UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

RESVERATROL: INCORPORAÇÃO EM NANOCÁPSULAS E NEUROPROTEÇÃO NA TOXICIDADE INDUZIDA PELO PEPTÍDEO β-AMILÓIDE

RUDIMAR LUIZ FROZZA

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Orientadora: Profa. Dra. Christianne Gazzana Salbego

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"A mente que se abre a uma nova idéia

jamais voltará ao seu tamanho original."

Albert Einstein

Àqueles que são as minhas bases:

meus pais, Antônio e Lúcia,

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SUMÁRIO

APRESENTAÇÃO

Esta Tese está organizada em seções dispostas da seguinte maneira: *Introdução, Objetivos, Capítulos* (I, II e III – referentes a artigos científicos), *Discussão, Conclusões, Perspectivas* e *Referências Bibliográficas.*

A seção *Introdução* apresenta o embasamento teórico que nos levou a formular as propostas da Tese, as quais estão descritas na seção *Objetivos*.

A seção *Capítulos* contém os artigos científicos publicados, submetidos ou a serem submetidos, os quais estão apresentados de acordo com os objetivos específicos. Esta seção também apresenta os materiais, os métodos e as referências bibliográficas específicas de cada artigo e está dividida em *Capítulos I, II* e *III.* Estes trabalhos foram realizados no Laboratório de Neuroproteção e Sinalização Celular, coordenado pela Profa. Dra. Christianne Gazzana Salbego; no Laboratório de Sistemas Nanoestruturados para a Administração de Fármacos e no Laboratório de Micro- e Nanopartículas Aplicadas na Terapêutica, coordenados pelas Profas. Dra. Sílvia Stanisçuaski Guterres e Dra. Adriana Raffin Pohlmann, todos na Universidade Federal do Rio Grande do Sul (UFRGS).

A seção *Discussão* contém uma interpretação geral dos resultados obtidos nos diferentes artigos científicos.

A seção *Conclusões* aborda as conclusões gerais obtidas na Tese.

A seção *Perspectivas* aborda as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos dando continuidade a esta linha de pesquisa.

A seção *Referências Bibliográficas* lista as referências citadas na Introdução e Discussão da Tese.

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DISCUSSÃO

LISTA DE ABREVIATURAS

Aβ – peptídeo beta-amilóide (***A**myloid-**beta** peptide*)

Aβ1-42 – peptídeo beta-amilóide contendo 42 aminoácidos

AD – Doença de Alzheimer (***A**lzheimer **D**isease*)

ALT – alanina aminotransferase (A***l**anine amino**t**ransferase*)

AST – aspartato aminotransferase (***As**partate amino**t**ransferase*)

AMPA – α-amino-3hidroxi-5-metil-4-isoxazol (*α-amino-3hydroxy-5-methyl-4-isoxazole*)

ApoE ε4 – apolipoproteína E4 (***Apo**lipoprotein**E** ε4)*

APP – proteína precursora amilóide (***A**myloid **P**recursor **P**rotein*)

BACE-1 – β-secretase (***B**eta-Site **A**myloid Precursor Protein-**C**leaving **E**nzyme-1*)

BBB – barreira hemato-encefálica *(**B**lood-**B**rain **B**arrier)*

BHE – **B**arreira **H**emato-**E**ncefálica

CaMKII – cinase dependente de cálcio/calmodulina II (**C*a**lcium/cal**m**odulin-dependent Protein **K**inase **II**)*

cdk5 – proteína cinase dependente de ciclina 5 (***c**yclin-**d**ependent protein **k**inase **5**)*

CK-1α – caseína cinase-1alfa (***C**asein **K**inase-**1alpha**)*

CNS – sistema nervoso central *(**C**entral **N**ervous **S**ystem)*

COX-2 – ciclooxigenase-2 (**C***ycl**oo**xygenase-**2***)

DA – Doença de **A**lzheimer

DAMPs – padrões moleculares associados ao dano (***D**amage **A**ssociate **M**olecular **P**attern**s***)

DCF – diclorofluoresceína (D***ich**loro**f**luorescein*)

DCFH2-DA – 2',7'-diclorofluoresceína diacetato (***2',7'-dich**loro**f**luorescin **d**iacet**a**te*)

ELISA – ensaio de imunoabsorção enzimática (E*nzyme-**l**inked **i**mmunosorbent **a**ss**a**y)*

ERK – proteína cinase regulada por sinais extracelulares *(**E**xtracellular Signal-**R**egulated*

***K**inase)*

EtOH – etanol (ethanol)

FDA - ***F**ood and **D**rug **A**dministration*

GFAP - proteína glial fibrilar ácida (***G**lial **F**ibrillary **A**cidic **P**rotein*)

HBSS - solução salina balenceada de Hank's *(**H**ank's **B**alanced **S**alt **S**olution)*

GSK-3β - glicogênio sintase cinase-3beta (***G**lycogen **S**ynthase **K**inase-**3beta**)*

γ-GT – Gama-glutamil transferase *(***Gamma-g**lutamyl**t**ransferase)

HPLC – cromatografia líquida de alta eficiência *(**H**igh-**P**erformance **L**iquid **C**hromatography)*

IB_4 – isolectina B_4 *(**I**solectin **B_4**)*

IDE – enzima que degrada insulina (***I**nsulin-**D**egradin **E**nzyme*)

ILs – interleucinas (***I**nter**l**eukine**s***)

IL-1 – interleucina-1 *(**I**nter**l**eukin**-1**)*

IL-1β – interleucina-1beta *(**I**nter**l**eukin**-1beta**)*

Il-6 – interleucina-6 *(**I**nter**l**eukin**-6**)*

IL-10 – interleucina-10 *(**I**nter**l**eukin**-10**)*

iNOS – óxido nitrico sintase induzível *(**i**nducible **N**itric **O**xide **S**ynthase)*

INF-α – interferon-alfa (***In**ter**f**eron-**alpha***)

JNK – proteína cinase c-Jun N-terminal *(c-**J**un **N**-terminal **K**inase)*

LDL – lipoproteínas de baixa-densidade *(**L**ow **D**ensity **L**ipoprotein)*

LI – índice lesional *(**L**esional **I**ndex)*

LNC – nanocápsulas sem a droga *(load-off **L**ipid-core **Nanoc**apsules)*

LRPs - receptor relacionado à proteína de baixa densidade (***L**DL-**R**eceptor-related **P**rotein*)

LRP-1 – receptor relacionado à proteína de baixa densidade-1 (***L**DL-**R**eceptor-related **P**rotein-**1**)*

LTP – potencial de longa duração (***L**ong-**T**erm **P**otentiation*)

MAPs – proteínas associadas aos microtúbulos (***M**icrotubule **A**ssociated **P**roteins family)*

MAPKs – proteínas cinase mitógeno-ativadas (***M**itogen-**A**ctivated **P**rotein **K**inase)*

MCP-1 – proteína quimioatraente de monócitos (***M**onocyte **C**hemoattractant **P**rotein-**1***)

MEM – meio essencial mínimo *(**M**inimum **E**ssential **M**edium)*

NF-κB – fator nuclear kappa B (**N***uclear **F**actor **κ**-light-chain-enhancer of activated **B** cells*)

MIP-1 – proteína-1 inflamatória de macrófagos (***M**acrophage **I**nflammatory **P**rotein-**1***)

NMDA – receptores glutamatérgicos (***N**-**m**ethyl-**D**-**a**spartate*)

MRPs – proteínas relacionadas à resistência a múltiplas drogas (***M**ultidrug **R**esistance-Related **P**roteins*)

NAD – nicotinamida adenina dinucleotídeo (***N**icotinamide **A**denine **D**inucleotide*)

NO – óxido nítrico *(**N**itric **O**xide)*

NLRs – receptores do tipo NOD (***NOD-l**ike **r**eceptors*)

PAMPs – padrões moleculares associados a patógenos (***P**athogen-**A**ssociated **M**olecular **P**atterns*)

PBS – tampão fosfato *(**P**hosphate **B**uffered **S**aline)*

PCL – polímero poli ε-caprolactona *[**P**oly(ε-**c**apro**l**actone)]*

PEG – poli**e**tileno**g**licol

PGP – glicoproteína P *(**P**-**g**lico**p**rotein)*

PI – iodeto de propídeo *(**P**ropidium **I**odide)*

PI3K – fosfatidilinositol 3-cinase *(**P**hospho**i**nositide 3-**k**inase)*

PKA – proteína cinase A (***P**rotein **K**inase **A**)*

RAGEs – receptores para produtos finais de glicação avançada (***R**eceptor for **A**dvanced **G**lycation **E**nd Products*)

ROS – espécies reativas de oxigênio (***R**eactive **O**xygen **S**pecies*)

RSV – *trans*-resveratrol (free *trans*-resveratrol)

RSV-LNC – *trans*-resveratrol em nanocápsulas lipídicas (*trans*-**r**e**s**veratrol-loaded **l**ipid-core **n**ano**c**apsules)

SAPKs – proteínas cinases ativadas por estresse (***S**tress-**A**ctivated **P**rotein **K**inases*)

SIRT1 – sirtuína-1 (***Sirt**uin-**1***)

SNC - sistema **n**ervoso **c**entral

TGF-β – fator de crescimento tumoral-beta (***T**umoral **G**rowth **F**actor-**beta***)

TLRs - receptores do tipo Toll (***T**oll-**l**ike **R**eceptors*)

TLR2 - receptores do tipo Toll 2 (***T**oll-**l**ike **R**eceptor 2*)

TLR4 - receptores do tipo Toll 4 (***T**oll-**l**ike **R**eceptor 4*)

TNF-α - fator de necrose tumoral- alfa *(**T**umor **N**ecrosis **F**actor **alpha**)*

RESUMO

A doença de Alzheimer (DA) é uma devastadora desordem neurológica que afeta mais de 37 milhões de pessoas ao redor do mundo, caracterizada pelo progressivo dano cognitivo e pela perda da memória. Estas alterações clínicas são acompanhadas por alterações histológicas cerebrais características da doença, as quais incluem atrofia cerebral, perda de neurônios e disfunção sináptica secundárias à deposição extracelular do peptídeo beta-amilóide (Aβ) e à deposição intracelular de emaranhados neurofibrilares constituídos da proteína *tau*. Fármacos disponíveis para a terapia da DA apresentam efeito limitado e tratamentos utilizando um único medicamento ou a combinação de terapias que possam efetivamente parar ou modificar o curso da doença ainda não se encontram disponíveis. O resveratrol, um polifenol de origem vegetal, tem atraído considerável interesse devido aos seus potenciais benefícios à saúde humana. Diversos estudos têm demonstrado que o resveratrol possui propriedades anti-amiloidogênicas; entretanto, os efeitos biológicos *in vivo* do resveratrol são fortemente limitados devido a sua baixa biodisponibilidade, a qual representa uma barreira no desenvolvimento de aplicações terapêuticas do resveratrol. Neste contexto, o objetivo deste estudo foi desenvolver uma nova formulação capaz de superar a baixa solubilidade, a limitada estabilidade, a elevada metabolização e a baixa biodisponibilidade do resveratrol e avaliar o seu efeito frente à toxicidade induzida pelo Aβ em modelos *in vitro* e *in vivo*. Inicialmente, o isômero *trans* do resveratrol foi incorporado em nanocápsulas poliméricas de núcleo lipídico (RSV-LNC) e a distribuição destas nanocápsulas nos tecidos cerebral, hepático e renal foi avaliada após a administração intraperitoneal (i.p.) ou por gavagem em ratos saudáveis. As nanocápsulas apresentaram elevada capacidade de encapsulamento do resveratrol e animais tratados com RSV-LNC exibiram maior concentração do resveratrol no cérebro, no fígado e no rim quando comparados aos animais tratados com resveratrol livre (RSV) após administrações diárias pelas vias i.p. ou gavagem. Na sequência, nós comparamos o efeito do tratamento com RSV e RSV-LNC frente à toxicidade induzida pelo Aβ através da exposição de culturas organotípicas de hipocampo ao Aβ1-42 por 48 h. Tanto o tratamento prévio quanto o simultâneo das culturas com RSV ou RSV-LNC reduziram significativamente a morte celular induzida pelo Aβ, com o RSV-LNC apresentando efeito mais pronunciado. O pré-tratamento com ambos, RSV ou RSV-LNC, preveniu a produção de espécies reativas de oxigênio; entretanto, o tratamento simultâneo com RSV não protegeu as culturas do dano oxidativo. Ambos os tratamentos, prévio e simultâneo, com RSV-LNC bloquearam a neuroinflamação desencadeada pelo Aβ de maneira sustentada. Além disso, apenas os tratamentos com RSV-LNC foram capazes de aumentar a liberação da IL-10 mesmo na presença do Aβ e prevenir/reduzir a ativação glial e a fosforilação da JNK. Finalmente, nós avaliamos o efeito do resveratrol frente à toxicidade induzida pelo Aβ através da injeção intracerebroventricular do Aβ1-42 em ratos. Nós observamos que os animais que foram injetados com o Aβ1-42 exibiram um significativo défcit na memória, o qual foi acompanhado pela significativa redução nos níveis da sinaptofisina no hipocampo. É importante ressaltar que através do uso das nanocápsulas o resveratrol foi capaz de reduzir estes efeitos deletérios do Aβ1-42 enquanto o tratamento com RSV não foi capaz de proteger da toxicidade induzida pelo Aβ, o que pode ser explicado pelo robusto aumento na biodisponibilidade cerebral do resveratrol atingida pelo uso das nanocápsulas. Adicionalmente, a ativação astrocitária e microglial, bem como fosforilação da JNK desencadeada pelo Aβ foram reduzidas somente após o tratamento com RSV-LNC, enquanto ambos os tratamentos com RSV e RSV-LNC foram capazes de reestabelecer os distúrbios na sinalização mediada pela GSK-3β e a desestabilização da β-catenina desencadeadas pelo Aβ. Juntos, nossos resultados não somente confirmam o potencial do resveratrol no tratamento dos processos neurodegenerativos como também oferecem uma via efetiva para melhorar o efeito neuroprotetor do resveratrol através de um sistema nanocarreador. Estes resultados fornecem suporte para futuros estudos objetivando o entendimento dos mecanismos envolvidos no efeito neuroprotetor do resveratrol. Além disso, a combinação do resveratrol com o sistema de entrega mediado por nanocápsulas poliméricas de núcleo lipídico abrem novas possibilidades para o tratamento da doença de Alzheimer.

