UNIVERSIDADE FEDERAL DO PAMPA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

EFEITO PROTETOR DE NANOCÁPSULAS POLIMÉRICAS CONTENDO CRISINA EM MODELO DE DOENÇA DE ALZHEIMER INDUZIDA POR INJEÇÃO INTRACEREBROVENTRICULAR DO PEPTÍDEO β-AMILÓIDE 1-42

DISSERTAÇÃO DE MESTRADO

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Uruguaiana, RS, Brasil.

2015

EFEITO PROTETOR DE NANOCÁPSULAS POLIMÉRICAS CONTENDO CRISINA EM MODELO DE DOENÇA DE ALZHEIMER INDUZIDA POR INJEÇÃO INTRACEREBROVENTRICULAR DO PEPTÍDEO β-AMILÓIDE 1-42

por

Renata Giacomeli

Dissertação apresentada ao Programa de Pós-Graduação Bioquímica, da Universidade Federal do Pampa (UNIPAMPA, RS),

como requisito parcial para a obtenção do grau de

Mestre em Bioquímica.

Orientador: Prof. Dr. Cristiano Ricardo Jesse

Coorientadora: Profª Drª Letícia Marque Colomé

Uruguaiana, RS, Brasil.

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Universidade Federal do Pampa

Programa de Pós-Graduação em Bioquímica

A Comissão Examinadora, abaixo assinada, aprova a

Dissertação de Mestrado

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AGRADECIMENTOS

A Deus, por tudo.

Aos meus queridos pais Paulo e Loreci, por todo apoio nesta caminhada, vocês são a base de tudo, amo infinitamente vocês!

Ao meu irmão, fonte de inspiração, obrigada pelo "empurrãozinho", quando eu crescer quero ser que nem tu! Te amo.

Ao meu orientador Prof. Dr. Cristiano Ricardo Jesse pela oportunidade, por toda dedicação, paciência e exemplo de competência, muito obrigada.

A minha coorientadora Letícia Marques Colomé, minha eterna "prof", que me acompanha desde a graduação, obrigada!

Aos colegas do laboratório Laftambio Pampa pela colaboração e auxilio no desenvolvimento deste trabalho, principalmente à Fran, obrigada!

Aos colegas do laboratório NanoFar, em especial à Jéssica, pela ajuda e incentivo.

Aos meus amigos, parceiros da vida, agradeço por todos os momentos, pela torcida e principalmente pelo apoio nesta caminhada, em especial à Fiama (minha irmã do coração, sempre torcendo por mim), ao Marcus Vinicius (por todas as palavras), ao Willian e à Jéssica (que acompanharam tão de perto e muito me ajudaram e escutaram), à Tici e Mari Freitax (por toda a parceria nesses últimos tempos, foram muito importantes nessa caminhada!) eu amo todos vocês!

À Universidade Federal do Pampa pela oportunidade oferecida de realizar o mestrado.

PARTE I

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

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EFEITO PROTETOR DE NANOCÁPSULAS POLIMÉRICAS CONTENDO CRISINA EM MODELO DE DOENÇA DE ALZHEIMER INDUZIDA POR INJEÇÃO INTRACEREBROVENTRICULAR DO PEPTÍDEO β-AMILÓIDE 1-42

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Data e Local da Defesa: Uruguaiana, 23 de Julho de 2015.

A doença de Alzheimer (DA) é uma desordem neurodegenerativa crônica caracterizada clinicamente pela perda progressiva de função cognitiva, distúrbios neuropsiquiátricos e comportamentais. Patologicamente esta doença caracteriza-se pelo acúmulo anormal do peptídeo β-amilóide (Aβ) no córtex e no hipocampo, emaranhados neurofibrilares intracelulares formados por Tau hiperfosforilada, disfunção progressiva sináptica e, posteriormente perda neuronal. As opções terapêuticas disponíveis melhoram os sintomas, mas não impedem a progressão da doença, portanto, ainda está faltando uma estratégia terapêutica efetiva para DA. A Crisina (5, 7-dihidroxiflavona) é um flavonoide natural encontrada em extratos de plantas (tais como *Passiflora caerulea* e *Populus tremula*), própolis e mel que apresenta propriedades farmacológicas relevantes, incluindo efeito antioxidante, antiinflamatório, hipolipidêmico, anti-aterogênico, anticâncer e, de forma mais significativa, efeito neuroprotetor. Porém, existem algumas desvantagens que podem limitar a sua potencial aplicação na terapêutica tais como baixa solubilidade e má absorção intestinal. Somando-se a isso, um fato determinante na neuroterapia é o efeito restritivo da barreira hematoencefálica (BHE), a qual limita a eficácia de tratamentos. Muitos estudos têm-se centrado sobre este problema fundamental através da concepção de estratégias diferentes para

facilitar a passagem de ativos em todo a BHE. Entre estes, as abordagens baseadas em nanotecnologia ganharam impulso significativo, já que podem efetivamente transportar substâncias ativas através da BHE. Assim, o objetivo deste trabalho foi preparar um sistema baseado em nanopartículas, capaz de veicular a crisina, bem como investigar os efeitos biológicos em um modelo de DA induzida por injeção intracerebroventricular (icv) do peptídeo beta amiloide$_{1-42}$ (Aβ_{1-42}) em camundongos Swiss fêmeas com idade entre 18 e 22 meses. Para tanto, determinou-se parâmetros de estresse oxidativo, neuroinflamação e níveis do fator neurotrófico derivado do cérebro (BDNF) no córtex pré-frontal e hipocampo, também verificou-se os efeitos comportamentais cognitivos dos camundongos. Os animais foram divididos em 10 grupos: (1) veículo/tampão phosphate-buffered saline (PBS); (2) veículo/nanocápsula (NC)-branca; (3) veículo/crisina livre (5 mg/kg); (4) veículo/NC-crisina (1 mg/kg); (5) veículo/NC-crisina (5 mg/kg); (6) Aβ_{1-42}/PBS; (7) Aβ_{1-42}/NC-branca; (8) Aβ_{1-42}/crisina livre (5 mg/kg); (9) Aβ_{1-42}/NC-crisina (1 mg/kg) e; (10) Aβ_{1-42}/NC-crisina (5 mg/kg). O peptídeo Aβ_{1-42} ou o veículo foram infundidos por injeção icv e, um dia depois, iniciou-se o tratamento, por via oral, durante 14 dias. Após o fim do tratamento, os animais foram submetidos aos testes comportamentais. Os resultados demonstraram que os efeitos neuroprotetores do crisina foram mais elevados quando administrada em nanopartículas. O nanossistema melhorou as concentrações de crisina nos tecidos cerebrais, bem como a eficácia farmacológica. O presente estudo demonstrou que o tratamento com crisina, principalmente na formulação de nanopartículas, foi eficaz em atenuar as seguintes alterações resultantes da exposição de camundongos à Aβ_{1-42}: o comprometimento da memória em testes de comportamento, o aumento dos níveis de espécies reativas (RS), fator de necrose tumoral α (TNF-α) e interleucina-1β (IL-1β), a redução dos níveis de tióis não-proteicos (NPSH), BDNF e IL-10; o aumento da atividade de glutationa peroxidase (GPx) e glutationa redutase (GR) em córtex pré-frontal e hipocampo. Em conclusão, esses resultados demonstram que a atenuação da neuroinflamação e do estresse oxidativo está envolvido no efeito neuroprotetor da crisina neste modelo de DA, além disso, sugerem que a formulação de nanopartículas potencializa seus efeitos, o que pode fornecer uma nova abordagem terapêutica para o tratamento e prevenção de DA.

Palavras-chave: doença de Alzheimer; estresse oxidativo; neuroinflamação; crisina; nanopartículas.

ABSTRACT

Dissertation of Master

Program of Post-Graduation in Biochemistry

Federal University of Pampa

PROTECTIVE EFFECT OF CHRYSIN LOADED POLYMERIC NANOCAPSULES IN ALZHEIMER'S DISEASE MODEL INDUCED BY INTRACEREBROVENTRICULAR INJECTION OF β-AMYLOID PEPTIDE 1-42

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Site and Date of Defence: Uruguaiana, July 23, 2015.

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized clinically by progressive loss of cognitive function, neuropsychiatric and behavioral disorders. Pathologically the disease is characterized by abnormal accumulation of β-amyloid peptide (Aß) in cortex and hippocampus, intracellular neurofibrillary tangles consisting of hyperphosphorylated *Tau*, and synaptic dysfunction progressively later neuronal loss. The therapeutic options available improve symptoms but did not prevent disease progression, therefore, is still missing an effective therapeutic strategy for AD. Chrysin (5, 7-dihidroxiflavone) is a flavonoid found in natural plant extracts (such as *Passiflora caerulea* and *Populus tremula*), honey and propolis which has significant pharmacological properties including antioxidant, anti-inflammatory, hypolipidemic, anti-atherogenic, anti-cancer effects and, more significantly, neuroprotection. However, there are some disadvantages that may limit their potential application in therapeutics such as low solubility and intestinal malabsorption. Adding to this, a key fact in Neurotherapy is the restrictive effect of the blood-brain barrier (BBB), which limits the effectiveness of treatments. Many studies have focused on this fundamental problem by designing different strategies to facilitate the transition of assets across the BBB. Among these, nanotechnology-based approaches have gained significant momentum as they can effectively carry active substances through the BBB. The

objective of this work was to prepare a system based on nanoparticles, capable of relaying chrysin, as well as investigating the biological effects in a model of AD induced by intracerebroventricular injection (icv) of Beta amyloid$_{1-42}$ peptide (Aβ_{1-42}) in swiss mice females aged between 18 and 22 months. Therefore, it was determined parameters of oxidative stress, neuroinflammation and levels of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex and hippocampus, it was also observed cognitive behavioral effects in mice. The animals were divided into 10 groups: (1) vehicle/phosphate-buffered saline (PBS); (2) Vehicle/blank-nanocapsule (NC); (3) vehicle/free chrysin (5 mg / kg); (4) Vehicle/NC-chrysin (1 mg / kg); (5) vehicle/NC-chrysin (5 mg / kg); (6) Aβ_{1-42}/PBS; (7) Aβ_{1-42}/blank-NC; (8) Aβ_{1-42}/free chrysin (5 mg / kg); (9) Aβ_{1-42}/NC-chrysin (1mg / kg) and; (10) Aβ_{1-42}/NC-chrysin (5 mg / kg). The Aβ_{1-42} peptide or vehicle were infused icv injection and, one day later, treatment began, orally, for 14 days. After the end of treatment, animals were subjected to behavioral testing. The results showed that the neuroprotective effects of chrysin were higher when administered in nanoparticles. The nano system improved chrysin concentrations in the brain tissue as well as the pharmacological effectiveness. The present study demonstrated that treatment with chrysin, especially in nanoparticle formulation was effective in attenuating the following shortcomings arising from exposure of mice to Aβ_{1-42}: the memory impairment in behavioral tests; the increased reactive oxygen species (RS), tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) levels, reduction non-thiol protein (NPSH), BDNF, and IL-10 levels; increasing the glutathione peroxidase (GPx) and glutathione reductase (GR) activity in the prefrontal cortex and hippocampus. In conclusion, these results demonstrate that blocking neuroinflammation and oxidative stress is involved in the neuroprotective effect of chrysin in this model, moreover, suggest that the nanoparticle formulation potentiates their effects, which may provide a new therapeutic approach for the treatment and prevention of AD.

Key words: Alzheimer's disease; oxidative stress; neuroinflammation; chrysin; nanoparticles.

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PARTE II

LISTA DE ABREVIATURAS E SIGLAS

DA = Doença de Alzheimer

APP = *Amyloid precursor protein* = Proteína precursora de amilóide

Aβ = *Amyloid-beta* = Beta-amilóide

NFT = *Neurofibrilary tangles* = Emaranhados neurofibrilares

BBB = blood-brain barrier (barreira hematoencefálica)

Aβ1-40 = isoforma do peptídeo beta-amilóide com 40 aminoácidos

Aβ1-42 = isoforma do peptídeo beta-amilóide com 42 aminoácidos

GSK3β = *Glycogen synthase kinase 3-beta* = glicogênio sintase quinase 3-beta

CDK5 = *Cyclin-dependente kinase-5* = quinase dependente de ciclina-5

ERK2 = *Extracellul*ar *signal regulated kinase-2* = quinase regulada por sinal extracelular tipo 2

p-ERK = forma fosforilada da proteína quinase regulada por sinal extracelular

Icv = intracerebroventricular

TNF-α = *tumor necrosis factor-alpha* = fator de necrose tumoral-alfa

IL-1β = interleucina 1-beta

IL-6 = interleucina 6

IL-10 = interleucina 10

SNC = Sistema nervoso central

BDNF = *Brain-derived neurotrophic factor* = fator neurotrófico derivado do cérebro

RS = *reactive species* = espécies reativas

NPSH = *non-protein thiols* (tióis não-protéicos)

CAT = catalase

GPx = glutationa peroxidase

GSSG = glutationa oxidada

GSH = glutationa reduzida

GR = glutationa redutase

GST = glutationa s-transferase

ORT = *object recognition test* = teste de reconhecimento de objetos

MWM = *Morris Water Maze* = Labirinto aquático de Morris

LTM = *long-term memory* = memória de longo prazo

STM = *short-term memory* = memória de curto prazo

HPLC = High Performance Liquide Chromatography = cromatografia líquida de alta eficiência

BN = nanocapsule blank

CLN = chrysin loaded nanocapsules = nanocápsulas contendo crisina

NC = nanocápsulas

SUMÁRIO

PARTE I

APRESENTAÇÃO

Os resultados que compõem essa dissertação apresentam-se divididos em 3 partes. Na **parte I** encontram-se a **Introdução** e os **Objetivos**. A **parte II** refere-se aos resultados deste trabalho sob a forma de **Manuscrito** que abrange as seções introdução, materiais e métodos, resultados, discussão dos resultados e referências bibliográficas. O item **Conclusão** encontra-se na **parte III** desta dissertação e apresenta conclusões gerais do manuscrito contido nesse trabalho. O item **Referências** inclui somente as citações que aparecem no item introdução desta dissertação.

