UNIVERSIDADE FEDERAL DO PARANÁ

LEONARDO DE CASTRO E SOUZA

ESTUDO DOS EFEITOS DE ESTRATÉGIAS DE NEUROPROTEÇÃO EM UM MODELO ANIMAL DE DOENÇA DE ALZHEIMER ESPORÁDICA

CURITIBA

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Tese de doutorado apresentada ao curso de Pós-Graduação em Farmacologia, no Setor de Ciências Biológicas, na Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Farmacologia

Orientadora: Profa. Dra. Maria Aparecida Barbato Frazão Vital

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ATA Nº316

ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM FARMACOLOGIA

No dia vinte e oito de abril de dois mil e vinte e dois às 09:00 horas, na sala Anfiteatro da Pós Graduação em Farmacologia., Setor de Ciências BiológicasDepartamento de Farmacologia, foram instaladas as atividades pertinentes ao rito de defesa de tese do doutorando **LEONARDO DE CASTRO E SOUZA**, intitulada**: ESTUDO DOS EFEITOS DE ESTRATÉGIAS DE NEUROPROTEÇÃO EM UM MODELO ANIMAL DE DOENÇA DE ALZHEIMER ESPORÁDICA**, sob orientação da Profa. Dra. MARIA APARECIDA BARBATO FRAZÃO VITAL. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: MARIA APARECIDA BARBATO FRAZÃO VITAL (UNIVERSIDADE FEDERAL DO PARANÁ), TAYSA BERVIAN BASSANI (MAASTRICHT UNIVERSITY), BRUNO JACSON MARTYNHAK (UNIVERSIDADE FEDERAL DO PARANÁ), ROBERTO ANDREATINI (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutor está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, MARIA APARECIDA BARBATO FRAZÃO VITAL, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 28 de Abril de 2022.

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MARIA APARECIDA BARBATO FRAZÃO VITAL
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TAYSA BERVIAN BASSANI
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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LEONARDO DE CASTRO E SOUZA** intitulada: **ESTUDO DOS EFEITOS DE ESTRATÉGIAS DE NEUROPROTEÇÃO EM UM MODELO ANIMAL DE DOENÇA DE ALZHEIMER ESPORÁDICA**, sob orientação da Profa. Dra. MARIA APARECIDA BARBATO FRAZÃO VITAL, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Dedico esse trabalho aos meus pais, Cássia e Sidenei, aos meus avós, Creonice, Mário e Rosa, e à minha namorada Mirian. Por tudo o que vocês são para mim, chegar até aqui também foi culpa de vocês.

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RESUMO

A doença de Alzheimer (DA) é uma desordem neurodegenerativa progressiva caracterizada clinicamente pelo declínio gradual das funções cognitivas. A infusão intracerebroventricular (ICV) de estreptozotocina (STZ) é usada como um modelo da forma esporádica da doença, causando várias alterações que são importantes no curso da DA, como o prejuízo cognitivo, a agregação de proteínas, danos na sinalização de insulina, neuroinflamação e estresse oxidativo. No presente trabalho investigamos os efeitos do andrografolide (ANDRO) no modelo STZ-ICV com relação a memória espacial de curto prazo (Testes de Localização de objetos (TLO) e Labirinto em Y), a memória de reconhecimento de curto prazo (Teste de reconhecimento de objetos (TRO)), a atividade locomotora (Campo aberto), a expressão da proteína precursora amiloide (APP) e a ativação de astrócitos (expressão de GFAP) e da micróglia (imuno-histoquímica para Iba-1). Ratos Wistar foram submetidos a infusão ICV de 3 mg/kg de STZ ou veículo, e foram tratados com ANDRO (2 mg/kg, i.p.) ou veículo três vezes por semana por quatro semanas, com início 1h após a cirurgia. O tratamento com ANDRO atenuou o prejuízo de desempenho nos testes do Labirinto em Y e TRO, e o aumento da expressão de GFAP no córtex pré-frontal dos animais causados pela STZ. Curiosamente a STZ não piorou o desempenho dos animais no TLO, mas o ANDRO sim. Além disso, o número de células da micróglia ativadas nas regiões CA1 e CA3 do hipocampo dos animais STZ que receberam ANDRO diminuiu em relação aos animais STZ que receberam veículo. Foram observadas correlações entre a neuroinflamação e o desempenho cognitivo. Com relação a APP, nem a infusão de STZ e nem o tratamento com ANDRO alteraram significativamente sua expressão. Os resultados sugerem efeitos parcialmente positivos do ANDRO na memória espacial de curto prazo e na memória de reconhecimento de curto prazo. Além disso, o ANDRO atenuou a ativação de astrócitos e da micróglia causada pelo modelo STZ.

Palavras-chave: Alzheimer; andrografolide; memória; micróglia; astrócitos; estreptozotocina

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder clinically characterized by a gradual decline in cognitive functions. The intracerebroventricular infusion (ICV) of streptozotocin (STZ) is used as a model of the sporadic form of the disease, causing several changes important to the course of AD, such as cognitive impairment, protein aggregation, damage to insulin signaling, neuroinflammation and oxidative stress. In the present study we investigated the effects of andrographolide (ANDRO) in the ICV-STZ model relative to short-term spatial memory (Object location test (OLT) and Y-Maze test), short-term recognition memory (Object recognition test (ORT)), locomotor activity (Open field), amyloid precursor protein (APP) expression and activation of astrocytes (GFAP expression) and microglia (immunohistochemistry for Iba-1). Wistar rats were given an ICV infusion of 3 mg/kg of STZ or vehicle and were treated with ANDRO (2 mg/kg, i.p.) or vehicle three times per week for four weeks, starting 1 h after the infusion. Treatment with ANDRO was capable of attenuate the impairments in the Y maze and ORT performance, and the increase in GFAP expression in the PFC induced by the ICV-STZ model. Interestingly, STZ did not worsen the animals' performance in OLT, but ANDRO did. In addition, the number of activated microglia cells in the CA1 and CA3 regions of the hippocampus of STZ animals that received ANDRO decreased in relation to STZ animals that received vehicle. Correlations were observed between neuroinflammation and cognitive performance. Regarding APP, neither STZ infusion nor ANDRO treatment significantly altered its expression. The results suggest partially positive effects of ANDRO on short-term spatial memory and short-term recognition memory. Furthermore, ANDRO attenuated the astrocyte and microglia activation caused by STZ.

Keywords: Alzheimer’s; andrographolide; memory; microglia; astrocyte; streptozotocin.

SUMÁRIO

1 INTRODUÇÃO

Demência é um termo genérico para um grupo particular de sintomas. Os sintomas característicos da demência são dificuldades com memória, linguagem, solução de problemas e outras capacidades cognitivas que afetam a habilidade de uma pessoa em realizar tarefas cotidianas. A demência tem várias causas, a doença de Alzheimer (DA) é a causa mais comum (ALZHEIMER'S ASSOCIATION, 2020). Em 2018 estimava-se que 44 milhões de pessoas viviam com demência no mundo, sendo que de 50 a 75% dos casos se deviam a DA. Reconhecida pela Organização Mundial da Saúde como uma prioridade global de saúde pública, a DA é uma importante causa de dependência, incapacitação e mortalidade (LANE; HARDY; SCHOTT, 2018).

A doença de Alzheimer é uma desordem neurodegenerativa progressiva relacionada a idade (CORREIA *et al.*, 2011). Dados de pessoas centenárias mostram que a DA não é necessariamente o resultado do envelhecimento, mas a idade é seu maior fator de risco. A incidência na população dobra a cada 5 anos de idade após os 65 anos, assim, as chances de receber o diagnóstico da doença após os 85 anos é de pelo menos 1 em 3 (CUMMINGS, 2004; QUERFURTH; LAFERLA, 2010). Na DA os sintomas clínicos mais característicos são prejuízo da memória episódica, deterioração da linguagem, e déficits visuoespaciais. Distúrbios funcionais, comportamentais, e neuropsiquiátricos são comuns, e progridem com o curso da doença. Problemas motores, sensoriais, e convulsões ocorrem em fases mais tardias (CUMMINGS, 2004; SALARDINI, 2019). A DA leva à morte dos pacientes de 3 a 9 anos após o diagnóstico (QUERFURTH; LAFERLA, 2010).

Apesar de ter sido descrita pela primeira vez por Alois Alzheimer em 1907, a etiologia da doença ainda não é bem conhecida, o que limita seu tratamento farmacológico (CISTERNAS; INESTROSA, 2017).

1.1 FISIOPATOLOGIA DA DOENÇA DE ALZHEIMER

As mudanças neuropatológicas da DA incluem características positivas e negativas. As características positivas clássicas consistem nas placas amiloides, constituídas de agregados extracelulares de proteína beta-amiloide (Aβ), e dos emaranhados neurofibrilares (ENF), formados por agregados intracelulares da

proteína tau hiperfosforilada, que são acompanhados por astrogliose e ativação celular da micróglia (SERRANO-POZO *et al.*, 2011a).

Alguns estudos clínico-patológicos de diferentes grupos têm estabelecido que os ENF se correlacionam com a severidade e a duração da DA. As principais regiões cerebrais que se degeneram na DA são o prosencéfalo basal, hipocampo, amígdala, e áreas associativas do isocórtex (neocórtex). O padrão espaço-temporal da progressão dos ENF é bastante estereotípico e previsível, eles se distribuem topograficamente de uma forma seletiva e não generalizada. Em seu estudo clínico-patológico, Braak e Braak (1991) distinguiram seis estágios que podem ser resumidos em três: entorrinal, límbico e isocortical (Fig. 1). A degeneração neurofibrilar começa no alocórtex do lobo temporal medial (córtex entorrinal e hipocampo) e se espalha para o isocórtex. Todas as áreas isocorticais são afetadas, com as áreas associativas sendo afetadas antes e mais severamente que as áreas sensoriais, motoras e visuais primárias (BRAAK; BRAAK, 1991; SERRANO-POZO *et al.*, 2011a).

FIGURA 1 - PADRÃO ESPAÇO-TEMPORAL DA DEGENERAÇÃO NEUROFIBRILAR NA DA

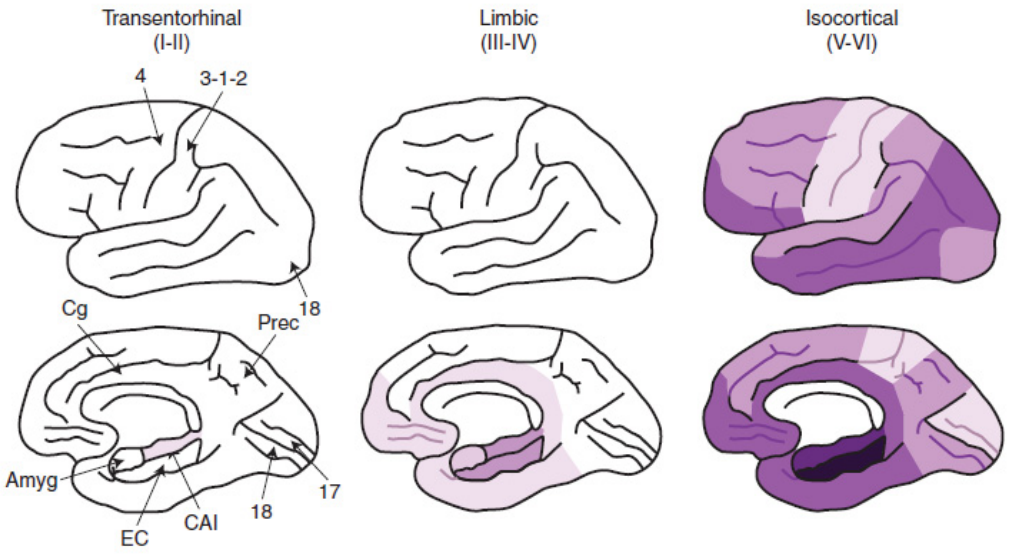

FIGURA 1. O sombreamento indica a distribuição dos emaranhados neurofibrilares com as cores mais escuras representando densidades crescentes. Amyg = Amígdala; EC = Córtex entorrinal; CA1 = Área CA1 do hipocampo; Cg = Córtex cingulado; Prec = Precuneus; 4 = Córtex motor primário; 3-1-2 = Córtex sensorial primário; 17 = Córtex Visual Primário; 18 = Córtex visual associativo. FONTE: SERRANO-POZO *et al*. (2011).

Esse padrão espaço-temporal dos ENF faz correspondência com o perfil neuropsicológico hierárquico típico da DA. O prejuízo proeminente inicial da memória episódica, a capacidade de lembrar conscientemente de episódios e experiencias pessoais, característico da doença é explicado pelo isolamento do córtex entorrinal e hipocampo em relação ao isocórtex associativo e aos núcleos subcorticais em função da degeneração massiva causada pelos ENF (SERRANO-POZO *et al.*, 2011a; TULVING, 2002). Em seguida, o envolvimento das áreas isocorticais associativas de alto grau é responsável pela deterioração progressiva de domínios cognitivos adicionais, incluindo disfunção executiva (córtex pré-frontal), apraxias (córtex parietal), déficits de navegação visuoespacial (córtex occipital), déficits visuoperceptivos (córtex occipitotemporal), e memória semântica (córtex temporal anterior), que se refere ao conhecimento adquirido por um indivíduo sobre fatos do mundo e conceitos, dando origem à síndrome demencial plena (BRAAK; BRAAK, 1991; MATTHEWS, 2015; SERRANO-POZO *et al.*, 2011a). Em contrapartida, o envolvimento tardio da áreas isocorticais motoras, sensoriais e visuais explica por que as funções motoras, sensoriais e visuais primárias são poupadas. Entretanto, se a formação dos ENF é necessária para a morte neuronal ou representa uma resposta protetora dos neurônios danificados ainda é controverso (BRAAK; BRAAK, 1991; BRAAK; DEL TREDICI, 2012).

Diferente dos ENF, as placas amiloides se acumulam principalmente no isocórtex. Ainda que o padrão espaço-temporal da progressão dos depósitos amiloides seja muito menos previsível do que o padrão dos ENF, em geral, o alocórtex (incluindo o córtex entorrinal e hipocampo), os núcleos da base, núcleos do tronco encefálico, e do cerebelo estão envolvidos em menor extensão e mais tardiamente que o isocórtex associativo. A dissociação entre a sobrecarga amiloide e dos emaranhados no lobo temporal é notável. Estudos tem demonstrado que as lesões causadas pelas placas amiloides não tem correlação com a severidade e a duração da DA. De fato, no isocórtex associativo, região de acúmulo amiloide inicial, as lesões chegam a um platô logo após o início dos sintomas cognitivos ou ainda na fase pré-clínica da doença e nem mesmo o tamanho das placas aumenta significativamente com a progressão da doença (BRAAK *et al.*, 2006; BRAAK; BRAAK, 1991; BRAAK; DEL TREDICI, 2012; SERRANO-POZO *et al.*, 2011).

Já as principais características neuropatológicas negativas da DA são a perda de sinapses e neurônios, esse processo ocorre através de danos iniciais nas sinapses seguidos pela degeneração retrógrada dos axônios e eventual atrofia da árvore dendrítica e do corpo celular neuronal. O padrão regional da perda neuronal se correlaciona ao padrão dos ENF, mas, dentro das mesmas regiões a perda neuronal excede o número de emaranhados, tendo assim uma correlação maior com os déficits cognitivos. Da mesma forma, o padrão da perda sináptica se correlaciona também ao da perda neuronal. A perda sináptica não é causada apenas pela morte neuronal, podendo excedê-la em determinadas áreas corticais. Isso indica que a perda sináptica é anterior a perda neuronal e que os neurônios remanescentes se tornam menos bem conectados aos seus parceiros de sinapse (GÓMEZ-ISLA *et al*., 1996; INGELSSON *et al*., 2004; SERRANO-POZO *et al*., 2011).

A evolução da perda sináptica e neuronal na DA, portanto, se correlaciona com a deposição dos emaranhados neurofibrilares, se espalhando pelas vias septo-hipocampal e prosencéfalo basal-isocortical. E ambos os eventos podem ser correlacionados com o prejuízo cognitivo dos pacientes. Assim, pode-se associar também o dano à cognição na DA com a transmissão de acetilcolina, já que essas vias neuronais atingidas são colinérgicas. Isso corrobora as análises neuroquímicas feitas no tecido de cérebros com DA que mostram uma redução substancial de enzimas que produzem e metabolizam a acetilcolina (AULD *et al.*, 2002; SELKOE, 2002). A “hipótese colinérgica” da disfunção de memória na DA também é apoiada por evidências farmacológicas, que mostram que a administração de antagonistas dos receptores colinérgicos (e.g. escopolamina) interferem com a aquisição e desempenho de roedores e primatas não-humanos em várias tarefas de memória (AULD *et al.*, 2002). Além disso, drogas que potencializam a função colinérgica no sistema nervoso central (SNC) são, até hoje, as terapias mais efetivas contra o declínio cognitivo na DA. Porém, essa efetividade das estratégias de reposição colinérgica é bastante limitada (O’BRIEN; BURNS, 2011).

Atualmente, existem três inibidores da acetilcolinesterase (enzima que degrada a acetilcolina) usados como tratamento para a DA, donepezila, galantamina, e rivastigmina, bem como o antagonista glutamatérgico, memantina. Apesar de serem amplamente prescritas, essas drogas possuem efeitos sintomáticos limitados e temporários quando comparadas com o placebo, sem um impacto de longo prazo no

processo neurodegenerativo (O'BRIEN; BURNS, 2011; STELLA *et al.*, 2015). Assim, esforços para o desenvolvimento de agentes farmacológicos com propriedades para prevenir ou interromper efetivamente os estágios iniciais das vias de neurodegeneração da DA ainda são feitos e continuam um problema crucial ao redor do mundo (STELLA *et al.*, 2015). Em junho de 2021, a *Food and Drug Administration* (FDA) nos Estados Unidos aprovou o aducanumab, um anticorpo monoclonal humano que reage com oligômeros e agregados de Aβ, mas essa aprovação ainda é controversa. Tanto os resultados em modelos animais quantos os ensaios clínicos iniciais eram promissores, sugerindo uma possível desaceleração na formação das placas amiloides e no quadro clínico. Entretanto, nos testes clínicos de fase III o aducanumab diminuiu significativamente as placas, mas não mostrou resultados importantes na cognição dos pacientes (ALEXANDER *et al*., 2021; DUNN *et al*., 2021; SEVIGNY *et al*., 2016).

1.2 ETIOLOGIA DA DOENÇA DE ALZHEIMER

Evidências sugerem fortemente que os mecanismos neuropatológicos da DA começam muito antes das manifestações clínicas, com o acúmulo de Aβ no córtex e dos emaranhados neurofibrilares no lobo temporal medial podendo preceder os sinais clínicos em décadas. Porém, ainda existe um amplo debate sobre como, onde e quando o processo neurodegenerativo começa (CALDERON-GARCIDUEÑAS; DUYCKAERTS, 2018; STELLA *et al.*, 2015).

A vasta maioria dos casos de DA ocorre de forma esporádica (>95%) e com um início tardio, após os 65 anos de idade. Mas existe uma rara forma familiar de DA (1-5% dos casos), de início precoce, podendo começar entre os 30 e 50 anos (LANE; HARDY; SCHOTT, 2018). Clinicamente são indistinguíveis, mas a DA familiar geralmente é associada a um ritmo mais acelerado de progressão e um padrão Mendeliano de herança. Três genes (APP, PSEN1 e PSEN2) que codificam proteínas envolvidas na quebra da APP e geração de Aβ são associados à DA familiar. Mutações nesses genes são em maioria herdados de forma autossômica dominante, e levam rigorosamente a agregação de Aβ e início precoce da doença (REITZ; MAYEUX, 2014).

Em contraste, a DA esporádica é multifatorial, e ocorre por uma complexa interação entre fatores genéticos e ambientais. Os genes envolvidos na DA esporádica aumentam o risco da doença de forma não Mendeliana. Familiares de primeiro grau de pacientes com DA esporádica possuem o dobro da probabilidade de desenvolver a doença durante a vida em comparação às pessoas que não possuem familiares de primeiro grau com DA. Além disso, a DA esporádica ocorre mais em gêmeos monozigóticos do que em dizigóticos, sugerindo uma contribuição genética substancial (60-80%) para a doença. Apenas um fator de risco genético foi estabelecido como gene de susceptibilidade, e apenas em pessoas brancas de origem europeia, o alelo APOEε4, localizado no gene 19q13. A APOE é uma proteína de ligação a lipídios e é expressa em humanos como três isoformas comuns codificadas por três alelos, APOEε2, ε3 e ε4. Um único alelo APOEε4 é associado com uma probabilidade de duas a três vezes maior de risco (KUUSISTO *et al.*, 1994; REITZ; MAYEUX, 2014). Na população o risco de possuir o alelo APOEε4 é de 20 a 50%, a presença do APOEε4 não é necessária e nem suficiente para o desenvolvimento da doença. Em outros grupos étnicos além dos brancos de origem europeia a associação entre o alelo e a doença é bastante inconsistente (MYERS *et al.*, 1996; REITZ; MAYEUX, 2014; SLOOTER *et al.*, 1998).