ABSTRACT

Alzheimer's disease (AD) is a devastating neurological disorder that affects more than 37 million people worldwide, characterized clinically by progressive impairments in cognition and memory. These clinical features are accompanied by characteristic histological changes in the brain, which include brain atrophy, loss of neurons and loss of synaptic function secondary to extracellular deposition of amyloid-beta peptide (Aβ) and intracellular deposition of neurofibrillary tangle composed of the microtubule-associated protein tau. Available drugs for AD therapy have small effect sizes and we still not have a single treatment or combination therapy that can effectively stop or reverse the relentless progression of AD. Resveratrol, a naturally occurring polyphenol, has attracted considerable interest for its beneficial potentials for human health. Several studies have been shown that resveratrol is associated with anti-amyloidogenic properties; however, the *in vivo* biological effects of resveratrol appear strongly limited by its low bioavailability, which is a barrier to the development of therapeutic applications. In this context, the present study was designed to develop a novel resveratrol formulation to overcome its poor solubility, limited stability, high metabolization and weak bioavailability, and to evaluate the effects of resveratrol against *in vitro* and *in vivo* Aβ-induced toxicity. Initially, *trans*-resveratrol was loaded into lipid-core nanocapsules (RSV-LNC) and the nanocapsule distribution in brain, liver and kidney tissues was evaluated by intraperitoneal (i.p.) and gavage routes in healthy rats. Lipid-core nanocapsules showed high entrapment of resveratrol and animals treated with RSV-LNC displayed a higher resveratrol concentration in the brain, the liver and the kidney than those treated with free resveratrol (RSV) after daily i.p. or gavage administration. Next, we compared the effects of RSV and RSV-LNC treatment against Aβ-induced toxicity by expositing organotypic hippocampal cultures to Aβ1-42 by 48 h. Pre- and co-treatment of cultures with both, RSV and RSV-LNC, significantly attenuated Aβ-induced cell death, with RSV-LNC showing somewhat higher potency. Reactive oxygen species formation was prevented by pretreatment with both RSV or RSV-LNC; however, co-treatment with RSV failed to protect cultures from oxidative damage. Pre- and co-treatment with RSV-LNC was able to block the neuroinflammation triggered by Aβ in a sustained pattern. Furthermore, only RSV-LNC treatments were able to increase IL-10 release even in the presence of Aβ, and prevent/decrease glial activation and JNK phosphorylation. Finally, we evaluated the effects of resveratrol against Aβ-induced toxicity by using an intracerebroventricular injection of Aβ1-42 model in rats. We found that Aβ1-42-injected animals showed a significant impairment on learning-memory ability, which was paralleled by a significant decrease in hippocampal synaptophysin levels. Noteworthy, by using lipid-core nanocapsules, resveratrol was able to rescue these deleterious effects of Aβ1-42 while treatment with RSV failed to protect against Aβ-induced toxicity, which can be explained by robust increase of brain bioavailability of resveratrol achieved by lipid-core nanocapsules. Additionally, activated astrocytes and microglial cells, as well as JNK phosphorylation triggered by Aβ was reduced only after RSV-LNC treatment, while both RSV and RSV-LNC treatments were able to restore the disturbance in GSK-3β signaling and destabilization of β-catenin triggered by Aβ. Taken together, our results not only confirm the potential of resveratrol in treating neurodegenerative processes but also offer an effective way to improve the neuroprotective efficiency of resveratrol by nanocarrier delivery system. These findings provide further support for future studies aiming at precisely understanding of mechanisms involved in the neuroprotective effects of resveratrol. Furthermore, the combination of resveratrol and lipid-core nanocapsules-based delivery system may open new avenues for the treatment of Alzheimer’s disease.

INTRODUÇÃO

1. Doença de Alzheimer

A doença de Alzheimer (DA) é a mais comum forma de doença neurodegenerativa relacionada à idade e constitui um dos mais devastadores diagnósticos que um paciente e seus familiares podem receber. Caracterizada pela perda da memória, desorientação espacial e redução da capacidade intelectual, a DA foi relatada pela primeira vez há mais de 100 anos atrás, mais precisamente em 1906, quando o psiquiatra e patologista alemão Alois Alzheimer, no XXXVII Congresso Germânico de Psiquiatria em Tübingen, Alemanha, apresentou o primeiro caso de uma paciente, Auguste D, 50 anos de idade, a qual havia falecido em abril do mesmo ano, apresentando uma doença mental incomum (Burns e cols., 2002). Durante a análise patológica do tecido cerebral *post-morten* desta paciente, Alzheimer observou uma acentuada atrofia cerebral acompanhada por densos depósitos extracelulares de diversas fibrilas e emaranhados intracelulares (Burns e cols., 2002; Blennow e cols., 2006). Quatro anos após, em 1910, Emil Kraepelin, também psiquiatra alemão, cunhou o epônimo doença de Alzheimer, na oitava edição do seu Manual de Psiquiatria (Burns e cols., 2002). Embora mais de um século tenha se passado desde o primeiro relato da DA, a pesquisa em seus sintomas, causas, fatores de risco e tratamento só ganhou impulso nos últimos 30 anos. Enquanto a pesquisa revelou muito sobre a DA, com a exceção de certas formas hereditárias da doença, sua(s) causa(s) ainda permanecem desconhecidas.

1.1. Incidência

Os avanços na área biomédica proporcionaram um aumento na expectativa de vida humana, a qual tem passado de aproximadamente 49 para mais de 70 anos. Dessa forma, um crescente número de indivíduos alcança a idade em que as doenças neurodegenerativas tornam-se comuns. Dentre estas, as demências têm crescido a níveis alarmantes, sendo a DA

responsável por mais que 60% dos casos (Ritchie e Lovestone, 2002; Blennow e cols., 2006). A maioria dos casos de DA são esporádicos, onde o maior fator de risco é o envelhecimento. Em menor número, cerca de 5%, encontram-se os casos da DA de origem familiar, ocasionados por mutações genéticas, nas quais o início da doença ocorre a partir dos trinta anos de idade (Mattson, 2004; Selkoe, 2004). Progressivamente incapacitante, o paciente com a DA pode conviver com esta desordem durante muitos anos, sendo a média de oito, mas podendo chegar até vinte anos (Alzheimer's Association, 2011). Mundialmente, estima-se que o número de pessoas afetadas pela DA ultrapasse a marca de 35 milhões (Querfurth e LaFerla, 2010). Atualmente, estima-se que apenas nos Estados Unidos 5,4 milhões de pessoas tenham DA. Destes, cerca de 5,2 milhões possuem mais de 65 anos de idade, configurando a maior prevalência de DA esporádica, enquanto 200 mil estejam com idade inferior a 65 anos, representando os casos da DA de origem familiar (Alzheimer's Association, 2011). Esta elevada incidência configura uma alarmante proporção onde uma em cada oito pessoas acima dos 65 anos ou quase uma em cada duas pessoas acima dos 85 anos esteja afetada pela DA (Alzheimer's Association, 2011). Sob o ponto de vista econômico, foi projetado que o custo anual de mais de 180 bilhões de dólares em 2011 ultrapasse 1,1 trilhão de dólares em 2050 apenas nos Estados Unidos (Alzheimer's Association, 2011). Em virtude do envelhecimento global da população, estima-se que 2/3 dos portadores da DA vivam em países em desenvolvimento. No Brasil, a incidência e a prevalência da DA seguem as estimativas mundiais, estimando-se que até 2025 em torno de 1,2 milhões de pessoas desenvolverão a DA (Lopes e Bottino, 2002; Aprahamian e cols., 2009).

1.2. Aspectos fisiopatológicos

A análise histopatológica *post-mortem* de cérebros de pacientes com DA revela, como previamente descrito por Alois Alzheimer, a presença de alterações moleculares

características, as quais incluem os emaranhados neurofibrilares intracelulares compostos por agregados fibrilares da proteína *tau (τ)* hiperfosforilada e oxidada e as placas senis ou neuríticas, as quais são constituídas por depósitos extracelulares de fibrilas e por agregados amorfos do peptídeo β-amiloide (Aβ) (Figura 1) (Hamdame e cols., 2003; Forman e cols. 2004; Mattson, 2004; Selkoe, 2004).

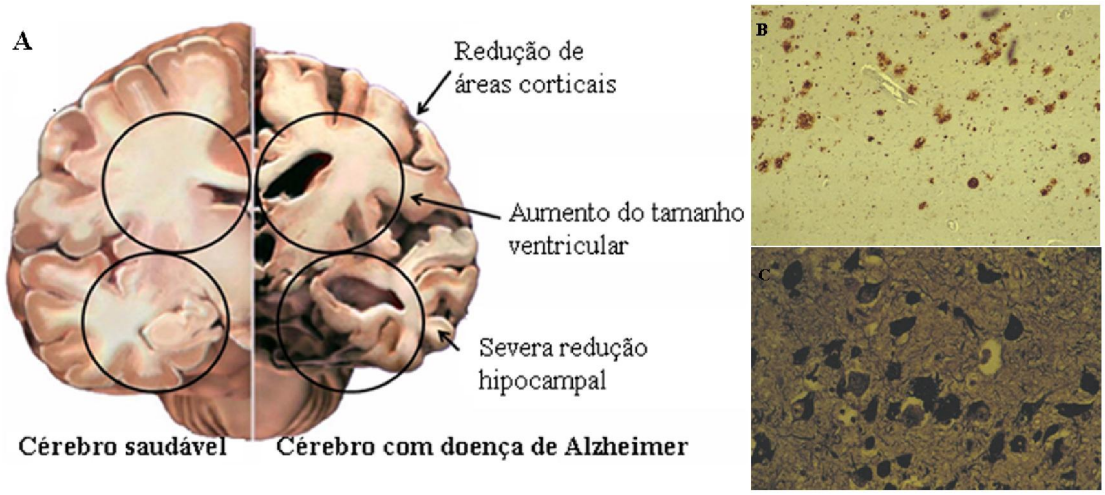

Figura 1. Alterações morfológicas observadas na doença de Alzheimer. (A) Comparação entre cérebro de paciente com DA (direita) e cérebro saudável (esquerda). É possível observar uma considerável redução cortical, aumento no tamanho dos ventrículos e redução hipocampal no cérebro com DA. (B) Placas senis constituídas por depósitos extracelulares do Aβ. (C) Emaranhados intracelulares constituídos pela proteína *tau* hiperfosforilada. Adaptado de Lee e cols., 2010.

A deposição do Aβ nas regiões cerebrais envolvidas com as funções cognitivas desencadeia uma cascata de eventos patológicos resultando na disfunção sináptica, na perda sináptica e na morte neuronal (Walsh e Selkoe, 2004). Além da perda neuronal e de substância branca, angiopatia congofílica (amilóide), inflamação e dano oxidativo também estão presentes (Querfurth e LaFerla, 2010). É importante ressaltar que na prática clínica, mesmo após os avanços dos últimos anos, o diagnóstico da DA é feito por exclusão de outras possíveis causas de demências, sendo o diagnóstico definitivo da DA realizado *post-mortem*.

Os emaranhados neurofibrilares são inclusões filamentosas encontrados em neurônios piramidais que ocorrem tanto na DA quanto em outras doenças neurodegenerativas

denominadas taupatias (Lee e cols., 2001). O principal componente destes emaranhados é uma forma anormalmente hiperfosforilada e agregada da proteína *tau (τ)*, uma fosfoproteína constituinte da família das proteínas associadas aos microtúbulos (*Microtubule Associated Proteins family* – MAPs) e que apresenta múltiplos sítios de fosforilação (Mandelkow e cols., 2003; Forman e cols., 2004; Gong e cols., 2005; Gong e cols., 2006). Abundante na sua forma solúvel nos axônios, a *tau* promove a estabilização dos microtúbulos e do transporte vesicular, mas quando hiperfosforilada torna-se insolúvel, perde sua afinidade pelos microtúbulos e acumula-se anormalmente em filamentos helicoidais pareados (Goerdt e cols., 2006; Querfurth e LaFerla, 2010), como pode ser observado na Figura 2.

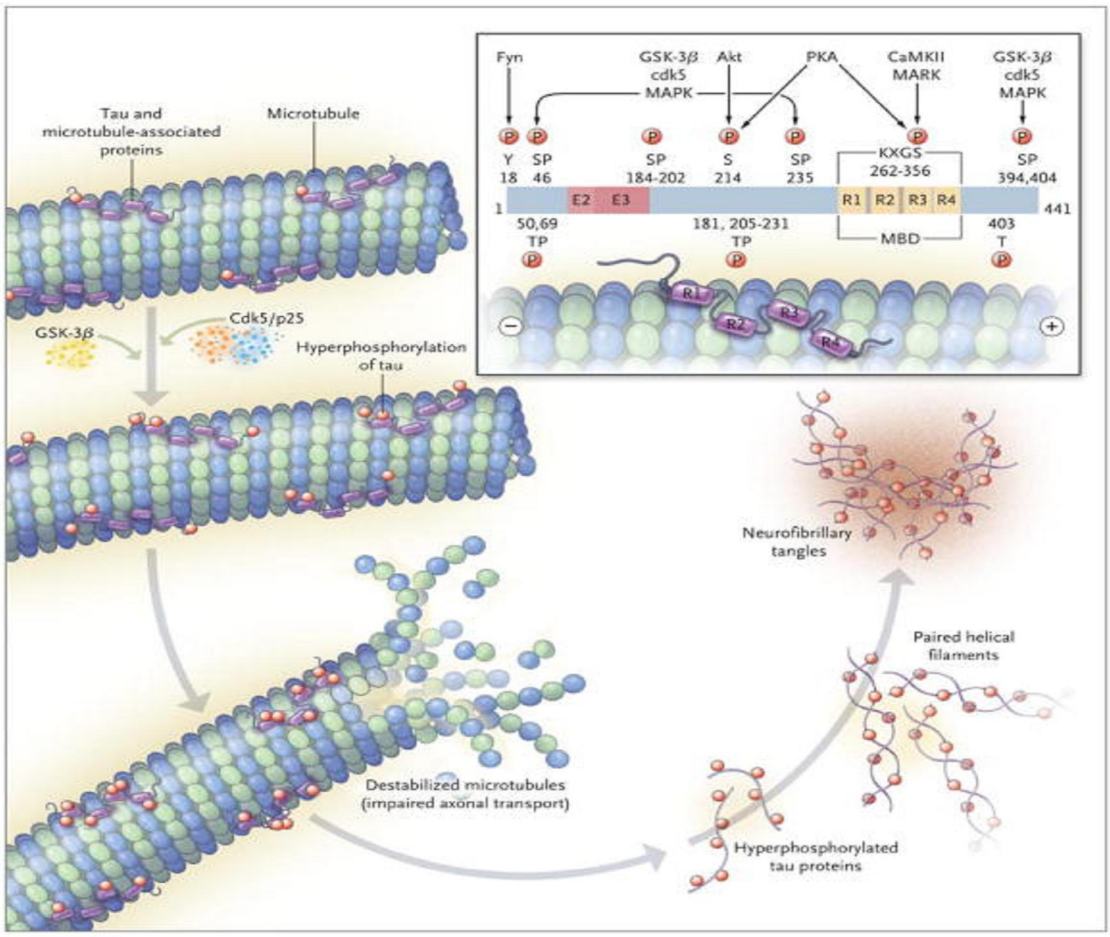

Figura 2. Estrutura e função da *tau*. A hiperfosforilação da *tau* altera sua afinidade pelos microtúbulos, levando a sua agregação e formação dos filamentos helicoidais pareados que se depositam nos emaranhados neurofibrilares intracelulares. Extraído de Querfurth e LaFerla, 2010.

Assim como o Aβ, agregados intermediários da *tau* hiperfosforilada são citotóxicos e alteram os processos cognitivos (Santacruz e cols., 2005; Oddo e cols., 2006). Acredita-se que a fosforilação anormal da *tau* deve-se a eventos diretos, tais como a elevada ativação de proteínas cinases ou a diminuição da atividade de proteínas fosfatases, mutações genéticas e modificações covalentes; ou eventos indiretos, como a toxicidade mediada pelo Aβ, o estresse oxidativo e a inflamação (Iqbal e cols., 2005; Ballatore e cols., 2007; McNaull e cols., 2010). Estudos sugerem que várias enzimas, tais como a glicogênio sintase cinase-3β (GSK-3β - *Glycogen Synthase Kinase-3β*), a proteína cinase dependente de ciclina 5 (cdk5 - *cyclin-dependent protein kinase 5*), a proteína cinase A (PKA - *Protein Kinase A*), a proteína cinase dependente de cálcio/calmodulina II (CaMKII – C*alcium/calmodulin-dependent Protein Kinase II*), a caseína cinase-1 (CK-1 - *Casein Kinase-1*), as proteínas cinase mitógeno-ativadas (MAPKs -*Mitogen-Activated Protein*) ERK 1/2 e as proteínas cinases ativadas por estresse (SAPKs - *Stress-Activated Protein Kinases*) participam na fosforilação da *tau* (Iqbal e cols., 2005, Iqbal e cols., 2009), como mostrado na Figura 2. A perda da estabilização dos microtúbulos pode levar a distúrbios na função estrutural e regulatória do citoesqueleto, dessa forma, comprometendo o transporte axonal e, assim, contribuindo para a disfunção sináptica e para a neurodegeneração (Roy e cols., 2005; Trojanowski e cols., 2005). Além disso, o aumento nos níveis de emaranhados neurofibrilares pode exacerbar a resposta imunológica e levar ao estresse inflamatório (Lee e cols., 2010). O nível de emaranhados neurofibrilares e o aumento dos níveis da *tau* no líquido cefalorraquidiano constituem potenciais marcadores patológicos da DA (Mattsson e cols., 2009; Hampel e cols., 2010; Humpel, 2011).