1. INTRODUÇÃO

1.1. Doença de Alzheimer

A doença de Alzheimer (DA) é uma desordem neurodegenerativa crônica, sendo a forma mais comum de demência em idosos, o que representa 50 a 75% de todos os casos (Choi et al., 2014). Atualmente, a DA afeta cerca de 36 milhões de pessoas no mundo, prevendo-se que esse número chegue a 100 milhões até 2050 (Zhang et al., 2014; Gregori et al., 2015).

A DA foi primeiramente descrita em 1906 pelo médico alemão Alois Alzheimer, durante o 37° Congresso do Sudoeste da Alemanha de Psiquiatria, na cidade de Tübingen. Durante sua conferência intitulada "Eine eigenartige Erkrankung der Hirnrinde" (Uma Doença Peculiar dos Neurônios do Córtex Cerebral), Alzheimer definiu seu achado como uma patologia neurológica, não reconhecida, que cursa com demência, dando destaque aos sintomas de déficit de memória, alterações comportamentais e incapacidade para as atividades rotineiras. Posteriormente, Alzheimer ainda viria a descrever os aspectos anatomopatológicos da doença, cujas principais características eram o acúmulo de placas senis e emaranhados neurofibrilares e a perda neuronal (Medeiros, 2010).

A apresentação clínica típica da DA é a perda progressiva da memória e das funções cognitivas (Chen et al., 2015), levando a uma diminuição considerável da independência funcional e, consequentemente, proporcionando sofrimento ao paciente, familiares e cuidadores. DA pode levar à morte dentro de 3 a 9 anos após o seu diagnóstico, sendo a idade o principal fator de risco, com sua incidência dobrando a cada 5 anos após os 65 anos de idade (Souza, 2012). Aspectos genéticos, doenças cardiometabólicas, traumatismo cerebral e fatores ambientais como o consumo de cigarro e exposição ao alumínio são outros fatores considerados de risco (Ballard et al., 2011). Embora uma pequena porcentagem de casos seja de fundo genético, conhecida como a doença de Alzheimer familiar (FAD), podendo ser de início precoce ou tardio, a maioria dos casos é causada por mutações na proteína precursora de amilóide (APP) (Ordóñez-Gutiérrez et al., 2015).

Apesar da etiologia da DA ainda não ter sido bem elucidada, há um substancial corpo de evidências demonstrando o papel central do peptídeo beta-amilóide (Aβ) na patogênese da doença (Laferla et al., 2007; Prediger et al., 2007). Nesse sentido, a clivagem descontrolada de APP, por fatores desconhecidos, provoca o acúmulo intra e extracelular do peptídeo Aβ no cérebro, o que gera a formação de placas amilóides extracelulares e emaranhados neurofibrilares intracelulares (NFT) (Lakey-Beitia et al., 2015). A APP, uma proteína

transmembrana consistindo de 695-770 aminoácidos, é degradada por várias proteases, incluindo α-, β-, e γ-secretases, sendo que um desequilíbrio entre os mecanismos de produção e degradação leva ao acúmulo de Aβ (Kung, 2012). Aβ contém principalmente 40 ou 42 resíduos de aminoácidos ($A\beta_{1-40}$ e $A\beta_{1-42}$, respectivamente) e pode formar placas fibrilares, monômeros e oligômeros, sendo estes os mais tóxicos (Ordóñez-Gutiérrez et al., 2015). Apesar de mais abundante (cerca de dez vezes), a isoforma $A\beta_{1-40}$ é mais solúvel e menos propensa a formar placas amiloides (Ballard et al., 2011). O fragmento $A\beta_{1-42}$ é dito ser mais patogênico com uma maior tendência para formar depósitos fibrilares em parênquima neural (Castellani et al., 2008).

Outro evento importante a destacar é a relação entre o peptídeo Aβ e a proteína Tau, uma proteína relativamente abundante nos neurônios, presente em todas as células nucleadas, sendo responsável pela estabilização dos microtúbulos do citoesqueleto (Castellani et al, 2008). Em situações normais, há um equilíbrio entre as formas fosforilada e desfosforilada da proteína tau. No entanto, o aumento dos níveis de peptídeos Aβ pode induzir a hiperfosforilação desta proteína através da ativação de fosfoquinases específicas, nomeadamente glicogênio sintase quinase 3β (GSK3β), quinase dependente de ciclina (CDK5) e quinase dependente de sinal extracelular (ERK2). Uma vez fosforiladas, as proteínas tau perdem sua afinidade pelos microtúbulos, agregando-se no corpo dos neurônios na forma de pares de filamentos helicoidais, que são os NFT, desestruturando o citoesqueleto neural e, consequentemente, prejudicando a transmissão neural (Souza, 2012).

Além disso, baixos níveis de acetilcolina (ACh) também são implicados na etiologia da DA, recebendo a denominação de "hipótese colinérgica". A acetilcolinesterase (AchE) atua principalmente como uma enzima reguladora de sinapses colinérgicas, enquanto que a butirilcolinesterase (BChE), uma enzima intimamente relacionado com AChE, serve como um co-regulador da neurotransmissão colinérgica por hidrólise de ACh. A inibição de AChE e BChE foram documentados como alvos críticos para a gestão eficaz da DA por um aumento da disponibilidade da ACh em regiões do cérebro (Liu et al, 2015).

Como mais um fator importante a considerar, tem-se o estresse oxidativo, o qual tem sido implicado numa variedade de doenças humanas, incluindo desordens neurodegenerativas. O cérebro é particularmente suscetível ao estresse oxidativo devido à sua alta taxa de consumo de oxigênio, além de ser rico em lipídios insaturados e ter uma relativa abundância de íons metálicos de transição com capacidade redox e uma relativa baixa disponibilidade de enzimas antioxidantes em comparação a outros órgãos (Zhu et al., 2008). Há evidências que sugerem que o estresse oxidativo está envolvido nos mecanismos de neurotoxicidade induzida

por Aβ na patogênese de DA (Prediger et al., 2007; Piermatini et al., 2010; Souza et al.; 2013).

Além do estresse oxidativo, a neuroinflamação é atualmente reconhecida como uma característica proeminente do tecido cerebral de DA, sendo que respostas inflamatórias desempenham um papel importante na modulação da progressão da doença (Bernardi et al., 2012), tanto para contribuir na neurodegeneração quanto para produção e acúmulo da proteína Aβ (Begum et al., 2008). Evidências crescentes mostram que a neuroinflamação é, também, um fator importante na patogênese da DA (Nazem et al., 2015; Tai et al., 2015), que inclui a ativação de células da glia (por exemplo, a microglia e astrócitos) e expressão de mediadores inflamatórios, tais como fator de necrose tumoral-α (TNF-α), interleucina 1β (IL-1β), interleucina-6 (IL-6) e interleucina-10 (IL-10) (Bernardi et al., 2012, Bungart et al., 2014; Guo et al., 2015).

A relação entre o peptídeo Aβ e os eventos de estresse oxidativo e neuroinflamação é inconclusiva, porém, o que está claro é que respostas oxidativas e inflamatórias estão presentes tanto em estágios iniciais quanto finais da doença (antes e depois do acúmulo amilóide) e, portanto, são importantes potenciais alvos terapêuticos para a DA. Embora um século tenha se passado após a sua descoberta, o tratamento para DA continua representando um desafio para a indústria farmacêutica (Liao et al., 2015). As opções terapêuticas atuais disponíveis para o tratamento da DA incluem os inibidores da colinesterase (tais como donepezil, rivastigmina, e galantamina) e antagonistas do receptor N-metil-D-aspartato (NMDA) (memantina, por exemplo), os quais proporcionam uma melhora nos sintomas clínicos da doença, porém não impedem a sua evolução (Liu et al., 2015). Nenhuma droga ou tratamento realmente efetivo para DA foi descoberto até o momento e muitas fármacos falharam em testes clínicos. Nesse contexto, a investigação tem começado a centrar-se sobre os produtos naturais como alternativas para o tratamento da DA (Lakey-Beitia et al., 2015).

1.2. Crisina

Atualmente, há um crescente interesse no estudo de antioxidantes naturais, dentre estes, incluem-se os flavonoides, os quais compreendem o grupo mais comum de compostos polifenólicos presentes na dieta humana. As principais fontes alimentares de flavonoides incluem frutas, legumes, cereais, chá, vinho e sucos de fruta. Historicamente, as ações biológicas de flavonoides, incluindo aquelas sobre o cérebro, têm sido atribuídas à sua capacidade de exercer ações antioxidantes, através da sua capacidade de sequestrar espécies reativas, ou através de uma possível influência sobre o estado redox intracelular (Spencer et

al., 2009). Nesse contexto, flavonoides isolados de plantas, tais como luteolina, hespiridina, apigenina, crisina, rutina e quercetina têm demonstrado efeitos protetores em diversas patologias, incluindo doenças cardiovasculares, renais, hepáticas, cerebrais e neoplásicas (Pietta, 2000; Sequeto et al., 2012).

A Crisina (5,7-dihidroxiflavona, Fig. 1) pertence à classe flavona de flavonoides. É encontrada naturalmente em mel, própolis e várias espécies de plantas, incluindo espécies do gênero Pelargonium, Passiflora e da família Pinaceae (Pichichero et al., 2010; Medic-Saric, 2011). A principal fonte natural de crisina é a planta *Passiflora coerulea* (regionalmente conhecida como maracujá do mato), da qual foi isolada em 1990 por Medina e colaboradores e apresentada como um composto com propriedades anticonvulsivantes (Medina et al., 1990).

Da mesma forma como outros flavonoides, a crisina demonstrou efeitos biológicos relevantes, incluindo efeito antioxidante e antiinflamatório (Kahn et al., 2012; Darwish et al., 2014; Yao et al., 2014; Shen et al., 2015), anti-hiperlipidêmico (Zarzecki et al., 2014), anti-aterogênico (Anandhi et al., 2014), anticâncer (Lirdprapamongkol et al., 2013) e, de forma mais significativa, efeito neuroprotetor (Arribas et al., 2010; Jiang et al., 2014; Kandhare et al., 2014). Também há relatos que sugerem que a crisina aumenta os níveis de testosterona pela inibição da enzima aromatase (Kao et al., 1998), que converte a testosterona em estradiol, e em decorrência disso, a crisina já está disponível no mercado como um suplemento dietético (500 mg por cápsula) (iHerbInc., Monrovia, Califórnia; VitaDigest, Walnut, Califórnia).

No entanto existem algumas desvantagens que limitam significativamente a sua potencial aplicação na terapêutica, sendo as principais a sua baixa solubilidade, relativa má absorção intestinal e rápido metabolismo de glicosilação. Estudos de biodisponibilidade mostraram que a crisina absorvida foi detectada no sangue e sistema vascular na forma de ácido glicurônico e sulfato de ácidos conjugados devido ao grupo hidroxila não protegido nas posições 5- e 7- da estrutura química de crisina (Liu et al., 2014).

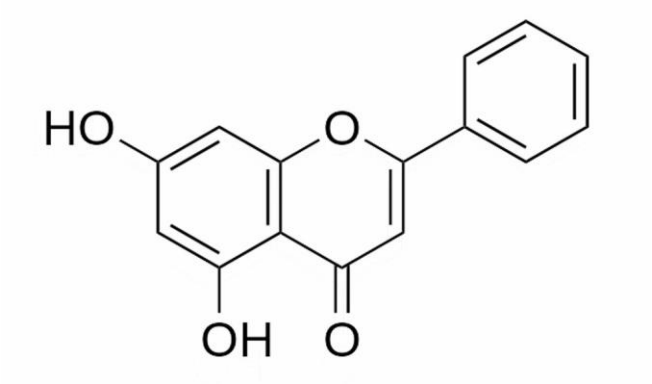

Figura 1. Estrutura química do flavonoide crisina.

1.3. Barreira hematoencefálica

Originalmente descrita por Paul Ehrlich em 1885, a barreira hematoencefálica (BHE) é primariamente composta por células endoteliais não fenestradas, que consiste de uma rede de microvasos bem diferenciados caracterizados pela presença de junções apertadas, que formam uma contínua barreira celular quase impermeável, a qual regula o tráfego de células imunitárias (macrófagos de vigilância), xenobióticos e compostos endógenos (Argaw et al., 2006; Wong et al., 2012). A BHE é composta por diferentes tipos de células, tais como células endoteliais, astrócitos e células microgliais, consistindo de uma estrutura essencial para a homeostase do sistema nervoso central (SNC). Entretanto, esse efeito restritivo acaba limitando a terapia de diversas patologias do SNC, incluindo a DA. As junções apertadas altamente restritivas entre as células endoteliais capilares do cérebro são as principais estruturas responsáveis pelas propriedades de barreira para limitar a transferência de quase todos os fármacos.

Além de compostos essenciais para a homeostase cerebral, tais como aminoácidos, hexoses, neuropeptídios e proteínas que são transportadas para o cérebro através de transportadores específicos, somente pequenas moléculas lipofílicas de menos de 500 Da têm capacidade de atravessar esta barreira complexa. Além disso, mesmo após a absorção bem sucedida de células endoteliais, os mecanismos de efluxo ativo podem bombear estas moléculas de volta para a corrente sanguínea (Wohlfart et al., 2012). Com isso, a presença da BHE torna difícil a efetividade de tratamentos novos ou já estabelecidos para doenças neurodegenerativas.

