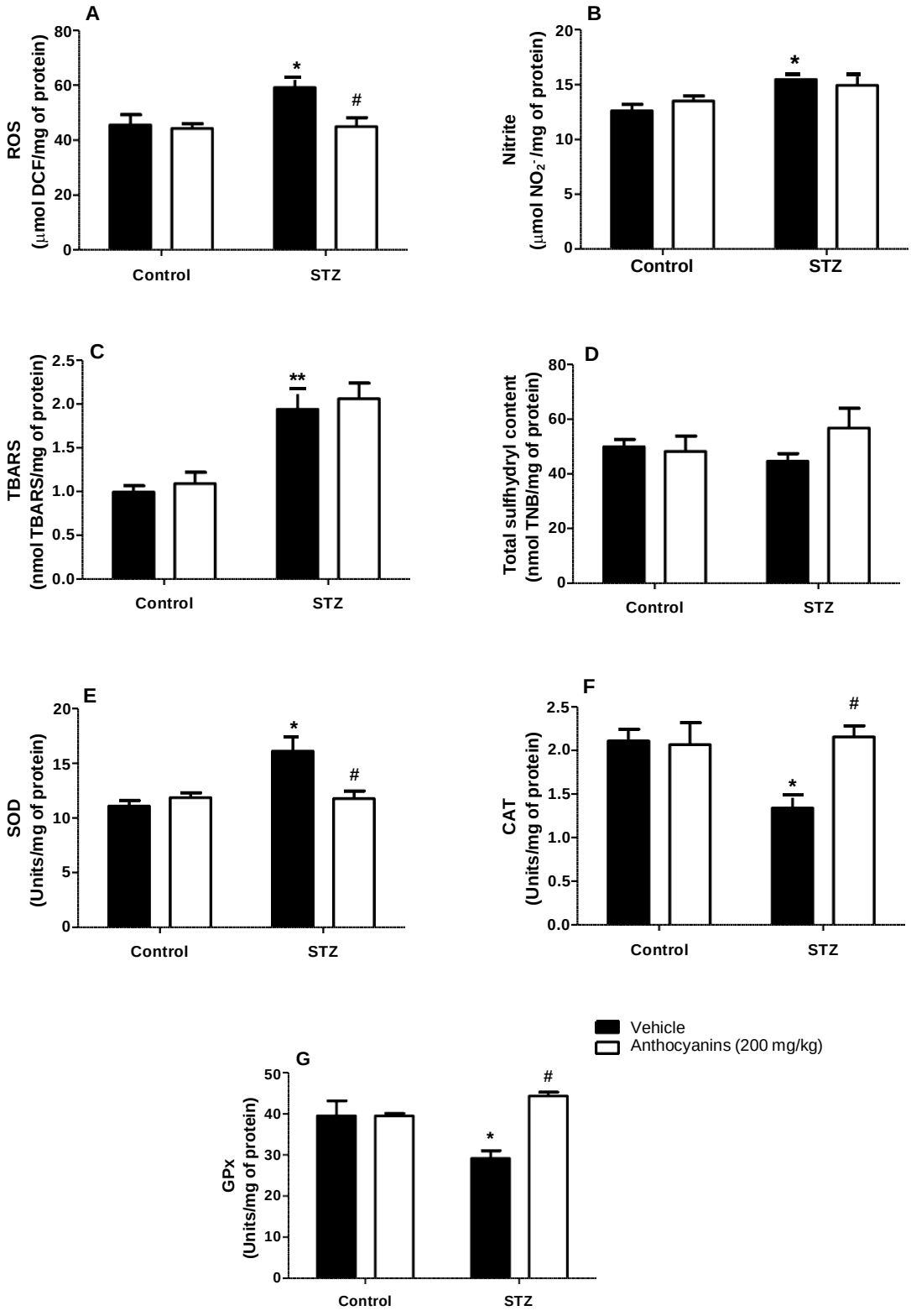
A
ROS
(µmol DCF/mg of protein)
80
60
40
20
0
*
#
Control
STZ
B
Nitrite
(µmol NO2- /mg of protein)
20
15
10
5
0
*
Control
STZ
C
TBARS
(nmol TBARS/mg of protein)
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
STZ
D
Total sulfhydryl content
(nmol TNB/mg of protein)
80
60
40
20
0
Control
STZ
E
SOD
(Units/mg of protein)
20
15
10
5
0
*
#
Control
STZ
F
CAT
(Units/mg of protein)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Control
STZ
Vehicle
Anthocyanins (200 mg/kg)
G
GPx
(Units/mg of protein)
50
40
30
20
10
0
*
#
Control
STZ

Figure 7

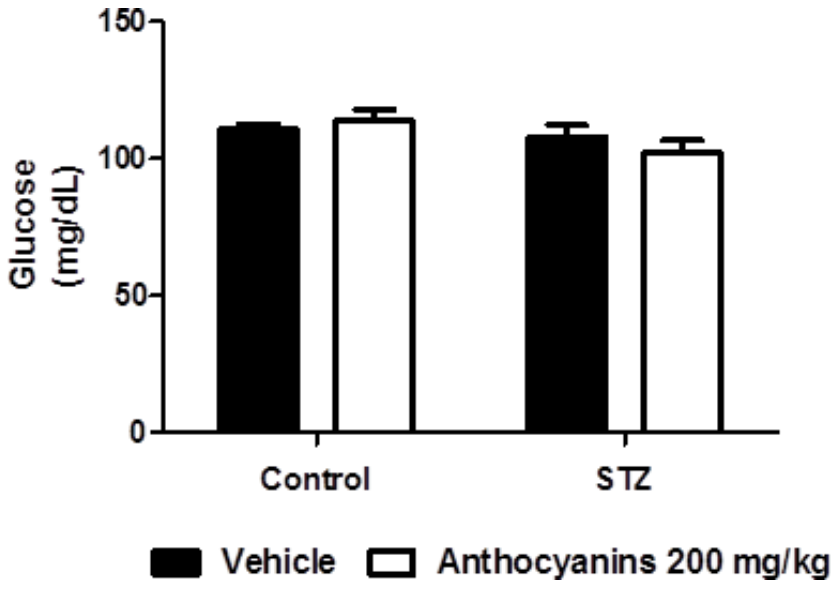
150
100
50
0
Glucose (mg/dL)
Control
STZ
Vehicle
Anthocyanins 200 mg/kg