O Aβ, principal componente das placas senis, é um fragmento de 36 a 43 aminoácidos gerado pelo processamento proteolítico da proteína precursora amilóide (APP – *Amyloid Precursor Protein*) (Mattson, 2004; LaFerla e cols., 2007; Querfurth e LaFerla, 2010). A APP é uma glicoproteína amplamente expressa por todos os tipos celulares e em diferentes

isoformas, alcançando um tamanho de 695 a 770 aminoácidos. A APP constituída de 695 (APP695) é a isoforma mais abundante no SNC e produzida principalmente por neurônios (Mattson, 2004). A clivagem e o processamento da APP podem ser divididos em duas vias: a via não-amiloidogênica e a via amiloidogênica. Na via de processamento prevalente, a via não-amiloidogênica, a APP sofre processamento inicial pela α-secretase onde a clivagem ocorre na região central do domínio do peptídeo Aβ (entre os resíduos 10 e 17), dessa forma prevenindo sua geração. Alternativamente, a geração do Aβ ocorre a partir do processamento proteolítico amiloidogênico da APP pela ação seqüencial das enzimas β-secretase ou BACE-1 (*Beta-Site Amyloid Precursor Protein-Cleaving Enzyme-1*), a qual processa a APP na porção N-terminal; e a γ-secretase, a qual é um complexo enzimático com a presenilina 1 no centro catalítico do complexo e processa a APP na porção C-terminal (Mattson, 2004; Haass e Selkoe, 2007; LaFerla e cols., 2007; Querfurth e LaFerla, 2010). Ao final do processamento da APP pela via amiloidogênica, o Aβ é gerado (Figura 3).

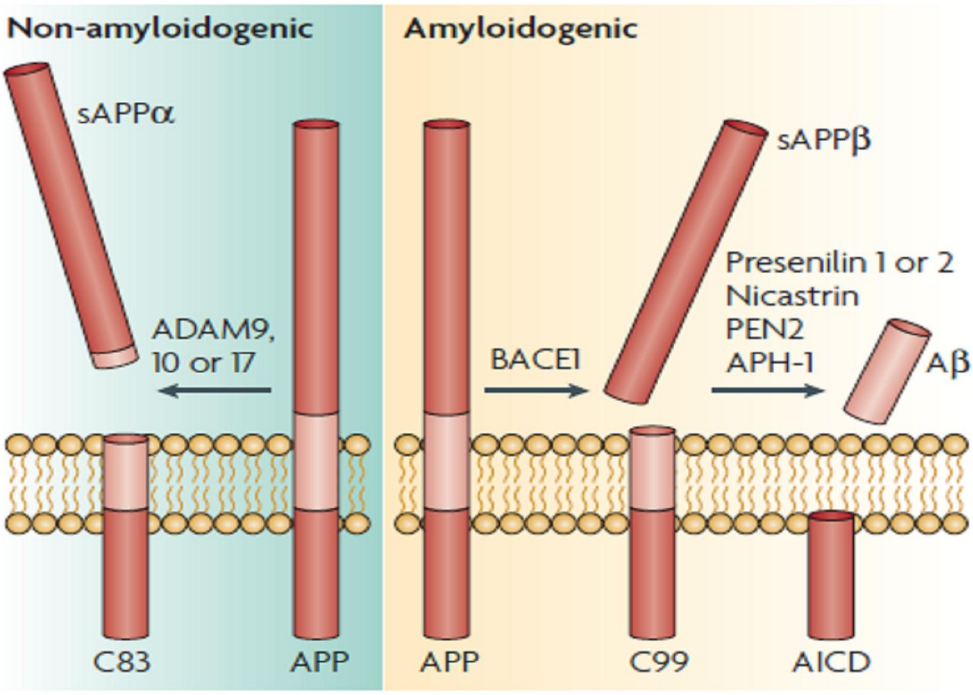

Figura 3. Processamento da Proteína Precursora Amilóide (APP). No processamento não-amiloidogênico a APP sofre ação inicial da α-secretase onde a clivagem ocorre na região central do domínio do peptídeo Aβ, dessa forma prevenindo sua geração. No processamento

amiloidogênico, a APP sofre ação inicial da β-secretase gerando ao final da seqüência proteolítica o peptídeo Aβ. Extraído de LaFerla e cols., 2007.

A maior quantidade de peptídeo Aβ produzido possui 40 aminoácidos (Aβ1-40), enquanto uma pequena porção, aproximadamente 10%, contém 42 aminoácidos (Aβ1-42). O peptídeo Aβ1-42 é a forma mais hidrofóbica e mais propensa à agregação, além de ser a isoforma predominante nas placas senis (Jarrett e cols., 1993; Mattson, 1997; Younkin, 1998; Selkoe, 2001). Ainda no estado monomérico, o peptídeo Aβ parece não ser neurotóxico, entretanto; espécies oligoméricas e protofibrilares são consideradas potentes bloqueadores de potenciais de longa duração (LTP) (Lambert e cols., 1998; Laferla e cols., 2007). Ainda nesta forma, o peptídeo Aβ pode sofrer degradação proteolítica por ação de peptidases como a gelatinase A (Yamada e cols., 1995), a gelatinase B (Howell e cols., 1995), a enzima que degrada insulina (IDE – *Insulin-Degradin Enzyme*) (Kurochkin e Goto, 1994; Carro e Torres-Aleman, 2004) e a neprilisina (Howell e cols., 1995). Além disso, níveis elevados da apolipoproteína E4 (ApoE ε4) contribuem para o acúmulo do Aβ. A principal hipótese do envolvimento da ApoE é a formação de um complexo ApoE/Aβ promovendo a retirada do peptídeo Aβ via proteína relacionada ao receptor de lipoproteína de baixa densidade – 1 (LRP-1 – *LDL-Receptor-related Protein-1*). Entretanto, a ApoE ε4 possui menor afinidade pelo peptídeo, reduzindo a capacidade de remoção do Aβ e promovendo, dessa maneira, o acúmulo e a formação de oligômeros do Aβ e das placas senis (Martins e cols., 2006; Bu, 2009). Um desequilíbrio entre a produção, a degradação e agregação do peptídeo leva ao acúmulo do Aβ e este excesso pode ser o fator iniciante da DA. Dessa forma, o acúmulo do Aβ desencadeia uma série de eventos celulares e moleculares que culminam na disfunção sináptica e na morte neuronal.

1.3. A hipótese da cascata amilóide

Isolado de cérebro de pacientes com DA a mais de 25 anos atrás (Glenner e Wong, 1984), o Aβ é reconhecido pela sua capacidade de agregação assumindo múltiplas formas físicas. A hipótese da cascata amilóide surgiu no final dos anos 80 (Allsop e cols., 1988; Selkoe, 1989) e foi formalizada em 1992, por Hardy e Higgins (Hardy e Higgins, 1992). A hipótese original atribui que a deposição de grandes fibrilas insolúveis formadas a partir da agregação do Aβ é um evento obrigatório, desencadeando diversas alterações celulares e moleculares que levam à morte celular e ao estabelecimento da demência (Ferreira e Klein, 2011; Karran e cols., 2011). Esta hipótese teve suporte a partir da co-localização das placas senis com neurônios em degeneração e da descoberta que a DA pode ser causada por mutações autossômicas dominantes nas presenilinas e no gene da APP, levando ao aumento na produção e na agregação do Aβ (Hardy e Higgins, 1992; Jarret e Lansbury, 1993; Karran e cols., 2011). É importante lembrar que essas mutações são observadas nos casos da DA de origem familiar e, embora estes casos representem a menor proporção de incidência da doença, em ambas as formas, esporádica e familiar, as alterações histopatológicas são as mesmas, dando suporte à hipótese. Na primeira versão da hipótese, acreditava-se que o Aβ induzia toxicidade somente quando na forma fibrilar, de modo que, *in vivo,* depósitos de placas de Aβ insolúvel constituíam uma marca da DA. Dessa forma, animais transgênicos que apresentavam anormalidades comportamentais características do modelo animal de DA, mesmo na ausência da deposição de placas senis, não eram considerados bons modelos para o estudo da DA (Klein e cols., 2001).

Devido aos estudos que sucederam essa proposta inicial, aliado à fraca correlação entre a quantidade das placas senis e à disfunção cognitiva, além das observações de que os depósitos de Aβ geralmente encontravam-se distantes dos sítios de perda neuronal (Terry e cols., 1994), uma nova versão foi elaborada. Na nova versão, a disfunção sináptica e os níveis de formas oligoméricas solúveis do Aβ constituem a melhor correlação com os distúrbios

cognitivos associados com a DA (Hardy e Selkoe, 2002). Essa correlação deve-se principalmente ao fato das formas oligoméricas do Aβ apresentarem a capacidade de se ligarem com grande especificidade a sítios sinápticos excitatórios causando uma intensa perda sináptica e uma grande diminuição na densidade de espinhos dendríticos (Lacor e cols., 2007). Além disso, os oligômeros solúveis do Aβ desencadeiam a ativação de células inflamatórias, como os astrócitos e a microglia, levando a uma intensa resposta inflamatória (Querfurth e LaFerla, 2010), além de desencadearem a produção de espécies reativas de oxigênio e de nitrogênio (De Felice e cols., 2007), alterações em diversas vias de sinalização celular (Lacor e cols., 2007), hiperfosforilação da *tau* (De Felice e cols., 2008), perda sináptica e redução da plasticidade sináptica hipocampal (Hsieh e cols., 2006; Selkoe, 2008). Diversos receptores protéicos têm sido apontados como capazes de se ligarem às várias formas do Aβ, incluindo o receptor nicotínico da acetilcolina 7α (α7nAChR -*α7 nicotinic acetylcholine receptor*) (Lilja e cols., 2011), os receptores glutamatérgicos NMDA (*N-methyl-D-aspartate*) e AMPA (*α-amino-3hydroxy-5-methyl-4-isoxazole*) (Snyder e cols., 2005; De Felice e cols., 2007; Shankar e cols., 2007), os LRPs (LRP – *LDL-Receptor-related Protein*) (Bu e cols., 2006), os receptores para produtos finais de glicação avançada (RAGEs - R*eceptor for Advanced Glycation End Products*) (Origlia e cols., 2009), os receptores de insulina (De Felice e cols., 2009), à proteína celular príon (Caetano e cols., 2011) e os receptores efrina (Selkoe, 2011), contribuindo para a alteração na sinalização celular. Além da ligação a esses receptores, a exposição de aminoácidos hidrofóbicos nos oligômeros de Aβ fazem com que estes apresentem uma maior afinidade à ligação em superfícies hidrofóbicas, como os lipídeos de membrana, ao invés de grandes ectodomínios hidrofílicos dos receptores protéicos. A sustentada ligação dos oligômeros às membranas celulares pode perturbar a fina estrutura da bicamada fosfolipídica, levando assim a um efeito biofísico secundário na estrutura e na

função dos receptores, o que pode contribuir para a resultante alteração da sinalização celular (Selkoe, 2011).

Embora a reformulação da cascata amilóide tenha colocado os oligômeros solúveis do Aβ exercendo um papel central no desenvolvimento e na progressão da DA, isso não significa que as placas não tenham um papel na degeneração neuronal progressiva, como evidenciado por oligômeros difusíveis do Aβ encontrados cercando as placas e por estarem intimamente associados à perda local de espinhos dendríticos e à distrofia neurítica (Knowles e cols., 1999; Koffie e cols., 2009). Além disso, a presença de dímeros e oligômeros maiores "presos" junto às placas do Aβ insolúvel sugere fortemente que estas placas servem como uma reserva local de pequenos oligômeros, e estes podem difundir para locais mais distantes das placas causando dano sináptico/neurítico (Shankar e cols., 2008; Selkoe, 2011).

1.4. Neuroinflamação na doença de Alzheimer

A neuroinflamação tem sido conhecida por exercer um papel central na patogenia de muitas doenças neurodegenerativas crônicas em geral e, particularmente, na DA. A presença do Aβ e dos emaranhados neurofibrilares no cérebro desencadeia a ativação de células inflamatórias e o aumento nos níveis teciduais de mediadores pró- e antiinflamatórios, incluindo citocinas e quimiocinas, os quais permanecem permanentemente alterados. Evidências da resposta inflamatória na DA incluem mudanças na morfologia das células microgliais, as quais passam de uma morfologia ramificada (em repouso) a uma amebóide (ativa), e da astrogliose reativa, manifestada pelo aumento no número, tamanho e mobilidade dos astrócitos (Wyss-Coray e Mucke, 2002; Cameron e Landreth, 2010; Glass e cols., 2010).

A microglia é composta ou representa as principais células do sistema imunológico residentes no sistema nervoso central (SNC), onde elas "vigiam" constantemente o microambiente cerebral e produzem fatores que influenciam os astrócitos e os neurônios

circunjacentes. Estas células atuam com a primeira e a principal defesa do tecido cerebral, controlando a resposta imunológica no SNC e protegendo as funções neurais (Heneka e O'Banion, 2007; Cameron e Landreth, 2010; Glass e cols., 2010). Entretanto, a microglia não possui apenas funções neuroprotetoras tais como a fagocitose, mas também exerce efeitos neurotóxicos (Streit e cols., 1999). Os astrócitos são células gliais com morfologia estrelar característica, sendo as células mais abundantes no SNC. Os astrócitos têm diversas capacidades funcionais, incluindo o suporte bioquímico das células endoteliais da bareira hemato-encefálica (BHE), o suprimento de nutrientes ao tecido nervoso, a manutenção da homeostasia iônica extracelular e a cicatrização após uma lesão traumática (Lee e cols., 2010). Assim como a microglia, os astrócitos também podem secretar uma variedade de moléculas pró- e antiinflamatórias, tais como interleucinas (ILs - *Interleukines*), prostaglandinas, leucotrienos, tromboxanos, fatores de coagulação, proteínas do sistema complemento, proteases e inibidores de proteases (Heneka e O'Banion, 2007; Whitney e cols., 2009; Lee e cols., 2010).

Na DA, a ativação prolongada e disseminada da microglia e dos astrócitos correlaciona-se com a extensão da atrofia cerebral (Cagnin e cols., 2001) e com o declínio cognitivo (Parachikova e cols., 2007). A maioria dos modelos da DA usando roedores transgênicos também exibe substancial gliose reativa e acúmulo de astrócitos ativados nas regiões cerebrais afetadas (Schwab e cols., 2010; Noble e cols., 2010). Essas características geralmente são observadas antes do surgimento das placas e dos emaranhados neurofibrilares (Schindowski e cols., 2006; Garwood e cols., 2010) e o grau de ativação das células inflamatórias correlaciona-se com o desenvolvimento da patologia e com a morte neuronal (Garwood e cols., 2010; Parachikova e cols., 2010). Além dos modelos transgênicos, os modelos onde a injeção do Aβ é realizada no cérebro de animais, camundongos ou ratos,

também levam ao desenvolvimento de uma intensa resposta inflamatória (Medeiros e cols., 2007; Ryu e McLarnon, 2008; Passos e cols., 2009).