1.4. Nanopartículas

Em uma tentativa de superar essas limitações, um caminho promissor para entrega de fármacos ao SNC é o emprego de sistemas nanoparticulados, os quais apresentam vantagens

únicas em relação à administração do fármaco livre, tais como: o aumento da concentração do fármaco no tecido alvo por meio de direcionamento ativo, reduzindo os efeitos colaterais tóxicos em tecidos normais; a melhoria da solubilidade, farmacocinética e farmacodinâmica dos medicamentos e; melhoria da estabilidade do ativo, reduzindo sua degradação na circulação sistêmica (Modi et al., 2010; Gregori et al., 2015).

As nanopartículas variam em tamanho de 10 a 1000 nm (Soppimath et al., 2001), sendo que o mais comumente obtido está na faixa de 100-500 nm (Mora-Huertas et al., 2010). Dependendo do método utilizado, podem ser obtidas nanocápsulas ou nanoesferas. As nanoesferas são constituídas por um tipo de matriz onde o fármaco pode estar localizado na superfície da esfera ou internalizado na partícula. Já as nanocápsulas são sistemas vesiculares onde o fármaco está dissolvido em um núcleo líquido cercado por uma membrana polimérica, ou ainda, adsorvido nesta última (Soppimath et al., 2001; Schaffazick et al., 2003; Reis et al., 2006; Guterres et al., 2007). Quando comparadas aos lipossomas, que são sistemas carreadores de fármacos constituídos de fosfolipídios, as nanopartículas constituídas por polímeros biodegradáveis têm atraído maior atenção dos pesquisadores, devido às suas potencialidades terapêuticas, à maior estabilidade nos fluídos biológicos, à diminuição dos efeitos colaterais e à maior estabilidade durante o armazenamento (Schaffazick et al., 2003; Reis et al., 2006).

Apesar de não existir nenhum nanocarreador "ideal" para o tratamento de desordens neurodegenerativas, várias classes de nanocarreadores favoráveis para sistemas de administração de fármaco no SNC têm sido desenvolvidos na última década (Wong et al., 2012). A maioria dos sistemas de distribuição de drogas baseados em nanotecnologia para o tratamento de desordens neurodegenerativas estão na forma de nanopartículas poliméricas. Nanopartículas poliméricas são promissoras para o tratamento da DA, uma vez que podem passar através de junções celulares apertadas, sendo capazes de atravessar a BHE e alcançar o SNC (Modi et al., 2010).

Um exemplo disto é o estudo realizado por Fornaguera e colaboradores (2015), no qual desenvolveram nanopartículas poliméricas utilizando o polímero biodegradável poli (ácido láctico-co-glicólico) (PLGA) utilizando método de nanoemulsificação por inversão de fase. Os autores utilizaram loperamida como droga modelo sem efeito de analgesia, uma vez que esta não tem capacidade de atravessar a BHE. As nanopartículas carregadas com loperamida foram funcionalizadas com um anticorpo monoclonal contra o receptor de transferrina e a travessia da BHE foi estudada *in vivo* pelo teste de placa quente. Os resultados

evidenciaram que as nanopartículas de PLGA carregadas com loperamida foram capazes de atravessar a BHE de forma eficaz, com alta eficiência de passagem.

Neste contexto, Bernardi e colaboradores (2012) investigaram o efeito potencial de proteção de nanocápsulas de núcleo lipídico carregadas com indometacina (IndOH-LNCs) comparativamente ao fármaco livre contra os danos celulares, neuroinflamação e alterações comportamentais em modelos *in vitro* e *in vivo* de DA induzidos por beta-amilóide $(A\beta)_{1-42}$. Os resultados mostraram que as IndOH-LNCs foram capazes de atenuar a morte celular e bloquear a neuroinflamação desencadeada por $A\beta_{1-42}$ em culturas organotípicas de hipocampo. Além disso, o tratamento com IndOH-LNCs foi capaz de aumentar a liberação de IL-10 (citocina anti-inflamatória) e diminuir a ativação glial. *In vivo*, apenas o tratamento com as IndOH-LNCs atenuou significativamente as deficiências comportamentais desencadeadas pela injeção de $A\beta_{1-42}$, evidenciando a potencialidade dos nanossistemas para direcionamento de ativos ao SNC.

Diante do exposto, justifica-se a relevância do desenvolvimento de um nanossistema capaz de garantir adequada distribuição no sistema nervoso central de crisina e, com isso, constituir-se em potencial tratamento para a DA.

2. OBJETIVOS

2.1 Objetivo geral

Preparar e caracterizar nanocápsulas poliméricas contendo crisina e investigar seu efeito protetor em um modelo de DA induzida pela infusão intracerebroventricular (icv) de $A\beta_{1-42}$ em camundongos.

2.2 Objetivos específicos

Preparar suspensões de nanocápsulas poliméricas contendo crisina.

Caracterizar as formulações em termos de parâmetros físico-químicos como tamanho de partícula, distribuição de tamanho, potencial zeta e pH.

Desenvolver e validar metodologia analítica para análise da crisina nas suspensões de nanocápsulas poliméricas.

Avaliar o teor de crisina, bem como sua eficiência de encapsulação nas nanoestruturas.

Avaliar os efeitos da crisina nanoencapsulada e compara-la à forma livre em estruturas encefálicas (córtex pré-frontal e hipocampo) de camundongos em parâmetros de estresse oxidativo, neuroinflamação e níveis do fator neurotrófico derivado do cérebro (BDNF).

Verificar os comportamentos cognitivos dos camundongos nos testes do labirinto aquático de Morris, esquiva passiva e reconhecimento de objeto novo.

PARTE II

Improved neuroprotective effects of chrysin loaded nanocapsules in a model of Alzheimer's disease induced by β-amyloid$_{1-42}$ peptide in aged mice

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A ser submetido à Nanomedicine

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ABSTRACT

AIM:

This study investigated the neuroprotective effects of chrysin loaded nanocapsules (CLN) in a model of Alzheimer's disease (AD) induced by β-amyloid$_{1-42}$ (Aβ_{1-42}) peptide in aged mice, and compared these effects with those from free chrysin.

METHODS:

Aged mice received chrysin, free (5 mg/kg, per oral, p.o.) or loaded nanocapsules (1 or 5 mg/kg, p.o.) for 14 days before Aβ_{1-42} administration.

RESULTS:

Aβ_{1-42} induced significant impairments on memory (Morris Water Maze task, object recognition and Step-Down-Type Passive Avoidance Tests), as well as caused oxidative stress and reduced the levels of neuroinflammation and brain-derived neurotrophic factor (BDNF) in prefrontal cortex and hippocampus of mice. Chrysin loaded nanocapsules displayed significant neuroprotection against Aβ_{1-42}-induced behavioral and neurochemical changes in a model of AD.

CONCLUSION:

These results point to Chrysin loaded nanocapsules a promising nanomedical tool and therapeutical approach for the treatment and prevention of AD.

KEYWORDS:

Alzheimer's disease; antioxidant; nanoparticles; flanovonoid

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder (Lee et al., 2011), affecting 36 million people worldwide (Gregori et al., 2015). AD is characterized clinically by progressive loss of cognitive function, neuropsychiatric and behavioral disorders (McGeer and McGeer, 2013). Its main pathologic characteristics are extracellular plaques and intracellular neurofibrillary tangles consisting of β-amyloid (Aβ) and hyperphosphorylated Tau respectively (Zhang et al., 2014).

Oxidative stress is a key component of the pathogenesis of AD (Liao et al., 2015). Measured by various parameters, including lipid peroxidation, protein oxidation and the formation of reactive species (RS), oxidative stress has been observed in brain tissue of AD patients (Salgado-Puga and Pena-Ortega, 2015; Moneim, 2015). The neuroinflammation is also an important factor in the pathogenesis of AD (Nazem et al, 2015; Tai et al, 2015), which includes the glial cell activation (for example, microglia and astrocytes) and expression of inflammatory mediators such as TNF-α, IL-1β and IL-10 (Bungart et al, 2014; Guo et al, 2015.). Furthermore, changes in activity levels of brain-derived neurotrophic factor (BDNF) have been associated with the pathogenesis of AD. Studies suggest that a decrease in BDNF levels may be associated with disease progression (Fukumoto et al, 2014; Prakash and Kumar, 2014).

Although a century has passed since its discovery, treatment for AD still represents a challenge for the pharmaceutical industry. There is little current therapeutic options consisting of agents that improve symptoms but do not prevent disease progression (Kung, 2012; Liao et al, 2015). Thus, the treatment of AD still lacks an effective therapeutic strategy. Chrysin (5, 7-dihydroxyflavone) is a natural flavonoid found in plant extracts (such as *Passiflora caerulea* and *Populus tremula*), honey and propolis (Borawska et al., 2014). Chrysin is an important nutritional supplement because of its many pharmacological properties including anti-

inflammatory (Shen et al, 2015; Yao et al, 2014), hypolipidemic (Zarzecki et al, 2014), anti-atherogenic, anti-cancer (Yang et al, 2013). Accumulating evidence demonstrated that the potential neuroprotective effects of chrysin might be due to alleviation of oxidative stress in prefrontal cortex and hippocampus of mice (Filho et al., 2015; Souza et al., 2015).

Adding to this, the blood-brain barrier (BBB), in its role of protection, limits the delivery of many potentially therapeutic drugs (Ordones-Gutiérrez et al, 2015), undermining the effectiveness of treatment. During the last decade, several attempts have focused on this fundamental problem by devising different strategies to facilitate the passage of drugs across the BBB. Among these, nanotechnology-based approaches have gained significant momentum, as it can effectively carry active ingredients through the BBB (Brambilla et al., 2011).

Thus, the objective of this study was to prepare a system based on polymeric nanocapsules, capable of conveying chrysin, as well as to investigate the effects of treatment for 14 days in a mice model of AD induced by intracerebroventricular (i.c.v.) injection $A\beta_{1-42}$. Therefore, we determined parameters of oxidative stress, neuroinflammation and derived neurotrophic factor (BDNF) levels of the brain in the prefrontal cortex and hippocampus, and verify that the nanocapsules formulation were able to protect against cognitive impairment caused by $A\beta_{1-42}$, compared to free chrysin.

Materials and methods

Preparation of polymeric nanocapsules

Nanocapsules (NC) suspensions were prepared by interfacial deposition of polymer (Jäger et al., 2009). At 40°C, chrysin (0.005 g), poly(ε-caprolactone) (0.100 g), pomegranate oil (0.33 mL), and sorbitan monostearate (0.077 g) were dissolved in acetone (27 mL) at

40°C. In a separate flask, polysorbate 80 (0.077 g) was added to 53 mL of water. The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 minutes, the acetone was eliminated and the suspensions were concentrated under reduced pressure. The final volume was adjusted to 10 mL for a drug concentration of 0,5 mg/mL. The control formulation (blank nanocapsules - BN) was prepared, as described above without adding chrysin.

Caracterization of polymeric nanocapsules

After preparation, the pH values of the NC suspensions were determined using a potentiometer (Hanna® Instruments, São Paulo, BR). The particle size and polydispersion profile of the suspensions were determined using a Matersizer® 2000 model (Malvern Instruments, Malvern, UK). The samples were then diluted with prefiltered water. Zeta potential of the suspensions were determined using a ZetaPlus (Brookhaven Instruments Corporation, NY, USA) after dilution 1:500 (v / v) suspension of nanocapsules in 10 mM NaCl solution aqueous previously filtered through a 0.45 uM membrane. The measurements were made in triplicate to assure accuracy. The morphology the samples was examined by atomic force microscopy (AFM) (Agilent Technologies 5500 equipment, CA, USA). AFM images were acquired at room temperature, in non-contact mode using high resolution probes SSS-NCL (Nanosensors, force constant = 48 N/m, resonance frequency = 154 kHz). Images were captured and analyzed using PicoView 1.14.4 software from Molecular Imaging Corporation and were analyzed using PicoImage 5.1. For the analysis, the solution was diluted in MilliQ water (1:100) and were dropped onto freshly cleaved mica substrate.

Drug content ($mg.ml^{-1}$) was determined after dissolution of nanocapsules in methanol (0,2 ml of suspension to 10 ml of methanol), left in ultrasound for 50 minutes and assayed by high performance liquid chromatography, HPLC. The chromatographic system consisted of a

Gemini RP-18 column (100mm×4.60 mm, 5µm, São Carlos, BRA) and a Shimadzu instrument (LC-20AT Pump, SIL-20A autosampler, SPD-20AT PDA Detector, LC Solution V. 1.24 SP1 system software, Shimadzu, Tokyo, Japan). The mobile phase at a flow rate of 0,5 ml min^{-1} consisted of acetonitrile/water (70:30, v/v) containing 0,2% of phosphoric acid. The volume injected was 20µl and chrysin was detected at 260 nm. The method was linear ($r2$ = 0.998) in the range of 1–25µg.ml^{-1}, accurate (recovery: 102±3%) and precise (R.S.D.: <2% for repeatability and <4,8% for intermediate precision). The specificity was tested in presence of the colloidal suspension adjuvants and demonstrated that these factors did not alter the chrysin assay. Free drug was determined in the ultrafiltrate (HPLC) after the separation of the nanoparticles by an ultrafiltration-centrifugation technique (Ultrafree-MC® 10,000MW, Millipore, Bedford, USA), at 12000 rpm for 5 min. Encapsulation efficiency (%) was calculated by the difference between the total and free drug concentrations.