Como mencionado, depois do fator de risco genético, a idade é o fator de risco mais importante na doença de Alzheimer. Existem ainda os fatores de risco ambientais (também chamados de adquiridos ou modificáveis) que antecedem parte do curso da doença, e podem ser associados com sua etiologia ou o seu desenvolvimento. A maior parte desses fatores de risco ambientais estão relacionados com fatores cardiovasculares (doença cerebrovascular, diabetes, hipertensão e obesidade) ou estilo de vida (e.g. tabagismo, atividade física, dieta e atividade mental e social) (CROUS-BOU *et al.*, 2017; REITZ; BRAYNE; MAYEUX, 2011). Como exemplo, estudos observacionais mostraram que o diabetes tipo 2 quase dobra o risco de desenvolver a DA. Foi sugerido que o diabetes pode aumentar o risco de DA afetando diretamente o acúmulo de Aβ no cérebro já que a hiperinsulinemia pode levar a insulina a competir com a Aβ pela enzima degradadora de insulina, que tem um papel na degradação de Aβ, prejudicando a depuração de Aβ no cérebro. Em outros estudos observacionais, as pessoas que tinham dietas com grande ingestão de vitaminas E e C (ambas antioxidantes) mostraram ter uma chance menor de desenvolver declínio

cognitivo e DA do que os indivíduos que tinham baixa ingestão dessas vitaminas (CRAFT, 2007; CROUS-BOU *et al.*, 2017; REITZ; BRAYNE; MAYEUX, 2011).

1.3 PATOGÊNESE DA DOENÇA DE ALZHEIMER

A “hipótese da cascata amiloide” da DA, que é a hipótese dominante da patogênese da doença, sugere que a acumulação patológica de Aβ produzida por clivagens sequenciais da proteína precursora amiloide (APP) pelas enzimas β- e γ-secretase no cérebro é o processo patológico inicial, gerando um desbalanço entre a produção e a degradação de Aβ. A formação dos emaranhados neurofibrilares e a disfunção neuronal e neurodegeneração, mediadas pela inflamação, são provavelmente os processos subsequentes. A forma familiar da DA dá grande suporte a essa hipótese (LANE; HARDY; SCHOTT, 2018).

Originalmente se pensava que as placas amiloides de núcleo denso eram críticas para o desenvolvimento da DA, porém, mais recentemente, algumas linhas de investigação têm apoiado a visão de que níveis aumentados de oligômeros solúveis de Aβ1-42 são as formas mais patológicas. Em modelos experimentais, foi observado que a distribuição transsináptica de Aβ, por exemplo do córtex entorrinal para a camada molecular do giro denteado do hipocampo, promove a neurodegeneração caracterizada pela perda de sinapses. Isso é acompanhado de disfunção da circuitaria e inervação aberrante do hipocampo. Os oligômeros de Aβ secretados por cultura de neurônios inibem a potenciação de longa duração (LTP) e danificam espinhas dendríticas. Esses estudos indicam que os oligômeros podem ser responsáveis pelos déficits de memória, pois eles acumulam nos cérebros de pacientes com DA, ainda que a sua relação com a severidade do prejuízo cognitivo seja incerta (LANE; HARDY; SCHOTT, 2018; SERRANO-POZO *et al.*, 2011a).

Ainda que um diagnóstico neuropatológico da DA seja baseado na avaliação da distribuição e abundância dos emaranhados neurofibrilares e placas amiloides, as características de neurodegeneração ainda incluem perda neuronal, neuroinflamação, gliose, degeneração vascular e de matéria branca. Além disso, déficits no metabolismo cerebral (utilização de glicose e oxigênio) são reconhecidos na DA há décadas e frequentemente avaliados (DE LA MONTE, 2017)

1.3.1 Neuroinflamação

A neuroinflamação desempenha um papel crítico na patofisiologia da DA, e é uma característica precoce da doença (DE LA MONTE, 2017). Neuroinflamação geralmente se refere a resposta inflamatória no SNC que pode ser causada por vários insultos patológicos, incluindo infecção, trauma, isquemia e toxinas (DISABATO; QUAN; GODBOUT, 2016). Na DA, citocinas inflamatórias têm sua expressão aumentada nas proximidades das placas amiloides e dos emaranhados neurofibrilares e são conhecidas por aumentar a produção de peptídeos Aβ. As citocinas como interleucina-1β (IL-1β), fator de necrose tumoral alfa (TNF-α), e IL-6 agravam o processo neuroinflamatório pela deposição de placas amiloides e assim causam efeitos citotóxicos (KAUR; SHARMA; DESHMUKH, 2019). O processo ainda é marcado pela produção de quimiocinas como CCL1, CCL5 e CXCL1, pequenas moléculas mensageiras, incluindo prostaglandinas e óxido nítrico, e espécies reativas de oxigênio. Esses mecanismos são desencadeados principalmente por células da imunidade inata no SNC, as células envolvidas nesse processo são primariamente a micróglia e os astrócitos (DISABATO; QUAN; GODBOUT, 2016).

A liberação de moléculas pró-inflamatórias pode levar à disfunção sináptica e morte neuronal com concomitantemente prejuízo cognitivo. A IL-1β induz a perda sináptica através do aumento da produção de prostaglandina E2, que leva à liberação de glutamato pré-sináptico e forte ativação de receptores N-metil-D-aspartato (NMDA), levando a efeitos tóxicos e possível perda sináptica (MISHRA *et al.*, 2012). O TNF causa a morte neuronal ao ativar o receptor 1 de TNF (TNFR1) e recrutar a caspase 8 quando a via do fator nuclear-κB (NF-κB) está inibida. Além disso, o sistema complemento pode ser ativado, promovendo a função fagocítica da micróglia, o que pode resultar no corte inapropriado de sinapses (LENG; EDISON, 2021). A neuroinflamação ainda promove a disfunção colinérgica e a inibição da neurogênese. Além disso, a inflamação crônica é conhecida por exacerbar a resistência à insulina associada com estados patogênicos sistêmicos, e pode também ter um papel importante na resistência à insulina e ao fator de crescimento semelhante à insulina tipo 1 (IGF-1) cerebral que ocorrem na DA (DE LA MONTE, 2017). Citocinas anti-inflamatórias, como a IL-4, IL-10 e IL-11, também são produzidas durante o processo neuroinflamatório e podem ser parte de um mecanismo sofisticado de prevenção da neuroinflamação excessiva. Entretanto, no contexto de doenças neurodegenerativas,

a neuroinflamação tende a ser crônica, incapaz de se resolver por si só e é considerado um motor da doença (CALSOLARO; EDISON, 2016; HENEKA *et al*., 2015).

1.3.1.1 Astrócitos e Micróglia

Sabe-se que os astrócitos respondem a insultos patológicos através da gliose reativa, que é parte do processo neuroinflamatório e manifestada por um aumento no número, tamanho e motilidade dos astrócitos (PEKNY; WILHELMSSON; PEKNA, 2014). Astrócitos reativos e células ativadas da micróglia são comumente associados com placas amiloides de núcleo denso, indicando que a Aβ é um grande desencadeador da resposta glial. Entretanto, têm-se observado um aumento linear dos astrócitos reativos e células ativadas da micróglia por todo o curso da DA apesar do platô inicial da acumulação de Aβ no córtex temporal associativo. De fato, foi visto uma correlação positiva entre ambas a astrogliose e a microgliose e as lesões pelos emaranhados neurofibrilares, mas não entre essas células e a Aβ, sugerindo que respostas gliais também estão relacionadas com a degeneração pelos ENF (INGELSSON *et al*., 2004; SERRANO-POZO *et al*., 2011b).

Os astrócitos são as células da glia mais abundantes no SNC e possuem funções importantes na organização e manutenção cerebral. Essas células interagem com neurônios e estão envolvidas na liberação e reciclagem de neurotransmissores, homeostase iônica, regulação do metabolismo energético, remodelamento sináptico e modulação do estresse oxidativo, processamento de informações e transmissão de sinais (MERAZ-RÍOS *et al.*, 2013). É proposto que o insulto inflamatório induz o fenótipo de astrócitos A1 através da via NF-κB, enquanto a isquemia induz o fenótipo A2, sendo as células A1 nocivas caracterizadas por expressar mediadores inflamatórios, e as células A2 protetoras marcadas por expressar fatores neurotróficos. Os astrócitos A1 perdem sua função homeostática e são capazes de induzir a apoptose de neurônios e oligodendrócitos. Porém, se os astrócitos adotam múltiplos estados de ativação ou apenas essas duas conformações, ainda está em discussão (LENG; EDISON, 2021; LIDDELOW *et al*., 2017; LIDDELOW; BARRES, 2017).

Dadas suas numerosas funções de manutenção, seria esperado que os astrócitos tentassem recuperar a homeostase nos estágios iniciais da DA. De fato, grânulos contendo Aβ já foram observados em astrócitos ao redor de placas amiloides em cérebros humanos, sugerindo uma tentativa dessas células de remover os depósitos amiloides durante o processo da doença (FUNATO *et al.*, 1998; THAL *et al.*, 2000). Experimentos já mostraram que astrócitos migram em direção das placas de Aβ e degradam a proteína amiloide *in vitro* e *in vivo* (WYSS-CORAY *et al.*, 2003). Porém, os astrócitos A1, que são tóxicos ao SNC, são encontrados em abundância em tecido cerebral *post-mortem* de indivíduos com DA, sugerindo um papel prejudicial dessas células (LIDDELOW *et al.*, 2017). Em um modelo animal de DA, foi visto que astrócitos reativos liberavam GABA e glutamato em excesso, levando ao prejuízo de memória e perda sináptica (JO *et al.*, 2014). Além disso, essas células contribuem para desregular a microcirculação e desestabilizar a barreira hematoencefálica, o que facilita a acumulação de Aβ e a progressão da doença (Fig. 2). Os astrócitos reativos podem ainda preparar o caminho para as primeiras placas amiloides. Os astrócitos atuam próximos a micróglia e podem mediar alguns dos efeitos tóxicos da micróglia em estados patogênicos (LENG; EDISON, 2021).

A micróglia são células da imunidade inata de linhagem mieloide que residem no SNC. A atividade microglial parece ser sincronizada com o desenvolvimento, maturação e senescência do SNC por toda a vida através de diferentes redes regulatórias e tem papéis importantes na poda sináptica durante o desenvolvimento, apoptose neuronal, manutenção da plasticidade sináptica e vigilância imune. Uma função de vigilância imune comprometida parece estar bastante relacionada com doenças neurodegenerativas (SALTER; STEVENS, 2017). Têm se observado que a micróglia ramificada inspeciona continuamente o microambiente do SNC com seus processos, detectando sinais de dano, e mediando uma resposta em direção ao local da lesão. Na presença de insultos patológicos endógenos ou exógenos, classes de receptores microgliais de membrana, denominados padrões moleculares associados a danos e padrões moleculares associados a patógenos, podem reconhecer patógenos, restos celulares ou proteínas anormais (incluindo Aβ) e induzir uma resposta microglial (HENEKA *et al.*, 2015). A micróglia ativada internaliza os patógenos via pinocitose, fagocitose ou endocitose mediada por receptor e tenta degradar eles através de várias vias endocíticas, bem como ativar a expressão de

módulos de genes relevantes, incluindo receptores de quimiocinas e interferons, que são importantes componentes do processo neuroinflamatório. Esse processo usualmente se conclui uma vez que o estímulo imune é eliminado, porém, a micróglia em cérebros envelhecidos possui prejuízos funcionais e é propensa a ativação crônica, o que pode contribuir para a patogênese de doenças neurodegenerativas (NORDEN; GODBOUT, 2013; SOLÉ-DOMÈNECH *et al*., 2016; SPITTAU, 2017).

A micróglia possui uma papel complexo na trajetória da DA devido aos seus fenótipos diversos e a variedade de vias de ativação. Micróglia altamente ramificada pode mudar para uma forma ameboide por estimulação patológica. A micróglia em cérebros envelhecidos diminui a sua ramificação, o que reduz a sua área de vigilância, o que pode contribuir para o prejuízo das funções homeostáticas. De maneira interessante, a morfologia microglial em cérebros doentes também varia dependendo de sua localização espacial e do estágio da doença (DAVIES *et al.*, 2017; LENG; EDISON, 2021). A micróglia associada as placas amiloides passa por mudanças morfológicas e eletrofisiológicas dramáticas, enquanto as células microgliais distantes das placas demonstram apenas pequenas alterações ao longo do tempo. Além disso, a micróglia em cérebros nos estágios V-VI de Braak possuem diferenças morfológicas muito mais profundas do que as dos cérebros em fases anteriores (NAVARRO *et al.*, 2018). A diversidade temporal da morfologia microglial pode estar atribuída a intensidade e duração do ambiente patológico, mas pode também estar relacionada a diferentes respostas da micróglia a diferentes estímulos como a Aβ e os agregados de tau. O aparecimento da micróglia distrófica, conhecida como micróglia escura, precede o desenvolvimento da patologia da tau. A tau hiperfosforilada solúvel pode levar a mudança fenotípica da micróglia, causando a perda de vigilância imune e facilitando a progressão da doença através da formação dos emaranhados neurofibrilares. Esses achados implicam que as mudanças de fenótipo na micróglia, incluindo alterações morfológicas, e assinaturas proteômicas e comportamentais, estão associadas a progressão da doença (BISHT *et al.*, 2016; LENG; EDISON, 2021).

Estudos de transcriptômica com modelos de DA em camundongos mostraram que a progressão da doença é paralela à ativação da micróglia em uma transição gradual de um estado homeostático para um estado associado a doença. A transição para a micróglia ativada é relacionada com a diminuição da transcrição de genes

homeostáticos e o aumento da transcrição de genes relacionados a DA, incluindo o gene da APOE (KEREN-SHAUL *et al*., 2017).

FIGURA 2 – UNIDADE NEUROVASCULAR-GLIAL E MUDANÇAS INICIAIS NA DA

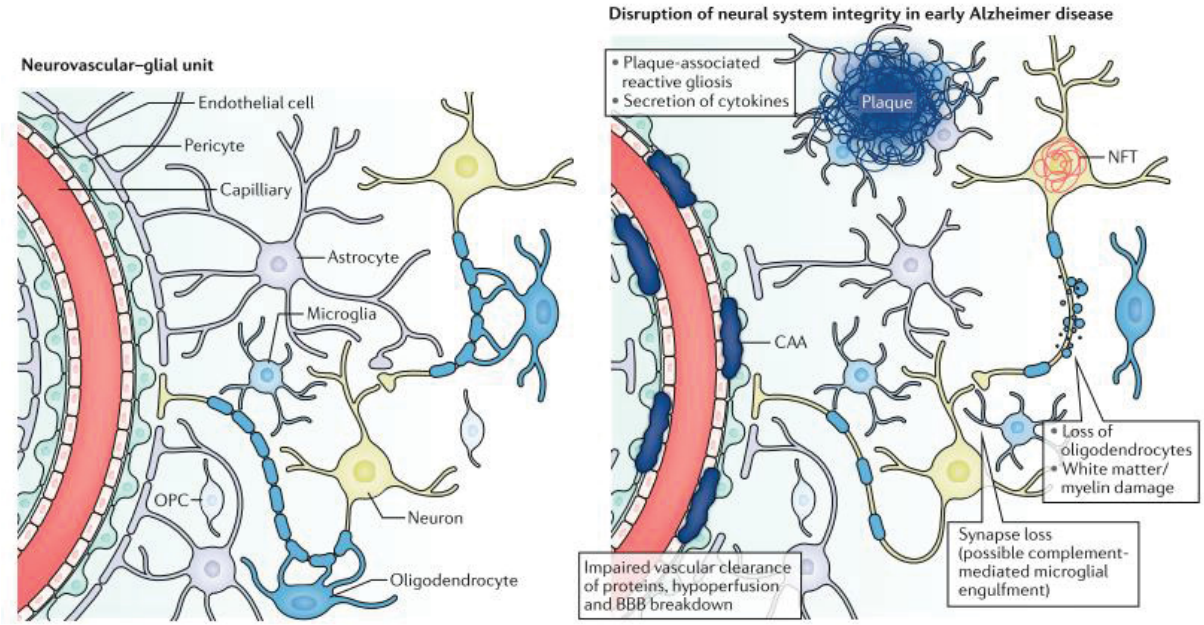

FIGURA 2. Todos os tipos celulares no cérebro interagem em uma rede complexa para manter o funcionamento cerebral. Durante os estágios iniciais da DA, vários desses processos homeostáticos estão prejudicados, e sinapses e neurônios são comprometidos. As placas amiloides ficam cercadas por oligômeros de Aβ, que danificam as sinapses e os processos neuronais próximos. As placas também ficam cercadas de astrócitos e micróglia, os quais inicialmente servem para remover Aβ, mas após a exposição a Aβ se tornam reativos e secretam citocinas. Dentro dos neurônios a proteína tau se acumula nos ENF, que estão associados com a acumulação de células da glia, disfunção neuronal e morte. As sinapses se tornam disfuncionais e desaparecem em um processo que provavelmente envolve a micróglia. FONTE: HENSTRIDGE; HYMAN; SPIRES-JONES (2019).

Estudos de neuroinflamação sugerem que a micróglia, os astrócitos e os neurônios trabalham de maneira sincronizada para promover a neurodegeneração. A Aβ ativa a via do NF-κB em astrócitos, resultando na liberação aumentada do complemento C3, que por sua vez atua nos receptores C3a nos neurônios e na micróglia causando disfunção neuronal e ativação da micróglia. Por outro lado, a micróglia ativada induz os astrócitos A1 ao expressar IL-1α, C1q e TNF. A interferência celular entre a micróglia e astrócitos pode formar um loop de feedback positivo sob o ambiente inflamatório na DA, resultando em uma resposta inflamatória desregulada e auto amplificada (LIAN *et al*., 2016; LIDDELOW *et al*., 2017).

Em modelos animais de DA foi observada a ativação microglial em estágios anteriores a formação de placas amiloides e um estudo de neuroimagem em humanos reportou o aumento da ativação da micróglia em pessoas com Comprometimento cognitivo leve com ausência de traços de Aβ. Esses dois achados sugerem que a neuroinflamação é um evento precoce na DA (LENG; EDISON, 2021). Evidências de um estudo *post-mortem* em humanos não mostraram mudanças morfológicas importantes na micróglia em indivíduos assintomáticos com carga amiloide elevada em várias idades, sugerindo fortemente que a neuroinflamação, além da deposição de Aβ, é um pré-requisito para a patogênese da DA (STREIT *et al.*, 2009).

1.3.2 Metabolismo energético e resistência à insulina

A glicose é de longe o substrato energético mais importante, e é essencial para a manutenção do metabolismo cerebral. A glicose é metabolizada para adenosina trifosfato (ATP) via glicólise, ciclo do ácido tricarboxílico e cadeia transportadora de elétrons (BUTTERFIELD; HALLIWELL, 2019). Como os neurônios não são capazes de sintetizar ou estocar glicose, eles são dependentes do transporte de glicose através da barreira hematoencefálica, que é mediado pelos transportadores de glicose (GLUTs). Imagens de tomografias por emissão de pósitrons revelaram um prejuízo na glicose cerebral de pacientes com DA que precede o prejuízo neuropsicológico e a atrofia (CORREIA *et al.*, 2011). Foi sugerido que esse prejuízo possa ser a causa, mais do que a consequência, da degeneração na DA. De fato, a utilização de glicose pelo cérebro se mostrou reduzida em 45%, e o fluxo sanguíneo cerebral em 20%, nos estágios iniciais da DA. Já nos estágios mais tardios, as anormalidades metabólicas e fisiológicas se agravam e a redução no fluxo de sangue chega a reduzir em 55-65% (HOYER, 2004). Adicionalmente, observou-se que tanto GLUT-1 quanto -3, que são responsáveis pela absorção de glicose nos neurônios, estavam diminuídas no cérebro com DA, essa redução foi associada com a hiperfosforilação da tau, e a densidade dos emaranhados neurofibrilares em cérebros humanos (LIU *et al.*, 2008). Bubber *et al*. (2005) verificaram que todas as mudanças nas atividades do ciclo do ácido tricarboxílico podiam ser relacionadas com o grau de incapacidade dos pacientes com DA, sugerindo uma alteração mitocondrial coordenada.

Dados da literatura sugerem um papel central da resistência à insulina e/ou redução das ações da insulina na diminuição da glicose cerebral/metabolismo energético na patogênese da DA. Análises *post-mortem* de cérebros humanos com DA revelaram uma redução do mRNA e dos níveis de insulina, expressão de receptores de insulina (IR), níveis de IRS-1 e IRS-2 e atividade de fosfatidilinositol 3-quinase (PI3-K) e quinase regulada por sinais extracelulares 1/2 (ERK1/2), essas anormalidades correlacionaram-se com a severidade e progressão da neurodegeneração e da demência. Isso sugere um dano na cascata de transdução de sinal da insulina, assim como ocorre no diabetes do tipo 2, o que alguns pesquisadores chamam de “estado cerebral de resistência à insulina”. Uma questão importante é saber se o estado de resistência à insulina central causa todas as características neuropatológicas típicas da DA. Características da disfunção endotelial e do dano vascular têm sido extensivamente documentadas na DA, o que aponta para um componente vascular como fator unificador em potencial ligando a sinalização cerebral de insulina prejudicada a patologia da DA (CORREIA *et al.*, 2011; MOLONEY *et al.*, 2010).