A função da microglia e dos astrócitos no desenvolvimento e/ou na progressão da DA permanece controversa. Inicialmente, foi atribuída a essas células a função de formar uma cicatriz no tecido cerebral após uma lesão, bem como fagocitar depósitos de Aβ e resíduos de neurítos distróficos. Entretanto, tem sido amplamente reconhecido que os astrócitos e a microglia estão envolvidos ativamente na dinâmica dos eventos inflamatórios e neurodegenerativos que caracterizam a DA (Meda e cols., 2001). Astrócitos e microglia expressam uma classe de receptores de reconhecimento capazes de detectar agentes infecciosos e danos no SNC por meio da ligação destes a padrões moleculares associados a patógenos (PAMPs - *Pathogen-Associated Molecular Patterns*) e a padrões moleculares associados ao dano (DAMPs – *Damage Associate Molecular Patterns*), como evidenciado pelos receptores do tipo Toll (TLRs – *Toll-like Receptors*) (Glass e cols., 2010, Hensley, 2010). Além disso, astrócitos e microglia expressam receptores purinérgicos, os quais são capazes de responder ao ATP liberado pelas células em processo de morte (Di Virgilio e cols., 2009), bem como expressam uma série de receptores sequestradores, os quais estão envolvidos na captação de diversos substratos tais como lipídeos e proteínas oxidados e corpúsculos apoptóticos (Husemann e cols., 2002). A ligação dos padrões de reconhecimento a estes receptores leva à ativação de diversas vias de transdução de sinal que regulam vários processos transcricionais e pós-transcricionais, como a expressão da COX-2 (*Cyclooxygenase-2*), da iNOS (*inducible Nitric Oxide Synthase*), de membros da família NFκB (*Nuclear Factor kappa B*) e a produção de citocinas e quimiocinas, as quais amplificam a inflamação e recrutam células do sistema imunológico, respectivamente (Glass e cols., 2010; Hensley e cols., 2010).

A fagocitose inicial do Aβ pela microglia parece ser um mecanismo protetor; entretanto, a ativação crônica da microglia leva à liberação de quimiocinas e uma gama de citocinas pró-inflamatórias, principalmente IL-1, IL-6 e fator de necrose tumoral-α (TNF-α – *Tumor Necrosis Factor-α*), além de proteínas do sistema complemento (Akiyama e cols., 2000). Além disso, através da expressão de RAGEs, nos quais o Aβ pode se ligar, ocorre uma resposta de amplificação na produção de citocinas pró-inflamatórias, na liberação de glutamato e na produção de óxido nítrico (NO – *Nitric Oxide*) por meio da ativação da iNOS (Yan e cols., 1996; Floden e cols., 2005; Tuppo e Arias, 2005). Além da influência da microglia, os astrócitos exercem um importante papel na regulação da morte neuronal e na resposta inflamatória induzida pelo Aβ (Meda e cols., 2001; Lee e cols., 2010; Hensley, 2010; Garwood e cols., 2011). Ao mesmo tempo em que os astrócitos produzem fatores pró-inflamatórios ocorre uma alteração no seu fenótipo metabólico, gerando um déficit energético e elevando a liberação de peróxido de hidrogênio, o que pode aumentar a vulnerabilidade neuronal (Gavillet e cols., 2008). Como pode ser observado na Figura 4, ambos, astrócitos e microglia, liberam uma gama de citocinas pró- e antiinflamatórias, incluindo TNF-α, TNF-β, IL-1β, IL-6, IL-10, interferon-α (INF-α), INF-β, bem como as quimiocinas IL-8, MIP-1 (*Macrophage Inflammatory Protein-1*) e MCP-1 (*Monocyte Chemoattractant Protein-1*), além de NO, espécies reativas de oxigênio (ROS – *Reactive Oxygen Species*) e prostaglandinas (Meda e cols., 2001; Wyss-Coray e cols., 2003; Cartier e cols., 2005; Glass e cols., 2010). A liberação desses mediadores leva ao recrutamento de monócitos e de linfócitos através da BHE (Lossinsky e Shivers, 2004; Simard e cols., 2006), bem como estimula o estabelecimento um processo de reatro-alimentação positiva, estimulando a ativação microglial e astrocitária, promovendo a sua proliferação e resultando na liberação de mais fatores inflamatórios.

Os neurônios também parecem contribuir para a propagação da resposta inflamatória na DA, dado que eles podem secretar diversas citocinas e quimiocinas, as quais atuam como mensageiros entre os neurônios e as células gliais (Li e cols., 2000; Friedman, 2001; de Haas e cols., 2007; Biber e cols., 2008). Os mediadores pró-inflamatórios secretados pelos neurônios podem, de fato, acentuar o processo neuroinflamatório e levar ao dano neuronal observado na DA. Além disso, neurônios expressam CD200 na sua superfície o qual interage com seu ligante correspondente, CD200L, na microglia. Esta interação atua inibindo a produção de mediadores pró-inflamatórios pela microglia. Entretanto, os níveis de ambos CD200 e CD200L estão reduzidos no córtex e no hipocampo de pacientes com DA (Walker e cols., 2009).

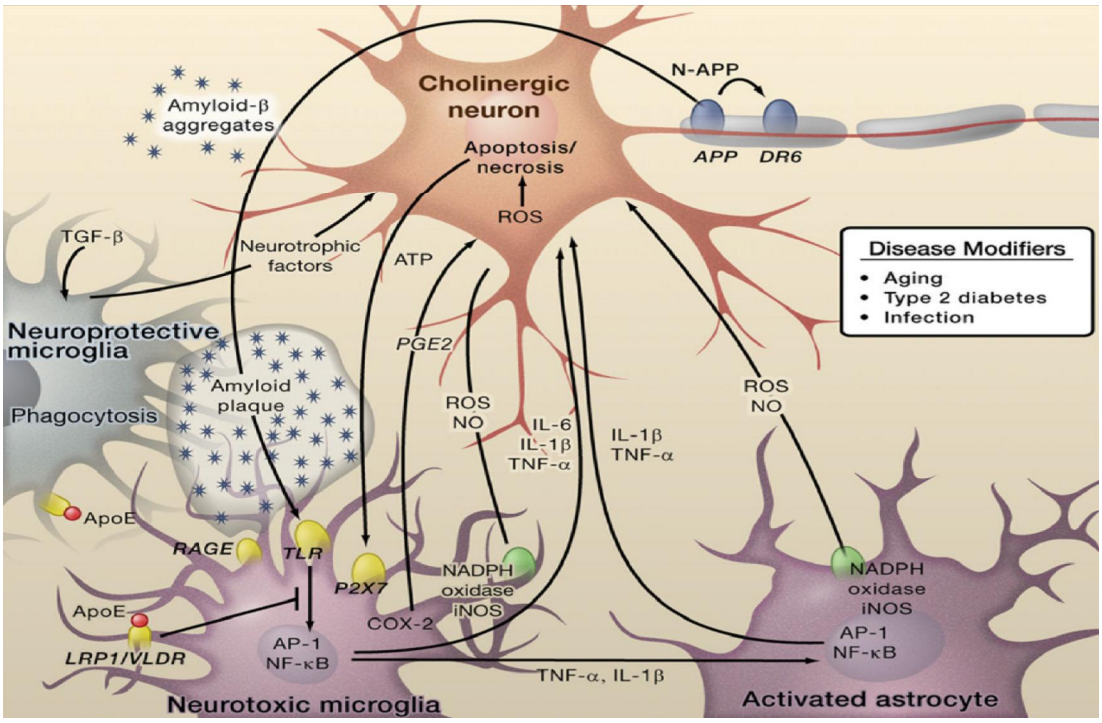

Figura 4. Inflamação na doença de Alzheimer. O peptídeo Aβ desencadeia a ativação da microglia levando a secreção de diversas quimiocinas e citocinas pró-inflamatórias e a produção de espécies reativas de oxigênio. Esses fatores inflamatórios atuam diretamente sobre os neurônios e estimulam os astrócitos, os quais amplificam a resposta inflamatória levando ao efeito neurotóxico. A morte neuronal resulta na liberação de ATP, o qual ativa a microglia levando a formação de um ciclo vicioso. Extraído de Glass e cols., 2010.

1.5. Terapia

Alterar o curso da DA poderia levar a um significativo benefício de saúde pública. Uma intervenção que poderia retardar o início da DA em dois anos diminuiria a incidência de tal maneira que em cinquenta anos haveria dois milhões a menos dos casos que estão sendo atualmente projetados (Brookmeyer e cols., 1998). A principal consequência da alteração do curso da DA seria a diminuição da necessidade dos cuidados exigidos pela doença, além de permitir que o paciente permaneça com suas atividades normais por um período maior de tempo. Considerando que os custos associados aos cuidados necessários aos pacientes com DA aumentam com a severidade da doença (Zu e cols., 2006), se os pacientes permanecessem nos estágios iniciais haveria um grande benefício para o paciente, para sua família e para a sociedade. Entretanto, apesar do progresso na terapia sintomática, um método terapêutico que interfira com o processo neurodegenerativo progressivo envolvido na DA ainda não se encontra disponível. Além disso, a eficácia de um fármaco que atue no SNC depende da sua capacidade em atravessar a BHE e de alcançar concentrações terapêuticas no cérebro após sua administração sistêmica, o que tem retardado o desenvolvimento de novas modalidades terapêuticas para doenças neurodegenerativas.

Atualmente, apenas cinco medicamentos estão aprovados pelo FDA (*Food and Drug Administration*) para o tratamento da DA. Quatro destes são inibidores da acetilcolinesterase (donepezil, galantamina, rivastigmina e tacrina - esta com uso limitado devido aos efeitos adversos) e o quinto é um inibidor dos receptores NMDA (memantina). É importante ressaltar que esses medicamentos apenas melhoram os sintomas clínicos e os sintomas comportamentais, funcionais e cognitivos, mas eles não curam nem modificam o curso da DA. Embora novas abordagens terapêuticas para a DA apontem para a utilização de antioxidantes, estatinas, agentes antiinflamatórios, quelantes de metais, inibidores das secretases, moduladores da agregação e da remoção do Aβ e da *tau* e imunoterapia (Scarpini e cols., 2003; Van Marum, 2008; Creed e Milgram, 2010; Neugroschl e Sano, 2010), os ensaios

clínicos baseados na biologia subjacente da doença têm revelado resultados insatisfatórios (Blennow, 2011). Além dos inúmeros ensaios clínicos utilizando um arsenal de novas drogas com potencial terapêutico, o papel da dieta na prevenção da DA vem ganhando atenção nos últimos anos. Dessa forma, a enorme diversidade de funções de compostos naturais, dentre estes o resveratrol, pode fornecer uma nova geração de drogas com potencial capacidade de prevenção e/ou o tratamento da DA (Howes e cols., 2003; Howes e Houghton, 2003; Bastianetto e Quirion, 2004; Anekonda e Reddy, 2005).

2. Resveratrol

O resveratrol (3,4',5-trihidroxiestilbeno) é um metabólito secundário produzido por mais de 70 espécies vegetais e encontrado em muitos alimentos naturais tais como uva, frutas vermelhas, amendoim, nozes, entre outras (Harikumar e Aggarwal, 2008). Desde a sua descoberta, o resveratrol tem demonstrado diversas propriedades farmacológicas que podem ser empregadas na medicina.

Isolado pela primeira vez de raízes de heléboro branco (*Veratrum grandiflorum O. Loes*) em 1940 (Takaoka, 1940) e, mais tarde, em 1963, da raiz de *Polygonum cuspidatum*, uma planta usada na medicina oriental (Nomura e cols., 1963), o resveratrol atraiu pouco interesse até 1992, quando a ele foram atribuídas algumas propriedades cardioprotetoras associadas ao consumo de vinho tinto (Siemann e Creasy, 1992). Quimicamente, trata-se de um composto polifenólico derivado da fenilalanina, contendo dois anéis aromáticos com hidroxilas reativas em sua estrutura apresentando-se nas orientações *cis* e *trans* (Figura 5), sendo o isômero *trans* mais estável e responsável pelos efeitos biológicos (Soleas e cols., 1997).

O resveratrol é sintetizado nas plantas em resposta a estresses ambientais, como radiação UV, ataque de fungos e alterações climáticas bruscas, sendo classificado como uma fitoalexina (Langckage e Pryce, 1976). Em relação à uva, elevadas concentrações do resveratrol são produzidas em resposta ao ataque pelo fungo *Botrytis Cinera*, principalmente na epiderme da folha da planta e na casca e na semente do fruto. Entretanto, a concentração do resveratrol no vinho depende principalmente do método de produção (Creasy e Coffee, 1988; Signorelli e Ghidoni, 2005).

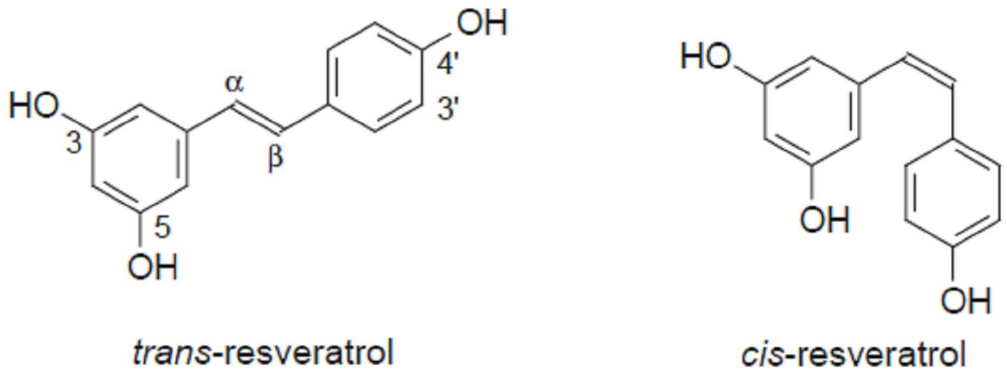

Figura 5. Estrutura química do resveratrol.

2.1. Efeitos biológicos e biodisponibilidade

Desde a década de 90, diversos estudos científicos têm relatado que o resveratrol apresenta um amplo espectro de atividades biológicas, incluindo efeitos cardioprotetores (Richard, 1987; Renaud e Lorergil, 1992), quimioprotetores (Jang e cols., 1997; Zamin e cols., 2009), neuroprotetores (Wang e cols., 2002; Zamin e cols., 2006; Simão e cols., 2011), antiinflamatórios (Birell e cols., 2005; Saiko e cols., 2008) e antioxidantes (Jang e Surh, 2001; Ovesna e cols., 2006), além de prolongar a sobrevivência de várias espécies (Howitz e cols., 2003; Valenzano e cols., 2006; Baur e cols., 2006).

O principal evento que desencadeou o intenso interesse pelo resveratrol está relacionado ao Paradoxo Francês. Este paradoxo teve origem a partir dos resultados de estudos epidemiológicos revelando uma correlação inversa entre o consumo de vinho tinto e a incidência de doenças cardiovasculares na população francesa, apesar do elevado consumo de gorduras saturadas (Renaud e Lorergil, 1992), sugerindo que o resveratrol poderia ser o princípio ativo do vinho tinto responsável, pelo menos em parte, por estes resultados. Desde então, diversos estudos epidemiológicos têm sugerido que o consumo do resveratrol pode estar associado à redução do risco de desenvolvimento das doenças neurológicas relacionadas à idade, dentre elas a DA (Orgogozo e cols., 1997; Lindsay e cols., 2002; Vingtdeux e cols., 2008).

Recentemente, alguns estudos têm demonstrado que o resveratrol possui atividade anti-amiloidogênica *in vitro* e *in vivo* (Han e cols., 2004; Marambaud e cols., 2005; Vingtdeux e cols., 2008; Karuppagounder e cols., 2009; Vingtdeux e cols., 2010); entretanto, os mecanismos envolvidos nos efeitos neuroprotetores do resveratrol ainda não são completamente conhecidos. Alguns trabalhos atribuem ao resveratrol a capacidade de reduzir a toxicidade do Aβ através da sua atividade antioxidante (Jang e Surh, 2003), por restaurar a alteração na sinalização celular mediada pela via da PKC (Han e cols., 2004), por promover a remoção do Aβ através da degradação via sistema proteassômico (Marambaud e cols., 2005) e por remodelar formas oligoméricas solúveis do Aβ diminuindo a sua toxicidade (Ladiwala e cols., 2010), além de reduzir a inflamação mediada pela ativação microglial (Capiralla e cols., 2012). Outra propriedade importante do resveratrol deve-se ao fato deste polifenol mimetizar os efeitos da restrição calórica, um fenômeno caracterizado pelo aumento da atividade das sirtuínas, tendo como consequência a prevenção dos processos de envelhecimento e o aumento da longevidade (Baur e cols., 2006). As sirtuínas são histonas deacetilases dependentes de NAD (*Nicotinamide Adenine Dinucleotide*), que participam de uma série de

modificações relacionadas à idade e estariam relacionadas à longevidade, promovendo a sobrevivência e a resistência ao estresse frente a situações adversas (Anekonda, 2006). Recentemente, tem-se mostrado que a ativação/superexpressão da SIRT1 leva a um aumento na expressão do gene da α-secretase diminuindo a produção do Aβ em camundongos (Donmez e cols., 2010). Considerando que o resveratrol atua sobre as sirtuínas, estes resultados abrem a possibilidade de um novo caminho para o desenvolvimento de uma modalidade terapêutica no tratamento da DA.