Animals

Experiments were performed using female Swiss Albino mice (30-35g, 18-22 months old). Animals were maintained at 22-25ºC with free access to water and food, under a 12:12h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol #009/2015) of Federal University of Pampa, Brazil.

Amyloid peptide preparation

The $A\beta_{1-42}$ peptide was dissolved in phosphate buffered saline (PBS). The Aβ peptides were aggregated by incubation at 37°C for 72 hours before in vivo use.

Experimental design

Mice were randomly assigned into ten groups (n=8 per group): (1) vehicle/PBS; (2) BN/PBS; (3) Chrysin free (FC) (5 mg/kg)/PBS, (4) NC-chrysin (CLN) (1 mg/kg)/PBS, (5) CLN (5 mg/kg)/PBS, (6) vehicle/$A\beta_{1-42}$, (7) BN/$A\beta_{1-42,}$ (8) FC (5 mg/kg)/$A\beta_{1-42}$, (9) CLN (1 mg/kg)/$A\beta_{1-42}$, (10) CLN (5 mg/kg)/$A\beta_{1-42}$. In this experimental design, mice were treated with for 14 days after receiving intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ or vehicle. Day 15 after i.c.v. injection, mice underwent behavioural assessment and 24h after, finally, submitted to euthanasia (**Fig. 2**). The prefrontal cortex and hippocampus were removed for assays.

Behavioural assessment

Object recognition test (ORT)

In this ORT mice were placed in an open box in order to evaluate the preference for a novel object, where the short-term memory (STM) could be assayed. The ORT was performed as described by Ennaceur and Delacour (1988) with some modifications. Mice were allowed to explore two identical objects (sample phase) for 5 min and then returned to their home cage. To evaluate the short-term memory (STM), mice were returned to the open box, after a delay of 90 min, where they were exposed to two different objects (test phase), one identical to the one previously encountered in the sample phase, therefore now familiar, and the other is novel. The animals were allowed to explore both objects for more 5 min. After each trial, box and objects were cleaned with 70% ethanol. To evaluate the long-term memory (LTM), mice were tested in same conditions with a delay of 24h after sample phase.

The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploratory behavior was defined as sniffing or touching the object with the nose and/or forepaws. Any other behavior, such as sitting on or turning around the object was not considered as exploration. The amount of time each animal spent actively investigating the objects was manually scored and discrimination index was calculated as the time exploring novel or familiar object divided by the total time spent exploring both objects.

Step-Down-Type Passive Avoidance Test

Step-down-type passive avoidance tests were conducted using a platform to investigate the effects of chrysin and CLN on learning and memory according to a modification of the method reported by Sakaguchi et al., 2006. The experimental device is a 12 × 12 × 18-cm electronic avoidance-response chamber, three sides of which are made of blank Plexiglas and one side of which is hard black plastic. The floor of the chamber is composed of parallel stainless-steel grids. Electric shocks were delivered to the grids. A rubber platform (5 cm high, upper surface 4 cm in diameter) was fixed in a corner on the floor of the chamber.

The test consisted of a training session and a retention session (24 h after the training session). During the training session, each mouse was placed on the steel grids and then exposed to an electric shock (0,3 mV) until it stepped up onto the rubber platform. Escape latency (the time required for the mouse to escape from electric shock) and the number of errors (the number of times that the mouse stepped down from platform) were recorded. In the retention test, each mouse was placed on the platform. When the mouse stepped down and placed its paws on the grids, an electric shock was delivered. Step-down latency and the number of errors were recorded. The cut-off time in both sessions was 300 s.

Morris Water Maze (MWM) task

The apparatus was made of black painted fibreglass, 97 cm in diameter and 60 cm in height. For the tests, the tank was filled with water maintained at 23±2 ◦C. The target platform (10 cm×10 cm) was made of transparent Plexiglas and it was submerged 1–1.5 cm beneath the surface of the water. Starting points for animals were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant visual cues (55 cm×55 cm) were placed on the walls of the water maze room. They were all positioned with the lower edge 30 cm above the upper edge of the water tank and in the standard setting, the position of each symbol marked the midpoint of the perimeter of a quadrant (circle =NE quadrant, square = SE quadrant, cross = SW quadrant and diamond =NW quadrant). The apparatus was located in a room with indirect incandescent illumination. Mice were submitted to a spatial reference memory version of the water maze using a protocol that was described by Prediger et al (2007). The training session consisted of 10 consecutive trials during which the animals were left in the tank facing the wall and then allowed to swim freely to the submerged platform. The platform was located in a constant position (middle of the southwest quadrant), equidistant from the centre and the wall of the pool. If the animal did not find the platform during a period of 60 s, it was gently guided to it. The animal was allowed to remain on the platform for 10 s after escaping to it and was then removed from the tank for 20 s before being placed at the next starting point in the tank. This procedure was repeated 10 times, with the starting points (the axis of one imaginary quadrant) varying in a pseudo-randomized manner. The test session was carried out 24 h later and consisted of a single probe trial where the platform was removed from the pool and each mouse was allowed to swim for 60 s in the maze. Behavioral data were recorded and analyzed using ANY-maze video tracking software (Stoelting Co., IL, USA). Escape latency, latency to target platform location and the time

spent in correct quadrant (i.e. where the platform was located on the training session) were computed for subsequent analysis. The speed (cm/sec) and distance traveled (m) we also recorded, as indicators of locomotor activity.

Biochemical assays

After behavioral tests, mice were euthanized with barbiturate overdose (pentobarbital sodium 150mg/kg; intraperitoneal, i.p.). The brain was removed and the prefrontal cortex and hippocampus were separated. For determination of biochemical parameters these cerebral structures were homogenized in 50 mM Tris–HCl, pH 7.4 (1:5, w/v), and centrifuged at 4000 × g for 10 min to obtain the low-speed supernatant (S_1). S_1 was utilized for all determinations.

Non-protein thiols (NPSH) levels

NPSH levels were determined by the method described by Ellman (1959). S_1 was precipitated with 10% trichloroacetic acid and centrifuged at 3,000×g for 10 min. Sulphydril groups were determined using clear supernatant. An aliquot of supernatant was added in 1M potassium phosphate buffer (pH 7.4) and 10 mM 5, 5´-dithiobis-2-nitrobenzoic acid (DTNB). NPSH levels were measured spectrophotometrically at 412 nm and expressed as µmol NPSH/g tissue.

Reactive species (RS) levels

To estimate the RS production in the prefrontal cortex and hippocampus, S_1 was diluted (1:10) in 50mM Tris–HCl (pH 7.4) and incubated with 10 µl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1mM), at room temperature for 30 min. The RS levels were determined by a spectrofluorimetric method, using DCHF-DA assay **(**Loetchutinat et al. 2005). DCHF-DA is a nonfluorescent compound easily that crosses cell membranes and,

in the presence of RS is rapidly oxidized to its highly fluorescent derivative dichlorofluorescein (DCF). The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium. The RS levels were expressed as arbitrary unit (AU).

Glutathione reductase (GR) activity

GR activity was determined spectrophotometrically as described by Calberg and Mannervick (1985). In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. An aliquot of S_1 was added in the system containing 0.15 M potassium phosphate buffer (Ph 7.0), 1.5 mM EDTA, 0.15 Mm NADPH. After the basal reading, the substrate (GSSG 20 mM) was added. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

Glutathione peroxidase (GPx) activity

GPx activity in S_1 was assayed spectrophotometrically by the method described by Wendel (1981), through the GSH/NADPH/glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. S_1 was added to the medium containing GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2 (4 mM). In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase present in the assay media at the expenses of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

Glutathione S-transferase (GST) activity

GST activity was assayed through the conjugation of GSH with CDNB at 340 nm as described by Habig et al. (1974). An aliquot of S_1 was added in a medium containing 0.1 M potassium phosphate buffer (pH 7.4). After that, 100 mM CDNB and GSH were added to the medium. CDNB was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugated/min/mg protein.

Catalase (CAT) activity

CAT activity in S_1 was assayed spectrophotometrically by the method proposed by Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the presence of S_1 at 240 nm. Enzymatic reaction was initiated by adding S_1 and the substrate H_2O_2 (0.3 mM) in a medium containing 50 mM potassium phosphate buffer (pH 7.0). One unit of enzyme was defined as the amount of enzyme required for monitoring the disappearance of H_2O_2. The enzymatic activity was expressed as Units (U)/mg protein (1U decomposes 1μmol H_2O_2/min at pH 7 at 25 °C).

Brain-derived neurotrophic factor (BDNF) levels

Levels of BDNF were measured using a BDNF E_{max} ImmunoAssay System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The supernatants were collected for determination of BDNF levels. The content of BDNF levels was demonstrated as pg/μg total protein in the tissue.

Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-10 (IL-10) levels

Levels of TNF-α, IL-1β and IL-10 in the hippocampus and prefrontal cortex were determined using commercially available ELISA assays, following the instructions supplied

by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

HPLC analysis of chrysin levels in the brain

In order to determine the levels of chrysin in the cerebral tissue, HPLC analysis was performed as has been described and validated previously (Liang et al., 2015). Briefly, 1 h after the last administration, one group of animals was euthanized by a lethal dosage of anesthesia and blood samples were collected by cardiac puncture. The animals were perfused (0.9 % NaCl) to flush blood from the vascular system, the brain was weighed, and the hemispheres were separated. The right hemisphere was minced with scissors and placed in a homogenizer vessel; 5 mL of acetonitrile was added and tissues were subsequently homogenized. The homogenized samples were transferred to 50 mL conical glass tubes and vortexed for 5 min prior to centrifugation at 2,800×g for 40 min at 4 °C. The supernatant was placed into a clean tube, filtered (Millipore® 0.45 μm) and placed in a sealed amber vial for HPLC analysis. The injection volume used was 40 μL for all samples. The quantity of chrysin was calculated by comparing the peak area ratio from tissue samples of treated animals with those of the corresponding concentration standards of chrysin in acetonitrile injected directly into the HPLC system.

Measurement of hepatic enzymes in serum

In an attempt to evaluate whether treatments caused hepatic toxicity, the serum levels of hepatic enzymes were evaluated at the end of the treatments. The blood samples collected by cardiac puncture were analyzed by activities of hepatic enzymes γ-glutamyltransferase (γ-GT), lactate desydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were used as markers of metabolic and tissue toxicity.

Results

Physicochemical characterization of polymeric nanocapsules

NC formulations were prepared by interfacial deposition of poly(ε-caprolactone) and did not require subsequent purification. CLN were macroscopically homogeneous pale yellow opalescent liquids, and suspensions without the active (BN) had a similar appearance, but with opalescent white color. After preparation, the particle diameters were 368 nm (CLN) and 419 nm (BN). The both suspensions had relatively uniform particle size (narrow distribution) as indicate by the SPAN values below 1.4. The pH values were 7,43 ± 0,05 (CLN) and 7,27 ± 0,05 (BN) and the zeta-potential values were -37.65± 1.04 mV and -35.48 mV ± 0.79 mV, respectively. The chrysin content was 0.578 ± 0.011 mg/mL and the encapsulation efficiency was close to 84,82%. Images obtained by atomic force microscopy analysis showed the formation of a nanoparticulate bit polydisperse system, consisting of spherical particles (**fig. 1**). Figure 1 shows sizes in the same order of magnitude of the values obtained by the laser diffraction method.

Memory in ORT

Two-way ANOVA of total exploration time in the ORT revealed that was not a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 0.18$, $p< 0.94$), chysin ($F_{4,50} = 0.37$, $p< 0.83$) and $A\beta_{1-42}$ ($F_{1,50} = 1.12$, $p< 0.29$) (**fig. 3A**).

Two-way ANOVA of recognition index in the ORT revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 12.15$, $p< 0.001$), and a significant effect of chrysin ($F_{4,50} = 18.57$, $p< 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 67.68$, $p< 0.001$). Post-hoc comparisons demonstrated that injection of $A\beta_{1-42}$ reduced recognition index in mice, promoting impairment in memory in ORT. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5

mg/kg p.o.) reverted the impairment elicited by $A\beta_{1-42}$, improving the recognition index in ORT (**fig. 3B**).

Passive avoidance test

Two-way ANOVA revealed that training sessions in the step-down inhibitory avoidance task test was not changed significantly by $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 0.98$, $p < 0.08$), chrysin ($F_{4,50} = 0.76$, $p < 0.45$) and $A\beta_{1-42}$ ($F_{1,50} = 0.68$, $p < 0.16$) (**Fig. 4A**).

Two-way ANOVA of test sessions to step-down inhibitory avoidance task test showed no significant $A\beta_{1-40}$ × chrysin interaction ($F_{4,50} = 1.18$, $p < 0.33$) and a significant effect of chrysin ($F_{4,50} = 19.46$, $p < 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 12.22$, $p < 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly decreased the escape latency of Aβ-treated mice compared to control group. Treatment with chrysin (free at 5 mg/kg and loaded nanocapsules at 1 and 5 mg/kg) significantly protected against the decrease in scape latency elicited by $A\beta_{1-42}$ (**Fig. 4B**).

Spatial memory in MWM test

Spatial memory was measured in a probe test conducted 24h after the last training trial. During this test the platform was removed and the escape latencies and time in correct (target) quadrant was computed.

Two-way ANOVA of latency to target platform location yielded a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 5.21$, $p < 0.01$) and a significant effect of chrysin ($F_{4,50} = 3.58$, $p < 0.01$) and $A\beta_{1-42}$ ($F_{1,50} = 74.73$, $p < 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ (no difference was found between animals from vehicle and blank nanocapsules groups) significantly increased the escape latency of Aβ-treated mice compared to control group.

Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) significantly protected against the increase in scape latency elicited by $A\beta_{1-40}$ (**Fig. 5A**).

Two-way ANOVA of number of crossing showed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 5.86$, $p < 0.001$) and a significant effect of chrysin ($F_{4,50} = 2.62$, $p < 0.04$) and $A\beta_{1-42}$ ($F_{1,50} = 17.78$, $p < 0.001$). In line with this, post hoc analyses indicated that animals treated with $A\beta_{1-42}$ alone presented impaired memory (decrease the number of crossings of the platform location) compared with control animals (vehicle and blank nanocapsules). Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reverted the deficit in memory expressed by the number of crossing of the platform location in the MWM test (**Fig. 5B**).

Two-way ANOVA revealed that average speed of mice was not changed significantly by $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 0.18$, $p < 0.94$), chrysin ($F_{4,50} = 0.58$, $p < 0.67$) and $A\beta_{1-42}$ ($F_{1,50} = 0.01$, $p < 0.95$) (**Fig. 5C**).

Two-way ANOVA revealed that total distance travelled was not modified significantly by $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 0.27$, $p < 0.89$), chrysin ($F_{4,50} = 0.28$, $p < 0.89$) and $A\beta_{1-42}$ ($F_{1,50} = 2.54$, $p < 0.89$) (**Fig. 5D**).

NPSH levels

Two-way ANOVA of NPSH levels in prefrontal cortex revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 8.85$, $p < 0.001$) and a significant effect of chrysin ($F_{4,50} = 9.78$, $p < 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 33.15$, $p < 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly depleted NPSH levels in prefrontal cortex of mice compared to control group (vehicle and blank nanocapsules). Treatment with chrysin reversed the depletion of NPSH levels induced by $A\beta_{1-42}$. In addition, chrysin loaded nanocapsules 5 mg/kg demonstrated a significant different when compared to free chrysin 5 mg/kg (**Table 1**), indicating that the

protective effect of chrysin against $A\beta_{1-42}$-induced NPSH depletion depends upon its physicochemical features (free or nanoparticulate).

Statistical analysis of NPSH levels in hippocampus revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 9.32$, $p < 0.001$) and a significant effect of chrysin ($F_{4,50} = 5.51$, $p < 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 35.93$, $p < 0.001$) Post-hoc comparisons demonstrated that $A\beta_{1-42}$ significantly decreased NPSH levels in hippocampus of mice. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the depletion of NPSH levels induced by $A\beta_{1-42}$ in hippocampus of mice (**Table 2**). Similar effect of chrysin loaded nanocapsules was observed in hippocampus when compared to cortex, indicating that the protective effect of chrysin also depends upon its physicochemical features.

RS levels

Two-way ANOVA of RS levels in prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 6.61$, $p < 0.001$) and a significant effect of chysin ($F_{4,50} = 7.91$, $p < 0.001$), and $A\beta_{1-42}$ ($F_{1,50} = 65.81$, $p < 0.001$). Post-hoc comparisons revealed that $A\beta_{1-42}$ significantly increased RS levels in prefrontal cortex of mice. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the increase of RS levels induced by $A\beta_{1-42}$ in prefrontal cortex of mice. Interestingly, the protective effect of chrysin against $A\beta_{1-42}$- induced increase of RS levels occurs in high scale when it is given in its nanoparticulate form (**Table 1**).

Statistical analysis of RS levels in hippocampus revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 8.31$, $p < 0.001$) and a significant effect of chrysin ($F_{4,50} = 13.65$, $p < 0.001$), and $A\beta_{1-42}$ ($F_{1,50} = 144.25$, $p < 0.001$). Post-hoc comparisons revealed that $A\beta_{1-42}$ (vehicle and blank nanocapsules) significantly increased RS levels in hippocampus of mice. Treatment

with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the increase of RS levels induced by $A\beta_{1-42}$ in hippocampus of mice (**Table 2**).

GR activity

Two-way ANOVA of GR activity in prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × chrysin interaction (Interação: $F_{4,50} = 4.51$, $p< 0.003$) and a significant effect of chrysin ($F_{4,50} = 6.00$, $p< 0.005$), and $A\beta_{1-42}$ ($F_{1,50} = 28.33$, $p< 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly significantly increased the activity of GR activity in prefrontal cortex of mice compared to control group (vehicle and blank nanocapsules). Treatment with chrysin reversed the increase of the activity of GR activity induced by $A\beta_{1-42.}$ In addition, chrysin loaded nanocapsules 5 mg/kg demonstrated a significant different when compared to free chrysin 5 mg/kg (**Table 1**), indicating that the protective effect of chrysin against $A\beta_{1-42}$-induced GR activity increase are high in the nanocapsules form.

Statistical analysis of GR activity in hippocampus revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 8.61$, $p< 0.001$) and a significant effect of chrysin ($F_{4,50} = 6.30$, $p< 0.003$), and $A\beta_{1-42}$ ($F_{1,50} = 81.52$, $p< 0.001$ Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly significantly increased the activity of GR activity in prefrontal cortex of mice compared to control group . Treatment with chrysin reversed the increase of the activity of GR activity induced by $A\beta_{1-42.}$ In addition, chrysin loaded nanocapsules 5 mg/kg demonstrated a significant different when compared to free chrysin 5 mg/kg (**Table 2**).

GPx activity

Two-way ANOVA of GPx activity in prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 9.61$, $p< 0.001$) and a significant effect of chrysin ($F_{4,50} = 8.12$, $p< 0.001$), and $A\beta_{1-42}$ ($F_{1,50} = 75.14$, $p< 0.001$). Treatment with free chrysin (5 mg/kg

p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the increase of GPx activity induced by $A\beta_{1-42}$ in prefrontal cortex of mice . Interestingly, the protective effect of chrysin against $A\beta_{1-42-}$ induced increase of GPx activity with similar value of vehicle group when it is given in its nanoparticulate form (**Table 1**).

Statistical analysis of GPx activity in hippocampus revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 8.54$, $p < 0.001$) and a significant effect of chrysin ($F_{4,50} = 9.70$, $p < 0.001$), and $A\beta_{1-42}$ ($F_{1,50} = 175.23$, $p < 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly increased the GPx activity in hippocampus of mice compared to control group (vehicle and blank nanocapsules). Treatment with chrysin (free and nanocapsules) reversed the increase of GPx activity induced by $A\beta_{1-42}$ in hippocampus of mice (**Table 2**).

GST activity

Two-way ANOVA of GST activity in prefrontal cortex demonstrated a significant effect for $A\beta_{1-42}$ ($F_{1,50} = 104.19$, $p < 0.001$), but not for chrysin ($F_{4,50} = 0.30$, $p < 0.87$), and $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 0.54$, $p < 0.71$) (**Table 1**).

Statistical analysis of GST activity in hippocampus revealed a significant effect for $A\beta_{1-42}$ (Fator beta amilóide: $F_{1,50} = 78.00$, $p < 0.001$), but not for chrysin ($F_{4,50} = 0.13$, $p < 0.95$), and $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 0.59$, $p < 0.67$) (**Table 2**).

CAT activity

Two-way ANOVA revealed that CAT activity in prefrontal prefrontal cortex of mice was not changed significantly by $A\beta_{1-42}$ × chrysin interaction $F_{4,50} = 0.28$, $p < 0.88$), chrysin ($F_{4,50} = 1.35$, $p < 0.26$) and $A\beta_{1-42}$ ($F_{1,50} = 1.33$, $p < 0.25$) (**Table 1**).

Two-way ANOVA revealed that CAT activity in hippocampus of mice was not modified significantly by $A\beta_{1-42}$ × chrysin interaction ($F_{4,50}$ = 0.38, p< 0.82), chrysin ($F_{4,50}$ = 1.01, p< 0.41) and $A\beta_{1-42}$ ($F_{1,50}$ = 0.01, p< 0.95) (**Table 2**).

BDNF levels

Two-way ANOVA of BDNF levels in prefrontal cortex demonstrated a significant effect for chrysin ($F_{4,50}$ = 23.27, p< 0.001) and Aβ1-42 ($F_{1,50}$ = 51.31, p< 0.001), but not for $A\beta_{1-42}$ × chrysin interaction ($F_{4,50}$ = 0.61, p< 0.65). Post-hoc comparisons revealed that $A\beta_{1-42}$ significantly decreased the BDNF levels in prefrontal cortex of mice. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) (effect *per se*) increased the IL-10 levels in prefrontal cortex of mice. In addition, treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the decrease the IL-10 levels in prefrontal cortex induced by $A\beta_{1-42}$ (**Fig. 6A**).

A significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50}$ = 2.68, p< 0.05) and a significant effect of chrysin ($F_{4,50}$ = 11.77, p< 0.001) and $A\beta_{1-42}$ ($F_{1,50}$ = 44.41, p< 0.001) in BDNF levels in hippocampus of mice was observed. Post-hoc comparisons revealed that $A\beta_{1-42}$ significantly decreased the BDNF levels in hippocampus of mice. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) (effect *per se*) increased the IL-10 levels in hippocampus of mice. In addition, treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the decrease the IL-10 levels in hippocampus induced by $A\beta_{1-42}$ (**Fig. 6B**).

TNF-α and IL-1β

Statistical analysis of TNF-α levels in prefrontal cortex revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50}$ = 12.66, p< 0.001), and a significant effect of chrysin ($F_{4,50}$ = 13.90,

$p< 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 196.41$, $p< 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly significantly increased the TNF-α levels in prefrontal cortex of mice compared to control group (vehicle and blank nanocapsules). Treatment with chrysin reversed the increase of the activity of GR activity induced by $A\beta_{1-42}$. In addition, chrysin loaded nanocapsules 5 mg/kg demonstrated a significant different when compared to free chrysin 5 mg/kg (Table 1), indicating that the protective effect of chrysin against $A\beta_{1-42}$-induced GR activity increase are high in the nanocapsules form (**Fig. 7A**).

Two-way ANOVA of TNF-α levels in hippocampus demonstrated a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 13.83$, $p< 0.001$) and a significant effect of chrysin ($F_{4,50} = 12.95$, $p< 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 124.72$, $p< 0.001$ Post-hoc comparisons revealed that $A\beta_{1-42}$ (vehicle and blank nanocapsules) significantly increased the TNF-α levels in hippocampus of mice. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the increase of RS levels induced by $A\beta_{1-42}$ in hippocampus of mice (**Fig. 7B**).

Statistical analysis of IL-1β levels in prefrontal cortex revealed a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 35.75$, $p< 0.001$) and a significant effect of chrysin ($F_{4,50} = 36.86$, $p< 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 161.5$, $p< 0.001$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly increased the IL-1β levels in prefrontal cortex of mice compared to control group. Treatment with chrysin (higher protective effect in nanocapsules form) reversed the increase of IL-1β levels in prefrontal cortex induced by $A\beta_{1-42}$ in hippocampus of mice (**Fig. 7C**).

Two-way ANOVA of IL-1β levels in hippocampus demonstrated a significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 17.96$, $p< 0.001$) and a significant effect of chrysin ($F_{4,50} = 17.84$, $p< 0.001$) and $A\beta_{1-42}$ ($F_{1,50} = 142.6$, $p< 0.001$). Post-hoc comparisons revealed that $A\beta_{1-42}$ significantly increased the IL-1β levels in hippocampus of mice. Treatment with free chrysin

(5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the increase of IL-1β levels in hippocampus induced by $A\beta_{1-42}$ (**Fig. 7D**).

IL-10 levels

A significant $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 3.028$, $p< 0.02$) and a main effect of chrysin ($F_{4,50} = 3.38$, $p< 0.01$) and $A\beta_{1-42}$ ($F_{1,50} = 118.9$, $p< 0.001$) in prefrontal cortex IL-10 levels of mice was observed. Post-hoc comparisons revealed that $A\beta_{1-42}$ significantly decreased the IL-10 levels in prefrontal cortex of mice. Treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) (effect *per se*) increased the IL-10 levels in prefrontal cortex of mice. In addition, treatment with free chrysin (5 mg/kg p.o.) and chrysin loaded nanocapsules (1 or 5 mg/kg p.o.) reversed the decrease the IL-10 levels in prefrontal cortex induced by $A\beta_{1-42}$ (**Fig. 7E**).

Two-way ANOVA of IL-10 levels in hippocampus demonstrated a significant effect for chrysin ($F_{4,50} = 2.34$, $p< 0.07$), and $A\beta_{1-42}$ ($F_{1,50} = 82.7$, $p< 0.001$), but not for $A\beta_{1-42}$ × chrysin interaction ($F_{4,50} = 1.48$, $p< 0.22$). Post hoc comparisons demonstrated that $A\beta_{1-42}$ significantly decreased the IL-10 levels in hippocampus of mice compared to control group. Treatment with chrysin reversed the decrease of the the IL-10 levels in hippocampus induced by $A\beta_{1-42.}$ In addition, chrysin loaded nanocapsules 5 mg/kg demonstrated a significant different when compared to free chrysin 5 mg/kg (**Fig. 7F**), indicating that the protective effect of chrysin against $A\beta_{1-42}$-induced GR activity increase are high in the nanocapsules form.

Nanocapsules increase chrsyin concentration in the brain of mice

Post-hoc comparisons revealed that treatment with CLN5 significantly increased the quantity of chrysin in comparison with groups that received free chrysin or CLN1 (**Fig. 8**).