Figure 8

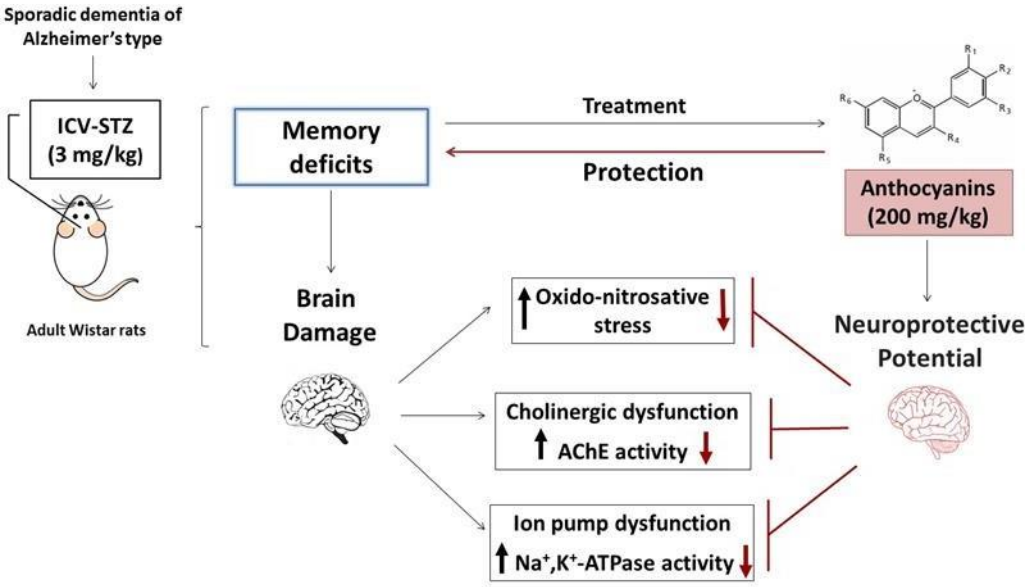
Sporadic dementia of Alzheimer's type
ICV-STZ (3 mg/kg)
Adult Wistar rats
Memory deficits
Brain Damage
Treatment
Protection
Anthocyanins (200 mg/kg)
Neuroprotective Potential
Oxido-nitrosative stress
Cholinergic dysfunction
AChE activity
Ion pump dysfunction
Na+,K+-ATPase activity

Figure 9

5 DISCUSSÃO

A DA é uma doença crônica com uma longa fase pré-clínica e assintomática e que culmina na demência. Seus sintomas característicos incluem dificuldades em relação à memória, à linguagem, à resolução de problemas e outras habilidades cognitivas (MASTERS et al., 2015; ALZHEIMER'S ASSOCIATION, 2018).

O tratamento atual da DA engloba a utilização de medicamentos que aumentam a cognição, restringindo-se a duas classes: inibidores da AChE e antagonista de receptores de NMDA. Entretanto, a resposta clínica desses fármacos é limitada, sendo que a maioria dos pacientes apresenta desaceleração do declínio cognitivo sem perda adicional por 6 a 9 meses (MASTERS et al., 2015). Recentemente, dois ensaios clínicos envolvendo fármacos promissores para o tratamento da DA não trouxeram benefícios significativos para os participantes dos estudos (MAKIN, 2018). Assim, a ausência de efetivas estratégias de prevenção e tratamento ainda estão entre os maiores desafios da doença (WANG et al., 2017).

Sabe-se que a maioria dos casos da DA são da forma esporádica na qual há associação de fatores ambientais, como os relacionados com o estilo de vida e de polimorfismos genéticos. Além disso, tem sido demonstrado associação positiva entre DA e patologias como diabetes mellitus, obesidade e doença cardiovascular. Cabe destacar que esses fatores de risco possuem uma base semelhante como aumento de marcadores do estresse oxidativo e da inflamação (HERMAN et al., 2018).

Estudo em cérebros de indivíduos portadores da DA já revelou a presença de estresse oxidativo e neuroinflamação nos mesmos. O mecanismo preciso que leva ao desenvolvimento dessas alterações não foi totalmente esclarecido, e ainda permanece o debate se são causa ou consequência da patologia. Contudo, evidências demonstram uma relação direta entre a produção anormal do peptídeo Aβ e desenvolvimento e/ou manutenção do estresse oxidativo e da neuroinflamação (GONZÁLEZ-REYES et al., 2017).

Mediante esse cenário, medidas que previnam, atenuem ou revertam esses danos são de fundamental importância. Dentre os inúmeros compostos neuroprotetores propostos em modelos *in vitro* e *in vivo* da DA, os compostos

fenólicos, especialmente, as antocianinas têm demonstrado resultados promissores. Diversos mecanismos podem estar envolvidos na neuroproteção promovida pelas antocianinas como a sua atividade antioxidante e anti-inflamatória, modulação da sinalização neuronal e da expressão gênica relacionadas com a plasticidade sináptica e aumento do fluxo sanguíneo cerebral (HE e GIUSTI, 2010; TSUDA, 2012; POJER et al., 2013; RENDEIRO, RHODES e SPENCER, 2015). Estudos prévios já demonstraram a ação das antocianinas em várias condições neurológicas como a doença de Parkinson (STRATHEARN et al., 2014), neuroinflamação (CARVALHO et al., 2017), desmielinização (CARVALHO et al., 2015), disfunção da memória (GUTIERRES et al., 2014a) e demência do tipo Alzheimer (GUTIERRES et al., 2014b).

A fim de dar prosseguimento à investigação da ação neuroprotetora das antocianinas em modelos experimentais da DA, o artigo I dessa tese avaliou os efeitos do extrato de *lingonberry* na viabilidade celular, na atividade da AChE e nos parâmetros de estresse oxidativo em cultura primária de astrócitos de ratos expostos ao LPS. Os astrócitos são fundamentais para os processos fisiológicos cerebrais como a homeostase do glutamato, fluxo sanguíneo e excitabilidade, suporte metabólico para os neurônios e plasticidade sináptica. Assim, alterações funcionais nos mesmos possuem peso importante para a progressão de doenças neurológicas (ACOSTA et al., 2017).

Nesse estudo, observou-se que o tratamento com o extrato, antes e após a exposição ao LPS, foi capaz de prevenir e reverter as alterações em relação à viabilidade e à proliferação celular. Também foi possível observar a diminuição da produção de ERO e ERN através da modulação da atividade da iNOS, prevenindo e revertendo as alterações causadas pelo LPS. É importante lembrar que o $NO^{\bullet}$, produzido em virtude do aumento da expressão da iNOS, pode contribuir para o estresse oxidativo e morte neuronal (WAKABAYASHI et al., 2010), uma vez que promoveria modificações na permeabilidade mitocondrial, favorecendo o extravasamento do seu conteúdo, o aumento da produção de ERO e a liberação de moléculas sinalizadoras de morte celular (STEWART e HEALES, 2003).

Além disso, o tratamento com o extrato de *lingonberry* foi associado com um aumento da viabilidade mitocondrial, o restabelecimento da atividade da SOD e da GPx, e aumento do conteúdo de sulfidrilas nesses astrócitos expostos ao LPS. A

manutenção do conteúdo tiólico da GSH é fundamental para a regulação do estado redox e da função proteica, uma vez que essa biomolécula é altamente suscetível ao dano oxidativo (MOSKAUG et al., 2005; POOLE, 2015). Estudos prévios já relataram que a liberação de GSH pelos astrócitos é essencial para a conservação dos níveis de sulfidrilas no SNC (WANG e CYNADER, 2000), e que os astrócitos protegem os neurônios contra a toxicidade do $NO^{\bullet}$ através de um mecanismo dependente de GSH (CHEN et al., 2001).