Em modelos animais da DA, a restrição calórica reduziu os sintomas da DA em camundongos, aumentou a produção de fatores neurotróficos e atenuou os déficits comportamentais em primatas (Saiko e cols., 2008). Ainda utilizando um modelo animal, camundongos transgênicos para a DA (Tg2576) exibiram uma redução na produção do Aβ e melhora no desempenho cognitivo após o consumo moderado de vinho Cabernet Sauvignon (Wang e cols., 2006). Considerando que os neurônios podem tornar-se protegidos frente a uma lesão mediante o aumento na expressão de sirtuínas, seja pelo efeito da restrição calórica ou mediado pelo tratamento com resveratrol (Sinclair, 2005; Anekonda e Reddy, 2006) e, que este composto tem apresentado efeitos neuroprotetores frente à toxicidade induzida pelo Aβ via redução da ativação microglial desencadeada pela ativação da sirtuína-1 (Chen e cols., 2005), o resveratrol tem sido colocado como uma molécula promissora na prevenção e no tratamento da DA. Entretanto, a aplicação terapêutica dos efeitos benéficos do resveratrol permanece muito limitada devido à sua baixa solubilidade, reduzida estabilidade, curta meia-vida, ao intenso metabolismo e à elevada taxa de excreção, levando a sua baixa biodisponibilidade.

O resveratrol apresenta baixa solubilidade em água (menos de 0,05 mg/ml), afetando sua distribuição nos tecidos; pode sofrer isomerização facilmente se exposto a luz, além de ser facilmente oxidado (Vian e cols., 2005; Piñeiro e cols., 2006). Grande parte dos

conhecimentos sobre a farmacocinética do resveratrol vem de estudos com animais; entretanto, estudos em humanos realizados nos últimos anos mostram resultados muito similares. As principais rotas de absorção, metabolização e eliminação do resveratrol estão ilustradas na Figura 6.

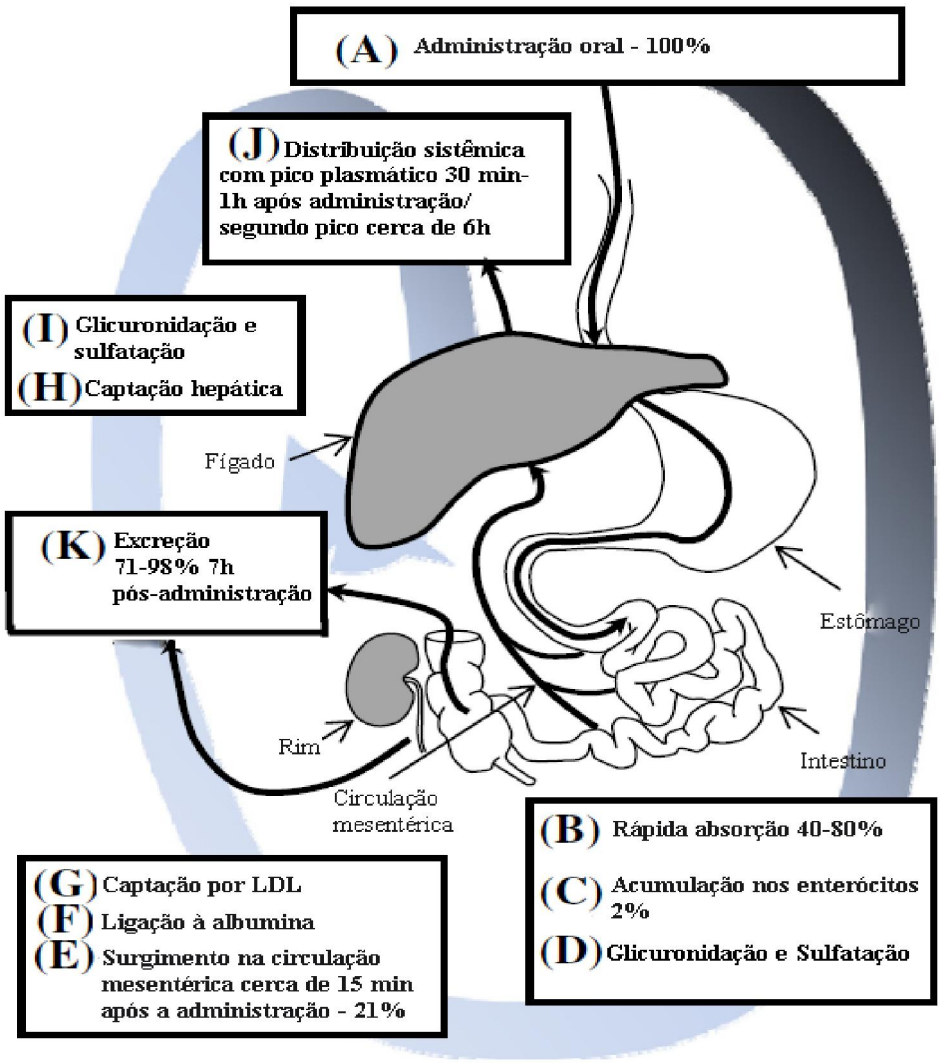

Figura 6. Destino do resveratrol após a administração oral. Esta ilustração tem como base resultados obtidos em estudos *in vitro*, *ex vivo* e *in vivo*, em animais e em humanos. O resveratrol administrado pela via oral **(A)** é absorvido pelo intestino como resultado da rápida difusão passiva **(B)** e apresenta baixa acumulação nos enterócitos **(C)**, onde inicia a formação de glicuroconjugados e sulfoconjugados **(D)**. O surgimento na circulação mesentérica ocorre em poucos minutos após a ingestão **(E)**, circula principalmente ligado a albumina **(F)** e a LDL **(G)**. Acumula-se no fígado **(H)** onde a formação de conjugados é acentuada **(I)**. O resveratrol atinge concentração plasmática máxima entre 30 e 60 minutos após a sua ingestão **(J)** e é excretado principalmente pela via renal **(K)**. Adaptado de Amri e cols., 2011.

A ingestão oral é a principal via de administração do resveratrol. Dessa forma, estima-se que cerca de 70% do resveratrol ingerido seja de absorvido no intestino humano como resultado da rápida difusão passiva (Walle e cols., 2004, Walle, 2011). Ainda no intestino, o resveratrol inicia o processo de metabolização, o qual consiste na formação de glicuroconjugados e sulfoconjugados (Andlauer e cols., 2000; De Santi e cols., 2000). A conversão do resveratrol nestes conjugados hidrofílicos pode facilitar sua entrada na circulação sanguínea, sua difusão através do corpo e, mais importante, sua excreção. Estes conjugados podem ser absorvidos pela vascularização ou eliminados no lúmen intestinal. Dessa maneira, devido ao intenso metabolismo a concentração plasmática do resveratrol livre é extremamente baixa (Walle e cols., 2004).

A maioria dos estudos focando na biodisponibilidade, na farmacocinética e no metabolismo do resveratrol revela que a concentração plasmática máxima é atingida entre 30 e 60 minutos após a sua ingestão (Walle e cols., 2004; Boocock e cols., 2007; Burkon e Somoza, 2008; Almeida e cols., 2009). Um segundo pico plasmático é observado 6 h após a ingestão do resveratrol, sugerindo recirculação enterohepática dos conjugados metabólicos por reabsorção seguido à hidrólise intestinal (Marier e cols., 2002; Walle e cols., 2004). Uma vez que o resveratrol apresenta baixa solubilidade em água, cerca de 90% do resveratrol absorvido na forma livre encontra-se fortemente ligado a proteínas plasmáticas, principalmente à albumina (Jannin e cols., 2004; Delmas e cols., 2011). Embora menos hidrofóbico que o resveratrol livre, grande parte de seus metabólitos é captada por LDL (*Low Density Lipoprotein*) na corrente sanguínea (Urpí-Sardà e cols., 2005). Pouco tempo após a ingestão oral, o resveratrol é encontrado no cólon, enquanto que a sua distribuição nos tecidos necessita de algumas horas. No fígado, o resveratrol acumula-se a uma concentração comparável àquela que exerce efeitos biológicos em ensaios *in vitro* (faixa micromolar) (Vitrac e cols., 2003) e a excreção renal é a principal rota de eliminação do resveratrol tanto

em animais quanto em humanos (Soleas e cols., 2001; Goldberg e cols., 2003; Vitrac e cols., 2003; Meng e cols., 2004; Urpí-Sardà e cols., 2005).

Baseado nos estudos em animais, o resveratrol é geralmente bem tolerado. Experimentos com humanos têm sido realizados apenas em curtos períodos de tempo e com curtas exposições ao resveratrol. Em um estudo em humanos, no qual 2 g de resveratrol foram administrados duas vezes por dia durante oito dias, alguns indivíduos apresentaram episódios de diarréia leve no início do tratamento e uma pessoa desenvolveu *rash* cutâneo e cefaléia (la Porte e cols., 2010). Em ratos, a administração de resveratrol por gavagem nas doses de 0,3, 1 e 3 g/kg/dia durante quatro semanas (doses equivalentes a 21, 70 e 210 g/dia em humanos de 70 kg) revelou efeitos adversos associados à nefrotoxicidade somente na maior dose (Crowell e cols., 2004).

Uma vez que o resveratrol é absorvido principalmente na forma conjugada e alguns trabalhos demonstram que estes metabólitos não apresentam efeitos biológicos (Wang e cols., 2004; Delmas e cols., 2011), alguns autores têm questionado os resultados descritos na literatura que atribuem a atividade anti-câncer e antiinflamatória ao resveratrol (Goldberg e cols., 2003). Dessa maneira, os potenciais terapêuticos do resveratrol poderão ser aplicados somente se as limitações associadas à baixa estabilidade, solubilidade e biodisponibilidade forem superadas. Atualmente tem se explorado diferentes meios para melhorar a biodisponibilidade do resveratrol, os quais incluem a co-administração com inibidores do seu metabolismo e o uso de análogos do resveratrol que apresentam melhor biodisponibilidade (Ndiaye e cols., 2011). Ainda neste contexto, um crescente número de estudos buscando o desenvolvimento de novas formulações tem surgido (revisado por Amri e cols., 2011). Entre estes, as estratégias baseadas na utilização da nanotecnologia têm recebido destaque.

3. Nanotecnologia

A nanotecnologia ou nanociência é um campo multidisciplinar da ciência aplicada e tem se tornado uma área prioritária na pesquisa científica e no desenvolvimento tecnológico nos últimos anos. O termo nanotecnologia refere-se à capacidade de medir, desenhar e manipular materiais na escala atômica, molecular e supramolecular, a fim de compreender, criar e aplicar estruturas e sistemas com funções específicas atribuídas a seu tamanho. Classicamente, a nanotecnologia aplica os princípios da engenharia, da eletrônica, da física e das ciências dos materiais na produção de sistemas nanoestruturados com tamanho compreendido na faixa entre 1 e 100 nm, mas muitas vezes é estendido para incluir materiais com tamanho inferior a 1 μm. O objetivo essencial da nanotecnologia é reunir nanopartículas e integrá-las em estruturas ordenadas de forma a obter materiais úteis. A nanotecnologia tem sido empregada por diversos setores industriais com múltiplas aplicações, entre as quais se destacam as áreas de sistemas de armazenamento eletrônico (Kang e cols., 1996), da biotecnologia (Pankhurst e cols., 2003) e de carreadores de genes e de fármacos (Sahoo e Labhasetwar, 2003; Dobson, 2006). A maioria dos nanomateriais atuais pode ser organizada em quatro tipos: materiais baseados em carbono; materiais baseados em metais; polímeros e dendrímeros; e compósitos (Lü e cols., 2009).

Os avanços na nanotecnologia têm levado ao desenvolvimento de novos nanomateriais cujas propriedades físico-químicas diferem dos seus homólogos maiores devido à sua maior relação superfície/volume, tornando-os excelentes candidatos para aplicações biomédicas (Boulaiz e cols., 2011). O emprego da nanotecnologia tem levado a abordagens inovadoras em diversas áreas da medicina. Suas aplicações na triagem, diagnóstico e tratamento de doenças são coletivamente referidas como nanomedicina, constituíndo um campo emergente e com potencial para revolucionar a área da saúde (Zang e cols., 2008). Com o emprego de

nanopartículas é possível fornecer terapia em um nível molecular, tratando a doença e aumentando a nossa compreensão sobre a sua patogênese.

O impacto da nanotecnologia na medicina pode ser observado principalmente nos métodos de diagnóstico, nas técnicas de entrega de fármacos e na medicina regenerativa. Métodos de diagnóstico são essenciais para a detecção precoce de doenças e para seu tratamento imediato, minimizando os possíveis danos ao resto do organismo. A importância dos métodos de imagem para diagnosticar, tratar e acompanhar o desenvolvimento do câncer e de pacientes com doenças cardiovasculares e neurológicas é bem conhecida. Técnicas de diagnóstico baseado no uso de nanopartículas oferecem maior sensibilidade e auxiliam na detecção precoce da doença, oferecendo um melhor prognóstico e maior possibilidade de sucesso do tratamento (Surendiran e cols., 2009). Medicamentos convencionais apresentam limitação, principalmente devido aos seus efeitos adversos, resultado da falta de especificidade da sua ação e da falta de eficácia, devido a doses inadequadas ou ineficazes, como é o caso da quimioterapia. Dessa forma, a nanotecnologia oferece a possibilidade de projetar novos medicamentos com maior especificidade celular e sistemas de liberação de fármacos que atuam seletivamente em alvos específicos, além de protegê-los da degradação. Isso permite a administração de doses menores porém mais eficazes, minimizando efeitos adversos. A nanotecnologia também pode ser utilizada para otimizar formulações de medicamentos, aumentando a solubilidade de um fármaco e alterando sua farmacocinética, promovendo uma liberação sustentada e, aumentando assim, a sua biodisponibilidade. As diversas plataformas da nanotecnologia podem ser utilizadas para o desenvolvimento de terapias mais sofisticadas, como o direcionamento de um fármaco para uma célula-alvo e a possibilidade de combinação de diferentes fármacos em um único nanocarreador promovendo uma terapia sinérgica (Zarbin e cols., 2010).

3.1. Nanotecnologia aplicada à terapêutica

A nanotecnologia aplicada à área farmacêutica teve inicio na década de 70. Inicialmente, as primeiras nanopartículas propostas como carreadores com aplicações terapêuticas eram compostas de gelatina ligada à albumina (Scheffel e cols., 1972; Marty e cols., 1978). A partir de então, com a finalidade de evitar o uso de proteínas que poderiam estimular o sistema imunológico, foram desenvolvidas nanopartículas poliméricas compostas de polímeros sintéticos biodegradáveis e nanopartículas lipídicas sólidas (Couvreur e cols., 1979). A partir de 1986, houve uma aceleração no desenvolvimento de novas metodologias para a preparação de vários tipos de nanopartículas (Fattal e Vauthier, 2002). Nos últimos 30 anos, o crescimento explosivo das aplicações da nanotecnologia na área farmacêutica trouxe inovações desafiadoras para a farmacologia, revolucionando a entrega de compostos biologicamente ativos. Desde os primeiros lipossomas propostos por Gregoriadis e cols. em 1974 (Gregoriadis e cols., 1974) até os dias de hoje, houve um aumento significativo no número de nanodispositivos disponíveis para a entrega de fármacos (Figura 7), os quais podem ser constituídos de lipídeos ou de polímeros (Jain, 2005; Vasir e cols., 2005). Recentemente, novos nanocarreadores a base de carbono também tem sido sugeridos (Bianco e cols., 2005; Venkatesan e cols., 2005).