Investigation of the possible side effects of chrysin treatment

Treatment with free (5 mg/kg p.o.) or loaded nanocapsules (1 or 5 mg/kg p.o.) chrysin, as well as the vehicles, respectively, did not induce mortality or alter body weight within 14 days of treatment (data not shown). The activities of hepatic enzymes γ-GT, LDH, ALT, and AST were assessed in serum. None of the treated animals presented with significant alterations in the investigated enzymes, suggesting no hepatic alterations or metabolic toxicity in the animals in the tested conditions (data not shown).

Discussion

The reaction induced by Aβ involves the release of damaging factors such as cytokines and RS, which promote activation of intracellular pathways that contribute to the progression of AD (Moneim, 2015; Butterfield et al., 2013; McGeer and McGeer, 2013). Aβ-induced inflammation and oxidative stress is thought to play a key role in the pathogenesis of cognitive impairment and AD (Salgado-Puga and Pena-Ortega, 2015). Therefore, a possible therapeutic strategy is to reduce inflammatory, oxidative stress and memory damages (Guo et al., 2015; Balducci and Forloni, 2014). Thus, antioxidants and anti-inflammatory have been considered to constitute promising potential neuroprotective drugs for AD treatment (Ianiski et al., 2012; Frozza et al., 2013). On the other hand, most of the clinical trials on this matter have shown unsatisfactory results, which seems to be related, at least in part, to the fact that antioxidants and anti-inflammatory have unfavorable pharmacokinetics as well as limited transport through the BBB (Gregori et al., 2015). In the present study, we provide compelling evidence that CLN were able to ameliorate memory and learning deficits by decreasing damage in the brain of mice and neuroprotective effect might be involved in reducing RS, pro-inflammatory cytokines and in increasing the levels of IL-10 and BDNF in a model of

AD. Additionally, neither free nor nanoencapsulated chrysin altered hepatic function in our experimental conditions.

Aβ species play a prominent role in the pathogenesis of AD, a pathology that disrupts memory performance (Prediger et al., 2008; Souza et al., 2013). This Aβ- induced cognitive disruption may result primarily from a synaptic dysfunction, which then spreads to include a pattern of neuronal death (Coleman et al., 2004). The acute i.c.v. injection of synthetic amyloid peptides such as $A\beta_{1-42}$ (analogous to peptides found in senile plaques in AD patients) has been a profitable experimental model for the characterization of Aβ-neurotoxicity and the study of neuroprotective interventions (Hatami et al., 2014; Yu et al., 2015). In this view, cognitive deficits induced by a single i.c.v. administration of $A\beta_{1-42}$ have been documented in mice (Chen et al., 2015; Meunier et al., 2015). The ameliorative effect of chrysin (free or nanoparticulate formulation) on nonspatial long-term memory was investigated in the step-down inhibitory avoidance task. Aβ-induced memory impairment was verified by decreasing the step-down latency, whereas chrysin prevented this impairment of nonspatial memory. In addition, the chrysin treatment increased the step-down latency in comparison with the control group. The MWM is usually accepted as an indicator of spatial learning and reference memory, which reflects long-term memory (Morris, 1984; Souza et al., 2015). In training trials (acquisition trials), $A\beta_{1-42}$-treated mice exhibited a progressive increase in the escape latency to find the platform compared to the control group. In the probe trial (retention trial), we observed that mice treated with $A\beta_{1-42}$ showed both an increase in the escape latency to target platform location in comparison to the respective control group. In contrast, chrysin blunted $A\beta_{1-42}$-induced acquisition and retention deficits as evidenced by chrysin/$A\beta_{1-42}$ group, which exhibited shorter escape latency in the training and probe trials. It is important to emphasize that no significant changes were observed in the speed and distance traveled in any of the groups ensuring that the locomotor activity did not change because the

treatments. Furthermore, chrysin improved the impairment of object recognition memory, which is dependent on the cortex and hippocampus (Bengoetxea et al., 2015; Grayson et al., 2015) in mice. These findings strengthen the notion postulated in existing literature that BDNF has an important role in memory formation, and may be involved in the pathology of AD, which is associated with Aβ production, accumulation and aggregation (Fukomoto et al., 2014). In our study, we demonstrated that Aβ administration caused a remarkable reduction in BDNF levels in the prefrontal cortex and hippocampus of mice. A reduction in BDNF levels in the cortex and hippocampus was also demonstrated by other researchers in rodents treated with $A\beta_{1-42}$ (Chen et al., 2015; Fukumoto et al., 2014). Treatment with chrysin was able to protect against the decrease in BDNF levels in the prefrontal cortex and hippocampus of $A\beta_{1-42}$-treated mice. Additionally, chrysin, *per se*, increased exceptionally BDNF levels in the brain of mice treated with PBS. Thus, our study presents primary evidence that chrysin may up-regulate BDNF levels in the brain of mice, modulating an important pathway for neuronal and cognitive protection against the neurotoxic effects of Aβ peptide. Notwithstanding, our data are in accordance with the results recently found, which demonstrated that chrysin ameliorated learning and memory functions in aged-mice (Souza et al., 2015), STZ-induced diabetes in rats (Li et al., 2014) and chronic cerebral hypoperfusion model in rats (He et al., 2012). Thus, our study presents primary evidence that chrysin, mainly in the nanocapsules formulation, up-regulated BDNF levels in the brain regions of mice, modulating an important pathway for neuronal and cognitive protection against the neurotoxic effects of Aβ peptide. Additionally, chrysin protect against the cognitive impairment induced by Aβ due to mechanisms such as antioxidant and anti-inflammatory.

Oxidative stress is a potential contributor to AD development, which is considered to be the state of imbalance between the antioxidant defense level and the production of free radicals that induce the potential brain damage. Overproduction of free radicals in

neurodegenerative diseases can alter protein and nucleic acid functions and induce lipid peroxidation, leading to eventual cell death (Moneim, 2015; Butterfield et al., 2013). Some neurotoxic insults, including oxidative damages, display an important role in the cognitive dysfunctional hippocampal-prefrontal pathway and may play an important role in the pathophysiology of AD (Rege et al., 2014; Butterfield et al., 2013). GSH redox cycling is extremely important in cellular free radical detoxification in the brain tissue. Its homeostasis is maintained by GSH synthesis and redox cycling. During detoxification, oxy-radicals are reduced by GPx to form GSSG. GSH is regenerated by redox recycling, in which GSSG is reduced to GSH by GR with the consumption of NADPH (Saharan and Mandal, 2014; Butterfield et al., 2013). We also demonstrated that the activity of CAT remained unaltered in all groups. This can be explained partly due to the lower levels of CAT naturally found in the brain (Aksu et al., 2009). Another explanation may be the higher K_m (low affinity) of CAT for H_2O_2 and its respective intracellular distribution in comparison with GPx. The reduction of RS levels is relevant since augmented ROS levels and associated damages have been implicated as primary factors associated with AD (Saharan and Mandal, 2014). Thus, our study demonstrated that chrysin protected against this neurotoxic mechanism of Aβ on cellular redox state of the brain. These results corroborate previous research that reported that chrysin is able to mitigate the decrease of GSH levels and the increase of ROS levels in the cerebral cortex and hippocampus of a rat model of diabetes (Li et al., 2014), stressed (Filho et al., 2015) and aged-mice (Souza et al., 2015). In the light of our results, we have suggested that free radical scavenger action of chrysin was the major mechanism of neuroprotection. In particular, Sathiavelu et al. (2009) supported that chrysin possibly confers a protective effect by dampening the generation of free radicals. These researchers also reported that the hydroxyl group present in the fifth and seventh positions possibly contributes to its potent antioxidant effect (free radical scavenging property). Thus, our study demonstrated that CLN,

probably due to a higher accumulation of the drug in brain tissue consequent to the nanocapsule targeting, may be potentially useful in counteracting the oxidative modifications involved in the AD pathogenesis. However, the mechanism behind the translocation of polymeric nanoparticles into the brain remains to be precisely determined in future studies.

Increasing evidence suggests that neuroinflammation significantly contributes to the pathogenesis of AD (McGeer and McGeer, 2013). In particular, the production and secretion of proinflammatory mediators may contribute to the initiation and progression of neurodegeneration by a variety of mechanisms. Proinflammatory cytokines, such as IL-1β and TNF-α, may play an important role in AD pathology and lead to increased production ROS, and further neuronal dysfunction and death (Cunningham and Hennessy, 2015; Balducci and Forloni, 2014). In addition, these authors have reported that TNF-α is an important mediator of the Aβ-induced inflammatory response and cognitive impairment. Our results clearly show that mice treated with CLN5 exhibited decreased levels of IL-1β and TNF-α after Aβ exposure. In view of these results, we sought to evaluate whether CLN5 administration would be able to induce release of anti-inflammatory cytokine IL-10 to facilitate its anti-inflammatory effects. Furthermore, *in vitro* studies have shown that IL-10 suppressed $A\beta_{1-42}$-induced inflammatory cytokine production in murine microglia (Rege et al. 2014). In this way, our data demonstrate that CLN5 administration was able to break the neuroinflammation response induced by Aβ, culminating in the protective effect on oxidative stress and neurotrophic factor and attenuating memory deficits. Therefore, the anti-inflammatory property of chrysin, mainly in the nanocapsules formulation, could represent an additional mechanism of protection against $A\beta_{1-42}$-induced neurotoxicity.

In this study, the neuroprotective effects of chrysin were higher when it administered as loaded nanoparticles. In this way, the CLN improved drug concentrations in brain tissues, as well as pharmacologic efficacy. In summary, the present study demonstrated treatment

with chrysin, mainly in the nanocapsules formulation, was effective in attenuating the following impairments resulting from $A\beta_{1-42}$ exposure in mice: (1) impairment on memory in behavior tests; (2) increased RS levels and decreased NPSH levels in prefrontal cortex and hippocampus; (3) rise in GPx, GR and GST activities in prefrontal cortex and hippocampus; (4), reduced BDNF levels in prefrontal cortex and hippocampus; and (5) decreased TNF-α and IL-1β levels and reduced IL-10 levels in hippocampus and prefrontal cortex. Thus, the data reported herein clearly demonstrate that blockage of neuroinflammation and oxidative stress is involved in the neuroprotective effect of chrysin in this model of AD, suggesting that the nanoparticles formulation may provide a novel therapeutical approach for the treatment and prevention of AD.

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Legends

Fig. 1. Chrysin loaded nanocapsules image obtained by atomic force microscopy.

Fig. 2. Overview of protocol of experimental procedures.

Fig. 3. Effect of free or nanoencapsulated chrsysin on $A\beta_{1-42}$-induced memory impairment in the object recognition test (total exploration time: **Panel A** and recognition index: **Panel B**) in mice. Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; f1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** $P<0.05$ when compared PBS/V and PBS/BN. **b** $P<0.05$ when compared $A\beta_{1-42}$/V and $A\beta_{1-42}$/BN. (one-way ANOVA and Newman-Keul's test).

Fig. 4. Effect of free or nanoencapsulated chrsysin on $A\beta_{1-42}$-induced memory impairment in the step-down inhibitory avoidance task test (training sessions: **Panel A** and test sessions: **Panel B**) in mice. Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** $P<0.05$ when compared PBS/V and PBS/BN. **b** $P<0.05$ when compared $A\beta_{1-42}$/V and $A\beta_{1-42}$/BN. (one-way ANOVA and Newman-Keul's test).

Fig. 5. Effect of free or nanoencapsulated chrsysin on $A\beta_{1-42}$-induced memory impairment in the Morris water maze task (time to locate the platform area: **Panel A**; number of crossings of the platform location: **Panel B**; average speed: **Panel C** and total distance travelled: **Panel D**) in mice. Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg

p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** P<0.05 when compared PBS/V and PBS/BN. **b** P<0.05 when compared Aβ $_{1-42}$/V and Aβ $_{1-42}$/BN. **c** P<0.05 when compared PBS/FC5. **d** P<0.05 when compared PBS/CLN1. **e** P<0.05 when compared PBS/CLN5. **f** P<0.05 when compared $A\beta_{1-42}$ + FC5 (one-way ANOVA and Newman-Keul's test).

Fig. 6. Effect of free or nanoencapsulated chrsysin on BDNF (**Panel A**: cortex; **Panel B**: hippocampus) levels of mice injected with $A\beta_{1-42.}$ Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** P<0.05 when compared PBS/V and PBS/BN. **b** P<0.05 when compared Aβ $_{1-42}$/V and Aβ $_{1-42}$/BN. **c** P<0.05 when compared PBS/FC5. **d** P<0.05 when compared PBS/CLN1. **e** P<0.05 when compared PBS/CLN5. **f** P<0.05 when compared $A\beta_{1-42}$ + FC5 (one-way ANOVA and Newman-Keul's test).

Fig. 7. Effect of free or nanoencapsulated chrsysin on TNF-α (**Panel A**: cortex; **Panel B**: hippocampus), IL-1β (**Panel C**: cortex; **Panel D**: hippocampus) and IL-10 (**Panel E**: cortex; **Panel F**: hippocampus) levels of mice injected with $A\beta_{1-42.}$ Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** P<0.05 when compared PBS/V and PBS/BN. **b** P<0.05 when compared Aβ $_{1-42}$/V and Aβ $_{1-42}$/BN. **c** P<0.05 when compared PBS/FC5. **d** P<0.05 when compared PBS/CLN1. **e** P<0.05 when compared PBS/CLN5. **f** P<0.05 when compared $A\beta_{1-42}$ + FC5 (one-way ANOVA and Newman-Keul's test).

Fig. 8. Nanocapsules improve the brain distribution of chrysin. Each column represents mean ± SEM from four per group. FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **f** $P<0.05$ when compared $A\beta_{1-42}$ + FC5 (one-way ANOVA and Newman-Keul's test).