Outro resultado importante desse trabalho demonstrou que o LPS induziu aumento da atividade da AChE nos astrócitos, sendo restaurada pelo tratamento com o extrato de *lingonberry*. A AChE é uma enzima também expressa em astrócitos (THULLBERY et al., 2005). Esse aumento da atividade da AChE pode contribuir para diminuir os níveis de ACh, sendo que sua síntese também pode ser inibida pelo H_2O_2 e $NO^{\bullet}$ (LÜTH et al., 2001). Além disso, outros estudos já demonstraram que aumento na atividade da AChE causa ativação da microglia, eleva os níveis de citocinas pró-inflamatórias (GNATEK et al., 2012) e aumenta a atividade da iNOS (VON BERNHARDI et al., 2003).

O artigo II dessa tese analisou o efeito neuroprotetor das antocianinas (extraídas e purificadas da casca da uva) em um modelo de demência esporádica do tipo Alzheimer induzida por STZ em ratos *Wistar* machos e adultos. Já é bem descrito que esse modelo promove alterações similares aquelas encontradas em pacientes com DA como diminuição do metabolismo de glicose, estresse oxidativo, alteração na sinalização colinérgica, neuroinflamação, morte neuronal e comprometimento do aprendizado e da memória (SALKOVIC-PETRESIC et al., 2007; 2013; 2014; KAMAT et al., 2016).

Conforme mostraram os resultados, o STZ alterou tanto a memória de curta duração quanto a memória espacial e não modificou a atividade locomotora desses animais o que está de acordo com estudos prévios (SHOHAM et al., 2007; ISHRAT et al., 2009b; SAXENA, PATRO e NATH, 2011; JAVED et al., 2012; EJAZ AHMED et al., 2013; GUTIERRES et al., 2014b; DESHMUKH et al., 2016; BIASIBETTI et al., 2017; RAVELLI et al., 2017). A administração ICV do STZ pode alcançar regiões do *septum* e do corpo caloso, além do fórnix, hipocampo anterior e estruturas periventriculares (SHOHAM et al., 2003; KRASKA et al., 2012). Essas estruturas estão envolvidas na memória, sendo que o córtex perirrinal está mais relacionado

com a memória de curto prazo e o hipocampo, com a memória de longo prazo e espacial (ANTUNES e BIALA, 2012). Além disso, o *septum* possui projeções colinérgicas para as regiões do córtex e hipocampo, também envolvidas na aquisição da memória, sugerindo que a administração do STZ pode interromper essas conexões, alterando a consolidação da memória (KRASKA et al., 2012; DE JAEGER et al., 2013).

As antocianinas foram capazes de proteger contra as alterações da memória de curto prazo, além de restabelecerem a atividade da AChE em córtex cerebral e hipocampo. Esses resultados estão de acordo com estudos anteriores que mostraram que as antocianinas promoveram melhora da memória de curto prazo e de trabalho em ratos idosos (RAMIREZ et al., 2005), da memória espacial em ratos tratados com D-galactose (REHMAN et al., 2017) e em um modelo transgênico para DA (VEPSÄLÄINEN et al., 2013). Também foi relatado que a ingestão de suco de cereja rico em antocianinas por 12 semanas poderia ter efeitos benéficos sobre o desempenho cognitivo em idosos com demência leve a moderada (KENT et al., 2017). Ainda, tratamento com mirtilo (*Vaccinium corymbosum* L.) durante 2 meses reduziu a atividade da AChE de ratos tratados com D-galactose (ÇOBAN et al., 2015) e achados prévios do nosso grupo de pesquisa demonstraram que o tratamento com antocianinas protegeu contra alterações na atividade dessa enzima e déficits de memória causados pela administração de escopolamina (GUTIERRES et al., 2014a).

Outro achado interessante do nosso estudo foi o aumento da atividade da Na^+-K^+-ATPase, incluindo suas isoformas α1 e α2/α3, induzido pelo STZ, diferentemente do encontrado em trabalhos anteriores nos quais foi observado diminuição dessa atividade (ISHRAT et al., 2009a; GUTIERRES et al., 2014b). A redução da atividade da Na^+-K^+-ATPase já foi associada com marcadores do estresse oxidativo, uma vez que essa enzima possui sulfidrilas, tornando-a suscetível à oxidação. Evidências também demonstraram que a mesma possui vários resíduos de aminoácidos como serina, treonina e tirosina que são alvos da fosforilação por cinases. Glicose e insulina também parecem ser importantes na regulação da Na^+-K^+-ATPase. Assim, é possível sugerir que as disfunções metabólicas causadas pela administração de STZ afetam principalmente as vias de sinalização dependentes de receptores de insulina, prejudicando a regulação da

Na+-K+-ATPase. Entretanto, nossos resultados mostraram um aumento na Na+-K+-ATPase no cérebro após a administração de STZ, demonstrando que outros mecanismos além da sinalização da insulina podem estar envolvidos nas alterações desta enzima nessa condição patológica (HERNÁNDEZ-R, 1992; LORES e BERSIER, 2014). Além disso, o tratamento com antocianinas foi capaz de restabelecer a atividade da Na^+-K^+-ATPase e, com isso, acredita-se que possam contribuir para a restauração dos níveis de ATP no cérebro.

Em relação aos parâmetros de estresse oxidativo, observou-se alterações desencadeadas pela administração do STZ. Já é bem estabelecido que o estresse oxidativo induzido pelo STZ ocorre por essa substância gerar ERO e ERN. Além disso, é capaz de promover a expressão da iNOS e da óxido nítrico sintase neuronal (nNOS) sendo uma possível fonte do elevado conteúdo de nitrito em córtex e hipocampo no modelo (RENDEIRO, RHODES e SPENCER, 2015). Assim, o excesso de $O_2^{\bullet-}$ pode se combinar rapidamente com o $NO^{\bullet}$, produzindo $ONOO^-$ que pode reagir com várias biomoléculas, gerando dano e morte neuronal (HALLIWELL, 2006; TIWARI et al., 2009; DARVESH et al., 2010; TOTA et al., 2010). Também foi observada, diminuição da atividade das enzimas antioxidantes. A diminuição da atividade da SOD, GPx e CAT pode prejudicar a remoção do $O_2^{\bullet-}$ e do H_2O_2, gerando $OH^{\bullet}$ e peroxidação lipídica, alterando as propriedades biológicas das membranas (ISHRAT et al., 2009b; JAVED et al., 2015; CERVELLATI et al., 2016). Curiosamente, foi observado aumento da atividade da SOD em hipocampo dos animais submetidos aos STZ o que pode indicar um mecanismo compensatório em resposta ao aumento do acúmulo de $O_2^{\bullet-}$. Nesse contexto, as antocianinas foram capazes de proteger contra o dano oxidativo induzido pela injeção ICV do STZ, sugerindo que esse efeito possa estar associado a melhora da disfunção na memória.

A ação antioxidante das antocianinas está relacionada com sua capacidade de eliminação direta das espécies reativas através da doação de hidrogênio que é dependente da sua estrutura química, especialmente a presença de grupos hidroxila no anel B (MIGUEL, 2011; POJER et al., 2013). Além disso, também atua por vias indiretas através da modulação de enzimas antioxidantes, redução da formação de adutos no DNA e formação endógena de ERO pela inibição da NADPH oxidase e da

xantina oxidase, ou, ainda, pela modificação da respiração mitocondrial e do metabolismo do ácido araquidônico (POJER et al., 2013).