A regulação da fosforilação da proteína tau é outro potencial mecanismo pelo qual a insulina parece estar implicada na patologia da DA. Tem se mostrado que a insulina ativa as principais quinases envolvidas na fosforilação da tau, incluindo a glicogênio sintase quinase-3β (GSK-3β), ERK1/2 e a quinase dependente de ciclina-5. Já foi mostrado que a insulina e o IGF-1 inibem a hiperfosforilação da tau estimulando a fosforilação/inativação da GSK-3β induzida pela quinase Akt tanto em neurônios humanos quanto animais. Foi previamente mostrado que a insulina e o IGF-1 reduzem a fosforilação da tau, promovendo a sua ligação a microtúbulos pela inibição da GSK-3β através da via PI3-K (DE LA MONTE; WANDS, 2006). Portanto, distúrbios na cascata de sinalização de insulina e/ou IGF-1 levam a um aumento da hiperfosforilação da tau, aumentando a formação de emaranhados neurofibrilares (CHENG *et al.*, 2005).

A sinalização de insulina também é prejudicada em modelos animais da doença. A resistência à insulina pode ser induzida por oligômeros de Aβ em culturas primárias de neurônios hipocampais e pela injeção intracerebroventricular (ICV) de oligômeros em camundongos e macacos. É mediada pela ativação de TNF-α e pela inibição de receptores de insulina, tendo importante impacto na disfunção sináptica,

no prejuízo à plasticidade sináptica, e perda de sinapses. Se sabe que drogas antidiabéticas exercem efeitos benéficos na cognição, proteção das sinapses, déficits da sinalização de insulina e outros mecanismos patológicos relacionados à DA, como estresse do retículo endoplasmático e inflamação crônica (FERREIRA *et al*., 2018).

1.3.3 Estresse oxidativo

A neurodegeneração é consistentemente associada com o estresse oxidativo em resultado a geração aumentada de espécies reativas de oxigênio e nitrogênio. Esses produtos exercem seus efeitos neurotóxicos ao reagir com macromoléculas incluindo lipídeos, ácidos nucleicos, e proteínas, causando sua disfunção. O dano oxidativo ocorre em estágios bem iniciais da neurodegeneração e tem sido ligado à disfunção mitocondrial na DA (DE LA MONTE, 2017). Por exemplo, os níveis de carbonilas de proteínas (biomarcador do dano oxidativo às proteínas) estão elevados nas regiões que são ricas em placas amiloides. Também já foi observado em pessoas com DA pré-clínica, que os níveis de peroxidação lipídica estão aumentados no hipocampo. Os oligômeros Aβ1-42 podem causar danos oxidativos às membranas sinápticas, em especial os oligômeros menores capazes de entrar em bicamadas lipídicas. Isso reforça a noção de que a peroxidação lipídica, e talvez outros tipos de danos oxidativos nas membranas sinápticas, são responsáveis pela redução da potenciação de longa duração (LTP) e outras funções sinápticas na memória e aprendizado (BUTTERFIELD; HALLIWELL, 2019).

Dados têm mostrado que a utilização ineficiente de glicose (e, portanto, a produção prejudicada de ATP) e danos oxidativos estão relacionados intimamente. Um grande contribuidor para a utilização ineficiente de glicose pode ser a modificação oxidativa, que frequentemente leva a diminuição da atividade de enzimas envolvidas com o metabolismo da glicose (BUTTERFIELD; BOYD-KIMBALL, 2018). O dano oxidativo ao DNA mitocondrial pode também contribuir para o prejuízo na produção de energia. De fato, disfunção mitocondrial e resistência à insulina são intimamente relacionadas. As consequências dessa produção reduzida de ATP na DA são profundas. Essa redução diminui a capacidade do neurônio em manter gradientes iônicos, dificultando a produção e propagação de potenciais de ação e por conseguinte a neurotransmissão. Além disso, a perda de gradientes iônicos pode

permitir a entrada de Ca^{2+} extracelular, que pode em seguida aumentar os níveis de Ca^{2+} intracelular livre, estimulando a atividade da endonuclease dependente de Ca^{2+}, fosfolipase e proteinases, contribuindo para a disfunção sináptica e eventual morte neuronal. O excesso de Ca^{2+} pode saturar a capacidade do retículo endoplasmático e da mitocôndria de tamponar o Ca^{2+}, causando a dilatação da mitocôndria e consequente abertura do poro de transição de permeabilidade mitocondrial, levando a liberação do citocromo c e do fator indutor de apoptose-1, provocando a morte neuronal apoptótica (BUTTERFIELD; HALLIWELL, 2019).

1.4 MODELO ANIMAL DE DOENÇA DE ALZHEIMER ESPORÁDICA INDUZIDO POR ESTREPTOZOTOCINA

Os modelos da DA com camundongos transgênicos são amplamente usados e têm permitido diversos *insights* com relação aos mecanismos moleculares da doença. A construção desses modelos passa pela manipulação de genes relacionados a Aβ, com mutações associadas com a forma familiar da doença. Portanto, esses modelos não podem ser considerados como modelos da DA esporádica (KAMAT *et al.*, 2016).

Com a DA esporádica sendo reconhecida como um estado cerebral de resistência à insulina, foi proposto um modelo não-transgênico da doença com essa propriedade. O modelo é desenvolvido com a injeção intracerebroventricular (ICV) da droga betacitotóxica estreptozotocina (STZ). A STZ, um composto glucosamina-nitrosoureia, é obtida de *Streptomyces achromogenes* e geralmente usada para a indução de modelos de diabetes do tipo 1 por ser tóxica as células β pancreáticas (KAMAT *et al.*, 2016). Por se assemelhar estruturalmente a glicose, a STZ parece ser transportada para dentro da célula pelo transportador de glicose GLUT2 (SALKOVIC-PETRISIC; HOYER; RIEDERER, 2014). Assim como outros agentes alquilantes da classe nitrosoureia, a STZ é citotóxica por formar carbocátions (CH^{3+}), o que causa danos ao DNA através da metilação. Ela também causa a morte celular pela fragmentação do DNA devido ao grupo nitrosoureia e outros mecanismos. O dano ao DNA induz a diminuição de NADH e ATP (KAMAT *et al.*, 2016).

Quando administrada de forma ICV, a STZ produz alterações metabólicas crônicas no cérebro dos animais. Alguns autores propõem que, assim como na DA, o

modelo de STZ-ICV leva os animais a desenvolverem um estado cerebral de resistência à insulina. Esse estado seria causado por danos na sinalização de receptores de insulina e uma consequente dessensibilização desses receptores (BASSANI *et al.*, 2017; KAMAT *et al.*, 2016). De fato, parece ocorrer uma inibição da função do receptor de insulina, e a expressão de genes relacionados a sinalização de insulina/IGF se mostrou prejudicada principalmente no hipocampo, córtex frontal e cerebelo de macacos que receberam a STZ-ICV, mas ainda não está muito bem definido se essa seria uma ação direta ou indireta da STZ (GRIEB, 2016).

Contudo, vários estudos mostram que uma injeção ICV de doses baixas e subdiabetogênicas (e.g. 3 mg/kg) de STZ em roedores causa além das perturbações na sinalização de insulina, reduções crônicas (10-30%) no metabolismo de glicose e glicogênio no córtex cerebral e no hipocampo, anormalidades que aparecem precocemente na DA esporádica (SALKOVIC-PETRISIC *et al.*, 2013). Essas alterações são acompanhadas de déficits colinérgicos progressivos, estresse oxidativo, neuroinflamação, neurodegeneração, danos na transmissão sináptica e prejuízos cognitivos (KRASKA *et al.*, 2012; LESTER-COLL *et al.*, 2006; SALKOVIC-PETRISIC *et al.*, 2009). Além disso, semanas após a infusão de STZ é possível observar a expressão aumentada da proteína tau e de Aβ tanto no hipocampo quanto no córtex cerebral dos animais (KAMAT *et al.*, 2016).

O modelo STZ-ICV também causa a alteração na expressão de genes relacionados a DA, como a APP, tau e a β-secretase em cérebros de ratos e macacos (KAMAT *et al.*, 2016). Mas a alteração neuropatológica mais proeminente no modelo parece ser a neuroinflamação (GRIEB, 2016). Ocorre uma proliferação significativa da micróglia reativa e da astroglia ativada, esse aumento da ativação da glia é prolongado e leva a liberação de citocinas pró-inflamatórias que exacerbam ainda mais a neuroinflamação e o estresse oxidativo (ZAPPA VILLAR *et al.*, 2018).

Essas alterações facilitam a perda sináptica e levam a prejuízos na memória de trabalho, episódica e de reconhecimento, como ocorre na DA. As drogas que são clinicamente usadas na DA também são efetivas no modelo da STZ, o que reforça sua validade. Assim, o modelo é uma estratégia adequada para mimetizar a condição humana de DA esporádica, sendo amplamente utilizado para avaliar propriedades neuroprotetoras de vários compostos (KRASKA *et al.*, 2012).

1.5 ESTRATÉGIAS NEUROPROTETORAS

Como explicitado, as desordens neurológicas são comuns e representam problemas de saúde importantes na população idosa. A inflamação e o estresse oxidativo possuem papéis cruciais em várias doenças neurodegenerativas como a doença de Alzheimer, assim, a busca por estratégias neuroprotetoras se faz necessária (HOSSAIN *et al*., 2022).

Recursos adjuvantes podem eventualmente ser recomendados na DA. Fatores nutricionais têm um papel importante na melhora da saúde e evidências clínicas estabelecem uma correlação direta entre deficiências nutricionais e disfunção cognitiva (YAMINI; RAY; CHOPRA, 2018). Nessa linha, recentemente, três suplementos alimentares (axona, cerefolin NAC e souvenaid) foram aprovados pela FDA. A axona fornece corpos cetônicos aos neurônios como alternativa energética, os corpos cetônicos cruzam a barreira hematoencefálica, adentram os neurônios e produzem ATP na mitocôndria pela fosforilação oxidativa. O cerefolin NAC, composto por L-metillfolato, vitaminas B12, B2 e B6, parece ter um papel favorável no estresse oxidativo relacionado a perda de memória. O souvenaid fornece uma combinação nutricional que parece melhorar a função sináptica, manter a integridade da membrana neuronal, aumentar a resistência ao estresse oxidativo e preservar a atividade metabólica no cérebro (STELLA *et al.*, 2015). Algumas outras dietas e suplementos alimentares também têm sido relacionados positivamente com a preservação da cognição e com a redução da proteína tau e da patologia amiloide em modelos animais da DA (MOURA *et al*., 2020).

Antioxidantes têm recebido bastante atenção na procura de novos tratamentos para a DA, em especial para a prevenção da doença. Além disso, tem acontecido um aumento na busca e no uso de drogas e suplementos alimentares obtidos de plantas. Em parte, pelos benefícios encontrados nos fitoquímicos aos quais o uso já é relatado na medicina tradicional (EL SAYED; GHONEUM, 2020). Estima-se que em uma única espécie de planta possa existir pelo menos 5 mil compostos químicos ou metabólitos secundários. Esses metabólitos secundários são conhecidos por possuir potencial terapêutico para tratar várias doenças (KISHORE *et al.*, 2017).

1.5.1 Andrografolide

Nesse contexto, investigamos os efeitos do andrografolide (ANDRO), uma molécula encontrada na planta *Andrographis paniculata,* no modelo animal de STZ-ICV. A *A. paniculata* é uma planta medicinal tradicionalmente usada na China e em outras partes da Ásia oriental para tratar várias enfermidades, como artrite reumatoide, febre, infecção do trato respiratório superior e diarreia. Esses benefícios da *A. paniculata* se devem principalmente aos seus efeitos farmacológicos multifatoriais, como o antibacteriano, antiviral, anti-inflamatório e hepatoprotetor. Hoje se tem conhecimento de que o ANDRO, uma lactona diterpênica, é o componente bioativo responsável pela maior parte das propriedades medicinais da *A. paniculata* (LU *et al.*, 2019). O ANDRO é amplamente conhecido por suas atividades anti-inflamatórias e antioxidantes (ZHANG *et al.*, 2021).

Existem diversos mecanismos em potencial para explicar as atividades antioxidantes e anti-inflamatórias do ANDRO. Esses mecanismos podem ser diretos ou indiretos. O ANDRO é capaz de prevenir a formação de radicais livres ao proteger a mitocôndria ou por inibir enzimas específicas que produzem espécies reativas de oxigênio. Ele também tem a capacidade de ativar antioxidantes enzimáticos e não-enzimáticos, principalmente através da ativação da via de sinalização do fator nuclear eritróide 2 relacionado ao fator 2 (Nrf2) (GUAN *et al.*, 2013; MUSSARD *et al.*, 2019). Foi observado que o ANDRO pode diminuir significativamente a expressão induzida por LPS de quimiocinas no córtex. Essa inibição neuroinflamatória é relacionada intimamente a ativação das vias de sinalização do fator NF-κB e da proteína quinase c-Jun terminal (JNK). Em astrócitos primários de rato o ANDRO reduziu a inflamação e o estresse oxidativo ao mediar as vias de sinalização de Nrf2-p38-MAPK e de ERK (DAI *et al.*, 2019). Além disso, estudos *in vitro* e *in vivo* sugerem que o ANDRO pode ativar a via Wnt/β-catenina, induzindo a transcrição dos genes Wnt e inibindo a GSK3-β por fosforilação (ZHANG *et al.*, 2019).

O ANDRO tem mostrado efeitos benéficos no SNC em modelos animais de isquemia cerebral, traumatismo cranioencefálico e doenças neurodegenerativas (CHAN *et al.*, 2010; GENG *et al.*, 2019; TAO *et al.*, 2018). No modelo de isquemia cerebral permanente, o ANDRO foi capaz de diminuir o volume do infarto e de déficits neurológicos. O modelo induziu a ativação da micróglia e aumentou os níveis de TNF-α, IL-1β e prostaglandina E2 nas regiões isquêmicas, e o ANDRO atenuou ou aboliu

esses efeitos. Além disso, o ANDRO suprimiu a ativação de NF-κB (CHAN *et al.*, 2010). Foi observado, em um modelo de traumatismo cranioencefálico, que a administração de ANDRO atenuou o déficit neurológico, e o edema cerebral e apoptose nos tecidos cerebrais também diminuíram. Tanto a ativação da micróglia quanto a expressão de citocinas pró-inflamatórias foram significativamente inibidas pelo ANDRO. Adicionalmente, o ANDRO inibiu a translocação da subunidade p65 do NF-κB e diminuiu os níveis de expressão da ERK e da proteína quinase ativada por mitógeno p38 (p38 MAPK) (TAO *et al.*, 2018). Ademais, o ANDRO, em um modelo da doença de Parkinson, melhorou os déficits comportamentais e atenuou a perda de neurônios dopaminérgicos em camundongos expostos a toxina MPTP. O ANDRO também reduziu os danos mitocondriais, inibindo a fissão mitocondrial, e reduziu a morte celular induzida por rotenona *in vitro* (GENG *et al.*, 2019).

Vários grupos de pesquisa já observaram que o tratamento com ANDRO causou uma redução do comprometimento cognitivo em diferentes modelos de DA (ARREDONDO *et al.*, 2021; CISTERNAS *et al.*, 2019; RIVERA *et al.*, 2016). Rivera *et al.* (2016) trataram com ANDRO indivíduos de *Octodon degus*, uma espécie de roedor sul-americano que desenvolve uma patologia tipo-Alzheimer em idades avançadas. Esses animais apresentam prejuízos na função cognitiva, que foram relacionados com uma diminuição das funções sinápticas e um aumento da expressão de tau e beta-amiloide. O ANDRO recuperou o desempenho da memória espacial e do aprendizado e a transmissão sináptica basal, também protegeu proteínas sinápticas e ainda reduziu a proteína tau fosforilada e a maturação de agregados de beta-amiloide. Ainda no modelo de DA com *Octodon degus,* Lindsay *et al*. (2020) observaram que o ANDRO foi capaz de reduzir a neuroinflamação no córtex e no hipocampo, os níveis da proteína ácida fibrilar glial (GFAP; marcador de astrócitos), JNK e IL-6 foram reduzidos, também foi visto uma diminuição do estresse oxidativo (lipoperoxidação e nitrosilação) causada pelo ANDRO nessas regiões (Fig. 3).

Em modelos transgênicos da DA (APPswe/PS1ΔE e J20), o ANDRO reduziu, além do prejuízo na memória, a deposição de Aβ, a ativação da micróglia, e a liberação de mediadores pró-inflamatórios no córtex pré-frontal e hipocampo. Adicionalmente, o ANDRO preveniu a redução dos marcadores do metabolismo energético celular e a diminuição da extensão das sinapses e promoveu a neurogênese hipocampal (ARREDONDO *et al.*, 2021; CISTERNAS *et al.*, 2019;

ZHANG *et al.*, 2021). Em linha com esses resultados, Patel *et al*. (2021) demonstraram recentemente que o ANDRO pode suprimir o prejuízo na memória espacial causado pelo modelo STZ-ICV em ratos. ANDRO protegeu o hipocampo contra o estresse oxidativo induzido por STZ, aumento de marcadores neuroinflamatórios, como IL-1β, IL-16 e TNF-α, e níveis de Aβ1-42 e tau fosforilada.

FIGURA 3 – VISÃO GERAL DOS MECANISMOS PRÓ-INFLAMATÓRIOS E PRÓ-OXIDANTES DE Aβ E OS EFEITOS DO ANDRO

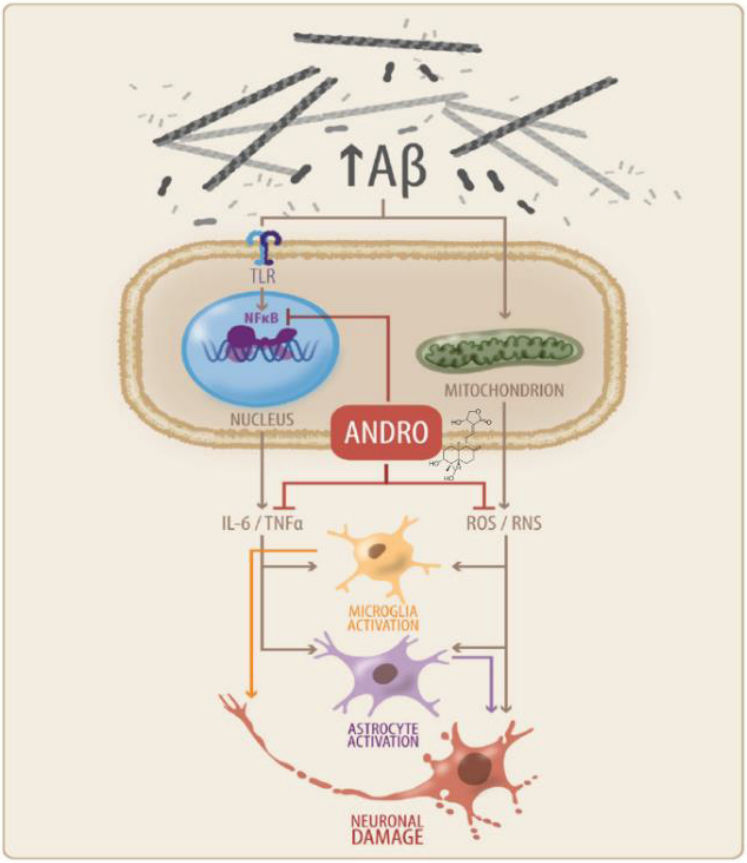

FIGURA 3. A Aβ é capaz de desencadear uma resposta inflamatória ao interagir diretamente com receptores 'toll-like' (TLR). A ativação de TLR leva a um aumento na produção de citocinas pró-inflamatórias, como a IL-6 e o TNF-α, através da via do NF-κB. Essas citocinas então ativam astrócitos e células da micróglia para lidar com Aβ e células danificadas, que em seguida vão produzir mais mediadores pró-inflamatórios, perpetuando um ciclo vicioso inflamatório. A Aβ pode ainda interagir diretamente com a mitocôndria, induzindo disfunção mitocondrial e levando a uma produção aumentada de espécies reativas de oxigênio e nitrogênio (ROS/RNS), que também ativam a micróglia e os astrócitos e causam alterações em macromoléculas. Por fim, as cascatas de danos desencadeadas por Aβ levam a falhas na rede neuronal. O ANDRO é capaz de bloquear a cascata do NF-κB, limitando a produção de citocinas e reduzindo os níveis de ROS/RNS, induzindo neuroproteção. FONTE: LINDSAY *et al*. (2019).

2 JUSTIFICATIVA

Muitos estudos vêm demonstrando que além da formação de oligômeros e placas da proteína beta-amiloide e dos emaranhados neurofibrilares de proteína tau, a neuroinflamação e o estresse oxidativo são bastante importantes no curso da doença de Alzheimer. Portanto, se fazem necessárias novas estratégias terapêuticas que abranjam essas características patológicas. O uso de antioxidantes e fitoquímicos parece ter um papel positivo na DA, com efeitos neuroprotetores promissores. O andrografolide está presente em uma tradicional planta medicinal, e tem diversas propriedades potencialmente terapêuticas, entre elas a anti-inflamatória e a antioxidante se destacam. O ANDRO já mostrou capacidade neuroprotetora em modelos animais de isquemia cerebral, traumatismo cranioencefálico e doenças neurodegenerativas. Em modelos de DA, foi visto que o ANDRO preserva a cognição ao diminuir os níveis de Aβ e tau fosforilada e de citocinas pró-inflamatórias, bem como reduz a ativação da micróglia e dos astrócitos e o dano causado pelo estresse oxidativo. Um recente trabalho mostrou efeitos benéficos do ANDRO no modelo de DA com STZ-ICV, mas ainda não foram observados no modelo os efeitos na memória de reconhecimento e na ativação da micróglia e de astrócitos, no presente estudo investigamos a hipótese de que o ANDRO poderia melhorar também esses aspectos.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Estudar os efeitos das estratégias neuroprotetoras andrografolide e formulação nutricional AZ1 no prejuízo de memória e na neuroinflamação causados pelo modelo animal da doença de Alzheimer esporádica induzido pela infusão ICV de STZ em ratos.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar em ratos Wistar infundidos ICV com STZ os efeitos do ANDRO nas memórias espacial e de reconhecimento através do desempenho dos animais no labirinto em Y, e nos testes de localização e de reconhecimento de objetos.