Figure 1

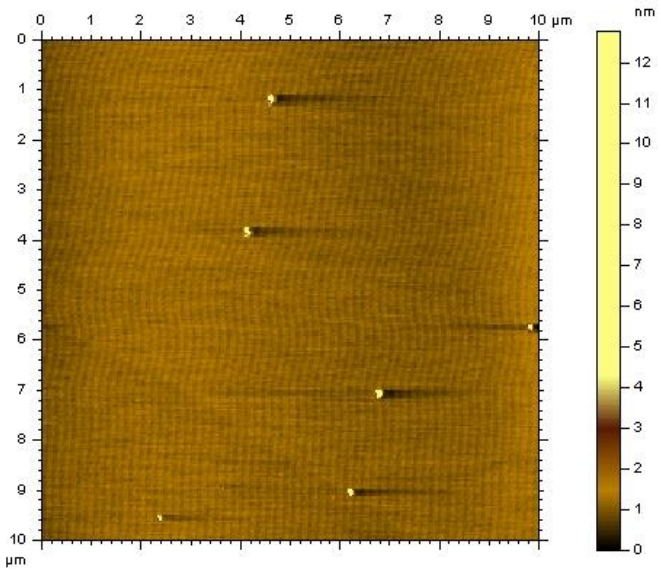


Figure 2

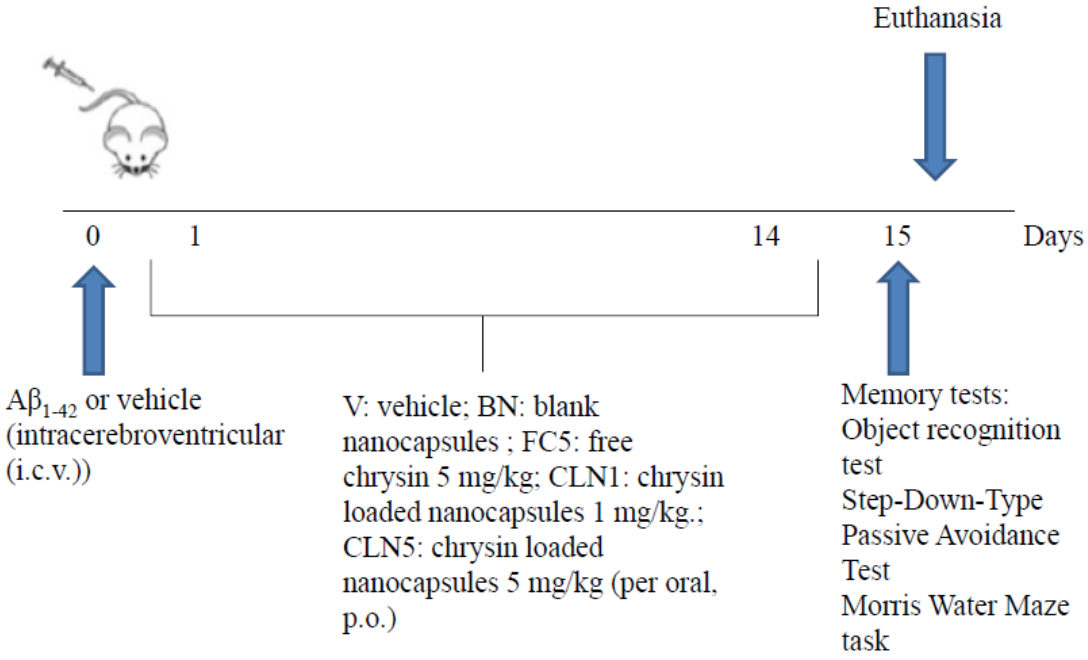

Euthanasia
0
1
14
15
Days
$A\beta_{1-42}$ or vehicle (intracerebroventricular (i.c.v.))
V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg; CLN1: chrysin loaded nanocapsules 1 mg/kg.; CLN5: chrysin loaded nanocapsules 5 mg/kg (per oral, p.o.)
Memory tests:
Object recognition test
Step-Down-Type Passive Avoidance Test
Morris Water Maze task

Figure 3

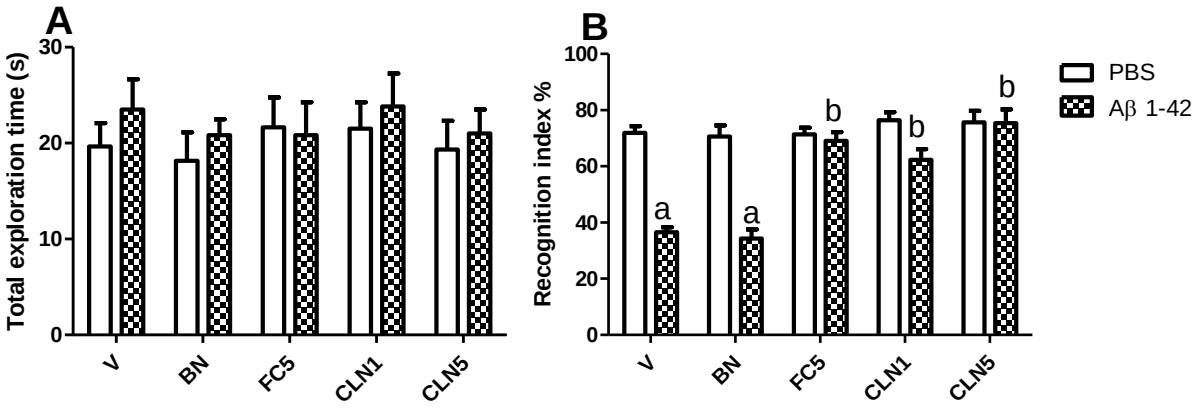
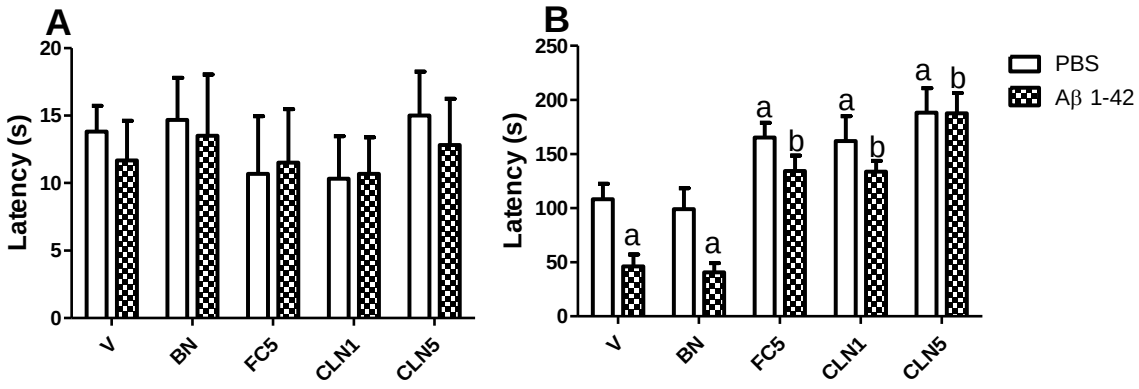

A
Total exploration time (s)
30
20
10
0
V
BN
FC5
CLN1
CLN5
B
Recognition index %
100
80
60
40
20
0
a
a
b
b
b
PBS
Aβ 1-42

Figure 4


A
Latency (s)
20
15
10
5
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V
BN
FC5
CLN1
CLN5
B
Latency (s)
250
200
150
100
50
0
a
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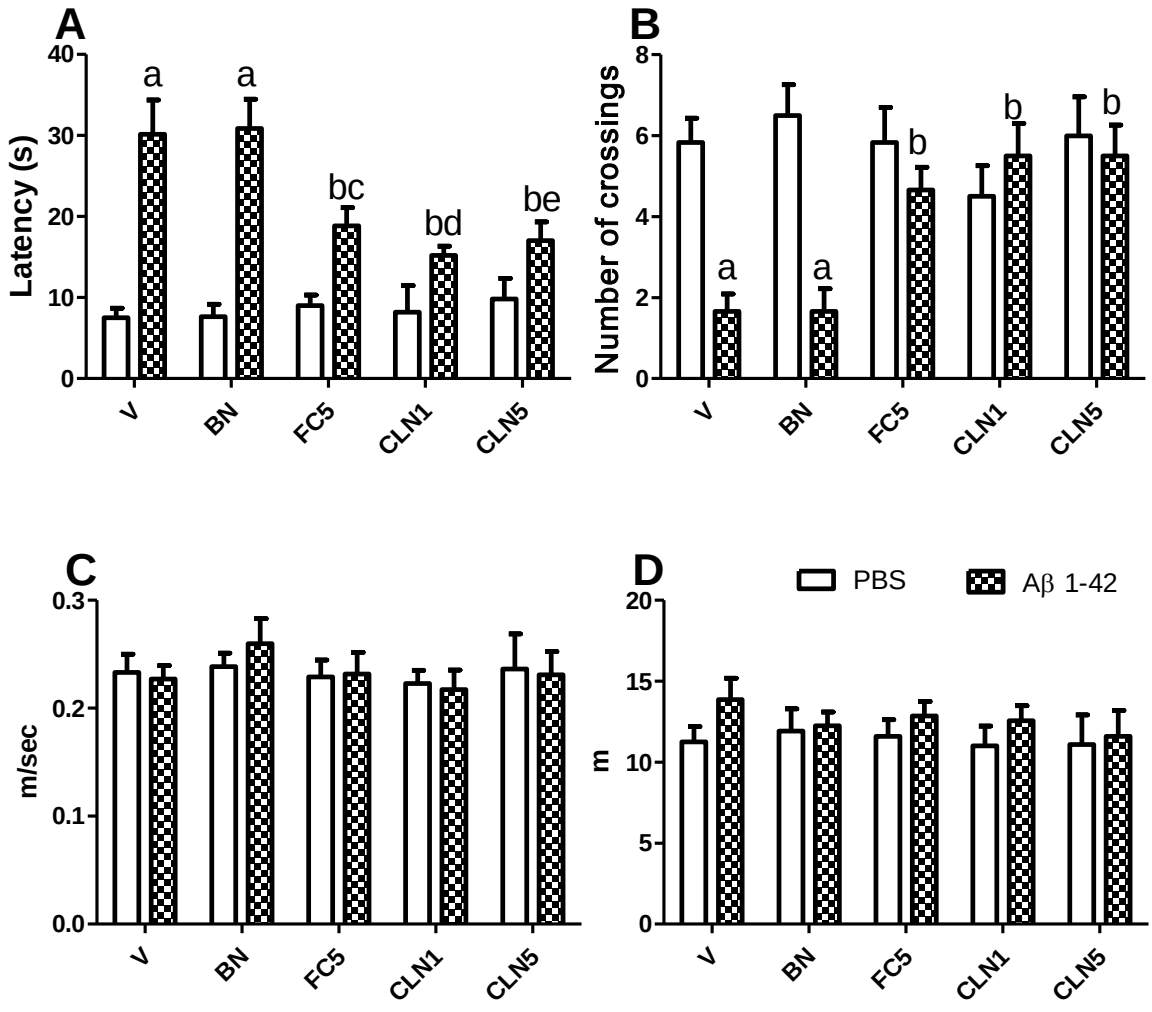
b
a
b
a
b
V
BN
FC5
CLN1
CLN5
PBS
Aβ 1-42

Figure 5


A
Latency (s)
40
30
20
10
0
a
a
bc
bd
be
V
BN
FC5
CLN1
CLN5
B
Number of crossings
8
6
4
2
0
a
a
b
b
b
V
BN
FC5
CLN1
CLN5
C
m/sec
0.3
0.2
0.1
0.0
V
BN
FC5
CLN1
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Aβ 1-42
m
20
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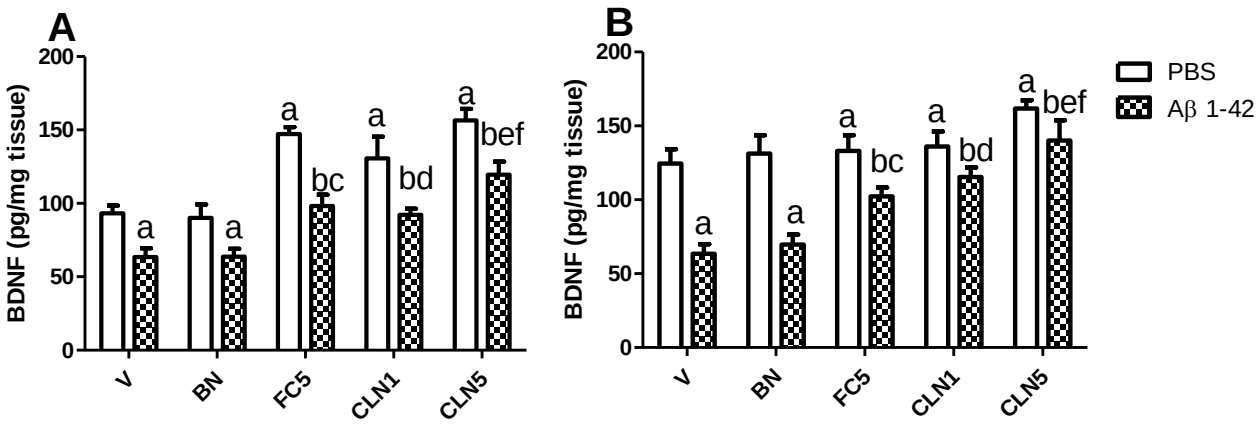
CLN1
CLN5