Assim, esses achados permitem observar que as antocianinas promoveram efeito glioprotetor, contribuindo para a manutenção da homeostase dos astrócitos, provavelmente, pelo seu efeito antioxidante e pela modulação da AChE, podendo, com isso, ter favorecido a proteção observada quanto à diminuição no déficit de memória, reforçando o. promissor potencial terapêutico das antocianinas.

6 CONCLUSÕES

Em relação ao protocolo *in vitro*, os resultados demonstram que o extrato de *lingonberry* foi capaz de prevenir e reverter os danos induzidos pelo LPS, exercendo um efeito glioprotetor através do aumento da viabilidade celular, modulação da via colinérgica e atividade antioxidante, contribuindo assim para a manutenção da homeostase dos astrócitos.

Em relação ao protocolo *in vivo*, os resultados demonstraram que o extrato rico em antocianinas (obtido da casca da uva) foi capaz de proteger contra os déficits de memória em um modelo de demência esporádica do tipo Alzheimer e que esse efeito pode ser atribuído ao seu potencial antioxidante e a capacidade de restaurar a sinalização colinérgica e as bombas iônicas no SNC.

7 PERSPECTIVAS

As principais perspectivas para a continuação desse trabalho envolvem o estudo de outros aspectos que possam contribuir para um maior esclarecimento dos mecanismos moleculares e bioquímicos associados à DA como os elencados a seguir:

- Avaliação do efeito do tratamento com extrato rico em antocianinas em cultura primária de astrócitos expostas ao peptídeo Aβ.
- Determinação dos níveis de citocinas IL-6 e IL-10 em astrócitos expostos ao LPS e ao peptídeo Aβ.
- Marcação de reatividade astrocitária através da GFAP.
- Determinação dos níveis de citocinas (IL-6 e IL-10), BDNF e neuropeptídeo Y no modelo de demência esporádica do tipo Alzheimer através da injeção ICV de STZ em ratos *Wistar* e tratados com extrato rico em antocianinas.

8 REFERÊNCIAS

ABOLHASSANI, N. et al. Molecular pathophysiology of impaired glucose metabolism, mitochondrial dysfunction, and oxidative DNA damage in Alzheimer's disease brain. *Mechanisms of Ageing and Development*, v. 161(Pt A), p. 95-104, 2017.

ACOSTA, C.; ANDERSON, H. D.; ANDERSON, C. M. Astrocyte dysfunction in Alzheimer disease. *Journal of Neuroscience Research*, v. 95, n. 12, p. 2430-2447, 2017.

AGOSTINHO, P.; CUNHA, R. A.; OLIVEIRA, C. Neuroinflammation, oxidative stress and pathogenesis of Alzheimer's disease. *Current Pharmaceutical Design*, v. 16, n. 25, p. 2766-2778, 2010.

AGRAWAL, R. et al. A study of brain insulin receptors, AChE activity and oxidative stress in rat model of ICV STZ induced dementia. *Neuropharmacology*, v. 56, n. 4, p. 779-787, 2009.

AL-AWAR, A et al. Experimental diabetes mellitus in different animal models. *Journal of Diabetes Research*, 2016: 9051426, 2016.

ALBERT, M. S. et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & Dementia*, v. 7, n. 3, p. 270-279, 2011.

ALI, T. et al. Natural dietary supplementation of anthocyanins via PI3K/Akt/Nrf2/HO-1 pathways mitigate oxidative stress, neurodegeneration, and memory impairment in a mouse model of Alzheimer's disease. *Molecular Neurobiology*, v. 55, n. 7, p. 6076-6093, 2018.

ALZHEIMER'S ASSOCIATION. 2017 Alzheimer's disease facts and figures. *Alzheimer's & Dementia*, v. 13, n. 4, p. 325-373, 2017.

ALZHEIMER'S ASSOCIATION. 2018 Alzheimer's disease facts and figures. *Alzheimer's & Dementia,* v. 14, n. 3, p. 367-429, 2018.

ANDRES-LACUEVA, C. et al. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutritional Neuroscience*, v. 8, n. 2, p. 111-120, 2005.

ANTUNES, M.; BIALA, G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive Processing*, v. 13, n. 2, p. 93-110, 2012.

ARNAIZ, G. R. L.; ORDIERES, M. G. L. Brain Na^+, K^+-ATPase activity in aging and disease. *International Journal of Biomedical Science*, v. 10, n. 2, p. 85-102, 2014.

AVILA-MUÑOZ, E.; ARIAS, C. When astrocytes become harmful: functional and inflammatory responses that contribute to Alzheimer's disease. *Ageing Research Reviews*, v. 18, p. 29-40, 2014.

AZIZI, G.; MIRSHAFIEY, A. The potential role of proinflammatory and anti-inflammatory cytokines in Alzheimer disease pathogenesis. *Immunopharmacology and Immunotoxicology*, v. 34, n. 6, p. 881-895, 2012.

BAGYINSZKY, E. et al. Role of inflammatory molecules in the Alzheimer's disease progression and diagnosis. *Journal of the Neurological Sciences*, v. 376, p. 242-254, 2017.

BASSANI, T. B. et al. Decrease in adult neurogenesis and neuroinflammation are involved in spatial memory impairment in the streptozotocin-induced model of sporadic Alzheimer's disease in rats. *Molecular Neurobiology*, v. 55, n. 5, p. 4280-4296, 2017.

BEKRIS, L. M. et al. Genetics of Alzheimer disease. *Journal of Geriatric Psychiatry and Neurology*, v. 23, n. 4, p. 213-227, 2010.

BIASIBETTI, R. et al. Hippocampal changes in STZ-model of Alzheimer's disease are dependente on sex. *Behavioral Brain Research*, v. 316, p. 205-214, 2017.

CALSOLARO, V. EDISON, P. Neuroinflammation in Alzheimer's disease: current evidence and future directions. *Alzheimer's & Dementia*, v. 12, n. 6, p. 719-732, 2016.

CAMPANARI, M. L. et al. Acetylcholinesterase protein level is preserved in the Alzheimer's brain. *Journal of Molecular Neuroscience*, v. 53, n. 3, p. 446-453, 2014.

CARVALHO, F. B et al. Anthocyanins suppress the secretion of proinflammatory mediators and oxidative stress, and restore ion pump activities in demyelination. *The Journal of Nutritional Biochemistry*, v. 26, n. 4, p. 378-390, 2015.

CARVALHO, F. B. et al. Anthocyanins control neuroinflammation and consequent memory dysfunction in mice exposed to lipopolysaccharide. *Molecular Neurobiology*, v. 54, n. 5, p. 3350-3367, 2017.

CASELLI, R. J. et al. Alzheimer Disease: Scientific Breakthroughs and Translational Challenges. *Mayo Clinic Proceedings*, v. 92, n. 6, p. 978-994, 2017.

CASTAÑEDA-OVANDO, A. et al. Chemical studies of anthocyanins: a review. *Food Chemistry*, v. 113, n. 4, p. 859-871, 2009.

CERVELLATI, C. et al. Oxidative challenge in Alzheimer's disease: state of knowledge and future needs. *Journal of Investigative Medicine*, v. 64, n. 1, p. 21-32, 2016.

CHEN, Y. et al. Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. *Journal of Neurochemistry*, v. 77, n. 6, p. 1601-1610, 2001.