Avaliar os efeitos do ANDRO na ativação de astrócitos no córtex pré-frontal e hipocampo dos ratos submetidos ao modelo STZ-ICV através da determinação da expressão da proteína GFAP no tecido cerebral dos animais.

Estudar os efeitos do ANDRO na ativação da micróglia nas regiões CA1 e CA3 do hipocampo dos ratos infundidos ICV com STZ através da avaliação imuno-histoquímica do tecido cerebral utilizando a marcação para a proteína Iba-1.

Avaliar os efeitos do ANDRO na expressão da proteína precursora amiloide no córtex pré-frontal e hipocampo dos ratos submetidos ao modelo STZ-ICV.

Investigar as correlações estatísticas entre a ativação dos astrócitos, a ativação da micróglia, e a expressão de APP e os prejuízos de memória dos animais submetidos ao modelo STZ-ICV.

Avaliar em ratos Wistar infundidos ICV com STZ os efeitos da formulação AZ1 nas memórias espacial e de reconhecimento através do desempenho dos animais no labirinto em Y e no teste de reconhecimento de objetos.

Estudar os efeitos da formulação AZ1 na ativação da micróglia nas regiões CA1, CA3 e giro denteado do hipocampo dos ratos infundidos ICV com STZ através

da avaliação imuno-histoquímica do tecido cerebral utilizando a marcação para a proteína Iba-1.

4 ARTIGOS CIENTÍFICOS

Os materiais e métodos, resultados e discussão do trabalho encontram-se nos artigos científicos a seguir.

Andrographolide attenuates short-term spatial and recognition memory impairment and neuroinflammation induced by a streptozotocin rat model of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder clinically manifested by a gradual decline in cognitive function. Intracerebroventricular injection (ICV) of streptozotocin (STZ), a model of Sporadic AD (sAD), shows many aspects of sAD abnormalities (i.e., neuroinflammation, oxidative stress, protein aggregation) resulting in memory impairment. Andrographolide (ANDRO), a diterpene lactone, has numerous bioactivities including anti-inflammatory and antioxidant properties. Studies in rodents revealed that ANDRO has neuroprotective properties and restores cognitive impairment. In the present study, we investigated the effects of ANDRO in the ICV-STZ model relative to short-term spatial memory (Object location test (OLT) and Y maze test) and short-term recognition memory (Object recognition test (ORT)), locomotor activity (Open field test (OFT)), expression of amyloid precursor protein (APP), and activation of astrocytes (GFAP expression) and microglia (immunohistochemistry for Iba-1) in the prefrontal cortex (PFC) and hippocampus (HIP). Wistar rats were injected ICV with STZ (3 mg/kg) or vehicle and treated with ANDRO (2 mg/kg, i.p.; three times per week). After four weeks, ANDRO was capable of attenuating the impairments in the Y maze and ORT performance, and the increase in the astrocyte activation in the PFC induced by the ICV-STZ model. In addition, ANDRO decreased the number of activated microglia cells in the HIP of STZ-injected rats. The APP expression was not altered, neither by the STZ nor ANDRO. ANDRO showed a beneficial effect on memory impairment and neuroinflammation in the STZ model of AD.

Keywords: Alzheimer, andrographolide, cognition, microglia, astrocyte, streptozotocin

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder and is mainly characterized by progressive and slow memory loss, especially memory related to recent events and spatial disorientation (Moura et al., 2020; Salardini, 2019). Late-onset AD, or sporadic AD (sAD), is the preponderant form of the disease, despite its unclear etiology many molecular disturbances have been detected in the sAD aging brain (Correia et al., 2011). The classical positive lesions consist of the accumulation of extracellular amyloid β (Aβ) and intracellular hyperphosphorylated tau (p-tau) and are accompanied by oxidative and inflammatory damage. These neuropathological aspects generate energy failure and ultimately synaptic and neuronal loss, resulting in cognitive impairment (Querfurth and LaFerla, 2010; Serrano-Pozo et al., 2011). In patients with sAD, the loss of synapses in the neocortex and hippocampus (HIP) appears to be substantial to sAD's pathology, since the slow decline in cognitive functions strongly correlates with the progression of the neuronal dysfunction in both regions (Cisternas et al., 2019; Serrano-Pozo et al., 2011).

An intracerebroventricular (ICV) injection of low, sub-diabetogenic doses of streptozotocin (STZ) in rodents can model sAD (Bassani et al., 2017a; Grieb, 2016; Salkovic-Petrisic and Hoyer, 2007). STZ can inhibit insulin receptors, inducing an insulin-resistant brain state and brain glucose hypometabolism, abnormalities that appear early in sAD (Salkovic-Petrisic et al., 2013). In the ICV-STZ model, as in the disorder, these features develop along with an increase in free radical formation, neuroinflammation, protein aggregation, cholinergic deficits, neurodegeneration, and cognitive decline (Kamat et al., 2016). Characteristics that resemble those of the human AD brain and have been documented after the ICV administration of STZ in rats. Thus, the model is a suitable strategy to mimic human sAD conditions, it is widely used to evaluate the neuroprotective properties of various compounds (Kraska et al., 2012).

Existent AD's therapeutic approaches are limited by the uncertainties about its etiology, there are still no disease-modifying treatments, and most agents provide only symptomatic relief from the cognitive deficit (Lane et al., 2018; Stella et al., 2015). Andrographolide (ANDRO), the main bioactive component of the medicinal plant *Andrographis paniculata*, is a labdane diterpenoid lactone that has multiple therapeutic uses and is largely known for its anti-inflammatory and antioxidative activities (Guan et

al., 2013; Li et al., 2015; Zhang et al., 2021). It has shown beneficial effects within the central nervous system (CNS) in animal models of cerebral ischemia, traumatic brain injury, and neurodegenerative diseases (Chan et al., 2010; Geng et al., 2019; Tao et al., 2018). The treatment with ANDRO reduced cognitive impairment in different models of AD, such as APPswe/PS1ΔE9 and J20 transgenic mouse models, as well as aged *Octodon degus,* used as a natural model of AD (Arredondo et al., 2021; Cisternas et al., 2019; Rivera et al., 2016).

ANDRO was found to be neuroprotective and improve learning and memory by different molecular and cellular mechanisms such as reduction of Aβ and tau levels, suppression of astrocyte and microglial activation, and promotion of hippocampal neurogenesis (Arredondo et al., 2021; Hossain et al., 2022; Serrano et al., 2014). Likewise, Patel et al. (2021) have recently demonstrated that ANDRO can suppress the impairment in spatial memory caused by the ICV-STZ model in rats. ANDRO protected the hippocampus against STZ induced oxidative stress, an increase in neuroinflammatory markers, such as interleukin-1β (IL-1β), IL-16, and tumor necrosis factor-alpha (TNF-α), and Aβ1-42 and p-tau levels. However, the effects of prolonged ANDRO treatment on astrocyte and microglia activation and recognition memory were not yet studied in the ICV-STZ model. Therefore, we hypothesized that ANDRO would be able to preserve spatial and recognition memory of ICV-STZ injected rats by decreasing the activation of astrocytes and microglia and reducing amyloid precursor protein (APP) expression in the prefrontal cortex and hippocampus.

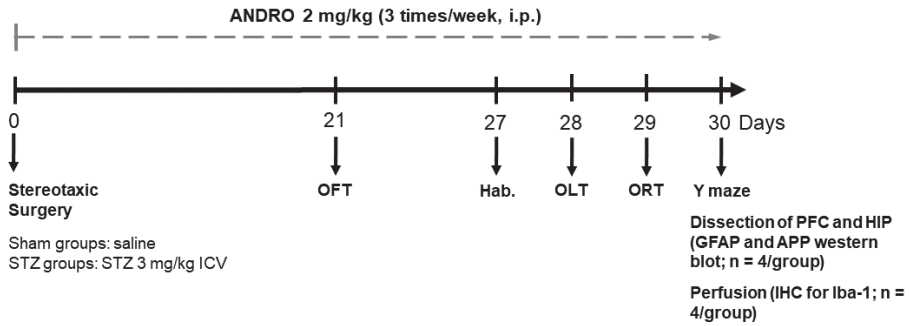

Figure 1. Experimental design. OFT, Open field test; Hab., habituation; OLT, Object location test; ORT, Object recognition test; PFC, prefrontal cortex; HIP, hippocampus; IHC, immunohistochemistry.

2. Results

2.1. ANDRO partially prevented the impairment in short-term spatial memory inflicted by the ICV-STZ

Short-term spatial memory was assessed in the Y maze on day 30 after surgery, with a 1 h interval between the training and test sessions. The STZ+VEH group showed a significant decrease in the percentage of time spent on the novel arm ($p < 0.05$) in the Y maze (Fig. 2A), in comparison with the SHAM+VEH group (treatment x lesion interaction [$F (1, 38) = 5.881$; $p = 0.020$]). The STZ+ANDRO group performed similarly to the SHAM+VEH group, suggesting that ANDRO prevented the memory disruption, however the percentage of time spent on the novel arm was not increased compared to the STZ+VEH group. There were no significant differences between the groups regarding the total entries to the arms of the maze (Fig. S1A) and the total distance travelled (Fig. S1B).

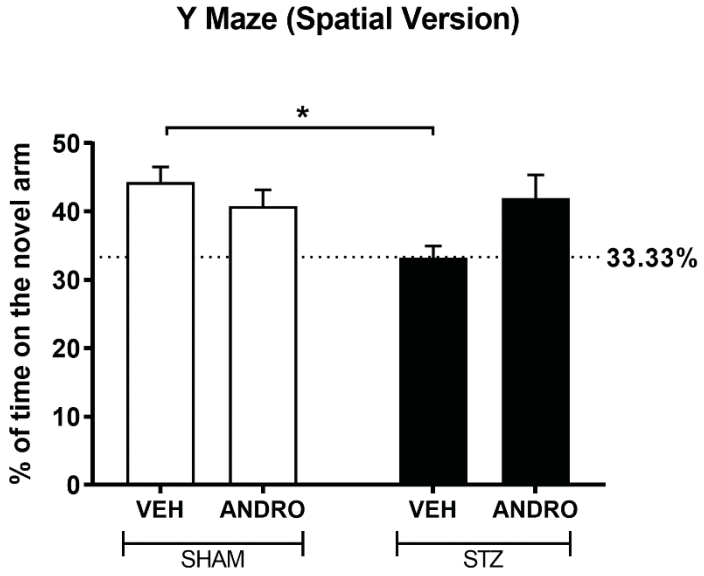

Figure 2. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on cognitive performance of STZ-injected animals compared with sham animals, assessed on the spatial version of the Y maze test. The data are expressed as mean ± SEM (n = 10-11/group); *$p < 0.05$, in comparison with the SHAM+VEH group (Two-way ANOVA followed by Tukey's post hoc test).

2.2. ANDRO worsened short-term spatial memory in the OLT and partially prevented the impairment in short-term recognition memory inflicted by the ICV-STZ

Short-term spatial memory and short-term recognition memory were assessed in the OLT on day 28 and in the ORT on day 29 after surgery, respectively, with a 1 h

interval between the training and test sessions. In contrast to the performance in the Y maze, in OLT (Fig. 3), the STZ+VEH group did not present a decrease in the index of spatial memory. And surprisingly, the STZ+ANDRO group showed a decrease in the OLT discrimination index compared to the STZ+VEH group ($p < 0.05$), suggesting that ANDRO had a negative impact on short-term spatial memory in this test (treatment x lesion interaction ([$F (1, 30) = 6.939$; $p = 0.0132$]; lesion factor [$F (1, 30) = 10.12$; $p = 0.003$]), as opposed to what was observed in the Y maze.

In the ORT (Fig. 4), STZ+VEH animals presented deficits in short-term memory, as indicated by treatment x lesion interaction [$F (1, 30) = 7.601$; $p = 0.009$], with a significant decrease in the discrimination index ($p < 0.05$) in comparison with the SHAM+VEH group. The STZ+ANDRO group performed similarly to the SHAM+VEH group, which indicates a protective effect of ANDRO, however, the discrimination index was not superior compared to the STZ+VEH group. There were no significant differences between the groups regarding the total time exploring the objects (e) both in the OLT (Fig. S2A) and in the ORT (Fig. 2S2B).

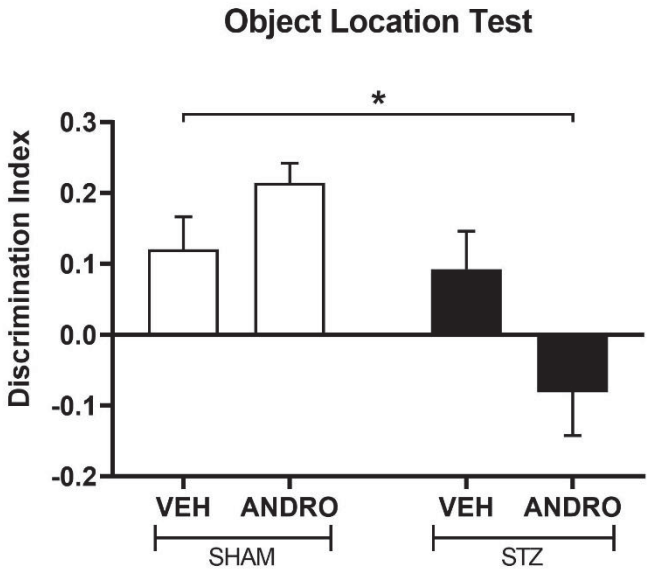

Figure 3. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on cognitive performance of STZ-injected animals compared with sham animals, evaluated on the Object Location Test. The data are expressed as mean ± SEM (n = 8-10/group); *$p < 0.05$, in comparison with the SHAM+VEH group (Two-way ANOVA followed by Tukey's post hoc test).

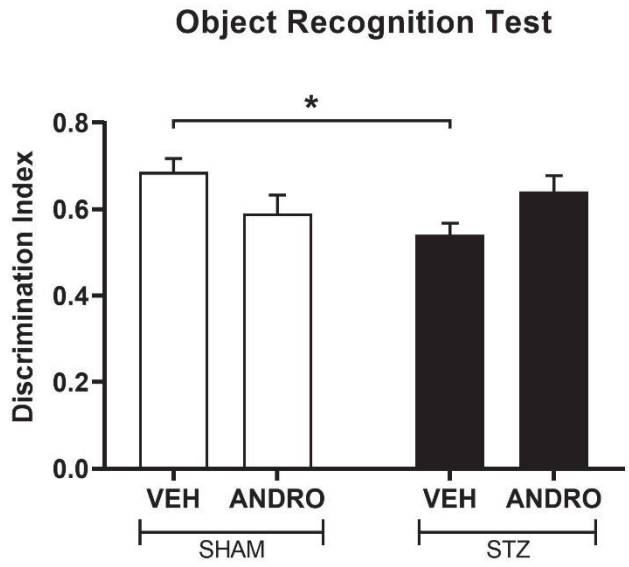

Figure 4. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on cognitive performance of STZ-injected animals compared with sham animals, evaluated on the Object Recognition Test. The data are expressed as mean ± SEM (n = 8-9/group); *p < 0.05, in comparison with the SHAM+VEH group (Two-way ANOVA followed by Tukey's post hoc test).

2.3. ANDRO diminished locomotion frequency in the Open Field Test

Regarding the performance in the OFT (Fig. 5), assessed on day 21 after surgery, there was only a significant difference in locomotion frequency between the STZ+ANDRO and the SHAM+VEH animals as indicated by the lesion factor [F (1, 38) = 9.126; p = 0.004].

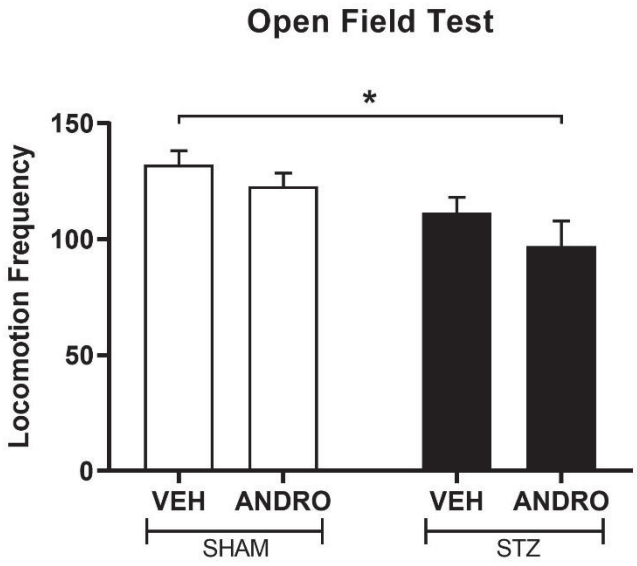

Figure 5. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on locomotion frequency of STZ-injected animals compared with sham animals, assessed on the Open Field Test. The data are expressed as mean ± SEM (n = 10-11/group); *p < 0.05, in comparison with the SHAM+VEH group (Two-way ANOVA followed by Tukey's post hoc test).

2.4. The treatment with ANDRO attenuated astrogliosis in the prefrontal cortex of ICV-STZ rats

GFAP expression in the PFC (Fig. 6A) significantly increased in the STZ+VEH group compared with the SHAM+VEH group 30 days after the surgery ($p < 0.05$), as showed by the post-hoc test, but not by the two-way ANOVA (treatment x lesion interaction [$F(1, 12) = 4.180$; $p = 0.063$], treatment [$F(1, 12) = 0.234$; $p = 0.637$], and lesion [$F(1, 12) = 3.965$; $p = 0.069$] factors). In the STZ+ANDRO group, GFAP expression in the PFC was comparable with SHAM+VEH animals, however there was also no significant difference in relation to the STZ+VEH group. In the hippocampus (Fig. 6B), there were no differences between the groups (treatment x lesion interaction [$F(1, 12) = 1.521$; $p = 0.241$]; treatment [$F(1, 12) = 3.350$; $p = 0.092$]; lesion [$F(1, 12) = 0.824$; $p = 0.381$]). These results suggest that the ICV-STZ model increases the GFAP expression in the PFC after 30 days, and that ANDRO attenuated the astrogliosis in this cerebral region.

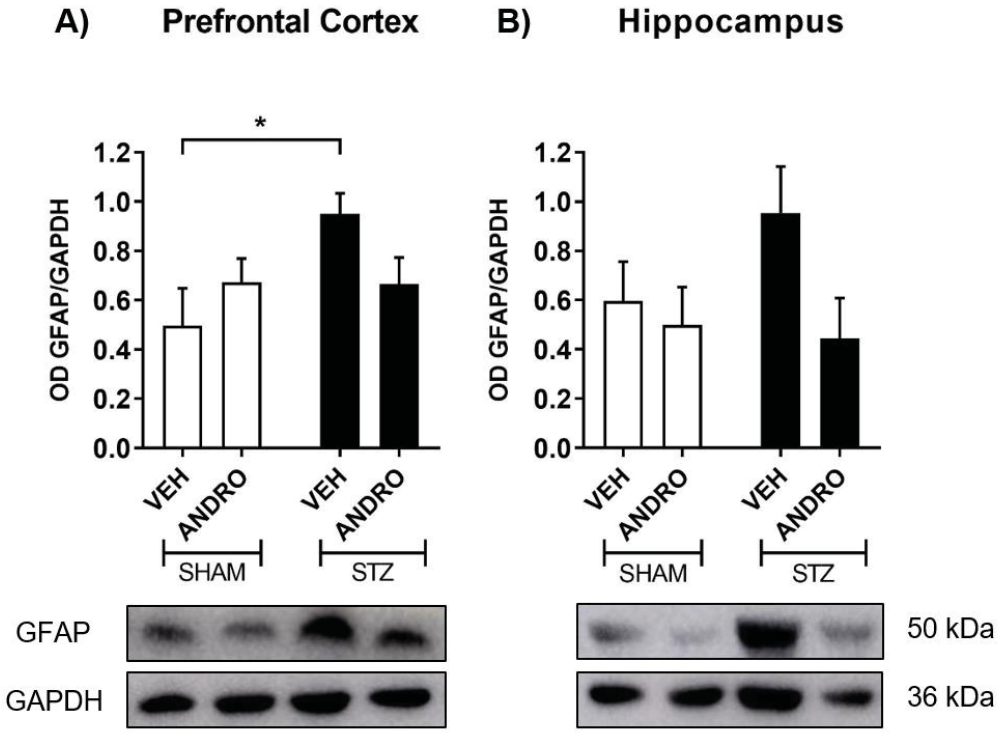

Figure 6. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on GFAP expression, determined by Western blot, in the (A) prefrontal cortex and (B) hippocampus of STZ-injected animals compared with sham animals 30 days after surgery. The data are expressed as mean ± SEM (n = 4/group); *p < 0.05, in comparison with the SHAM+VEH group (Two-way ANOVA followed by Tukey's post hoc test).