Figure 6


A
B
BDNF (pg/mg tissue)
200
150
100
50
0
a
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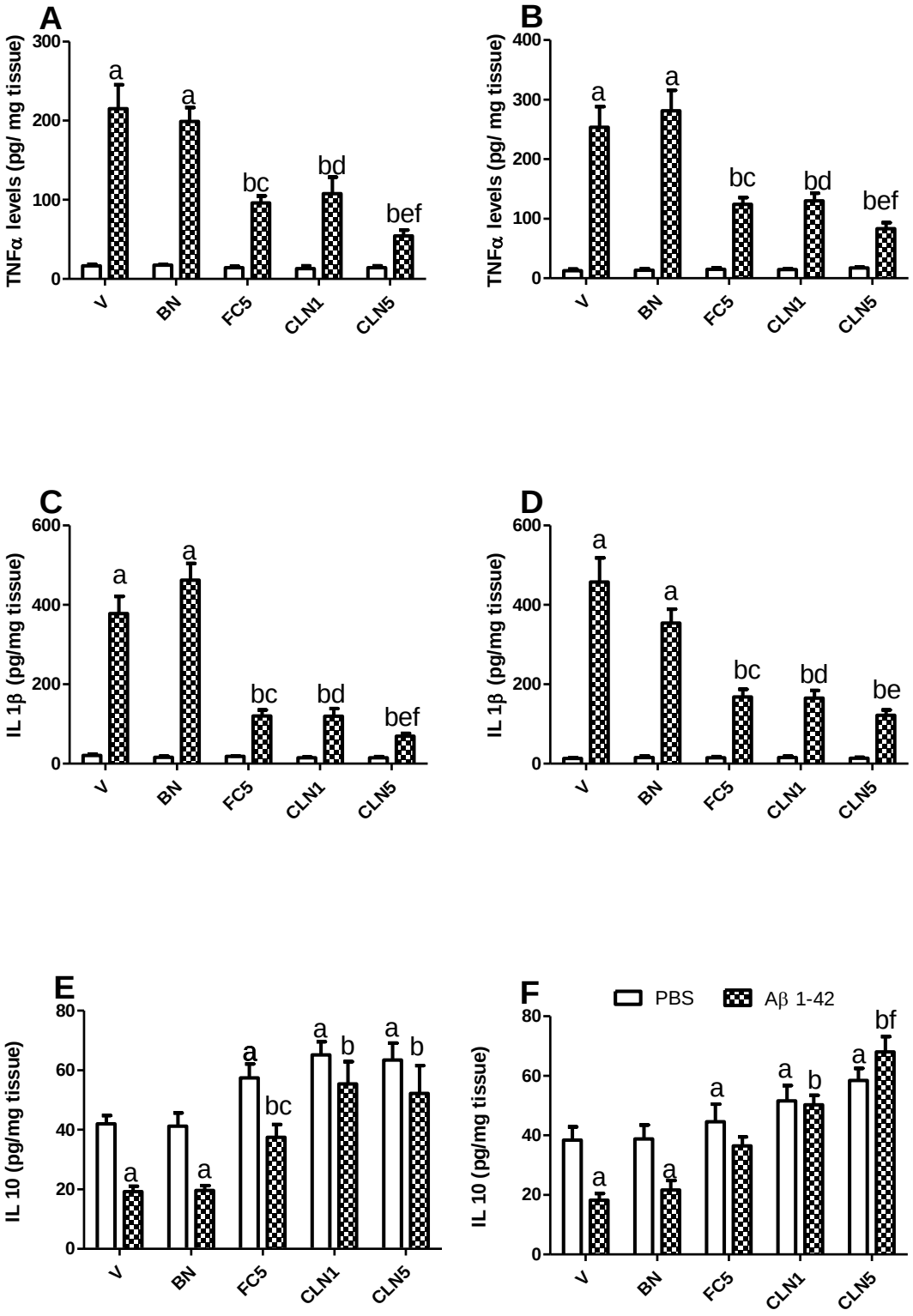
bef
V
BN
FC5
CLN1
CLN5
PBS
Aβ 1-42

Figure 7


A
TNFα levels (pg/ mg tissue)
300
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100
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a
a
bc
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FC5
CLN1
CLN5
B
TNFα levels (pg/ mg tissue)
400
300
200
100
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a
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bd
bef
V
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FC5
CLN1
CLN5
C
IL 1β (pg/mg tissue)
600
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IL 1β (pg/mg tissue)
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IL 10 (pg/mg tissue)
80
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CLN1
CLN5
F
PBS
Aβ 1-42
IL 10 (pg/mg tissue)
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FC5
CLN1
CLN5

Figure 8

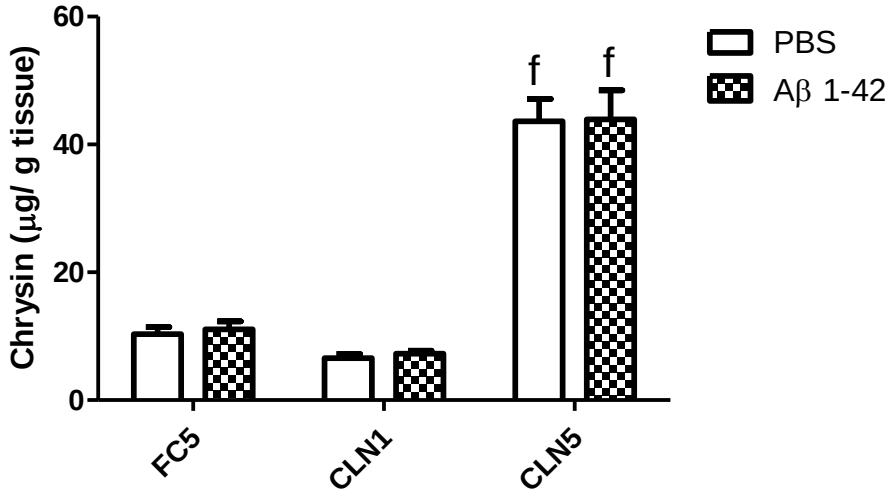

Table 1. Effect of treatment with free or nanoencapsulated chrsysin on NPSH and RS levels, and GR, GPx, GST and CAT activities in the prefrontal cortex of mice injected with $A\beta_{1-42}$.

Groups	NPSH (nmol GSH/g sue)	RS (RS levels (AU))	GR (nmolNADPH/min/ mg protein)	GPx (mmolNADPH/mi n/mg protein)	GST (nmolCDNB/min /mg protein)	CAT (Units/mg protein)
PBS + V	23.35 ± 1.50	14.31 ± 0.96	36.83 ± 2.33	47.33 ± 5.73	120.8 ± 7.19	6.21± 0.51
PBS + BN	23.07 ± 1.97	14.06 ± 1.33	38.83 ± 2.81	45.33 ± 5.46	117.0 ± 8.87	5.68 ± 0.87
PBS + FC5	21.87 ± 1.84	13.63 ± 1.23	38.50 ± 2.74	46.00 ± 4.19	115.3 ± 9.36	6.16± 0.53
PBS + CLN1	22.84 ± 1.84	16.08 ± 0.67	37.17 ± 3.68	46.17 ± 5.80	125.3 ± 16.7	6.78± 0.65
PBS + CLN5	26.17 ± 1.48	14.55 ± 1.29	35.00 ± 3.43	47.67 ± 3.94	118.0 ± 12.02	6.40 ± 0.64
$A\beta_{1-42}$ + V	13.65 ± 1.21[a]	40.07 ± 5.14[a]	60.83 ± 3.66[a]	103.7 ± 6.14[a]	220.7 ± 12.32[a]	6.96± 5.96
$A\beta_{1-42}$ + BN	10.78 ± 1.28[a]	41.15 ± 5.44[a]	55.67 ± 5.40[a]	108.7 ±11.33[a]	212.0 ± 13.56[a]	5.96 ± 0.76
$A\beta_{1-42}$ + FC5	19.65 ± 1.21[bc]	24.22 ± 2.28[bc]	48.50 ± 1.94[b]	69.67 ± 6.08[bc]	231.8 ± 10.31[a]	6.68± 0.45
$A\beta_{1-42}$ + CLN1	20.26 ± 1.15[bd]	23.02 ± 3.40[bd]	45.17 ± 2.27[bd]	65.67 ± 3.93[bd]	213.5 ± 8.99[a]	7.93 ± 0.49
$A\beta_{1-42}$ + CLN5	25.17 ± 1.32[bf]	17.70 ± 1.47[bf]	35.00 ± 2.12[bf]	51.83 ± 3.83[bf]	230.8 ± 11.25[a]	6.18 ± 0.81

Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** $P<0.05$ when compared PBS/V and PBS/BN. **b** $P<0.05$ when compared $A\beta_{1-42}$/V and $A\beta_{1-42}$/BN. **c** $P<0.05$ when compared PBS/FC5. **d** $P<0.05$ when compared PBS/CLN1. **e** $P<0.05$ when compared PBS/CLN5. **f** $P<0.05$ when compared $A\beta_{1-42}$ + FC5 (one-way ANOVA and Newman-Keul's test).

Table 2. Effect of treatment with free or nanoencapsulated chrsysin on NPSH and RS levels, and GR, GPx, GST and CAT activities in the hipocampus of mice injected with $A\beta_{1-42}$.

Groups	NPSH (nmol GSH/g tissue)	RS (RS levels (AU))	GR (nmolNADPH/min/mg protein)	GPx (mmolNADPH/min/mg protein)	GST (nmolCDNB/min/mg protein)	CAT (Units/mg protein)
PBS + V	25.32 ± 1.37	14.02 ± 0.91	26.33 ± 1.56	36.01 ± 3.03	122.0 ± 9.77	6.11± 0.56
PBS + BN	23.28 ± 2.08	14.08 ± 1.01	27.33 ± 3.45	38.67 ± 3.16	123.3 ± 9.57	7.01 ± 0.79
PBS + FC5	23.67 ± 2.27	12.55 ± 1.00	26.50 ± 2.23	33.50 ± 4.80	129.8 ± 6.07	6.90 ± 0.66
PBS + CLN1	22.98 ± 1.41	13.28 ± 1.04	29.50 ± 2.62	38.67 ± 2.69	107.0 ± 8.51	7.16 ± 0.43
PBS + CLN5	22.25 ± 1.92	12.08 ± 0.62	28.67 ± 3.57	37.05 ± 3.21	117.2 ± 12.82	6.05 ± 0.37
$A\beta_{1-42}$ + V	12.51 ± 1.22[a]	39.63 ± 2.75[a]	55.33 ± 3.35[a]	88.17 ± 3.52[a]	250.2 ± 10.85[a]	6.88 ± 0.65
$A\beta_{1-42}$ + BN	11.47 ± 1.12[a]	38.26 ± 4.29[a]	57.50 ± 4.46[a]	87.17 ± 4.80[a]	245.8 ± 11.82[a]	7.03 ± 0.65
$A\beta_{1-42}$ + FC5	19.72 ± 1.14[b]	26.13 ± 1.76[bc]	39.83 ± 1.53[bc]	65.17 ± 3.71[bc]	227.3 ± 15.93[a]	6.20± 0.69
$A\beta_{1-42}$ + CLN1	18.50 ± 1.06[b]	25.57 ± 2.14[bd]	36.00 ± 1.78[bd]	62.67 ± 5.03[bd]	271.2 ± 19.81[a]	7.13 ± 0.65
$A\beta_{1-42}$ + CLN5	25.89 ± 1.33[bf]	18.62 ± 1.80[bf]	33.33 ± 3.12[bf]	51.52 ± 4.62[be]	233.0 ± 15.65[a]	6.13± 0.39

Each column represents mean ± SEM from eight per group. V: vehicle; BN: blank nanocapsules ; FC5: free chrysin 5 mg/kg p.o.; CLN1: chrysin loaded nanocapsules 1 mg/kg p.o.; CLN5: chrysin loaded nanocapsules 5 mg/kg p.o. **a** $P<0.05$ when compared PBS/V and PBS/BN. **b** $P<0.05$ when compared $A\beta_{1-42}$/V and $A\beta_{1-42}$/BN. **c** $P<0.05$ when compared PBS/FC5. **d** $P<0.05$ when compared PBS/CLN1. **e** $P<0.05$ when compared PBS/CLN5. **f** $P<0.05$ when compared $A\beta_{1-42}$ + FC5 (one-way ANOVA and Newman-Keul's test).

PARTE III

CONCLUSÃO

O presente estudo demonstrou que os efeitos neuroprotetores da crisina foram potencializados quando esta foi administrada veiculada em nanocápsulas poliméricas, as quais foram capazes de melhorar as concentrações do ativo nas estruturas cerebrais. O tratamento via oral por 14 dias com crisina, principalmente na formulação de nanopartículas, foi eficaz em atenuar as seguintes deficiências resultantes da exposição dos camundongos à $A\beta_{1-42}$:

- O comprometimento da memória em testes comportamentais;
- O aumento dos níveis de RS, IL-1β, TNF-α no córtex pré-frontal e hipocampo;
- Diminuição dos níveis de NPSH, BDNF e IL-10 no córtex pré-frontal e hipocampo;
- O aumento da atividade de GR e GPx no córtex pré-frontal e hipocampo;

Em conclusão, os dados aqui relatados demonstram que a atenuação da neuroinflamação e do estresse oxidativo está envolvido no efeito neuroprotetor da crisina neste modelo de DA, além disso, sugerem que a formulação de nanopartículas potencializa seus efeitos, o que pode fornecer uma nova abordagem terapêutica para o tratamento e prevenção de DA.

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ANEXO

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **009/2015**

Título: **Avaliação dos efeitos de nanocápsulas poliméricas contendo crisina em modelo de alzheimer em camundongos**

Data da aprovação: **30/04/2015**

Período de vigência do projeto: De: **04/2015** Até: **04/2018**

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