CHOI, D. Y. et al. Antioxidant properties of natural polyphenols and their therapeutic potentials for Alzheimer's disease. *Brain Research Bulletin*, v. 87, n. 2-3, p. 144-153, 2012.

ÇOBAN, J. et al. Blueberry treatment decreased D-galactose-induced oxidative stress and brain damage in rats. *Metabolic Brain Disease*, v. 30, n. 3, p. 793-802, 2015.

CORREIA, S. C. et al. Mitochondrial abnormalities in a streptozotocin-induced rat model of sporadic Alzheimer's disease. *Current Alzheimer Research*, v. 10, n. 4, p. 406-419, 2013.

CRUNFLI, F. et al. NO-dependent Akt inactivation by S-nitrosylation as a possible mechanism of STZ-induced neuronal insulin resistance. *Journal of Alzheimer's Disease*, v. 65, n. 4, p. 1427-1443, 2018.

CUMMINGS, J. Alzheimer's disease diagnostic criteria: practical applications. *Alzheimers Res Ther*, 4(5):35, 2012.

DARREH-SHORI, T. et al. Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer's disease patients. *Journal of Neurochemistry*, v. 88, n. 5, p. 1102-1113, 2004.

DARVESH, A. S. et al. Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. *Expert Review of Neurotherapeutics*, v. 10, n. 5, p. 729-745, 2010.

DAVIS, K. L. et al. Cholinergic markers in elderly patients with early signs of Alzheimer disease. *Journal of the American Medical Association*, v. 281, n. 15, p. 1401-1406, 1999.

DEHGHAN-SHASALTANEH, M. et al. Determination of the best concentration of streptozotocin to create a diabetic brain using histological techniques. *Journal of Molecular Neuroscience*, v. 59, n. 1, p. 24-35, 2016.

DEKOSKY, S. T. et al. Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. *Annals of Neurology*, v. 51, n. 2, p.145-155, 2002.

DE LA MONTE, S. M.; TONG, M. Mechanisms of nitrosamine-mediated neurodegeneration: potential relevance to sporadic Alzheimer's disease. *Journal of Alzheimer's Disease*, v. 17, n. 4, p. 817-825, 2009.

DE LA MONTE, S. M.; TONG, M. Brain metabolic dysfunction at the core of Alzheimer's disease. *Biochemical Pharmacology*, v. 88, n. 4, p. 548-559, 2014.

DE JAEGER, X. et al. Decreased acetylcholine release delays the consolidation of object recognition memory. *Behavioral Brain Research*, v. 238, p. 62-68, 2013.

DESHMUKH, R. et al. Caffeic acid attenuates oxidative stress, learning and memory deficit in intra-cerebroventricular streptozotocin induced experimental dementia in rats. *Biomedicine & Pharmacotherapy*, v. 81, p. 56-62, 2016.

DUBOIS, B. et al. Preclinical Alzheimer's disease: definition, natural history, and diagnostic criteria. *Alzheimer's & Dementia*, v. 12, n. 3, p. 292-323, 2016.

EJAZ AHMED, M. et al. Amelioration of cognitive impairment and neurodegeneration by catechin hydrate in rat model of streptozotocin-induced experimental dementia of Alzheimer's type. *Neurochemistry International*, v. 62, n. 4, p. 492-501, 2013.

FANG, J. Classification of fruits based on anthocyanin types and relevance to their health effects. *Nutrition*, v. 31, n. 11-12, p. 1301-1306, 2015.

FARIA, A. et al. Flavonoid metabolites transport across a human BBB model. *Food Chemistry*, v. 149, p. 190-196, 2014.

FERREIRA-VIEIRA, T.H. et al. Alzheimer's Disease: targeting the cholinergic system. *Current Neuropharmacology*, v. 14, n. 1, p. 101-115, 2016.

GARCÍA-AYLLÓN, M. S. et al. Revisiting the role of acetylcholinesterase in Alzheimer's disease: cross-talk with P-tau and β-amyloid. *Frontiers in Molecular Neuroscience*, v. 4, article 22, p. 1-9, 2011.

GNATEK, Y. et al. Acetylcholinesterase loosens the brain's cholinergic anti-inflammatory response and promotes epileptogenesis. *Frontiers in Molecular Neuroscience*, v. 5, article 66, p. 1-10, 2012.

GONZÁLEZ-REYES, R. E. et al. Involvement of astrocytes in Alzheimer's disease from a neuroinflammatory and oxidative stress perspective. *Frontiers in Molecular Neuroscience*, v. 10, article 427, p. 1-20, 2017.

GRAHAM, W. V.; BONITO-OLIVA, A; SAKMAR, T. P. Update on Alzheimer's disease therapy and prevention strategies. *Annual Reviews of Medicine*, v. 68, p. 413-430, 2017.

GRIEB, P. Intracerebroventricular streptozotocin injections as a model of Alzheimer's disease: in search of a relevant mechanism. *Molecular Neurobiology*, v. 53, n. 3, p. 1741-1752, 2016.

GRILL, J. D. et al. Comparing recruitment, retention, and safety reporting among geographic regions in multinational Alzheimer's disease clinical trials. *Alzheimer's Research & Therapy*, v. 7, n. 39, 2015.

GRIMM, A.; FRIEDLAND, K.; ECKERT, A. Mitochondrial dysfunction: the missing link between aging and sporadic Alzheimer's disease. *Biogerontology*, v. 17, n. 2, p. 281-296, 2016.

GUTIERRES, J. M. et al. Neuroprotective effect of anthocyanins on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia in rats. *International Journal of Developmental Neuroscience*, v. 33, p. 88-97, 2014a.

GUTIERRES, J. M. et al. Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type. *Life Sciences*, v. 96, n. 1-2, p. 7-17, 2014b.

HALLIWELL, B. Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry*, v. 97, n. 6, p.1634-1658, 2006.

HAMPEL, H. et al. Perspective on future role of biological markers in clinical therapy trials of Alzheimer's disease: a long-range point of view beyond 2020. *Biochemical Pharmacology*, v. 88, n. 4, p. 426-449, 2014.

HE, J.; GIUSTI, M. M. Anthocyanins: natural colorants with health-promoting properties. *Annual Review of Food Science and Technology*, v. 1, p. 163-187, 2010.

HENEKA, M. T.; KUMMER, M. P.; LATZ, E. Innate immune activation in neurodegenerative disease. *Nature Reviews Immunology*, v. 14, n. 7, p. 463-477, 2014.

HEPPNER, F. L.; RANSOHOFF, R. M.; BECHER, B. Immune attack: the role of inflammation in Alzheimer disease. *Nature Reviews Neuroscience*, v. 16, n. 6, p. 358-372, 2015.

HERMAN, F. et al. Suppression of presymptomatic oxidative stress and inflammation in neurodegeneration by grape-derived polyphenols. *Frontiers in Pharmacology*, v. 9, article 867, p. 1-20, 2018.

HERNÁNDEZ-R, J. Na+/K(+)-ATPase regulation by neurotransmitters. *Neurochemistry International*, v. 20, n. 1, p. 1-10, 1992.

HIPPIUS, H.; NEUNDÖRFER, G. The discovery of Alzheimer's disease. *Dialogues in Clinical Neuroscience*, v. 5, n. 1, p. 101-108, 2003.