2.5. Correlation between short-term memory and GFAP expression in the HIP and PFC

The Pearson's correlation coefficients revealed negative correlations between the percentage of time spent on the novel arm in the Y maze and the expression of GFAP in the PFC (r = -0.711, p = 0.014; Fig. 7A), and between the discrimination index in the ORT and the expression of GFAP in the HIP (r = -0.653, p = 0.021; Fig. 7B). No correlation was found between the percentage of time spent on the novel arm in the Y maze and the expression of GFAP in the HIP (r = -0.137, p = 0.688; data not shown), and between the discrimination index in the ORT and the expression of GFAP in the PFC (r = -0.162, p = 0.615; data not shown).

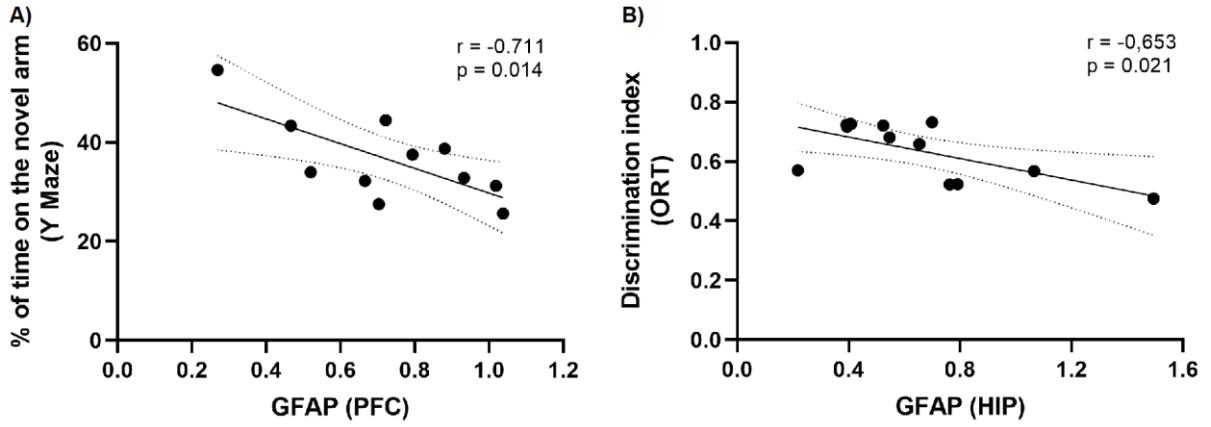

Figure 7. Correlations between (A) the percentage of time spent on the novel arm in the Y maze and the expression of GFAP in the PFC, and between (B) the discrimination index in the ORT and the expression of GFAP in the HIP. r: Pearson correlation coefficient; n = 11-12.

2.6. ANDRO attenuated the increase in hippocampus Iba-1+ cells of STZ-injected animals

The two-way ANOVAs showed significant differences between the groups variances regarding the number of Iba-1-positive cells in the CA1 (treatment x lesion interaction [F (1, 12) = 5.218; p = 0.041]; treatment [F (1, 12) = 5.671; p = 0.035]) and CA3 (treatment x lesion interaction [F (1, 12) = 7.378; p = 0.019]; treatment [F (1, 12) = 4.987; p = 0.045]) areas of the HIP, however, different from what we expected, there

was no difference between the STZ+VEH and SHAM+VEH groups (CA1, p = 0.176; and CA3, p = 0.069). Yet, in the comparison between the STZ-injected groups, the treatment with ANDRO reduced (p < 0.05) the Iba-1+ cells in CA1 (Fig. 8A) and CA3 (Fig. 8B), and the reactive Iba-1+ cells in CA3 (Fig. 8D). These results indicate a mild improvement by ANDRO against the increase in microglia cells and reactivity.

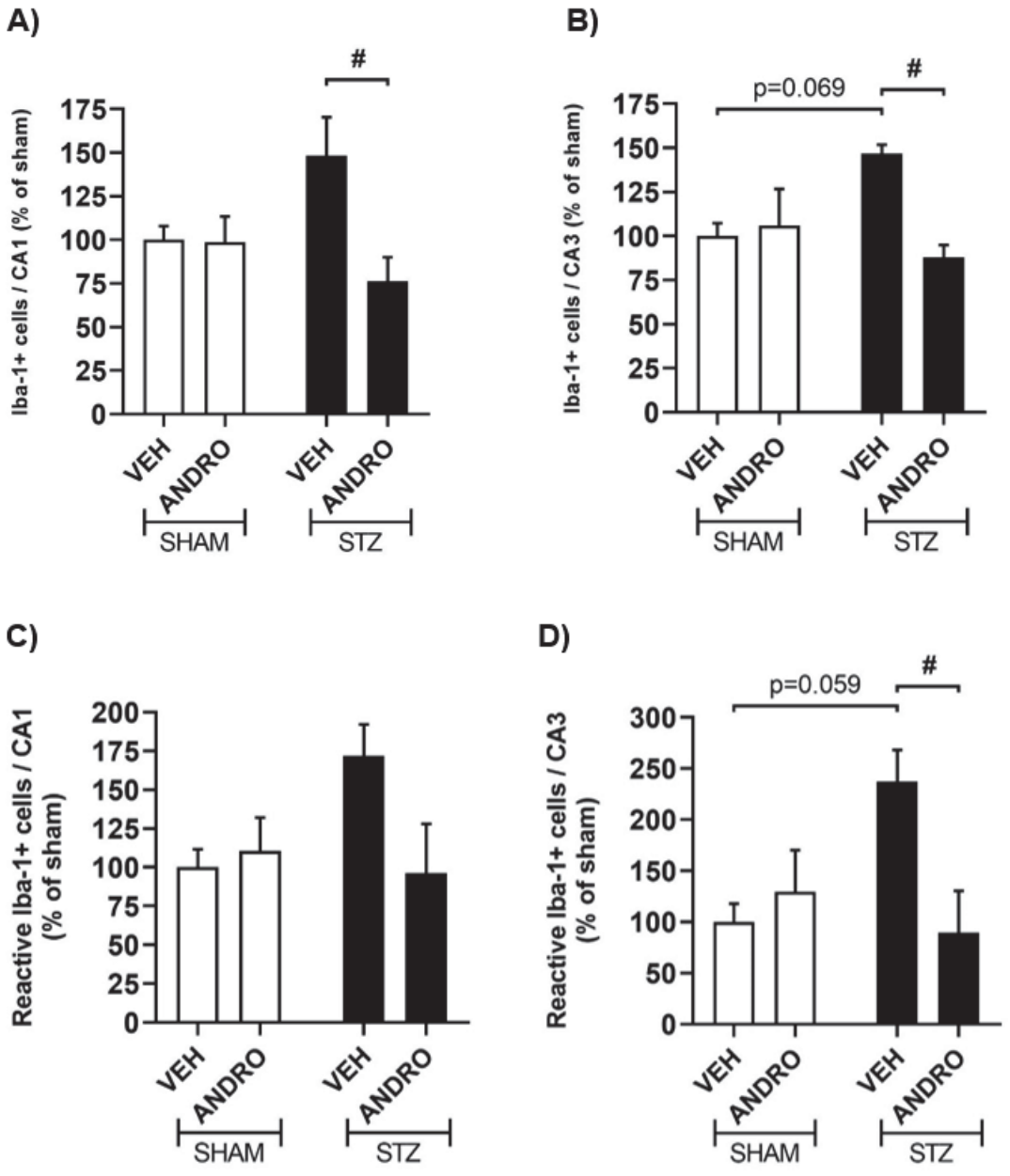

Figure 8. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on the number of Iba-1-positive cells in the (A) CA1 and (B) CA3 areas of the HIP, and on the number of reactive Iba-1-positive cells in the (C) CA1 and (D) CA3 areas of the HIP of STZ-injected animals compared with sham animals 30 days after surgery. The data are expressed as mean ± SEM (n = 4/group); *p < 0.05, in comparison with the SHAM+VEH group; #p < 0.05, in comparison with the STZ+VEH group (Two-way ANOVA followed by Tukey's post hoc test).

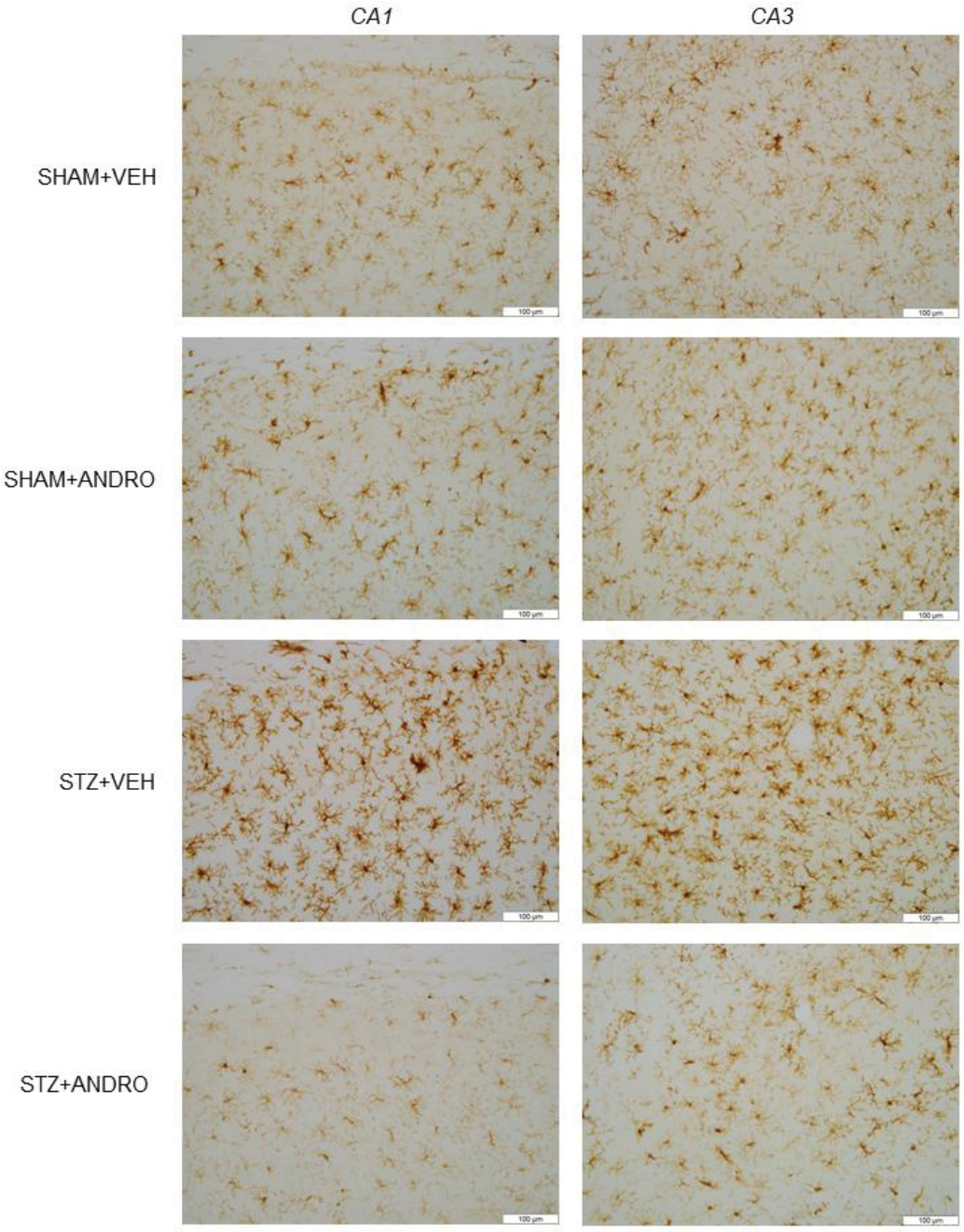

Figure 9. Representative photomicrographs of Iba1-positive cells in CA1 and CA3 hippocampal regions. Iba1-positive cells in the hippocampal CA1 and CA3 regions. Scale = 100 μm.

2.7. Correlation between short-term memory and the number of Iba-1+ cells in the HIP

The Pearson's correlation coefficients revealed negative correlations between the percentage of time spent on the novel arm in the Y maze and the number of Iba-1+ cells ($r = -0.530$, $p = 0.042$; Fig. 10A) and reactive Iba-1+ cells ($r = -0.567$, $p = 0.027$; Fig. 10B), in the CA3 area of the HIP.

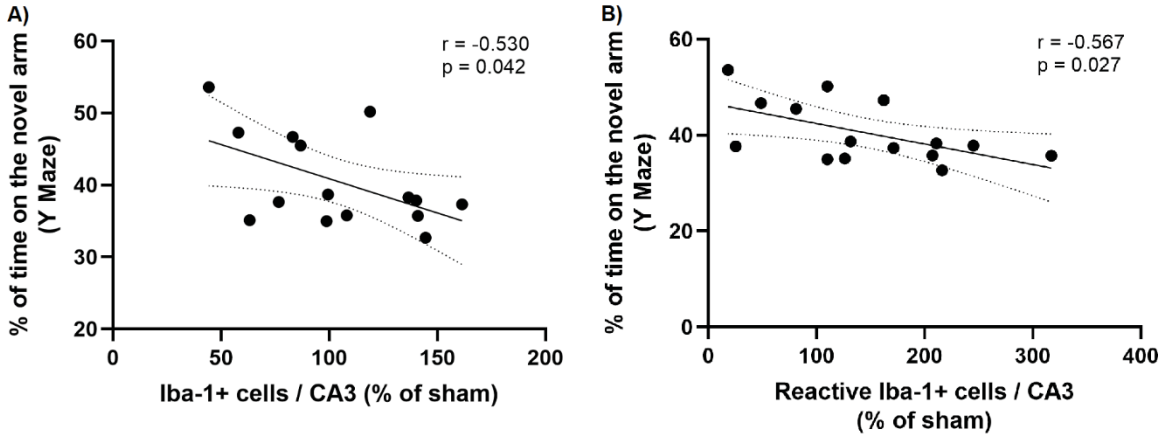

Figure 10. Correlations between the percentage of time spent on the novel arm in the Y maze and the number of (A) Iba-1-positive cells and (B) reactive Iba-1-positive cells in the CA3 area of the HIP. r: Pearson correlation coefficient; n = 15.

2.8. ICV-STZ infusion and ANDRO treatment did not affect the APP expression in the PFC and HIP

The two-way ANOVA did not show differences between the groups in the expression of the amyloid precursor protein, both in the PFC (treatment x lesion interaction [F (1, 11) = 0.008; p = 0.930]; treatment [F (1, 11) = 0.746; p = 0.406]; lesion [F (1, 11) = 0.725; p = 0.412]) and the HIP (treatment x lesion interaction [F (1, 12) = 0.122; p = 0.732]; treatment [F (1, 12) = 0.665; p = 0.430]; lesion [F (1, 12) = 0.006; p = 0.940]).

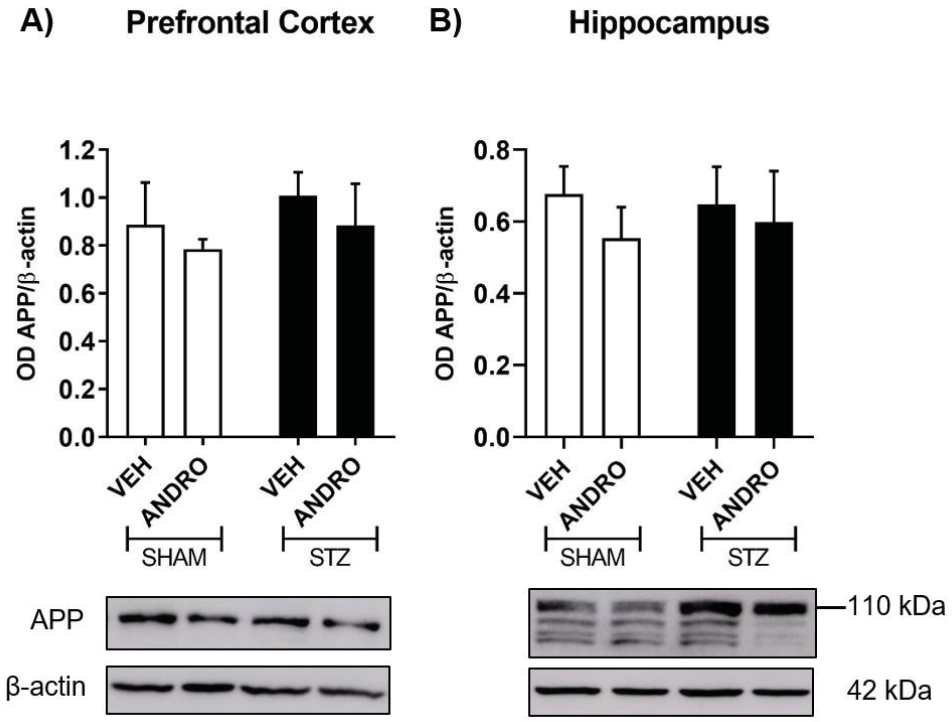

Figure 11. Effect of prolonged administration of andrographolide 2 mg/kg (ANDRO) on APP expression, determined by Western blot, in the (A) prefrontal cortex and (B) hippocampus of STZ-injected animals compared with sham animals 30 days after surgery. The data are expressed as mean ± SEM (n = 3-4/group) Two-way ANOVA followed by Tukey's post hoc test).

3. Discussion

The current study investigated the effects of prolonged andrographolide treatment (2 mg/kg, 30 days) in short-term spatial and recognition memory and neuroinflammation in the ICV-STZ model of sAD in rats. It is extensively described in the literature that the ICV infusion of STZ in rodents causes impairments in cognitive tasks that rely on the HIP (Li et al., 2020; Prickaerts et al., 1999; Rodrigues et al., 2010). This feature can mimic a well-documented early symptom of AD, spatial and temporal disorientation (Tu et al., 2015). The ability of rodents to remember a location in space defined by distal visual cues, which is essential for the Y maze task, is severely disturbed by hippocampal damage (Eichenbaum, 2017; Martin and Clark, 2007). In the present study, as indicated by the Y maze performance, the STZ-injected animals showed impairment in short-term spatial memory. It has been previously demonstrated that ANDRO can improve the performance of rodents in spatial memory tasks (Rivera et al., 2016; Serrano et al., 2014). Here, ANDRO exhibited an ambiguous role. In the Y maze test, the results suggest a positive effect of ANDRO in short-term

spatial memory. However, in the object location test, the STZ-animals treated with ANDRO had a significantly worse performance in comparison with the SHAM+VEH group, and the STZ+VEH group a similar one, suggesting a negative impact of ANDRO. The conflict between these results is unexpected since the two tasks have fundamental similarities in assessing short-term spatial memory, both are inherently not stressful and based on exploiting rodents' innate preference for novelty (Dellu et al., 1997; Vogel-Ciernia and Wood, 2014). Despite the worse performance of the STZ+ANDRO group, all the groups showed a low discrimination index in the OLT ($-0.1 < d < 0.2$), including the control group, which could have occurred due to performance confounds, like stress and anxiety (Vogel-Ciernia and Wood, 2014).

Along with spatial memory dysfunction, recognition memory dysfunction is proposed as an early marker in AD, been relevant for animal models of AD (Bassani et al., 2017b; Grayson et al., 2015). Therefore, we subjected the animals to the object recognition test. The ORT relies on different brain regions, including insular, perirhinal, and ventromedial prefrontal cortices. The PFC may be of particular importance for associative memory (Vann and Albasser, 2011). The HIP role in object recognition varies depending on the experimental setup, in the present protocol is suggested that the hippocampus is required for encoding the spatial and temporal context of the objects (Grayson et al., 2015; Vogel-Ciernia and Wood, 2014). In line with previous studies (Cisternas et al., 2019; el Sayed and Ghoneum, 2020), we observed a disruption in short-term recognition memory by STZ, and ANDRO was able to preserve it, having a protective effect on rats' ORT performance. Our study is the first to report an improvement in short-term recognition memory by ANDRO in the ICV-STZ model of sAD. In a recent report, it was observed that ANDRO (15, 30, and 60 mg/kg, 14 days) protected rats against STZ-induced learning and memory impairment (Patel et al., 2021). However, cognition was assessed 21 days after STZ infusion in the Morris Water Maze Test and in a modified version of the Elevated Plus Maze Test, which measures spatial long-term memory. In addition, the doses were substantially higher than the dose used in our study.

In the OFT, the diminished frequency of locomotion of the STZ+ANDRO group could suggest a negative effect of ANDRO on the spontaneous exploratory behavior of STZ-injected rats and an impairment on locomotor activity (Choleris, 2001; Walsh and Cummins, 1976). Yet, the lesion factor was the only factor showing significant

differences, indicating that the STZ infusion was the responsible for the decrease on locomotion frequency not the ANDRO treatment. It is known that changes in these parameters could decrease the animals' performance in subsequent cognitive tests (Moura et al., 2020). However, the exploratory behavior and/or ambulation seem to have been affected only in the OFT, the groups did not show differences on the total time spent exploring the objects in the OLT and in the ORT, in addition, there were no differences on the distance covered in the Y maze. An explanation is that the OFT was performed 8 to 9 days before the other behavioral tests and the animals could have recovered the exploratory and/or locomotor capacity before the cognitive tests.