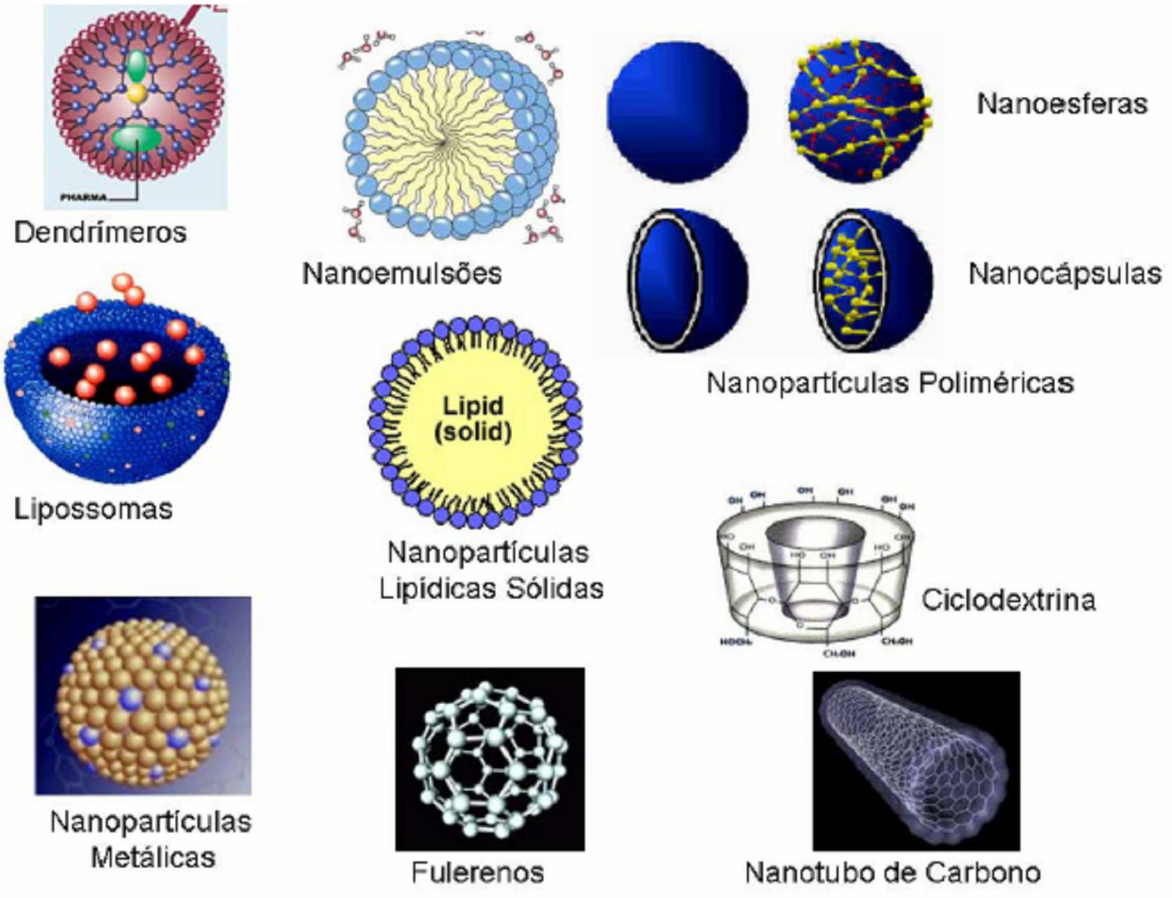

Figura 7. Sistemas nanométricos que podem ser utilizados para a vetorização de fármacos. Adaptado de Suh e cols., 2009 e de Faraji e cols., 2009.

De forma geral, o uso de nanocarreadores pode proteger o fármaco da degradação, acentuar sua absorção por facilitar a difusão através do epitélio, modificar a farmacocinética e o perfíl de distribuição nos tecidos, além de melhorar a penetração e distribuição intracelular (Couvreur e Vauthier, 2006). Carreadores coloidais de fármacos, incluindo nanoemulsões, nanoesferas, nanocápsulas, lipossomas e complexos lipídicos, apresentam-se como veículos promissores para administração intravenosa de fármacos lipofílicos, bem como para administração nas vias oral, ocular, cutânea e endovenosa, possibilitando a otimização da velocidade de cedência e do regime de dosagem das substâncias (Alvarez-Róman e cols., 2001; Fattal e Vauthier, 2002; Couvreur e cols., 2002). Estes sistemas nanométricos são

considerados vetores para a administração de substâncias lipofílicas, possibilitando uma liberação homogênea e, muitas vezes, controlada de fármacos, aumentando assim a resposta terapêutica no sítio de ação por tempo prolongado. A vetorização em órgãos, tecidos ou células específicas também apresenta como vantagem a diminuição de efeitos adversos apresentados sistemicamente pelo fármaco não vetorizado (Guterres e cols., 2001). Outro aspecto importante é a possibilidade de diminuição das doses e do número de aplicações, de modo que os pacientes apresentam maior adesão ao tratamento. Além disso, em alguns casos, o nanoencapsulamento pode ter como objetivo a melhora da biodisponibilidade de um fármaco através do aumento da estabilidade desse fármaco nos fluidos biológicos ou, ainda, o aumento de estabilidade da própria formulação farmacêutica (Couvreur e cols., 2002; Couvreur e Vauthier, 2006).

Dentre as nanopartículas poliméricas amplamente utilizadas para a entrega de fármacos destacam-se as nanocápsulas. Estas são formadas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica, dependendo das propriedades físico-químicas e da composição das nanocápsulas. Dessa forma, nanocápsulas podem ser consideradas um sistema "reservatório" (Couvreur e cols., 2002). As nanocápsulas apresentam alto potencial de encapsulamento de substâncias, especialmente as lipofílicas, são capazes de controlar a liberação de fármacos em sítios de ação específicos otimizando a velocidade de cedência e gerando respostas adequadas por prolongados períodos de tempo. Além disso, aumentam a estabilidade de armazenamento e no organismo, melhoram regimes de dosagens de substâncias e o índice terapêutico por aumentarem a eficácia e/ou reduzirem a toxicidade de fármacos, diminuindo a absorção sistêmica (Meier, 2000; Barrat, 2003; Schaffazick e cols., 2003; Guterres e cols., 2007).

Todos os sistemas de nanocareadores são explorados para fins terapêuticos com o objetivo de transportar o fármaco no organismo de uma maneira controlada a partir do sítio de administração até o alvo terapêutico e isso implica a passagem do fármaco e do sistema carreador através de diversas barreiras fisiológicas, as quais representam o maior desafio na vetorização de fármacos.

3.1.1. Nanotecnologia aplicada ao tratamento de doenças neurodegenerativas

O tratamento de doenças que atigem o SNC é bastante limitado, principalmente pela presença da BHE, a qual limita a passagem de fármacos mesmo em certas situações patológicas onde a BHE encontra-se parcialmente interrompida. Dessa maneira, o desenvolvimento de modalidades terapêuticas para o tratamento de doenças neurodegenerativas constitui um dos maiores desafios na pesquisa e no desenvolvimento de medicamentos na área da nanotecnologia farmacêutica.

O SNC não possui sistema linfático ou outra forma de drenagem. Por estar incluso dentro do crânio não sendo passível de expansão, um influxo de moléculas ao SNC poderia aumentar a osmolaridade permitindo a entrada de água a partir da vasculatura elevando a pressão intracranial. No entanto, a evolução da BHE tornou rara a ocorrência desta condição. Além disso, a BHE previne que toxinas potencialmente nocivas alcancem o cérebro. Entretanto, a complexidadde desta estrutura levou ao impedimento do tratamento de doenças neurodegenerativas de modo que apenas moléculas pequenas, com tamanho menor de 400 a 600 Daltons, lipofílicas e eletricamente neutras conseguem difundir passivamente através da BBB (Pardridge, 2010).

A BHE é constituída por uma monocamada de células endoteliais associadas aos pericitos e aos astrócitos. Entre os principais fatores que separam o parênquima cerebral do sistema circulatório limitando a penetração de drogas ao SNC incluem-se a falta de

fenestração com reduzida atividade pinocítica, embora com aumentado número e volume de mitocôndrias nas células endoteliais (Oldendorf e cols., 1977; Stewart, 2000; Abbott, 2005), a presença de *tight junctions* entre as células endoteliais adjacentes formando um intrincado complexo de proteínas transmebrana e a expressão de vários transportadores, tais como as glicoproteínas P (P-gp – *P-glycoprotein*) e as proteínas relacionadas à resistência a múltiplas drogas (MRPs – *Multidrug Resistance-Related Proteins*) (Abbott e cols., 2006; Rip e cols., 2009).

Além de limitar a penetração de drogas ao SNC, a BHE também afeta negativamente a eficácia e a tolerância ao tratamento das doenças neurodegenerativas, porque elevadas doses de medicamentos são necessárias para atingir a concentração mínima efetiva. Dessa forma, o desenvolvimento de ferramentas que permitam aprimorar o diagnóstico e a terapia para doenças do SNC tem sido direcionado a explorar a aplicabilidade de sistemas nanoparticulados. Apesar da grande variedade de nanocarreadores desenvolvidos até hoje, lipossomas e nanopartículas poliméricas têm sido amplamente explorados para a vetorização de fármacos ao SNC (Garcia-Garcia e cols., 2005; Bernardi e cols., 2009a; Modi e cols., 2009; Chen e Liu, 2011). Em relação ao tratamento da DA, algumas estratégias têm sido direcionadas ao nanoencapsulamento de diversas moléculas biologicamente ativas para a entrega no cérebro, como o nanoencapsulamento da tacrina e da rivastigmina, contornando a baixa biodisponibilidade cerebral e os efeitos adversos; enquanto outras têm centrado na utilização de nanodispositivos para reduzir a toxicidade do Aβ, promovendo sua remoção ou alterando sua cinética de agregação (Agyare e cols., 2008; Wilson e cols., 2008; Wilson e cols., 2010; Brambilla e cols., 2011).

Os mecanismos pelos quais as nanopartículas são capazes de atravessar a BHE ainda não são completamente compreendidos. Tem sido proposto que o tamanho da partícula, o tipo de polímero, bem como as características físico-químicas da superfície são fundamentais

para induzir uma estabilização estérica, aumentando assim o tempo de circulação, o que favorece a interação e penetração nas células endoteliais da BHE (Garcia-Garcia e cols., 2005). Alguns estudos têm demonstrado que nanopartículas revestidas com polissorbato 80 são capazes de adsorver apolipoproteínas plasmáticas atravessando a BHE via endocitose mediada por receptores de LDL (Friese e cols., 2000; Calvo e cols., 2001, Kreuter e cols., 2002). Além disso, nanocápsulas poliméricas podem aderir à membrana celular e "escapar" dos sistemas de efluxo mediado pelas P-gps e MRPs (Vauthier e cols., 2003). Outras estratégias utilizadas pela nanotecnologia para melhorar a passagem através da BHE incluem a modificação da superfície dos nanocarreadores como o revestimento com polietilenoglicol, permitindo o prolongamento do tempo de circulação, além da ligação de anticorpos específicos para determinados receptores permitindo a permeação das nanopartículas pelas células endotelias da BHE (Chen e Liu, 2011; Wong e cols., 2011).

OJETIVOS

1. Objetivo Geral

Avaliar o potencial efeito neuroprotetor do resveratrol em modelos *in vitro* e *in vivo* de toxicidade induzida pelo peptídeo β-amilóide (Aβ).

1.2 Objetivos Específicos

1. Incorporar o resveratrol em nanocápsulas poliméricas e avaliar a sua biodistribuição em ratos saudáveis.

2. Investigar o potencial efeito neuroprotetor do resveratrol, livre e nanoencapsulado, sobre a morte celular e a neuroinflamação induzida pelo Aβ em cultura organotípica de hipocampo de ratos.

3. Avaliar o efeito do tratamento com resveratrol livre e nanoencapsulado em um modelo *in vivo* de toxicidade induzida pelo Aβ.

CAPÍTULO I

Characterization of *trans*-Resveratrol-Loaded Lipid-Core Nanocapsules and Tissue Distribution Studies in Rats

Rudimar L. Frozza, Andressa Bernardi, Karina Paese, Juliana B. Hoppe, Thaline da Silva, Ana Maria O. Battastini, Adriana R. Pohlmann, Sílvia S. Guterres, and Christianne Salbego

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Characterization of *trans*-Resveratrol-Loaded Lipid-Core Nanocapsules and Tissue Distribution Studies in Rats

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Several studies have reported that orally ingested *trans*-resveratrol is extensively metabolized in the enterocyte before it enters the blood and target organs. Additionally, *trans*-resveratrol is photosensitive, easily oxidized and presents unfavorable pharmacokinetics. Therefore, it is of great interest to stabilize *trans*-resveratrol in order to preserve its biological activities and to improve its bioavailability in the brain. Here, *trans*-resveratrol was loaded into lipid-core nanocapsules and analyzed for particle size, polydispersity and zeta potential. The nanocapsule distribution in brain tissue was evaluated by intraperitoneal (i.p.) and gavage routes in healthy rats. The lipid-core nanocapsules had a mean diameter of 241 nm, a polydispersity index of 0.2, and a zeta potential of −15 mV. No physical changes were observed after 1, 2 and 3 months of storage at 25 °C. Lipid-core nanocapsules showed high entrapment of *trans*-resveratrol and displayed a higher *trans*-resveratrol concentration in the brain, the liver and the kidney after daily i.p. or gavage administration than that observed for the free *trans*-resveratrol. Because *trans*-resveratrol is a potent cyclooxygenase-1 inhibitor, gastrointestinal damage was evaluated. The animals that were administered with *trans*-resveratrol-loaded lipid-core nanocapsules showed significantly less damage when compared to those administered with free *trans*-resveratrol. In summary, lipid-core nanocapsules exhibited great *trans*-resveratrol encapsulation efficiency. *trans*-Resveratrol-loaded lipid-core nanocapsules increased the concentration of *trans*-resveratrol in the brain tissue. Gastrointestinal safety was improved when compared with free *trans*-resveratrol. Thus, *trans*-resveratrol-loaded lipid-core nanocapsules may be used as an alternative potential therapeutic for several diseases including Alzheimer's disease.

Keywords: *trans*-Resveratrol, Nanocapsule, Drug Delivery, Physical Stability, Biodistribution.

RESEARCH ARTICLE

1. INTRODUCTION

trans-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), was identified as a biologically active compound found in grapes, red wine, peanuts, and various berries. It is a polyphenol that has potential beneficial effects on health, presenting a wide range of pharmacological properties.[1,2] It is well-known for its antioxidant, anti-inflammatory, analgesic, cardio-protective, anti-diabetes, anti-obesity, neuroprotective, anti-aging and cancer chemoprevention activities.[1,3]

In view of the listed effects, *trans*-resveratrol may potentially target organs, and, therefore, its absorption, metabolism and distribution *in vivo* are considered to be important. Several studies have reported that orally ingested *trans*-resveratrol is extensively metabolized in the enterocyte[4,5] before its entrance into the blood and organs.[1] It has also been shown that *trans*-resveratrol can be glucuronidated or sulfated after absorption and that the conjugates predominantly circulate in plasma. However, if the conjugates are biologically active, their effects remain to be determined. The use of *trans*-resveratrol is limited because it is photosensitive and easily oxidized.[6]

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Additionally, previous pharmacokinetic evaluation of free *trans*-resveratrol has reported its unfavorable pharmacokinetics: a half-life of only 30–45 min,[1,7] its extensive metabolism[8] and its low bioavailability.[9] Therefore, it is of great interest to stabilize *trans*-resveratrol in order to preserve its biological activities and improve the bioavailability profile. To reach those objectives, a possible strategy includes the use of nanocarriers.