HRIBAR, U.; ULRIH, N. P. The metabolism of anthocyanins. *Current Drug Metabolism*, v. 15, n. 1, p. 3-13, 2014.

ISHRAT, T. et al. Effects of pycnogenol and vitamin E on cognitive deficits and oxidative damage induced by intracerebroventricular streptozotocin in rats. *Behavioral Pharmacology*, v. 20, n. 7, p. 567-575, 2009a.

ISHRAT, T. et al. Amelioration of cognitive deficits and neurodegeneration by curcumin in rat model of sporadic dementia of Alzheimer's type (SDAT). *European Neuropsychopharmacology*, v. 19, n. 9, p. 636-647, 2009b.

JACK, C. R. JR. et al. NIA-AA research framework: toward a biological definition of Alzheimer's disease. *Alzheimer's & Dementia*, v. 14, n. 4, p. 535-562, 2018.

JAVED, H. et al. Rutin prevents cognitive impairments by ameliorating oxidative stress and neuroinflammation in rat model of sporadic dementia of Alzheimer type. *Neuroscience*, v. 210, p. 340-352, 2012.

JAVED, H. et al. Effect of hesperidin on neurobehavioral, neuroinflammation, oxidative stress and lipid alteration in intracerebroventricular streptozotocin induced cognitive impairment in mice. *Journal of the Neurological Sciences*, v. 348, n. 1-2, p. 51-59, 2015.

KALT, W.et al. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *Journal of Agricultural and Food Chemistry*, v. 56, n. 3, p. 705-712, 2008.

KAMAT, P. K. et al. Streptozotocin intracerebroventricular-induced neurotoxicity and brain insulin resistence: a therapeutic intervention for treatment of sporadic Alzheimer's disease (sAD)-like pathology. *Molecular Neurobiology*, v. 53, n. 7, p. 4548-4562, 2016.

KARPPINEM, K. et al. On the developmental and environmental regulation of secondary metabolism in *Vaccinium* spp. berries. *Frontiers in Plant Science*, v. 7, article 655, p. 1-9, 2016.

KENT, K. et al. Consumption of anthocyanin-rich cherry juice for 12 weeks improves memory and cognition in older adults with mild-to-moderate dementia. *European Journal of Nutrition*, v. 56, n. 1, p. 333-341, 2015.

KENT, K. et al. Consumption of anthocyanin-rich cherry juice for 12 weeks improves memory and cognition in older adults with mild-to-moderate dementia. *European Journal of Nutrition*, v. 56, n. 1, p. 333-341, 2017.

KHAN, M. S. et al. Anthocyanins improve hippocampus-dependent memory function and prevent neurodegeneration via JNK/Akt/GSK3β signaling in LPS-treated adult mice. *Molecular Neurobiology*, 2018. doi:10.1007/s12035-018-1101-1

KIM, G. H. et al. The role of oxidative stress in neurodegenerative diseases. *Experimental Neurobiology*, v. 24, n. 4, p. 325-340, 2015.

KNEZOVIC, A. et al. Staging of cognitive deficits and neuropathological and ultrastructural changes in streptozotocin-induced rat model of Alzheimer's disease. *Journal of Neural Transmission*, v. 122, n. 4, p. 577-592, 2015.

KNEZOVIC, A. et al. Rat brain glucose transporter-2, insulin receptor and glial expression are acute targets of intracerebroventricular streptozotocin: risk factors for sporadic Alzheimer's disease? *Journal of Neural Transmission*, v. 124, n. 6, p. 695-708, 2017.

KONG, J. M. et al. Analysis and biological activities of anthocyanins. *Phytochemistry*, v. 64, n. 5, p. 923-933, 2003.

KOVINICH, N. et al. Not all anthocyanins are borne equal: distinct patterns induced by stress in *Arabidopsis*. *Planta*, v.240, n.5, p.931-940, 2014.

KRASKA, A. et al. In vivo cross-sectional characterization of cerebral alterations induced by intracerebroventricular administration of streptozotocin. *PLoS One*, v. 7, n. 9, e46196, 2012.

KRIKORIAN, R et al. Concord grape juice supplementation and neurocognitive function in human aging. *Journal of Agricultural and Food Chemistry*, v. 60, n. 23, p. 5736-5742, 2012.

LENZEN, S. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia*, v. 51, n. 2, p. 216-226, 2008.

LEUNER, K.; MÜLLER, W. E.; REICHERT, A. S. From mitochondrial dysfunction to amyloid beta formation: novel insights into pathogenesis of Alzheimer's disease. *Molecular Neurobiology*, v. 46, n. 1, p. 186-193, 2012.

LIN, M. T.; BEAL, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, v. 443, n. 7113, p. 787-795, 2006.

LORES, G. R.; BERSIER, M. G. Relationship between Na+, K+-ATPase and NMDA receptor at central synapses. *Current Protein & Peptide Science*, v. 15, n. 8, p. 761-777, 2014.

LÜTH, H.J. et al. Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. *Brain Research*, v. 913, n. 1, p. 57-67, 2001.

MAKIN, S. The amyloid hypothesis on trial. *Nature*, v. 559, S4-S7, 2018. doi: 10.1038/d41586-018-05719-4

MANOHARAM, S. et al. The role of reactive oxygen species in the pathogenesis of Alzheimer's disease, Parkinson's disease, and Huntington's disease: a mini review. *Oxidative Medicine Cellular Longevity*, v. 2016, article 8590578, p. 1-15, 2016.

MASTERS, C. L. et al. Alzheimer's disease. *Nature Reviews Disease Primers*, v.1, article 15056, 2015.

MAYEUX, R.; STERN, Y. Epidemiology of Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, v. 2, n. 8, p. 1-18, 2012.

MCKHANN, G. M. et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & Dementia*, v. 7, n. 3, p. 263-269, 2011.

MCDOWELL, I. Alzheimer's disease: insights from epidemiology. *Aging*, v. 13, n. 3, p.143-162, 2001.

MESULAM, M. The cholinergic lesion of Alzheimer's disease: pivotal factor or side show? *Learning & Memory*, v. 11, n. 1, p. 43-49, 2004.

MIGUEL, M. G. Anthocyanins: antioxidant and/or anti-inflammatory activities. *Journal of Applied Pharmaceutical Science*, v. 1, n. 6, p. 7-15, 2011.

MINTER, M. R.; TAYLOR, J. M.; CRACK, P. J. The contribution of neuroinflammation to amyloid toxicity in Alzheimer's disease. *Journal of Neurochemistry*, v. 136, n. 3, p. 457-474, 2016.

MORALES, I. et al. Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. *Front in Cellular Neuroscience*, v. 8, article 112, p. 1-9, 2014.

MOSELEY, A. E. et al. Deficiency in Na,K-ATPase α isoform genes alters spatial learning, motor activity, and anxiety in mice. *Journal of Neuroscience*, v. 27, n. 3, p. 616-626, 2007.

MOSKAUG, J. Ø. et al. Polyphenols and glutathione synthesis regulation. *American Journal of Clinical Nutrition*, v. 81, n. 1suppl, p. 277S-283S, 2005.

MUFSON, E. J. et al. Cholinergic system during the progression of Alzheimer's disease: therapeutic implications. *Expert Review of Neurotherapeutics*, v. 8, n. 11, p. 1703-1718, 2008.