As mentioned, the hippocampus and the prefrontal cortex are critical for spatial memory and recognition memory tasks (Akirav and Maroun, 2005; Barker and Warburton, 2011; Eichenbaum, 2017). The HIP and the PFC have distinct yet complementary roles in rats and humans and are part of a larger network of interconnected brain regions that contribute to mnemonic function (Vann and Albasser, 2011). The ICV-STZ infusion can compromise the optimal functioning of these areas by increasing the expression of APP, Aβ, and tau; oxidative stress; and neuroinflammation, as expressed by microgliosis and astrogliosis (Bassani et al., 2017a; Chen et al., 2013; Hira et al., 2019; Moura et al., 2020; Pierzynowska et al., 2019). It is known that ANDRO can protect the PFC and the HIP from these disturbances in rodent models of AD. In APP/PS1 transgenic mice, the treatment with 2 mg/kg of ANDRO (three times per week for 4 weeks) reduced Aβ deposition, inhibited microglial activation, and decreased the secretion of proinflammatory factors in the PFC and HIP (Zhang et al., 2021). In the *Octodon degus* model, ANDRO (2 mg/kg, three times per week for 3 months) significantly reduced the total Aβ burden, the oxidative stress, as well as astrogliosis in the HIP (Lindsay et al., 2020). Thus, in the current study, we analyzed the effects of the ICV-STZ infusion and ANDRO in markers of neuroinflammation regarding the microglia and astroglia, and in the APP expression in the PFC and HIP.

Neuroinflammation, as reflected by astrogliosis and microglial activation, is a pathological hallmark of AD (Guo et al., 2017). The formation of amyloid plaques and neurofibrillary in the vicinity of activated microglia and astrocytes in the brain is demonstrated in animal studies and AD patients. Aβ deposition promotes the activation of glial cells, stimulating reactive gliosis pro-inflammatory signaling cascade (Heneka

et al., 2015; Serrano-Pozo et al., 2013). Pro-inflammatory cytokines when chronically activated affect the processing of APP through beta-secretase, accelerating the production of Aβ. The result is a reduction in activated glial cells clearance of Aβ, leading to an increase in Aβ burden (Kaur et al., 2019). During immune responses, reactive microglia and astrocytes change their morphology and functionality. The activation of this reactive phenotype ensures the removal of injurious stimulus, protecting the brain. However, the diversification and perpetuation of activated glial cells can act as a double-edged sword that induces neurodegeneration and AD (Meraz-Ríos et al., 2013). Studies suggest a deleterious role of glial cells in the progression of multiple AD biomarkers (Furman et al., 2012).

Reactive astrocytes are characterized by increased expression of GFAP (Heneka et al., 2015). Several reports demonstrated that in the ICV-STZ model, the GFAP immunoreactivity and expression increased in the PFC and the HIP (Pilipenko et al., 2019; Rai et al., 2014; Rajasekar et al., 2017; Ravelli et al., 2017). In the present study, GFAP expression increased only in the PFC of the STZ+VEH group. ANDRO protected the PFC of the rats against astrocyte reactivity induced by STZ. In the HIP, there were no differences between groups, however, a negative correlation was identified between the performance in the ORT and the GFAP expression. Furthermore, we found a negative correlation between the performance in the Y maze and the GFAP expression in the PFC. These results corroborate the association between cognitive impairment and astrogliosis and the neuroprotective role of ANDRO in the PFC.

Distinctively from previous studies (Guo et al., 2017; Moura et al., 2020; Zappa Villar et al., 2018), we did not observe an increase in the number of cells and reactivity of microglia in the HIP of the STZ+VEH group in relation to the SHAM+VEH group, despite that, in the CA3 area of the HIP, there were important tendencies. On the other hand, there was a significant decrease in microglia cells in CA1 and CA3, and in reactive microglial cells in CA3 of the STZ+ANDRO group compared to the STZ+VEH group, agreeing with previous reports (Zhang et al., 2021) and reinforcing the capability of ANDRO to inhibit the hypertrophy and proliferation of microglia in regions of the HIP. In addition, we identified negative correlations between the performance in the Y maze and microgliosis in the CA3 area.

Regarding the expression of APP, no significant differences were observed between the groups both in the PFC and the HIP. There is evidence that the ICV infusion of STZ can increase APP levels. For example, Pierzynowska et al. (2019) observed augmented expression of APP in the PFC, HIP, and the rest of the brain of Wistar rats after 30 days of the STZ infusion (3 mg/kg). Retinasamy et al. (2020) observed in Sprague Dawley (SD) rats an increase in the gene expression of APP in the PFC and HIP 21 days post-surgery (STZ 3 mg/kg). However, there is also contrasting data, as in the study of Zappa Villar et al. (2018), in which the infusion of 1 and 3 mg/kg of STZ did not increase the APP expression in the HIP of SD rats after 25 days; in the work of Gupta et al. (2018), the gene expression of APP was increased by STZ infusion only in the cortex, but not in the HIP of SD rats 30 days after surgery. APP has been shown to have a key role in synapse formation and repair and is known to be upregulated during neuronal injury (Plummer et al., 2016). The increase in APP expression can be attributed to a regenerative attempt of the neurons to counteract the neuronal injury induced by STZ (Mishra et al., 2018). APP is a complex molecule that undergoes substantial posttranslational modification and at least ten different proteolytic fragments of APP have been identified. Several of these are suggested to be pathogenic, whereas others are neuroprotective (Wang et al., 2017). As the levels of the pathogenic fragments (e.g., Aβ 1-40, Aβ 1-42) were not measured in the present report it is difficult to state if the amyloidogenic pathway influenced the effects of STZ and ANDRO.

The mechanisms of andrographolide neuroprotection involve its anti-inflammatory and antioxidative activities (Lu et al., 2019). ANDRO can reduce glia-mediated oxidative damage and production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-18, nitric oxide) by down-regulating the nuclear factor kappa B (NF-κB) pathways and activating the NF-E2-related factor-2 (Nrf2), heme oxygenase-1 (HO-1), and the anti-inflammatory factor CD206, important molecules of the inflammatory response (Wong et al., 2016; Xu et al., 2019; Zhang et al., 2021). Furthermore, ANDRO reduces levels of tau protein, improves insulin resistance, restores glucose uptake, and inhibits glycogen synthase kinase-3 beta (GSK-3β) activity, activating the downstream Wnt/β-catenin pathway, recently implicated in regulating glucose metabolism in the brain, which is dysfunctional in AD (Cisternas et al., 2019; Gherardelli et al., 2020; Rivera et al., 2016; Tan et al., 2017). These mechanisms have

not been contemplated in the current report, further studies are necessary to better understand the ANDRO role in the ICV-STZ model.

In summary, ANDRO attenuated the impairment in short-term spatial memory and short-term recognition memory induced by the ICV-STZ model of sAD in rats. Additionally, ANDRO decreased astrogliosis in the prefrontal cortex and microgliosis in the hippocampus of the animals, which could be related with the memory improvement. Further investigations of the effects of ANDRO in the ICV-STZ model are necessary to elucidate the adjacent mechanisms of neuroprotection and the treatment optimal dose.

4. Material and methods

4.1. Animals

Male Wistar rats (60-90 days old, weighing between 300-350g), provided by the animal facility of the Federal University of Parana, were socially housed in groups of 3–4 in polypropylene cages with wood shavings as bedding. The rats were maintained in a temperature-controlled room (22 °C ± 2 °C) on a 12 h/12 h light/dark cycle (lights on at 7:00 AM), water, and standard laboratory chow were available ad libitum. Before the experiment, the rats were allowed to acclimatize for 1 week to reduce environmental stress. The experiments were performed following the Brazilian Law for Animal Experimental Ethics and Care (11.794/October 8, 2008) and the guidelines of the Federal University of Parana Committee on the Care and Use of Laboratory Animals. The experimental procedures were approved by the University Ethics Board (CEUA/BIO - protocol #1315). All efforts were made to minimize the number of animals used and their suffering.

4.2. Experimental Design and Treatments

The rats were randomly divided into four experimental groups: SHAM+VEH, SHAM+ANDRO, STZ+VEH, STZ+ANDRO (10-11 per group). All the animals were submitted to stereotaxic surgery, the STZ groups received a single bilateral injection of STZ (N-[methylnitrosocarbamoyl]-α-D-glucosamine, Santa Cruz Biotechnology, USA) in a total dose of 3 mg/kg, dissolved in sterile saline. The sham groups received

a single bilateral injection of sterile saline. The animals were treated three times per week (Monday, Wednesday, Friday) intraperitoneally (i.p.) with 2 mg/kg ANDRO (Sigma-Aldrich, USA) or its vehicle (0.9% saline and 2% dimethyl sulfoxide) for 4 weeks, starting 1h after the surgery (Kanazawa et al., 2021; Varela-Nallar et al., 2015).

After surgery, the rats were allowed to recover for three weeks, and the behavioral tests were conducted on the fourth week. The spontaneous locomotor activity was assessed in the open field test (OFT) on day 21 following surgery, and cognitive performance was evaluated in the object location test (OLT) on day 28, in the object recognition test (ORT) on day 29, and the spatial version of the Y maze on day 30 (Bassani et al., 2017b). The experimental design is presented in Fig. 1.

Posterior to the behavioral tests, a subset of animals (n = 4/group) was decapitated under chloral hydrate anesthesia (400 mg/kg, i.p.). The brains were extracted, and the whole HIP and PFC were dissected for the quantification of APP and glial fibrillary acidic protein (GFAP) by Western blot. Another subset of animals (n = 4/group) was deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and intracardially perfused for immunohistochemical evaluation of the microglia-specific marker for ionized calcium-binding adaptor molecule (Iba-1).

4.3. Stereotaxic surgery

Stereotaxic surgery was carried out as previously described (Bassani et al., 2017b; Bassani et al., 2017a; Moura et al., 2020). The animals were anesthetized with sodium thiopental (30 mg/kg, i.p.) and chloral hydrate (150 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf, USA). A 28- gauge stainless steel needle was lowered into each lateral ventricle (LV). The stereotaxic coordinates for ICV infusion, according to Paxinos and Watson (Paxinos and Watson, 2007), were measured: anterior/posterior, −0.8 mm from bregma; medial/lateral, ±1.5 mm from the midline; dorsal/ventral, −3.8 mm from the skull. An electronic pump (Insight, Ribeirão Preto, SP, Brazil) was used to control the flow of the injections at a rate of 1.0 µl/min over 4.5 min. The lesioned group received bilateral ICV injections of STZ (3 mg/kg total dose) dissolved in sterile 0.9% saline (4.5 µl per injection site). Sham surgery followed the same procedure, but sterile saline was injected instead of STZ. After surgery, all the rats were allowed to recover from anesthesia for 2–4 h in a heated and well-ventilated

room. Food and water were placed inside the cage for 10–15 days so that the animals could easily access it without physical trauma caused by head surgery.

4.4. Open Field Test

The OFT was performed 21 days after surgery. The open field apparatus was placed in a moderately lit room and consisted of a circular arena (97 cm diameter, 42 cm height) which was divided into three concentric circles and subdivided into 19 quadrants. A video camera was placed right above the arena to record the animal's behavior for posterior analysis. Individually the animals were placed in the center of the apparatus and allowed to freely explore it for 5 min. The total number of crossings from one quadrant to another was measured. A crossing was considered only when the animal entered another quadrant with its four paws. The apparatus was cleaned with a 20% ethanol-water solution between each test to eliminate possible odors left by other rats.

4.5. Object location test (OLT) and object recognition test (ORT)

The OLT and ORT were performed between days 27 and 29 following surgery to evaluate short-term spatial and short-term recognition memory, respectively. Both tests took place in a squared arena, 100 cm × 100 cm × 40 cm, made of wood and painted black. It was placed in a moderately lit room. A video camera was positioned over the arena, and the animals' behavior was recorded for later evaluation.

The first procedure consisted of the habituation of the animals in the box. Each animal was placed in the empty apparatus for 5 min for free exploration. Twenty-four hours later, a novel habituation session of 5 min was performed. After a 1 h delay, during the training session, two identical objects were placed in the apparatus in a symmetrical position about 10 cm away from the wall. The animals were allowed to explore them freely for 5 min and were then returned to their home cages. In the OLT (which was performed on day 28), after a 1 h delay, during the test session, each rat was put back into the box with one of the objects displaced 15 cm away from the original position (novel position); animals were allowed to freely explore the objects for 3 min. In the ORT (which was performed on day 29), a new training session was done

with the two identical objects placed in a symmetrical position about 10 cm away from the wall, and the animals were allowed to explore them freely for 5 min and were then returned to their home cages. After a 1 h interval, the rats were put back into the arena for the test session; but now with two dissimilar objects, a familiar one (the sample) and a new one, the animals were allowed to freely explore the objects for 3 min.

The objects were made of plastic or ceramic. To avoid an olfactory bias, before each trial, the objects were cleaned with a 20% ethanol solution. All objects and locations were balanced to reduce potential biases due to preferences for particular locations or objects. A rat could not displace the objects and the subjects were always placed into the box facing the same wall. Exploration was defined as sniffing at no more than 2 cm or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. The animals that spent less than 10 s exploring the objects were excluded from the test.

The measures for both the OLT and ORT were the time spent by the rats exploring each object during the test session. The time spent exploring the familiar and the new object (ORT)/displaced object (OLT) was represented by 'a' and 'b', respectively. The following variables were calculated: e = a + b, and d = (b − a)/e. The 'e' variable is a measure of the total exploration time of both objects during the test session. 'd' is considered a discrimination index between the new and the familiar objects/locations, and a relative measure of discrimination that corrects for the exploratory activity (e) (de Bruin et al., 2011).

4.6. Spatial Version of Y maze

A symmetrical Y maze was constructed of wood and painted black. It consisted of three arms. The arms (50-cm in length, 27- cm in height, 12-cm in width) were arranged at a 120° angle relative to each other. The test consisted of a training session and test session that were performed at 1-h intervals. In the training session, one arm was made inaccessible by a removable blockade that was placed in front of it. A rat was placed in one of the other arms (i.e., "start arm"). The start arm was randomized between groups. The animals were then allowed to explore these two arms for 5 min. The rat was then removed from the arena and returned to its home cage. After a 1-h interval, in the test session, the rat was returned to its corresponding start arm, but the

blockade that prevented access to the third arm (i.e., "novel arm") was removed, thus providing access to all three arms. The rat was allowed to explore the three arms for 3 min. The test session was video recorded for subsequent analysis. Short-term spatial memory was assessed as the percentage of time spent on the novel arm, which had to be significantly greater than 33.3% of the total time on the maze (corrected for the latency to move from the start arm to another arm and the time spent in the center of the maze). An arm entry was considered when both hind paws were placed completely inside an arm. The maze was cleaned between sessions with a 10% ethanol solution to reduce olfactory bias (Kraeuter et al., 2019).

4.7. Quantification by Western Blot

To investigate the effects of ANDRO on the astroglia in the HIP and PFC of ICV-STZ rats, we measured the level of GFAP, the commonly used marker for astrocytes, via western blot. The samples were homogenized and sonicated in lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl, pH 8.0, and protease inhibitors). After centrifugation at 20,000 rpm and 4 °C for 20 min, the supernatant was collected, and the protein concentration was determined by the Bradford method (Bio-Rad, Germany). The supernatants were boiled with Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HC with adjust final pH of buffer to 6.3) for 10 min at 95ºC. After reduction, the samples were subjected to electrophoresis and transferred to nitrocellulose membranes (Bio-rad, 0.45 µm). The membranes were blocked with 5% nonfat milk in TBS-Tween 20 solution, and then incubated overnight at 4ºC with mouse monoclonal anti-β-amyloid antibody (1:750; sc-28365, Santa Cruz Biotechnology, Santa Cruz, USA), rabbit polyclonal anti-GFAP antibody (1:1000; ab7260, Abcam), rabbit polyclonal anti-β-actin (1:500; sc-130656, Santa Cruz Biotechnology, Santa Cruz, USA), or mouse monoclonal anti-GAPDH antibody (1:5000; sc-32233, Santa Cruz Biotechnology, Santa Cruz, USA). After incubation with the primary antibody, the membranes were extensively washed with TBS-T and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma, USA) in a blocking solution for 1h at room temperature. The membranes were then washed, and the visualization was performed with Pierce ECL kit (Thermo scientific) chemiluminescence substrate on an Amersham ™ Imager 600 detection system (GE Healthcare, São Paulo, SP, Brazil). Protein levels

were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

4.8. Immunohistochemistry

The effects of ANDRO on the microglia in the HIP and PFC of ICV-STZ rats were explored, and immunostaining to identify microglia was carried out using Iba-1 primary antibody. Brain sample processing and immunohistochemistry reactions were developed as described by Moura et al. (2020). The animals were deeply anesthetized and then euthanized with intracardiac perfusion of saline phosphate-buffered solution (pH 7.4; 1,000 mL/kg; 4ºC), followed by 4% paraformaldehyde in phosphate-buffered solution (pH 7.4; 1,000 mL/kg, 4ºC). Then the brains were removed and placed in 30% sucrose solution for 4 days for tissue cryoprotection. After that, the tissue was protected with plastic, quickly frozen in liquid nitrogen, and stored in a freezer (–80ºC) for subsequent sectioning. The frozen tissue was cut into 30 µm semi-serial coronal sections along the dorsal hippocampus at a temperature of –25ºC using a cryostat (Leica Biosystems, Germany). The dorsal hippocampus was collected, and the sections were placed serially in ten well-plates using –2.56 to –4.52mm stereotaxic coordinates to the bregma, totaling six to eight 30µm cuts per well-plate. Brain sections were stored at –20ºC in a cryoprotectant solution containing 30% ethylene glycol and 15% sucrose in a 0.05 M phosphate-buffered solution for subsequent processing.

The free-floating method was used for immunohistochemical reactions. The brain sections were initially washed with buffer A (0.1 M PBS, pH 7.4 with 0.5% Triton X-100) and incubated in 0.1M citrate buffer, pH 6.0 in a water bath at 50ºC for 30 min. The steps described above characterize an antigen retrieval step for this marker. After that, the sections were cooled to room temperature, washed with buffer A, incubated with 0.5% H2O2 solution in 0.1M PBS for 30 min at room temperature and protected from light, washed again with buffer A and incubated with 2% bovine serum albumin (BSA) in buffer A (blocking buffer) at room temperature for 1 h. Subsequently, the plates containing the sections were incubated overnight at 4ºC with the primary antibody diluted in anti-IBA1 goat polyclonal blocking buffer (1:500, Abcam, Cambridge, USA).

The following day the sections were washed in buffer A and incubated with biotinylated secondary antibody at 4ºC for 2 h, washed again in buffer A and incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories, USA) in 0.1M PBS in room temperature for 2 h. After being washed with 0.1MPBS, the reaction was revealed by incubating the sections with 3,3'-diaminobenzidine (DAB, Vector Laboratories, USA) in 0.1MPBS at room temperature for 7 min. The sections were washed in 0.1M PBS, mounted on gelatinized slides, and air-dried. The brain slices were mounted on microscope slides, dehydrated in solutions of ascending ethanol concentrations, cleared in xylene, and coverslipped.

The areas of the hippocampus were photographed using a BX50 optical microscope (Olympus Optical, USA) at 200× magnification. Microglial cells were identified as Iba-1-positive (Iba1+) cells and manually counted using the Cell Counter of the ImageJ software. Iba1+ cells were morphologically classified as Types I, II, III, IV, and V as previously described by as (Diz-Chaves et al., 2012). Types I, II, and III were categorized as non-reactive glia, whereas Types IV and V were taken as reactive glia (Zappa Villar et al., 2018). Results were expressed as a percentage of the number of sham Iba-1+ cells and sham reactive Iba-1+ cells.

4.9. Statistical Analysis

The data are presented as mean ± standard error of the mean (SEM) and analyzed using a two-way analysis of variance (ANOVA) (factor lesion: STZ or vehicle injection; factor treatment: ANDRO or vehicle treatment). Pearson's correlation coefficient was used to evaluate the degree of association between variables. The level of significance was $p < 0.05$. The analysis was performed with the software GraphPad Prism 8.0 (GraphPad Software, Inc) and STATISTICA (data analysis software system) version 12 (StatSoft, Inc).

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CRediT authorship contribution statement

Leonardo C. Souza: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Marcos K. Andrade:** Formal analysis, Investigation, Data Curation. **Evellyn M. Azevedo:** Formal analysis, Investigation, Data Curation. **Daniele C. Ramos:** Investigation, Writing - Review & Editing. **Ellen L. Bail:** Investigation**. Maria A. B. F. Vital:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Supplementary material

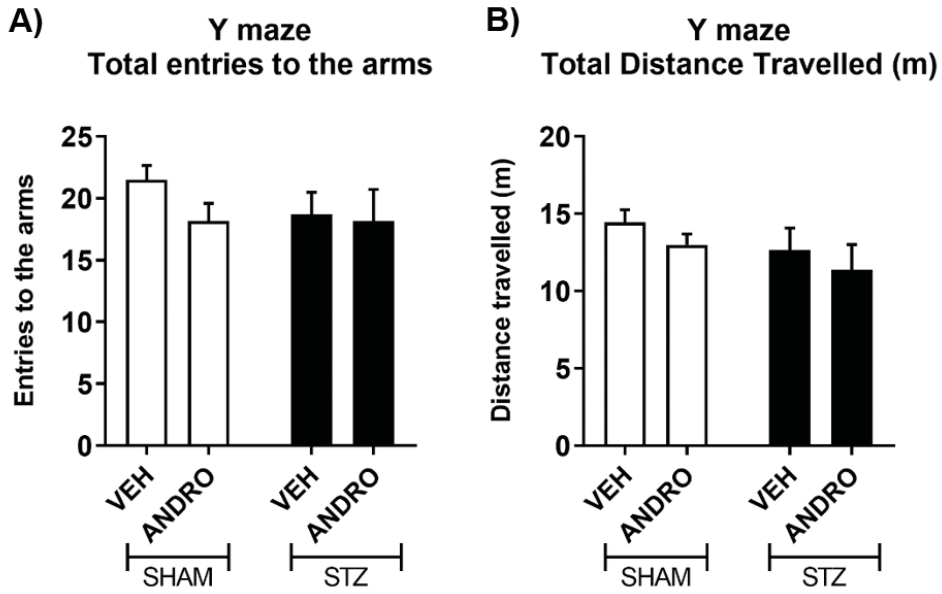

Figure S1. (A) Total number of entries to the arms and (B) total distance travelled of the STZ-injected animals compared with sham animals on the spatial version of the Y maze test. The data are expressed as mean ± SEM (n = 10-11/group).