Nanoparticles have become an important component of therapeutics research because they have the ability to deliver a wide range of drugs to the body for a sustained period of time.[10] Nanoparticles are loaded with drug either through incorporation of the drug during the preparation process or by adsorption onto the surface of the preformed particle.[11,12] A wide variety of drugs can be nanoencapsulated using a many routes. Nanoparticles can be used to deliver many therapeutics, such as hydrophilic drugs, hydrophobic drugs, proteins, vaccines and biological macromolecules.[10]

Polymeric nanoparticles are colloidal structures designed to encapsulate lipophilic drugs and are used to target tissues, avoid drug degradation, improve efficacy and circumvent toxicity.[11,13–18] Utilizing our knowledge of nanoparticles, we hypothesized that *trans*-resveratrol-loaded lipid-core nanocapsules might display an improvement in the biodistribution of *trans*-resveratrol. Therefore, the present study investigated the development of polymeric lipid-core nanocapsules containing *trans*-resveratrol and evaluated their distribution in healthy rat tissues. Additionally, we compared the gastrointestinal safety of *trans*-resveratrol-loaded lipid-core nanocapsules with the effects displayed by free *trans*-resveratrol.

RESEARCH ARTICLE

2. MATERIALS AND METHODS

2.1. Materials

Poly(ε-caprolactone) (PCL) (MW = 65,000) was supplied by Aldrich (Strasbourg, France). Caprylic/capric triglyceride and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). Span 60® (sorbitan monostearate) was obtained from Sigma (St. Louis, USA). *trans*-Resveratrol (>98% pure) was purchased from Gerbras (Anápolis, GO, Brazil). All other chemicals and solvents used were of analytical or chromatographic grade. All reagents were used as they were received.

2.2. Methods

2.2.1. Preparation of Lipid-Core Nanocapsules

Nanocapsule suspensions were prepared by interfacial deposition of the polymer as previously described.[19] At 40 °C, *trans*-resveratrol (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 ml) and sorbitan monostearate (0.038 g) were dissolved in acetone (27 ml). In a separate flask, polysorbate 80 (0.038 g) was added to 53 ml of water (MilliQ®). The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was evaporated and the suspensions were concentrated under reduced pressure. The final volume was adjusted to 10 mL. A control formulation (load off lipid-core nanocapsules) was prepared without *trans*-resveratrol.

2.2.2. Physicochemical Characterization of the Formulations

The pH values of the suspensions were determined using a potentiometer B-474 (Micronal, Brazil). Mean diameters (z-average), polydispersity and zeta potentials were measured at 25 °C using a Zetasizer® nano-ZS ZEN 3600 model (Nanoseries, Malvern, UK), after diluting the samples with MilliQ® water or with 0.01 mol/L NaCl aqueous solution, respectively. To avoid any sample selection, only the dilution media was filtered (Millipore 0.45 μm) prior to analysis. Measurements were carried out using 3 different batches for each formulation in triplicate. They were analyzed immediately, and at 30, 60 and 90 days after the lipid-core nanocapsule preparation; the samples remained at 25 °C. Calculation of the size and polydispersity indices were achieved using the software (Dispersion Technology Software–DTS Nano–Version 5.02, Malvern) provided by the manufacturer (Malvern Instruments Ltd).

2.2.3. Analytical Procedure

trans-Resveratrol was analyzed by high-performance liquid chromatography (HPLC) at 306 nm. The content (total concentration) of *trans*-resveratrol in the formulations (100 μl) was determined after dissolving the lipid-core nanocapsules containing *trans*-resveratrol into acetonitrile (10 ml) and filtering (Millipore 0.45 μm) for analysis. The system consisted of a UV-Vis detector, pump and auto-injector S200 Perkin-Elmer (PerkinElmer Instruments, Norwalk, CT) and a Shim-pack CLC-C8 (M) column (150 mm, 4.6 mm, 5 μm, Shimadzu Corporation, Japan) with a guard-column. The mobile phase was prepared by using Milli-Q® water and HPLC grade acetonitrile, and consisted of acetonitrile/water (40:60 v/v) with a pH of 3.0 ± 0.5 corrected with 10% (v/v) orthophosphoric acid (Merck, Darmstadt, Germany). The isocratic flow rate of the mobile phase was 1.2 ml/min and the retention time of *trans*-resveratrol was 3.45 min.

2.2.4. Optical Characterization Using Turbiscan Lab

The lipid-core nanocapsule suspensions were investigated using *Turbiscan Lab* (Formulaction, France), which allows the optical characterization of any type of dispersion.[20,21] The detection head, which moves up and down along a

flat bottomed cylindrical cell, is composed of a pulsed near infrared light source ($\lambda = 880$ nm) and two synchronous detectors. The transmission detector (at 180°) receives the light which goes through the sample, while the backscattering detector (at 45°) receives the light scattered backward by the sample. The detection head scans the entire height of the sample, acquiring transmission and backscattering data every 40 μm. The samples, *trans*-resveratrol-loaded lipid-core nanocapsules and load off lipid-core nanocapsules (20 ml), were poured into the glass cells without any dilution and analyzed at 25 °C up to 12 h.

2.2.5. Animal Maintenance

Healthy male *Wistar* rats (180–200 g) were obtained from in-house breeding colonies at the "Departamento de Bioquímica", "Universidade Federal do Rio Grande do Sul" (UFRGS–Porto Alegre, Brazil). Animals were housed in cages under optimum light conditions (12:12 h light–dark cycle), temperature (22 ± 1 °C), and humidity (50 to 60%), with food and water provided *ad libitum*. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23 and were approved by the local Ethics Committee on the Use of Animals (CEP-UFRGS, protocol number 2007977). All efforts were made to minimize the number of animals and their suffering.

2.2.6. Recovery

Recoveries were determined by spiking 50 μg of *trans*-resveratrol in each tissue sample (brain, liver and kidney). The animals were euthanized and the organs of five animals were excised, minced with scissors, and placed in a homogenizer vessel; acetonitrile was added and the tissues were subsequently homogenized. The homogenization process was adjusted for each tissue. The brain was homogenized in 5.5 mL, the liver in 10.5 mL and the kidney in 4.5 mL of acetonitrile. The homogenized samples were transferred to 50 ml conical glass tubes and vortexed for 5 min prior to centrifugation at $2{,}800 \times$ g for 30 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 20 μl for all samples. Absolute recoveries were calculated by comparing the peak area ratio from the spiked samples to those of the corresponding concentration standard of *trans*-resveratrol in acetonitrile injected directly into the HPLC system.

2.2.7. Tissue Distribution Study

Free *trans*-resveratrol was prepared at a concentration of 1 mg/ml in a saline solution (0.9% NaCl) with 30% DMSO, and *trans*-resveratrol-loaded lipid-core nanocapsules were prepared at 1 mg/ml as described above. One animal group received one dose (5 mg/kg) of *trans*-resveratrol-loaded lipid-core nanocapsules and another animal group received one dose (5 mg/kg) of free *trans*-resveratrol through the i.p. route. Additionally, four animal groups received a daily dose (5 mg/kg) of *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol for 14 days using i.p. or gavage. At 1 h or 3 h of i.p. administration (animals received only one dose) or 14 days (daily dose, 1 h after the last administration) of treatment via i.p. or gavage, animals were anaesthetized by i.p. injection of choral hydrate (325 mg/kg). The abdominal cavity and the skull were opened, and the liver, kidneys and brain were rapidly removed, weighed, and washed in ice cold 0.9% NaCl. The organs were minced with scissors and placed in a homogenizer vessel; acetonitrile was added and tissues were subsequently homogenized. The homogenization process was performed as described above. Subsequently, 20 μl of each filtered sample (Millipore 0.45 μm) was injected into an HPLC system. The quantity of *trans*-resveratrol was calculated by comparing the peak area ratio from tissue samples of treated animals with those of the corresponding concentration standards of *trans*-resveratrol in acetonitrile injected directly into the HPLC system.

2.2.8. Gastrointestinal Tolerance Study

The occurrence of gastrointestinal lesions was evaluated because *trans*-resveratrol is a potent cyclooxygenase-1 inhibitor. For this purpose, independent animals were randomly separated into five groups as follows: (1) untreated (Control group); (2) treated with 30% DMSO in 0.9% NaCl (Control Vehicle group); (3) treated with load off lipid-core nanocapsules (Control NC group); (4) treated with 5 mg/kg of free *trans*-resveratrol in 30% DMSO in 0.9% NaCl (RSV-Sol group); (5) treated with 5 mg/kg of *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC group). The treatments were performed by i.p. or gavage routes over 14 days. At the end of the administrations, the rats were euthanized and the excised intestine (duodenum, jejunum and ileum) was slit open opposite to the attached mesenteric tissue. The organs were washed with saline and the mucosal surfaces were macroscopically examined according to an arbitrary scale previously reported.[15] Accordingly, the number and the gravity of erosions were scored on a five-grade scale: grade 0, no lesion; grade 0.5, hemorrhagic point; grade 1, ulcer length <2 mm; grade 2, ulcer length >2 mm; grade 3, lesion with perforation and hemorrhage. Experimental data were obtained by multiplying the score by the number of lesions. The mean scores for each group were calculated and expressed as lesional indexes.

2.2.9. Determination of Hepatic Enzymes in Serum and Histological Analysis

The serum levels of hepatic enzymes and the histological analyses were evaluated in the same animals that the gastrointestinal tolerance was performed. At end of the treatments, blood samples were collected by cardiac puncture for hepatic enzymes analysis. The blood samples were centrifuged at 2,500 × g at 4 °C for 10 min. The blood serum was collected and frozen at −70 °C until the assays were performed. The activities of hepatic enzymes γ-glutamyltransferase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured according to the recommendations of the supplier (Labtest, MG, Brazil). The histological analysis was performed at the end of the treatments (14 days via i.p. or gavage); the animals were euthanized, and the brain, heart, stomach, liver and kidneys were removed, fixed in 10% formalin and embedded in paraffin. The sections (5 μm) were cut using a microtome, stained with hematoxylin and eosin (H&E) and analyzed under a microscope (40× magnification) (Nikon Eclipse TE 300).

2.3. Statistical Analysis

The results are presented as the mean ± SD of 3 to 5 animals per group. The statistical significance among groups was assessed by a one-way analysis of variance (ANOVA) followed by Tukey's test. P-values lower than 0.05 ($p < 0.05$) were considered significant. The amount of *trans*-resveratrol present in the tissues was determined in μg/g tissue, on the basis of the area under the curve. The statistical significance between groups treated with *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol for tissues\distribution of *trans*-resveratrol was assessed by means of unpaired Student's *t*-test. *p*-values lower than 0.05 ($p < 0.05$) were considered significant.

3. RESULTS

3.1. Physicochemical Characterization of Lipid-Core Nanocapsule Formulations

The lipid-core nanocapsule formulations were prepared by interfacial deposition of polymer without purification. *trans*-Resveratrol-loaded lipid-core nanocapsules and

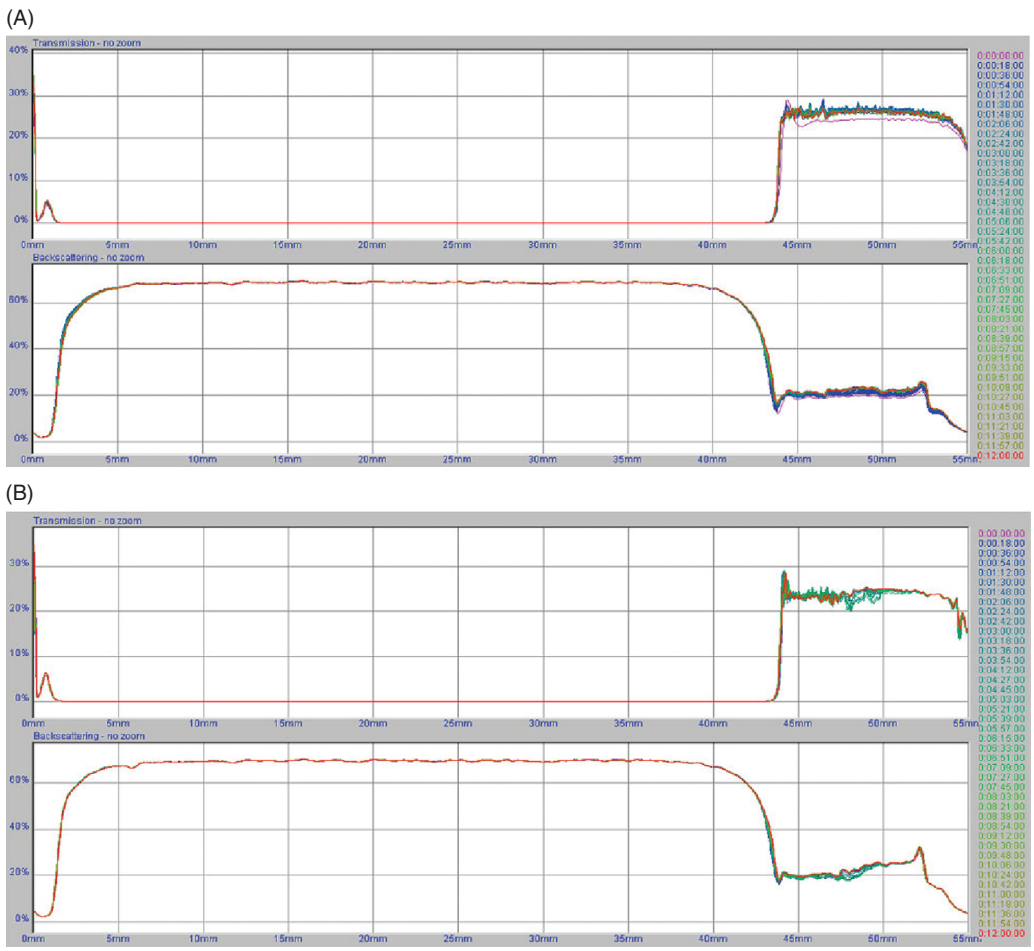

Fig. 1. Transmission and backscattering profiles of lipid-core nanocapsules using *Turbiscan Lab.* (A) Representative image of load off lipid-core nanocapsules (NC) analysis. (B) Representative image of *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC) analysis.

load off lipid-core nanocapsules presented a macroscopic homogeneous aspect, such as white bluish opalescent liquids. Immediately after the preparation, the *trans*-resveratrol-loaded lipid-core nanocapsules were 241 ± 7 nm, 0.16 ± 0.03, -14.1 ± 2.3 mV, and 5.2 ± 0.3, for particle size, polydispersity, zeta potential, and pH values, respectively. The analyses were performed again after 30 (260 ± 10 nm; 0.21 ± 0.07; -12.7 ± 1.6 mV; 4.8 ± 0.21), 60 (256 ± 4 nm; 0.25 ± 0.09; -13.2 ± 1.1 mV; 4.4 ± 0.19) and 90 (281 ± 30 nm; 0.27 ± 0.1; -13.2 ± 3.6 mV; 4.2 ± 0.15) days after nanocapsule preparation, which were stored at 25 °C. Similar results were obtained for load-off nanocapsules (data not shown). The suspensions showed stable monomodal size distributions and polydispersity indices lower than 0.3, indicating narrow size distributions. The HPLC method was validated presenting linearity between 2.5 and 17.5 μg/ml, ($r > 0.9999$), inter- and intraday variability lower than 2.0%, and accuracy from 96.19% to 97.21%. *trans*-Resveratrol content was 0.964 ± 0.037 mg/ml and the encapsulation efficiency was $99.89 \pm 1.3\%$.