NAZEM, A.; SANKOWSKI, R.; BACHER, M.; AL-ABED, Y. Rodent models of neuroinflammation for Alzheimer's disease. *Journal of Neuroinflammation*, v. 12, n. 74, p. 1-15, 2015.

NEHA et al. Animal models of dementia and cognitive dysfunction. *Life Science*, v. 109, n. 2, p. 73-86, 2014.

NOWRANGI, M. A.; LYKETSOS, C. G.; ROSENBERG, P. B. Principles and management of neuropsychiatric symptoms in Alzheimer's dementia. *Alzheimer's Research & Therapy*, v. 7, article 12, p. 1-10, 2015.

OHNISHI, T. et al. Na, K-ATPase α3 is a death target of Alzheimer patient amyloid-β assembly. *Proceedings of the National Academy of Sciences of the United States of America,* v. 112, n. 32, p. E4465-E4474, 2015.

ORTIZ, G. G. et al. Oxidative stress: love and hate history in central nervous system. *Advances in Protein Chemistry and Structural Biology*, v. 108, p. 1-31, 2017.

PERSSON, T.; POPESCU, B. O.; CEDAZO-MINGUEZ, A. Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail? *Oxidative Medicine and Cellular Longevity*, v. 2014, article 427318, 2014.

PETRONI, K.; TONELLI, C. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Science*, v. 181, n. 3, p. 219-229, 2011.

PETRUSHANKO, I. Y. et al. Direct interaction of beta-amyloid with Na,K-ATPase as a putative regulator of the enzyme function. *Science Reports*, v. 6, article 27738, p. 1-10, 2016.

PIERCE, A. L.; BULLAIN, S. S.; KAWAS, C. H. Late-onset Alzheimer disease. *Neurologic Clinics*, v. 35, n. 2, p. 283-293, 2017.

POJER, E. et al. The case for anthocyanin consumption to promote human health: a review. *Comprehensive Reviews in Food Science and Food Safety*, v. 12, n. 5, p. 483-508, 2013.

POOLE, L. B. The basics of thiols and cysteines in redox biology and chemistry. *Free Radical Biology & Medicine*, v. 80, p. 148-157, 2015.

QUERFURTH, H. W.; LAFERLA, F. M. Alzheimer's disease. *New England Journal of Medicine*, v. 362, n. 4, p. 329-344, 2010.

RAI, S. et al. A study on neuroinflammation and NMDA receptor function in STZ (ICV) induced memory impaired rats. *Journal of Neuroimmunology*, v. 254, n. 1-2, p. 1-9, 2013.

Ramirez, M. R et al. Effect of lyophilised *Vaccinium* berries on memory, anxiety and locomotion in adult rats. *Pharmacological Research*, v. 52, n. 6, p. 457-62, 2005.

RASKIN, J. et al. Neurobiology of Alzheimer's disease: integrated molecular, physiological, anatomical, biomarker, and cognitive dimensions. *Current Alzheimer Research*, v. 12, n. 8, p. 712-722, 2015.

RAVELLI, K. G. et al. Intracerebroventricular streptozotocin as a model of Alzheimer's disease: neurochemical and behavioral characterization in mice. Neurotoxicity Research, v. 31, n. 3, p. 327-33, 2017.

REHMAN, S. U. et al. Anthocyanins reversed D-galactose-induced oxidative stress and neuroinflammation mediated cognitive impairment in adult rats. *Molecular Neurobiology*, v. 54, n. 1, p. 255-271, 2017.

REITZ, C.; MAYEUX, R. Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochemical Pharmacology*, v. 88, n. 4, p. 640-651, 2014.

RENDEIRO, C. et al. Dietary levels of pure flavonoids improve spatial memory performance and increase hippocampal brain-derived neurotrophic factor. *PLoS One*, v. 8, n. 5, article e63535, 2013.

RENDEIRO, C.; RHODES, J. S.; SPENCER, J. P. The mechanisms of action of flavonoids in the brain: Direct versus indirect effects. *Neurochemistry International*, v. 89, p. 126-139, 2015.

RUBIO-PEREZ, J. MORILLAS-RUIZ, J. M. A review: inflammatory process in Alzheimer's disease, role of cytokines. *Scientific World Journal*, v. 2012, article 756357, p. 1-15, 2012.

SABBAGH, M.; CUMMINGS, J. Progressive cholinergic decline in Alzheimer's disease: consideration for treatment with donepezil 23 mg in patients with moderate to severe symptomatology. *BMC Neurology*, v. 11, article 21, p. 1-6, 2011.

SALKOVIC-PETRISIC, M.; HOYER, S. Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach. *Journal of Neural Transmission*, v. 72, p. 217-233, 2007.

SALKOVIC-PETRISIC, M. et al. What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research. *Journal of Neural Transmission*, v. 120, n. 1, p. 233-252, 2013.

SALKOVIC-PETRISIC, M. et al. Long-term oral galactose treatment prevents cognitive deficits in male Wistar rats treated intracerebroventricularly with streptozotocin. *Neuropharmacology*, v. 77, p. 68-80, 2014.

SANTOS, R. X. et al. Mitochondrial DNA oxidative damage and repair in aging and Alzheimer's disease. *Antioxidants & Redox Signaling*, v. 18, n. 18, p. 2444-2457, 2013.

SANTOS, J. P. A. et al. Early and persistent O-GlcNAc protein modification in the streptozotocin model of Alzheimer's disease. *Journal of Alzheimers Disease*, v. 61, n. 1, p. 237-249, 2018.

SAXENA, G.; PATRO, I. K.; NATH, C. ICV STZ induced impairment in memory and neuronal mitochondrial function: a protective role of nicotinic receptor. *Behavioral Brain Research*, v. 224, n. 1, p. 50-57, 2011.

SCHEFF, S. W.; ANSARI, M. A.; MUFSON, E. J. Oxidative stress and hippocampal synaptic protein levels in elderly cognitively intact individuals with Alzheimer's disease pathology. *Neurobiology of Aging*, v. 42, p. 1-12, 2016.

SCHELTENS, P. et al. Alzheimer's disease. *Lancet*, v. 388, n. 10043, p. 505-517, 2016.

SCHLIEBS, R.; ARENDT, T. The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. *Journal of Neural Transmission*, v. 113, n. 11, p. 1625-1644, 2006.

SCHLIEBS, R.; ARENDT, T. The cholinergic system in aging and neuronal degeneration. *Behavioral Brain Research*, v. 221, n. 2, p. 555-563, 2011.
SELFRIDGE, J. E. et al. Role of mitochondrial homeostasis and dynamics in Alzheimer's disease. *Neurobiology of Disease*, v. 51, p. 3-12, 2013.

SELKOE, D. J.; HARDY, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*, v. 8, n. 6, p. 595-608, 2016.

SHARMA, V. et al. Neuroprotective effect of RO-20-1724-a phosphodiesterase4 inhibitor against intracerebroventricular streptozotocin induced cognitive deficit and oxidative stress in rats. *Pharmacology, Biochemistry, and Behavior*, v. 101, n. 2, p. 239-245, 2012.