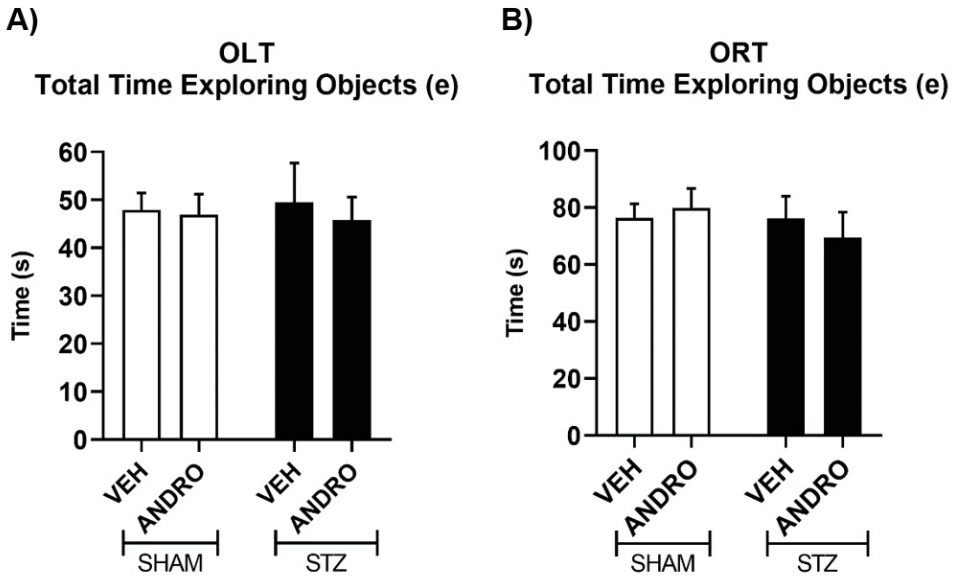

Figure S2. (A) Total time exploring the objects on the object location test and (B) on the object recognition test of the STZ-injected animals compared with sham. The data are expressed as mean ± SEM (n = 8-10/group).

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EFFECTS OF A NUTRITIONAL FORMULATION CONTAINING CAPRYLIC AND CAPRIC ACID, PHOSPHATIDYLSERINE AND DOCOSAHEXAENOIC IN STREPTOZOTOCIN-LESIONED RATS

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Background:

It has been studied that nutrition can influence Alzheimer's disease (AD) onset progression. Some studies on rodents using intraventricular brain streptozotocin (STZ) injection showed that this toxin changes cerebral glucose metabolism and insulin signaling pathways.

Objective:

The aim of the present study was to evaluate whether a nutritional formulation could reduce cognitive impairment in STZ-induced animals.

Methods:

The rats were randomly divided into two groups: sham and STZ. The STZ group received a single bilateral STZ-ICV injection (1 mg/kg). The sham group received a single bilateral ICV injection of 0.9% sterile saline solution. The animals were treated with AZ1 formulation (Instanth® NEO, Prodiet Medical Nutrition) (1 g/kg, PO) or its vehicle (saline solution) for 30 days, once a day starting one day after the stereotaxic surgery (n = 6-10 per group). The rats were evaluated using the open field test to evaluate locomotor activity at day 27 after surgery. Cognitive performance was evaluated at day 28 using the object recognition test and the spatial version of the Y-maze test. At day 30, the rats were deeply anesthetized with chloral hydrate (400 mg/kg, i.p) and euthanized in order to evaluate IBA1 in the hippocampus. The differences were analyzed using one-way ANOVA with Bonferroni's or Kruskal Wallis with Dunn's post-hoc test.

Results/Conclusion:

STZ-lesioned rats present memory impairment besides the increased microglial activation. The treatment with AZ1 formulation reversed the memory impairment observed in the object recognition test and Y-maze and also reduced IBA1 in CA1 and DG.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease and the most common form of dementia. It is mainly characterized by progressive and slow memory loss, especially sporadic memory (related to recent events) and spatial disorientation. AD presents common histopathological findings, evidenced by protein accumulations. These findings are described as intracellular neurofibrillary tangles in cortex and hippocampal neurons caused by tau protein hyperphosphorylation, and as β-amyloid extracellular filamentous aggregation, also called senile plaques [1–7]. Disease progression also causes hippocampal, neocortex and amygdala atrophy, mainly seen in postmortem examinations. Such protein dysfunctions also result in a cascade of events involving neuronal loss, neuroinflammation, glial reactivity, cognitive and functional disorders [1–6], and decreased cerebral glucose metabolic rate, which worsens the cognitive status [8–13].

Many of the signals seen in Alzheimer's disease, such as severe cognitive impairment, neuroinflammation, and oxidative stress, are also described after intracerebroventricular (ICV) injection of streptozotocin (STZ), being validated by several studies [14–18]. This toxin, produced by the bacterial strain *Streptomyces achromogenes*, is commonly used for animal models of type II diabetes for its ability to act on β-pancreatic cells and cause insulin resistance. However, STZ can cause cerebral glucose metabolic rate dysfunction by inhibiting insulin receptors when administered ICV at sub-diabetogenic doses. These factors trigger neuroinflammation, glial activation and neuronal death, as well as a cognitive decline related to loss of spatial and recognition memory, very similarly to AD symptoms [15–18].

AD also presents abnormal complications of neuronal membrane lipids with increased cholesterol to the detriment of phosphatidylserine [19–21]. In this context, oral phosphatidylserine supplementation can cross the blood-brain barrier and

increase the brain supply of this nutrient [21]. Some experimental studies showed that phosphatidylserine helped improve cognitive problems and performance on tasks testing short-term learning and memory skills [21].

Another important nutrient in the composition of the neuronal membrane is docosahexaenoic acid (DHA), which has a complementary function to phosphatidylserine supplementation. About 20-30% of phosphatidylserine in gray matter is combined with DHA [22,23]. Decreased phosphatidylserine DHA in cerebral cortex is associated with AD mild cognitive impairment progression [21,24]. In addition, the brain is vulnerable to lipid peroxidation, decreasing membrane fluidity, damaging membrane proteins and resulting in membrane breakdown [25]. In the STZ-induced AD model, Pardeshi et al. [26] demonstrated that DHA improved the efficacy of the pharmacological agent used in the study and increased survival and neuronal memory, decreasing oxidative stress and inflammation.

Diets that induce ketone body formation have been related to preserved cognition and reduced tau protein and β-amyloid pathology in AD animal models [27,28]. One of the mechanisms responsible for the formation of ketone bodies is the use of caprylic and capric fatty acids [29–31], even when added to regular meals, as they are rapidly absorbed by the portal system and beta-oxidized in the liver, generating excess acetyl-coA, which forms ketone bodies [31,32]. Ketone bodies cross the blood-brain barrier [33], enter the neurons and produce ATP in the mitochondria by oxidative phosphorylation [31]. Thus, caprylic and capric acids are also important nutrients in AD.

Although many nutrients seem to be important for brain health in AD, there are no studies evaluating these nutrients combined. The objective of the present study was to evaluate whether a formulation with a unique combination of capric acid, caprylic

acid, phosphatidylserine, DHA, vitamins and minerals can reduce cognitive impairment in STZ-induced animals in also the neuroinflammatory markers of the model.

2. Material and Methods

2.1. Animals

This study included thirty-two young adult (approximately three months old) Wistar male rats (*Rattus norvegicus*) from the Central Laboratory Animal Facility of the Federal University of Paraná (UFPR) weighing 290 - 320 g at the beginning of the experiment.

Each five animals were kept in a polypropylene box with *ad libitum* water and food intake in a room with controlled humidity and temperature (22 ± 2° C) and 12-hour light-dark cycle (7 a.m. - 7 p.m.). The procedures were approved by the Animal Ethics Committee (CEUA) of the UFPR under protocol number 1226.

2.2. Stereotaxic surgery

The animals were deeply anesthetized with sodium thiopental (30 mg/kg, i.p.) and chloral hydrate (150 mg/kg, i.p.) followed by atropine sulfate (0.4 mg/kg IP) as an anesthetic adjunct to reduce mucus production. The rats were placed in the stereotaxic equipment (David Kopf, 957L model) and positioned according to the following coordinates: anteroposterior (AP): - 0.8 mm from the bregma; mediolateral (ML): ± 1.5 mm from the midline; and dorsoventral (DV): - 3.8 mm from the skullcap [34]. The head region was shaved and a small skin incision mas made to expose the skull. Subsequently, two incisions were drilled in the skull with a dental surgical drill, followed by the insertion of a 30-gauge needle connected to a polyethylene tube adapted to a

10 µL micro syringe (Hamilton, USA) and connected to an infusion pump (Harvard Apparatus, USA).

The lesioned group received bilateral ICV injections of STZ (1 mg/kg total dose) dissolved in sterile saline (2 µl per injection site) into the lateral ventricles. The sham group animals underwent the same surgical procedure; however, they received only the diluent (0.9% sterile saline solution) instead of the STZ solution.

Microinfusion was programmed at a rate of 1 µL/minute for two minutes. The needle remained at the infusion site for two minutes after the end of the procedure to avoid reflux, and then the skin incision was sutured. After that, the animals received 1 mL of saturated sodium bicarbonate solution IP to accelerate anesthetic elimination and 0.1mL of penicillin G procaine 2.000 IU/mL IM to prevent possible infections.

At the end of the surgery, the rats were placed back in warmed boxes until complete recovery.

2.3. Drugs

Streptozotocin (STZ - Santa Cruz Biotechnology - Santa Cruz, CA, USA) 1 mg/kg and AZ1 formulation (Instanth® NEO, Prodiet Medical Nutrition, PR, Brazil). AZ1 formulation was administered by gavage (1g/kg) once a day for 30 days and its composition is in TABLE 1.

2.4. Experimental design

The animals were randomly divided into four experimental groups: STZ + saline (n = 6), STZ + AZ1 formulation 1 g/kg (n = 9), sham + saline (n = 10), and sham + AZ1 formulation 1 g/kg (n = 7). Animals in STZ groups received a bilateral 2µL microinfusion of STZ solution (1 mg/kg) dissolved in sterile saline solution. Animals in

sham groups underwent the same surgical procedure; however, they received the same amount of 0.9% sterile saline solution instead of the STZ solution. The groups were treated with AZ1 formulation (1 g/kg) or saline solution for 30 days, once a day in the afternoon, starting one day after surgery.

At day 27 after surgery the animals underwent the open field test (OFT) to evaluate spontaneous locomotion and rearing frequency. At day 28 the animals underwent the object recognition (ORT) and the Y-maze tests, which evaluated spatial memory and short-term recognition. Finally, at day 30 the animals were deeply anesthetized with hydrated chloral (400 mg/kg, IP) and euthanized with an intracardiac perfusion process. Subsequently, the brain structures were prepared and immunohistochemically evaluated with the expression of the microglia-specific marker for ionized calcium-binding adaptor molecule (IBA1).

2.5. Open field test (OFT)

The OFT is a behavioral test widely used to measure locomotor and behavioral characteristics in rodents. It is an easy-to-perform test based on physiological concepts well discussed in the literature that is also used to evaluate anxiety and exploratory behavior [35–37].

The test takes place in a white round arena (97 x 42 cm) divided by three concentric circles into three parts subdivided by straight segments into 19 quadrants with a central circle where the animal was carefully placed, allowing it to freely explore the entire area for five minutes. This study analyzed two motor parameters: locomotion (ambulation) and rearing frequency. Locomotion is one of the most analyzed factors [36] and considers the quadrants the animal crosses with all four paws as a measure of motor activity [35]. Rearing is the frequency with which the animal stands on its hind

legs and measures exploratory activity [35]. The arena was cleaned prior to each animal test with 20% water-ethanol solution to eliminate odors and substances left by the previous rat.

2.6. Object recognition test (ORT)

Object recognition memory is associated to the ability the animal has to distinguish between known and new objects. Rodents have an intrinsic tendency to spend more time exploring new than familiar objects [38]. This decreased novelty may be related to repeated exposure to a new stimulus that eventually becomes familiar; however, it should not be confused with decreased exploratory interest [38].

The ORT takes place in a square black (to avoid clues) box (100 x 100 x 40 cm) made of wood and positioned in a moderate light environment (20 lux). A camera was positioned above the box for subsequent image analysis in order to avoid interference due to the presence of the handler in the test environment. The test started with an adaptation session in which the animals were placed in the box for five minutes for free exploration of the environment. Another five-minute session was held 24 hours later and the test started one hour after [39]. Session one was a training session. The animals were placed in the test box with two identical objects and had five minutes to explore them before being placed back in the habitat box. One hour after the initial exposure, the animals were placed back in the test box with a new object for three minutes. This session was recorded for later evaluation of how much time the animal spent interacting with each object [40].

The objects used in the test were made of plastic or ceramic and cleaned with 20% ethanol (standardized by the laboratory) after each session to avoid the influence of the smell of other animals [40]. The objects were randomly arranged to reduce the

bias of preference for specific place or object. Weights have been added to objects so that the rat could not move or knock them down [17,41]. All animals were placed in the box facing the same wall in both sessions [39]. A chronometer was used to measure the time the animal explored the objects, which was considered only when the animal smelled an object at a distance less than two centimeters or touched the object with the muzzle. Sitting, rearing or walking around objects were not considered exploratory behaviors [42].

Exploratory behavior was measured by counting how much time the animal explored each object during the test session [40]. The time spent by the animal exploring the familiar object and the new object will be represented by ‘a’ and ‘b’, respectively. The variable ‘e’ is the sum of the total exploration time of the new and familiar objects during the test session. The variable ‘d’ is an index of discrimination between the new and the familiar objects and was considered a relative measure to correct the exploratory activity of the animal. Thus, the formula was: $e = a + b$; and $d = (b – a)/e$ [39].

2.8. Spatial version of the Y-maze test

In this test the animal was placed in a Y-shaped maze with three identical arms at an angle of 120°. These arms measured 50 x 12 x 27 cm. The maze was placed in a controlled light room (20 lux). A camera was positioned above the maze for subsequent image analysis in order to avoid interference due to the presence of the handler in the test environment.

The test consisted of two steps with a one-hour interval between them: a training and a test session. In the training session the animals were randomly placed in one arm of the maze but a removable wood door previously positioned restricted the

access to one of the other arms. At this stage the animals could freely explore two arms of the maze for five minutes and after were placed back in the habitat box. The test session took place one hour after. The animals were placed in the same arm where they started the training session but the barrier that prevented the animal from accessing one arm was previously removed, allowing free exploration of all arms for three minutes. This session was recorded and the parameter evaluated was the time the animal stayed in the unknown arm. The maze was cleaned with 20% ethanol between sessions to avoid the influence of the smell of other animals on the test [17,41,43].

The time each animal stayed in the unknown arm was corrected by latency to leave the initial arm and time spent in the center of the maze. Animal entrance in one of the arms was considered when it had all paws inside the arm [17,41,43].

2.9. IBA1 immunohistochemistry

Brain sample processing and immunohistochemistry reactions were developed as described by Bassani et al. (2017). The animals were deeply anesthetized and then euthanized with an intracardiac perfusion of saline phosphate buffered solution (pH 7.4; 1,000 mL/kg; 4º C), followed by 4% paraformaldehyde in phosphate buffered solution (pH 7.4; 1,000 mL/kg, 4º C). Then the brains were removed and placed in 30% sucrose solution for four days for tissue cryoprotection. After that, the tissue was protected with plastic, quickly frozen in liquid nitrogen and stored in a freezer (-80º C) for subsequent sectioning (BASSANI et al., 2017). The frozen tissue was cut into 30 µm semi-serial coronal sections along the dorsal hippocampus at a temperature of -25° C using a cryostat (Leica Biosystems, Nusslock, Germany). The dorsal hippocampus was collected and the sections were placed

serially in ten well-plates using -2.56 to -4.52 mm stereotaxic coordinates in relation to the bregma [34], totaling six to eight 300-µm cuts per well-plate. Brain sections were stored at -20° C in cryoprotectant solution containing 30% ethylene glycol and 15% sucrose in 0.05 M phosphate buffered solution for subsequent processing [17,41].

The free-floating method was used for immunohistochemical reactions with the IBA1 marker. The brain sections were initially washed with buffer A (0.1 M PBS, pH 7.4 with 0.5% Triton X-100) and incubated in 0.1 M citrate buffer, pH 6.0 in a water bath at 50° C for 30 minutes. The steps described above characterize an antigen retrieval step for this marker. After that, the sections were cooled to room temperature, washed with buffer A, incubated with 0.5% H_2O_2 solution in 0.1 M PBS for 30 minutes at room temperature and protected from light, washed again with buffer A and incubated with 2% bovine serum albumin (BSA) in buffer A (blocking buffer) at room temperature for one hour. Subsequently, the plates containing the sections were incubated overnight at 4° C with the primary antibody diluted in anti-IBA1 goat polyclonal blocking buffer (1:500, Abcam, Cambridge, MA, USA) [17,41].

The following day the sections were washed in buffer A and incubated with biotinylated secondary antibody at 4° C for two hours, washed again in buffer A and incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS at room temperature for two hours. After being washed with 0.1 M PBS, the reaction was revealed by incubating the sections with 3,3'-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS at room temperature. The sections were washed in 0.1 M PBS, mounted on gelatinized slides and air dried [17,41].

2.10. Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). The data were tested for a Gaussian distribution using the Kolmogorov-Smirnov test. Group differences were analyzed using one-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* test for parametric data or Kruskal Wallis (IBA-1 analysis in the CA1 region of the Hippocampus) with Dunn's post-hoc test for nonparametric data. Values of $p < 0.05$ were considered statistically significant.

3.RESULTS

3.1. Open field test

The analysis of number of quadrants traveled by the animals (FIGURE 1A) at day 27 after surgery showed no significant motor activity differences between groups. Thus, there were also no changes regarding motor capacity between groups. In addition, the OFT allowed the analysis of rearing behavior (FIGURE 1B), when the animals stood on both hind legs. This analysis showed no significant exploratory capacity differences between groups, so all animals in this study had similar exploratory activities.

3.2. AZ1 formulation effects on animal cognition

Animal recognition memory was evaluated using the ORT (FIGURE 2A) at day 28. The execution protocol requires a one-hour interval between training and test sessions.

Animals in the STZ + saline group showed a statistically significant decrease in the discrimination index compared to the sham + saline group, showing that STZ-lesioned animals were unable to discriminate new and familiar objects. Animals in the STZ + AZ1 group showed a statistically significant increase in the discrimination index

compared to the STZ + saline group, showing that AZ1 formulation was able to revert the memory loss caused by STZ. In addition, short-term spatial memory evaluated by spatial version of the Y-maze test (FIGURE 2B) showed that the rats in the STZ + saline group spent a significantly decreased percentage of time exploring the new arm compared to the sham + saline group. The AZ1 formulation treatment significantly reversed cognitive impairment in STZ-lesioned rats in the STZ + AZ1 group.

3.3. AZ1 formulation effects on neuroinflammation

IBA1 immunoreactivity (FIGURE 3) was used as a parameter to analyze the influence of AZ1 formulation treatment on glial reactivity and neuroinflammation. Our data showed increased IBA1 immunoreactivity in the three analyzed hippocampal regions (CA1, CA3, and DG - dentate gyrus) in the STZ + saline group compared to the sham + saline group (FIGURE 4). IR to IBA1 reduction in CA1 and DG regions (FIGURES 3A-C) showed that AZ1 formulation treatment significantly reduced glial activation in STZ-lesioned animals; however it did not change the marking in the CA3 region (FIGURE 3B) compared to sham + saline animals. The groups sham + saline and sham + AZ1 formulation showed no significant differences in IR to IBA1 in any hippocampal region.

4. DISCUSSION

Prolonged AZ1 formulation treatment reversed cognitive impairment in both the ORT and the Y-maze test and reduced neuroinflammation in CA1 and DG hippocampal regions.

This neuroprotective effect of AZ1 formulation may be due to the combination of several lipids, such as caprylic and capric acid, which produce ketone bodies and

are a source of brain energy, and the combination of DHA and phosphatidylserine, which restructure neuronal membranes and have an anti-inflammatory action. In addition, the presence of choline, acting on plasma phospholipid synthesis and structuring of cell membranes, vitamins and minerals is extremely important for brain health.

An increased inflammatory process is associated with increased activation of microglia cells and astrocytes and progressive cognitive decline in humans [44,45]. Postmortem studies in humans showed CA1 and CA3 hippocampal regions presenting decreased neuronal density in AD patients [46]. Thus, the results of the present study corroborate the neuroinflammation reported in other studies [16,17,41].

Our results show that the animals treated with STZ and the animals receiving only saline solution presented the short-term object recognition and spatial memory deficits already mentioned in the literature, respectively by decreasing the discrimination index in the ORT [47,48] and the time spent on the new arm in the Y-maze test [48]. In addition, STZ-related decreased spatial memory was already described in the Morris water maze test [49]. On the other hand, STZ-lesioned animals treated with the enteral AZ1 formulation showed significant improvement in the evaluated parameters when compared to STZ + saline animals, indicating that AZ1 formulation components reversed spatial and object recognition memory impairment.