SHIH, P. et al. Protective effects of anthocyanins against amyloid β-peptide-induced damage in neuro-2A cells. *Journal of Agricultural and Food Chemistry*, v. 59, n. 5, p. 1683-1689, 2011.

SHOHAM, S. et al. Intracerebroventricular injection of streptozotocin causes neurotoxicity to myelin that contributes to spatial memory deficits in rats. *Experimental Neurology*, v. 184, n. 2, p. 1043-1052, 2003.

SHOHAM, S. et al. Ladostigil prevents gliosis, oxidative-nitrative stress and memory deficits induced by intracerebroventricular injection of streptozotocin in rats. *Neuropharmacology*, v. 52, n. 3, p. 836-843, 2007.

SMALL, D. H.; CAPPAI, R. Alois Alzheimer and Alzheimer's disease: a centennial perspective. *Journal of Neurochemistry*, v. 99, n. 3, p. 708-710, 2006.

SMERIGLIO, A. et al. Chemistry, pharmacology and health benefits of anthocyanins. *Phototherapy Research*, v. 30, n. 8, p. 1265-1286, 2016.

SOHANAKI, H. et al. Pelargonidin improves memory deficit in amyloid β25-35 rat model ofAlzheimer's disease by inhibition of glial activation, cholinesterase, and oxidative stress. *Biomedicine & Pharmacotherapy*, v. 83, p. 85-91, 2016.

SPERLING, R. A. et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & Dementia*, v. 7, n. 3, p. 280-292, 2011.

STRATHEARN, K. E. et al. Neuroprotective effects of anthocyanin- and proanthocyanidin-rich extracts in cellular models of Parkinsons disease. *Brain Research*, v. 1555, p. 60-77, 2014

STEWART, V. C.; HEALES, S. J. Nitric oxide-induced mitochondrial dysfunction: implications for neurodegeneration. *Free Radical Biology & Medicine*, v. 34, n. 3, p. 287-303, 2003.

SZKUDELSKI, T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiological Research*, v. 50, p. 536-546, 2001.

TALAVÉRA, S. et al. Anthocyanins metabolism in rats and their distribution to digestive area, kidney, and brain. *Journal of Agricultural of Food Chemistry*, v. 53, n. 10, p. 3902-3908, 2005.

TAROZZI, A. et al. Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells. *Neuroscience Letters*, v. 424, n. 1, p. 36-40, 2007.

TAROZZI, A. et al. Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25–35) oligomer-induced toxicity. *Neuroscience Letters*, v. 473, n. 2, p. 72-76, 2010.

TATA, A. M. et al. Cholinergic system dysfunction and neurodegenerative diseases: cause or effect? *CNS & Neurological Disorders Drug Targets*, v. 13, n. 7, p. 1294-1303, 2014.

THULLBERY, M. D. et al. Differential localization of acetylcholinesterase in neuronal and non-neuronal cells. *Journal of Cellular Biochemistry*, v. 96, n. 3, p. 599-610, 2005.

TIWARI, V. et al. Chronic treatment with tocotrienol, an isoform of vitamin E, prevents intracerebroventricular streptozotocin-induced cognitive impairment and oxidative–nitrosative stress in rats. *Pharmacology, Biochemistry, and Behavior*, v. 93, n. 2, p.183-189, 2009.

TOTA, S. et al. Protective effect of quercetin against intracerebral streptozotocin induced reduction in cerebral blood flow and impairment of memory in mice. *Behavioral Brain Research*, v. 209, n. 1, p. 73-79, 2010.

TSUDA, T. Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. *Molecular Nutrition & Food Research*, v. 56, n. 1, p. 159-170, 2012.

VEPSÄLÄINEN, S. et al. Anthocyanin-enriched bilberry and blackcurrant extracts modulate amyloid precursor protein processing and alleviate behavioral abnormalities in the APP/PS1 mouse model of Alzheimer's disease. *The Journal of Nutritional Biochemistry*, v. 24, n. 1, p. 360-370, 2013.

VERKHRATSKY, A. et al. Astrocytes in Alzheimer's disease. *Neurotherapeutics*, v. 7, n. 4, p. 399-412, 2010.

VON BERNHARDI, R. et al. Acetylcholinesterase induces the expression of the beta-amyloid precursor protein in glia and activates glial cells in culture. *Neurobiology of Disease*, v. 14, n. 3, p. 447-57, 2003.

WAKABAYASHI, N. et al. When NRF2 talks, who's listening? *Antioxidants & Redox Signaling*, v. 13, n. 11, p. 1649–1663, 2010.

WANG, J. et al. A systemic view of Alzheimer disease - insights from amyloid-β metabolism beyond the brain. *Nature Reviews Neurology*, v. 13, n. 10, p. 612-623, 2017.

WANG, X. F.; CYNADER, M. S. Astrocytes provide cysteine to neurons by releasing glutathione. *Journal of Neurochemistry*, v. 74, n. 4, p. 1434-1442, 2000.

WILKINS, H. M.; CARL, S. M.; GREENLIEF, A. C. S.; FESTOFF, B. W.; SWERDLOW, R. H. Bioenergetic dysfunction and inflammation in Alzheimer's disease: a possible connection. *Frontiers in Aging Neuroscience*, v. 6, article 311, 2014.

WU, X. et al. Concentration of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry*, v. 54, n. 11, p. 4069-4075, 2006.

YAN, Z.; FENG, J. Alzheimer's disease: interactions between cholinergic functions and β-amyloid. *Current Alzheimer Research*, v. 1, n. 4, p. 241-248, 2004.

ZAKARIA, R.; WAN YACOOB, W. M. H.; OTHMAN, Z.; LONG, I.; AHMAD, A. H.; AL-RAHBI, B. Lipopolysaccharide-induced memory impairment in rats: a model of Alzheimer's disease. *Physiological Research*, v. 66, p. 553-565, 2017.

ZHANG, L. N. et al. Na^{+}-K^{+}-ATPase, a potent neuroprotective modulator against Alzheimer disease. *Fundamental & Clinical Pharmacology*, v. 27, n. 1, p. 96-103, 2013.

ZHAO, Y.; ZHAO, B. Oxidative stress and the pathogenesis of Alzheimer's disease. *Oxidative Medicine and Cellular Longevity*, v. 2013, article 316523, 2013.

ZILBERTER, Y.; ZILBERTER, M. The vicious circle of hypometabolism in neurodegenerative diseases: ways and mechanisms of metabolic correction. *Journal of Neuroscience Research*, v. 95, n. 11, p. 2217-2235, 2017.

ANEXO

UFPel

CEEA

Comissão de Ética em Experimentação Animal

Pelotas, 14 de janeiro de 2015

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professora Roselia Maria Spanevello

Centro de Ciências Químicas, Farmacêuticas e de Alimentos

Senhora Professora:

A *CEEA* analisou o projeto intitulado: **"Mecanismo neuroprotetores das antocianinas sobre a perda de memória e parâmetros bioquímicos em modelos experimentais para a doença de Alzhmeir",** processo n°23110.000179/2015-48, sendo de parecer **FAVORÁVEL** a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro nº **CEEA 0179-2015**).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva

Presidente da CEEA

Ciente em: 25 /02 /2015

Assinatura da Professora Responsável:

Roselia Maria Spanevello