Spontaneous locomotion and exploratory behavior are important in all test groups, since changes in these parameters could decrease the animals' performance in subsequent cognitive tests [17]. Performance comparison among the four experimental groups showed no significant noncognitive behavior differences, demonstrating that all animals had similar characteristics regarding exploratory capacity and locomotion.

Some experimental studies reported that phosphatidylserine increased interneuronal communication through increased cell membrane fluidity and attenuated acetylcholine reduction, in addition to improving performance on tasks that test short-term learning and memory skills [21]. In humans, phosphatidylserine increased glucose use and memory in information processing and ability to perform daily activities [50–56]. The incorporation of phosphatidylserine into human membranes depends on phosphatidylserine and DHA availability [21,22].

The brain is vulnerable to lipid peroxidation, which decreases cell membrane fluidity and damages membrane proteins, contributing to membrane breakdown. Thus, in addition to the ideal combination with phosphatidylserine, DHA increases synapses and membrane fluidity, resulting in a more effective neurotransmitter function; besides inhibiting free radical production and reducing oxidative stress [25]. The use of omega-3 fatty acids has been reported in numerous AD animal models [57–60]. In the STZ-induced AD model, Pardeshi et al. [26] showed that DHA improved the efficacy of the pharmacological agent used in the study by increasing survival and neuronal memory due to decreased oxidative stress and inflammation.

As for caprylic and capric acid and the consequent formation of ketone bodies, a clinical study reported better cognitive performance evaluated by logical memory and coding tests in individuals diagnosed with probable mild to moderate AD after ingesting 20 g of caprylic and capric acid. In addition, cognitive test scores were correlated with plasma ketone body levels [61].

Some antioxidant nutrients present in the AZ1 formulation, such as zinc, selenium, magnesium, vitamins C and E, may be related to neuronal protection, since oxidative stress leads to DNA damage, which in turn leads to neuronal apoptosis and consequent neurodegeneration [62,63]. In addition, AD-related decreased antioxidant

levels can be observed in brain regions of elderly people [64]. Furthermore, vitamin D3 treatment improved spatial learning and functional memory in STZ-lesioned animals [65]. Vitamin D can prevent cognitive impairment and dementia through neuronal protection [66].

It should be highlighted that the ICV administration of STZ (1 mg/kg) was able to cause memory deficits that could be related to the earlier stages of AD. This combination of components used in the nutritional AZ1 formulation, which had never been tested together, was able to reverse STZ-induced memory impairment.

However, a possible limitation of this study is that we did not evaluate neither the Aβ amyloid protein nor the Tau expression. According to Salkovic-Petrisic et al. [67], increased Aβ deposition in the wall of meningeal capillaries and cortical blood vessels was found in icv-STZ rats. Results suggest that cerebral amyloid angiopathy observed 6 and 9 months after the STZ-icv injection seems to be a continuation and progression of the amyloid pathology observed already 3 months following the STZ-icv treatment in this non-transgenic sAD animal model. In this line, Ravelli et al. [68] showed that icv injections of STZ increase Aβ expression in the hippocampus STZ and increased phosphorylation of Tau in mice.

In addition, according to Chen and collaborators [69], the deposition of beta amyloid protein in this model begins to be observed after 3 months of the ICV injection of STZ. In the present study, the evaluation of the prolonged administration of the AZ formulation was done during 30 days. Maybe, in our schedule the deposition of Tau and beta amyloid proteins might not be sufficient to obtain conclusive results. Future studies, some of them now in progress could help to answer these questions.

The present study showed that prolonged supplementation with AZ1 formulation reversed STZ-related cognitive impairment, in addition to decreasing the progression of neuroinflammation in CA1 and DG of the hippocampus.

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CONFLICT OF INTEREST

MABF Vital; TB Bassani and LC Souza declare no conflict of interest. ELR Moura was a collaborator on a grant supported by Prodiet.; H Santos is Scientific Specialist on Prodiet. APM Celes is Technical Director and Co-founder of Prodiet

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TABLES

TABLE 1: Composition per 100 g of the nutritional formulation AZ1

AZ1 Composition	*100g*
Caprylic acid	36g
Capric acid	27g
DHA	327 mg
Phosphatidylserine	546 mg
Minerals	
Magnesium	233 mg
Zinc	5.6 mg
Selenium	102 mcg
Vitamins	
Vitamin D	75 mcg
Vitamin E	56 mg α TE
Vitamin C	436 mg
Vitamin B6	36 mg
Folic Acid	526 mcg
Vitamin B12	12 mcg
Niacin	31 mg NE
Choline	835 mg

FIGURES

FIGURE 1: EFFECTS OF AZ1 FORMULATION ON NONCOGNITIVE PERFORMANCE OF STZ-ICV-INJECTED ANIMALS (1 mg/kg). Noncognitive performance was evaluated using the open field test. The parameters analyzed were spontaneous locomotor activity (a) and exploratory behavior (b). The data are shown as mean ± SEM; n = 6 - 10 per group. (One-way ANOVA with Bonferroni's *post-hoc* test).

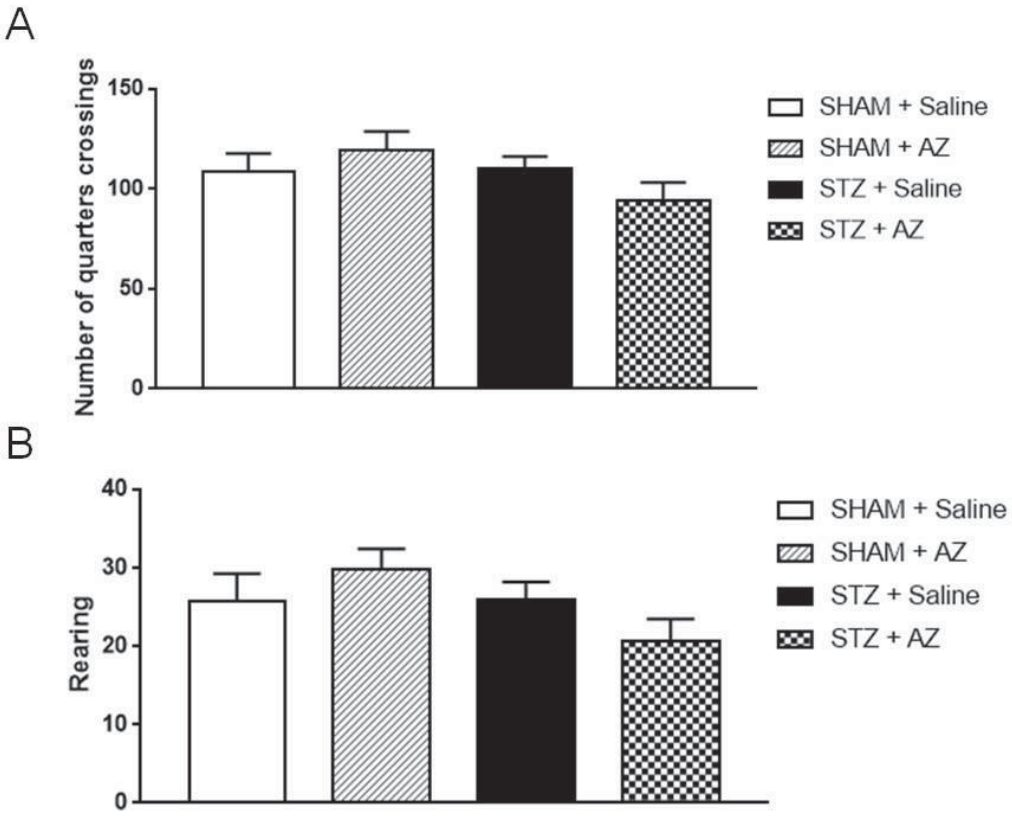


FIGURE 2: EFFECTS OF AZ1 FORMULATION ON COGNITIVE PERFORMANCE OF STZ-ICV-INJECTED ANIMALS. Cognitive performance was evaluated using the object recognition (a) and the spatial version of Y-maze (b) tests. The data are shown as mean ± SEM; n = 6 - 10 per group; *p < 0.05 vs. sham group; #p < 0.05; ## p < 0.01 vs. STZ group (one-way ANOVA with Bonferroni's *post-hoc* test).

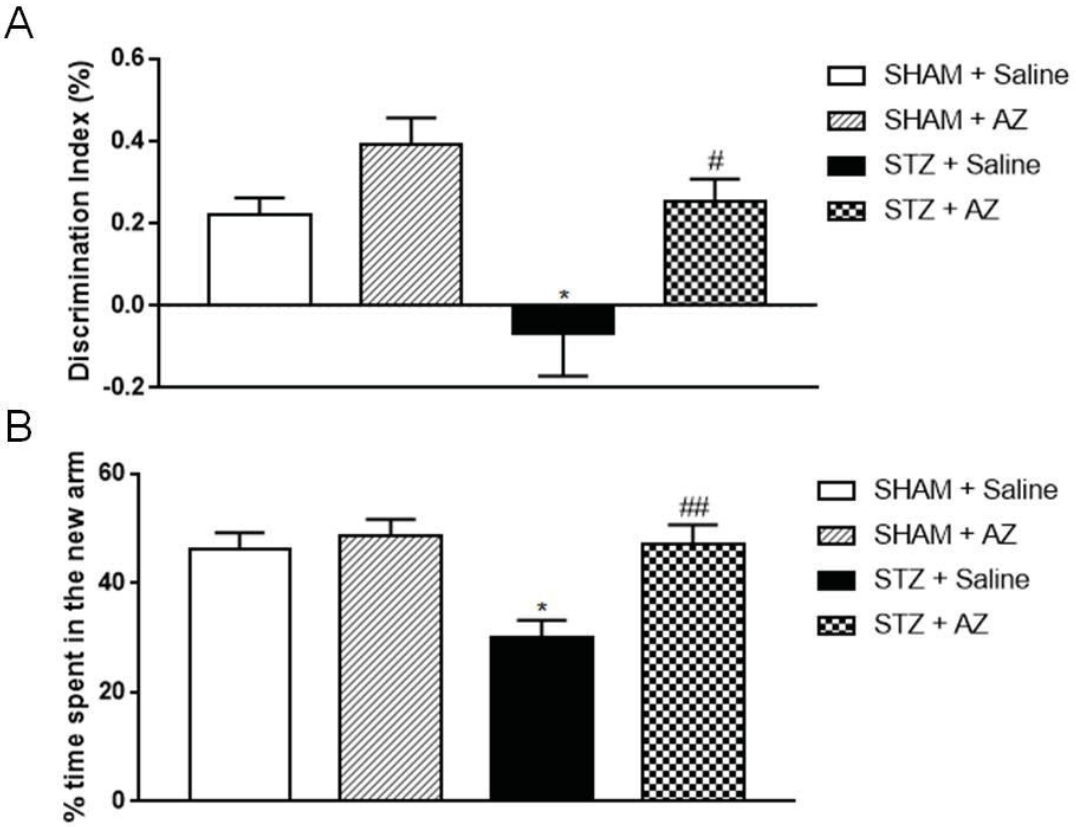


FIGURE 3: EFFECTS OF AZ1 FORMULATION ON MICROGLIAL CELLS (IBA1 IMMUNORREACTIVITY) ON STZ-ICV-INJECTED ANIMALS. IBA1-IR significantly increased in CA1 region 30 days after surgery in STZ-ICV-injected rats. The treatment with AZ1 formulation at a dose of 1 g/kg reduced IBA-IR in hippocampal regions CA1 (a) and DG (c) but showed no significant results in CA3 region (b). The data are shown as mean ± SEM; n = 4 - 6 per group; *p < 0.05; **p < 0.01 vs. sham + saline group; #p < 0.05 vs. STZ + saline group (one-way ANOVA with Bonferroni's *post-hoc* test on IBA1 immunoreactivity in the CA3 and GD regions, and Kruskal Wallis with Dunn's post-hoc test for the hippocampus CA1 region).

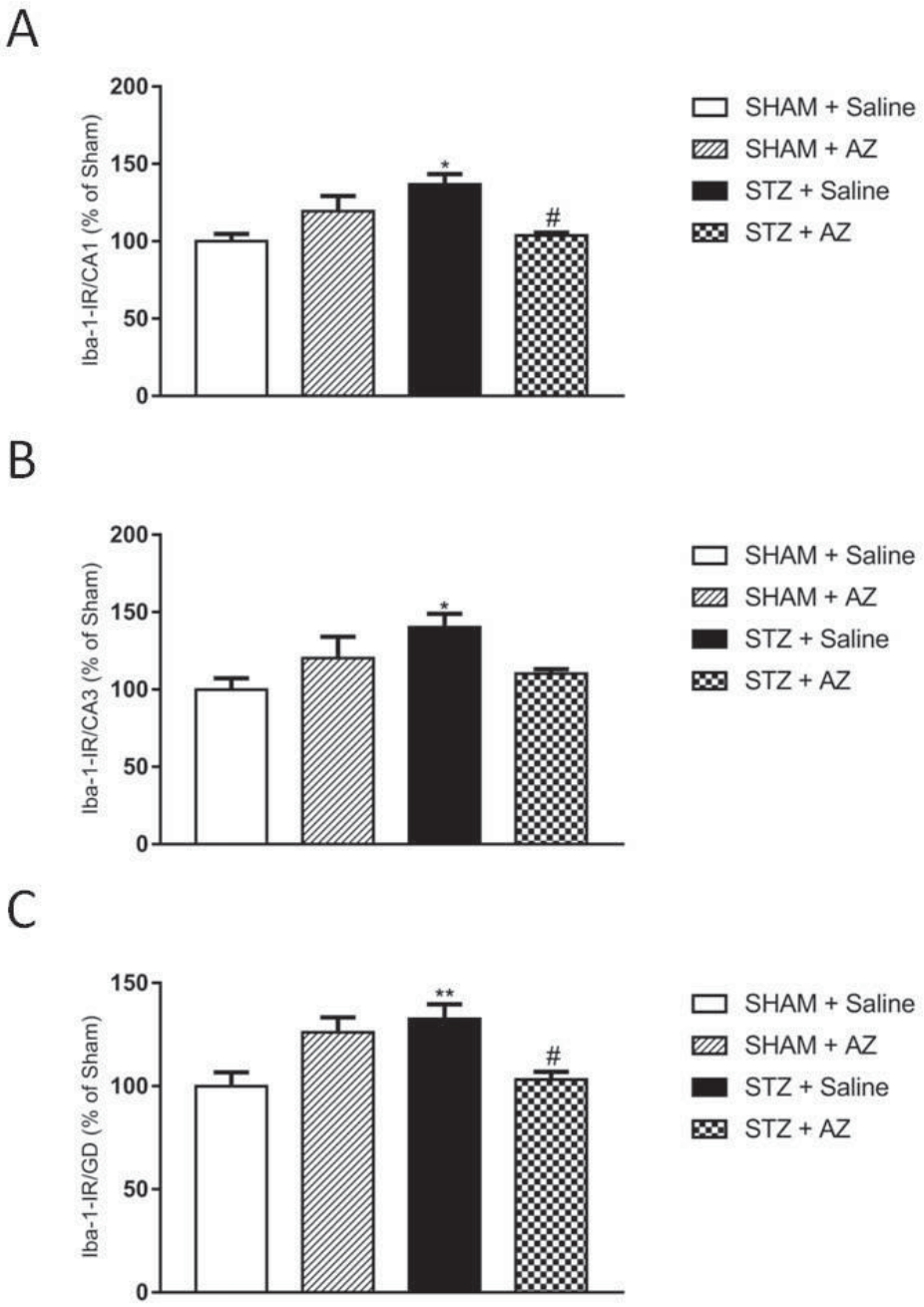
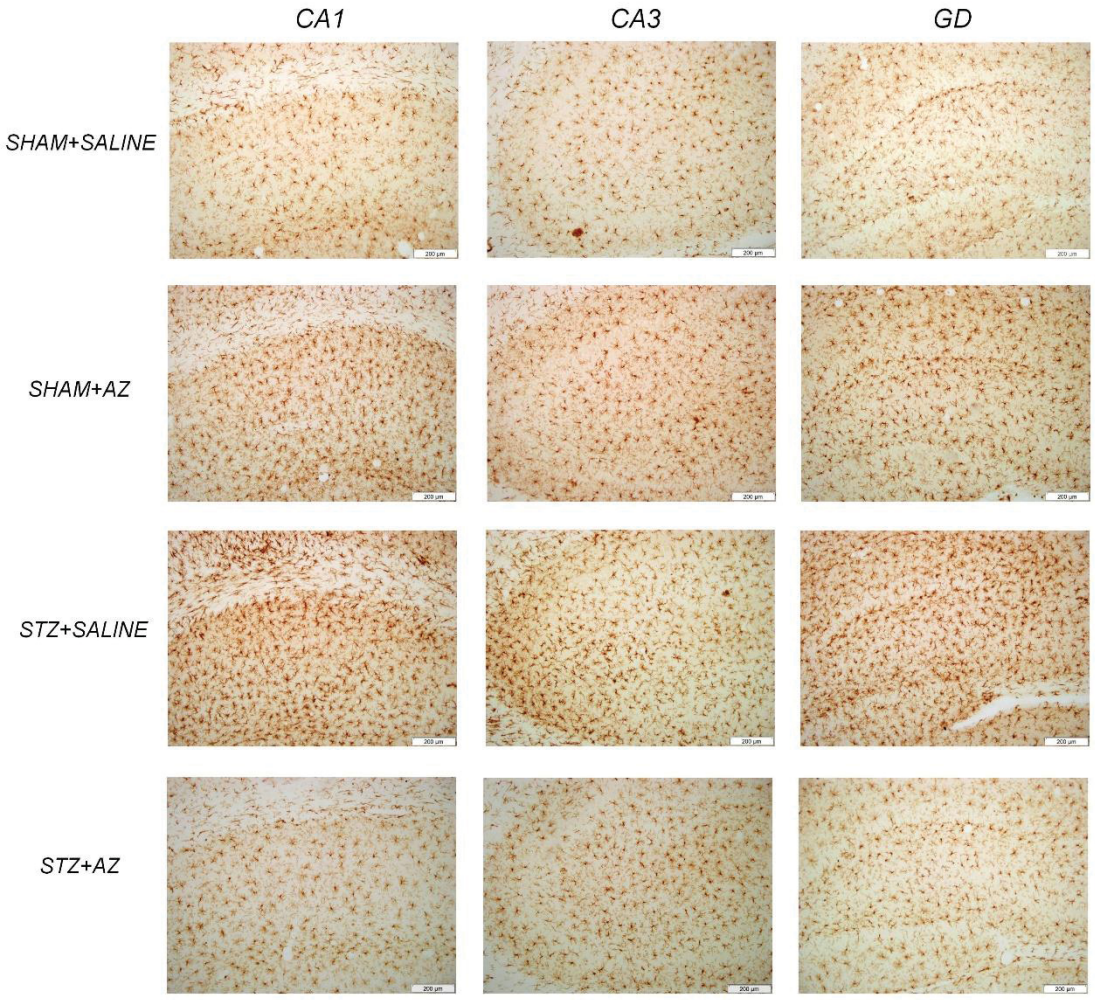


FIGURE 4: REPRESENTATIVE PHOTOMICROGRAPHS OF IBA1-POSITIVE CELLS IN CA1, CA3, AND DG HIPPOCAMPAL REGIONS. IBA1-positive cells in the hippocampal dentate gyrus (DG), CA1, and CA3 regions. Scale = 200 µm.



5 CONCLUSÃO

As estratégias neuroprotetoras empregadas no presente trabalho mostraram efeitos benéficos no modelo de DA esporádica induzido por injeção ICV de STZ em ratos Wistar. As propriedades anti-inflamatórias do AZ1 e do ANDRO pareceram ter um papel crítico para uma diminuição do prejuízo induzido pela STZ na memória espacial de curto prazo e na memória de reconhecimento de curto prazo, observamos uma diminuição da neuroinflamação em regiões relacionadas a essas funções, confirmando nossa hipótese inicial. O que reforça a contribuição da glia para a neurodegeneração e o prejuízo cognitivo na DA, como também mostram as correlações encontradas. Ainda que com limitações, os trabalhos indicam que compostos anti-inflamatórios e antioxidantes são importantes estratégias neuroprotetoras e terapêuticas na DA.

Entretanto, o ANDRO mostrou causar apenas uma melhora leve tanto na cognição (ainda que os resultados do OLT contradigam essa melhora) quanto na ativação da micróglia e dos astrócitos. Apesar da dose de 3 mg/kg de STZ ser considerada alta por alguns autores, a literatura mostra que o ANDRO é capaz de diminuir os danos causados por vários modelos animais de doenças. O esperado por nossa hipótese inicial eram efeitos mais pronunciados de neuroproteção e de proteção da cognição. A dose de 2 mg/kg de ANDRO pode ter sido um fator decisivo para os efeitos não serem mais significativos, estudos com outros modelos de DA usam essa mesma dose e mostram melhoras importantes, mas o único trabalho que encontramos que utilizou o ANDRO no modelo STZ-ICV administrou doses substancialmente maiores e observou efeitos mais significativos. Portanto, novos estudos com o ANDRO nesse modelo usando doses maiores podem ser importantes. Precisa-se também pontuar que apenas alguns dos mecanismos pelos quais a STZ induz danos ao cérebro foram explorados. Nossos dados indicam potenciais efeitos benéficos.

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