3.2. Physical Stability of Lipid-Core Nanocapsules

To evaluate the physical stability of the lipid-core nanocapsules, the optical characterization of *trans*-resveratrol-loaded and load off lipid-core nanocapsule formulations were investigated using *Turbiscan Lab* for 12 h. As observed in Figures 1(A and B), the transmission signal was zero. Both formulations exhibited a slight creaming phenomenon with an increase in backscattering at the top of the cell, probably due to a small portion of nanoemulsion present in the formulations; this was previously described in similar formulations.[19]

3.3. Recovery and Tissue Distribution of *trans*-Resveratrol-Loaded Lipid-Core Nanocapsules

First, the extraction recoveries of *trans*-resveratrol from different tissues were conducted in at least five animals per group. The recoveries (%) in the analysis from the brain, liver and kidney were 97.85 ± 6.3, 86.65 ± 5.06, and 96.66 ± 2.54, respectively. The results illustrated the high extraction efficiency of this procedure.

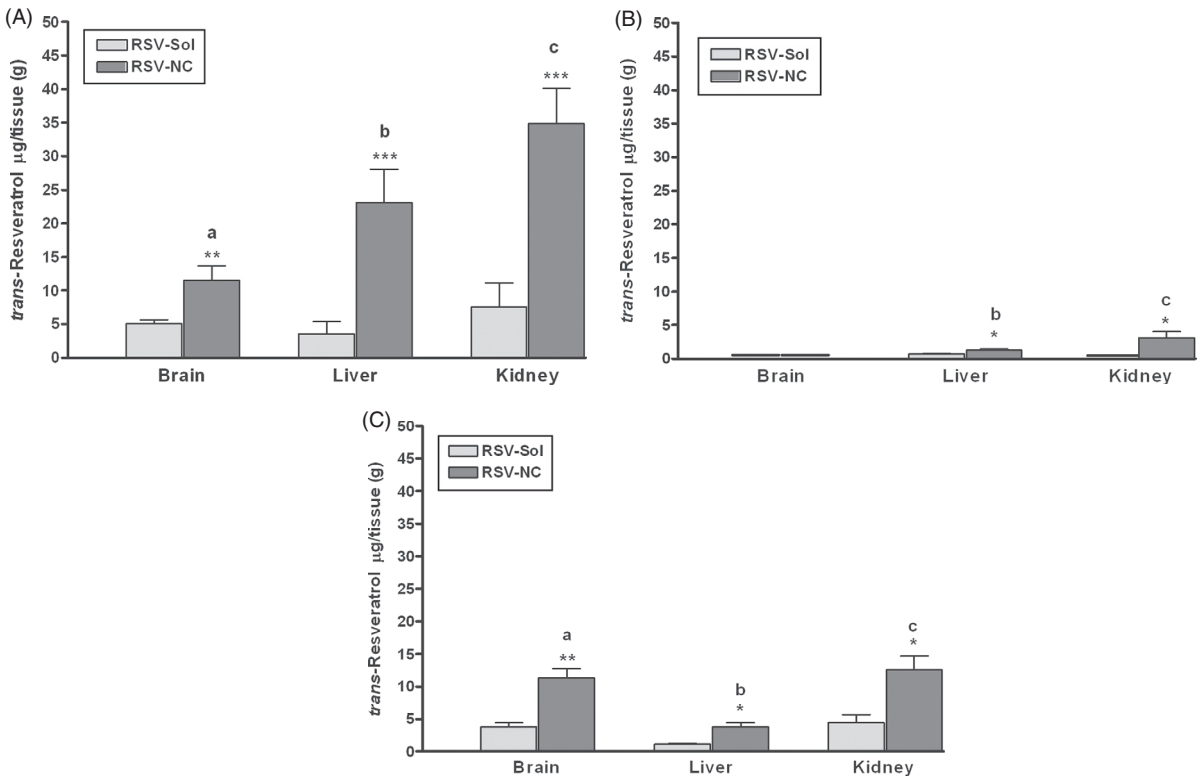

Fig. 2. *trans*-Resveratrol concentration in rat tissue. (A) *trans*-Resveratrol distribution in the brain, liver and kidney after 14 days of daily administration of *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC) or free *trans*-resveratrol (RSV-Sol) 5 mg/kg, i.p. (B) *trans*-Resveratrol distribution in the brain, liver and kidney 1 h after a single administration of *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC) or free *trans*-resveratrol (RSV-Sol) 5 mg/kg, i.p. (C) *trans*-Resveratrol distribution in the brain, liver and kidney 3 h after a single dose of *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC) and free *trans*-resveratrol (RSV-Sol) 5 mg/kg, i.p. The values are depicted as the means $\pm$ SD of five animals per group. The data were analyzed by unpaired Student's *t*-test. Asterisks denote the significance levels of *trans*-resveratrol-loaded lipid-core nanocapsules in comparison with free *trans*-resveratrol treatment values: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Next, *trans*-resveratrol-loaded lipid-core nanocapsules were administered to healthy *Wistar* rats for concentration analysis in the brain, liver and kidney tissues compared with free *trans*-resveratrol. The concentrations of *trans*-resveratrol (μg/g tissue) after 14 days of treatment with *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol via i.p. administration are shown in Figure 2(A). A significantly higher quantity of *trans*-resveratrol was found in all animal tissues treated with *trans*-resveratrol-loaded lipid-core nanocapsules in comparison with animals treated with free *trans*-resveratrol.

Additional investigations were carried out to evaluate *trans*-resveratrol accumulation in rat tissues. Rats were treated with *trans*-resveratrol-loaded lipid-core nanocapsules or with free *trans*-resveratrol (5 mg/kg) via i.p. in a single dose and analysis was performed 1 h after administration. As displayed in Figure 2(B), a significant amount of *trans*-resveratrol was detected in the liver. *trans*-Resveratrol was only observed in the kidneys in the treated group with the *trans*-resveratrol-loaded lipid-core nanocapsules. In both groups, *trans*-resveratrol was not detected in the brain. Because *trans*-resveratrol was not observed 1 h after administration in the brain, another animal group was submitted to the same treatment, but the analysis was performed 3 h after the single *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol i.p. injection (5 mg/kg). As observed in Figure 2(C), the amount of *trans*-resveratrol found in the brain was similar to those observed after 14 days of treatment. The liver and kidney presented a similar profile, although the concentrations of *trans*-resveratrol were lower.

trans-Resveratrol is found in natural products that are present in the diet, and it is extensively metabolized by enterocytes, slowing its bioavailability. Due to this phenomenon, we have also investigated the tissue distribution after oral administration of *trans*-resveratrol-loaded lipid-core nanocapsules compared to free *trans*-resveratrol. Five animals per group were administered daily with 5 mg/kg of *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol for 14 days by gavage. Figure 3 shows the concentrations of *trans*-resveratrol in the brain, liver and kidney. In the same way, the results present a similar profile to those observed in i.p. treatment (Fig. 2), although the concentrations were lower.

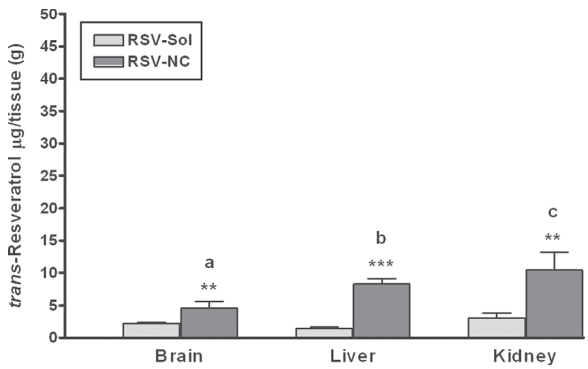

Fig. 3. *trans*-Resveratrol concentration in rat tissues after *trans*-resveratrol-loaded lipid-core nanocapsules and free *trans*-resveratrol administration by gavage. *trans*-Resveratrol-loaded lipid-core nanocapsules (RSV-NC) or free *trans*-resveratrol (RSV-Sol) 5 mg/kg gavage were daily administered for 14 days. The values are depicted as the means ± SD of five animals per group. The data were analyzed by unpaired Student's *t*-test. Asterisks denote the significance levels of *trans*-resveratrol-loaded lipid-core nanocapsules in comparison with free *trans*-resveratrol treatment values: $^{**}p < 0.01$, $^{***}p < 0.001$.

3.4. Evaluation of Gastrointestinal Damage

A series of long-term administration experiments were performed to evaluate the gastrointestinal damage of *trans*-resveratrol-loaded lipid-core nanocapsules when compared with free *trans*-resveratrol. For this purpose, the rat's

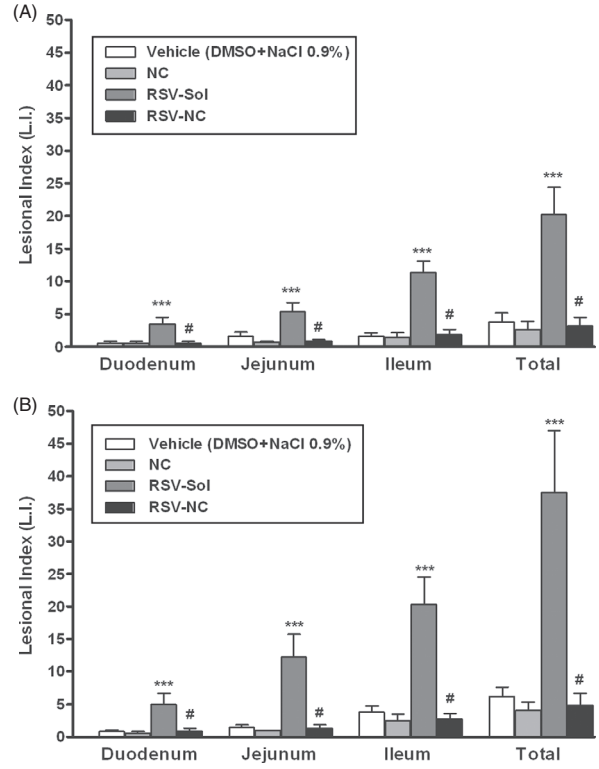

Fig. 4. Effect of *trans*-resveratrol-loaded lipid-core nanocapsules and free *trans*-resveratrol on the intestine lesional index. (A) The animals were treated daily via i.p., for 14 days, with vehicle (DMSO + NaCl 0.9%) or with 5 mg/kg *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC), load off lipid-core nanocapsules (NC), or free *trans*-resveratrol (RSV-Sol). (B) The animals were treated daily by gavage, for 14 days, with vehicle (DMSO + NaCl 0.9%) or with 5 mg/kg *trans*-resveratrol-loaded lipid-core nanocapsules (RSV-NC), load off lipid-core nanocapsules (NC), or free *trans*-resveratrol (RSV-Sol). Each point represents the means ± SD of 3 to 5 animals. The data were analyzed by ANOVA followed by Tukey's test. ***Significantly different from the control, vehicle, and load off lipid-core nanocapsules (NC) groups ($p < 0.001$). $^{\#}$Significantly different from the free *trans*-resveratrol (RSV-Sol) group ($p < 0.001$).

intestines were analyzed after 14 days of treatment via i.p. or gavage, and the lesional indexes were separately determined by duodenum, jejunum and ileum. As shown in Figures 4(A and B), the lesional indexes in the animals treated with *trans*-resveratrol-loaded lipid-core nanocapsules were significantly lower than when compared with the free *trans*-resveratrol group. The reduction was 7.0, 6.6 and 6.0 fold for i.p. treatment and 6.25, 9.35 and 7.8 fold for gavage treatment for duodenum, jejunum and ileum, respectively (Fig. 4).

3.5. Investigation of Possible Side Effects of *trans*-Resveratrol-Loaded Lipid-Core Nanocapsules Treatment

The treatment with *trans*-resveratrol-loaded lipid-core nanocapsules (5 mg/kg/day, i.p. or gavage for 14 days) did not induce mortality or alter the body weight when compared to the control group within a 14-day observation period (data not shown). Necropsy of the animals at the end of treatment did not show any macroscopic changes in the observed organs (liver, brain, kidney, heart and stomach) (data not shown). Microscopic analysis of these organs by H&E displayed an absence of necrosis (data not shown). Additionally, the weights of the brain, liver, and kidney did not differ between *trans*-resveratrol-loaded lipid-core nanocapsules (1.74 ± 0.1; 8.06 ± 0.72; 0.90 ± 0.03, respectively) and free *trans*-resveratrol groups (1.76 ± 0.1; 7.33 ± 0.51; 0.83 ± 0.06, respectively) after i.p. treatment. Similarly, no change was observed after gavage treatment (data not shown). Possible liver damage was evaluated in the rats treated with both *trans*-resveratrol-loaded lipid-core nanocapsules and free *trans*-resveratrol. The activities of hepatic enzymes γ-glutamyltransferase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were assessed in rat blood serum. None of the treated animals presented significant alterations in the investigated enzymes, suggesting no hepatic alterations in the animals in the tested conditions (data not shown).

4. DISCUSSION

Although many studies have been published regarding the beneficial effects of *trans*-resveratrol on health, limited information exists on this natural product, due to its extensive metabolism and unfavorable pharmacokinetics. Consequently, the aim of this study was to contribute the development of a nanocarrier system able to stabilize *trans*-resveratrol and improve its biodistribution.

Initially, *trans*-resveratrol was encapsulated into polymeric lipid-core nanocapsules through the interfacial deposition of the polymer approach,[19] which has been widely used for the production of nanocapsules. The method provided the formation of particles with high encapsulation efficiencies. The nanocapsule suspension presented a homogeneous shape with particle mean sizes lower than 300 nm. These values are in agreement with those usually observed for nanocapsules prepared with preformed polymers using the interfacial deposition method (from 100 nm to 500 nm).[11, 22, 23] It is recognized that the size of the nanoparticles plays a key role in their adhesion to and interaction with cells. Particles of nanoscale size have easy accessibility in the body, capable of transportation via circulation to different organs and tissues.[24] Therefore, the combination of *trans*-resveratrol and biodegradable lipid-core nanocapsules is a promising approach to control the rate of polyphenol administration that would prolong the duration of its beneficial effects.

Another important factor affecting nanoparticle stability is the zeta potential (surface charge). Zeta potential can greatly influence particle stability in suspension through the electrostatic repulsion between particles. The repulsive interactions between the particles are larger when the zeta potential increases, leading to the formation of more stable particles with a more uniform size distribution.[11] The zeta potential values of *trans*-resveratrol-loaded lipid-core nanocapsules and load off lipid-core nanocapsules in our study indicate the physical stability of the prepared formulations. Optical characterization of *trans*-resveratrol-loaded was also evaluated. The formulations exhibited a slight creaming tendency, probably due to a small portion of nanoemulsion presented in the formulations; this was described previously in similar polymeric formulations.[19] However, the creaming was reversible by means of stirring the nanocapsules, which is typical for suspensions and demonstrated the great stability of formulations. Additionally, it was observed that the initial drug content and the content after 1, 2 and 3 months of storage at 25 °C were similar, indicating that there were no significant changes in the physical and chemical characteristics of the formulations. Additionally, no significant changes were observed in the particle diameter, zeta potential and pH of formulations after 3 months of storage at room temperature. Considering all of the parameters evaluated, highly stabile formulations were obtained in the present study.

Several studies have demonstrated that the daily oral administration of high doses of *trans*-resveratrol in rats for 28 days is not harmful.[2] The addition of *trans*-resveratrol to the diet in daily doses that are feasible for humans improved the health and survival of mice fed with a high-caloric diet; it increased the insulin sensitivity, improved the mitochondrial number and improved the motor function.[25] Despite these advantages, no information was available concerning the organ distribution of *trans*-resveratrol in long-term experiments. Recently, Juan and co-workers developed a method for quantification of *trans*-resveratrol in rat plasma and tissues by HPLC,[26] but *trans*-resveratrol was administered intravenously through the tail vein and the analysis was performed only for 12 h after administration. Another previous study showed the

distribution of [^{3}H]*trans*-resveratrol in rat tissues after a single oral administration; however, *trans*-resveratrol was analyzed only 2 h and 18 h after administration.[27] In summary, these studies demonstrate the low bioavailability and the fast metabolism of *trans*-resveratrol.

In an attempt to improve the biodistribution and decrease the intensive metabolism of *trans*-resveratrol, a nanocarrier system was developed to stabilize *trans*-resveratrol. Initially, *trans*-resveratrol distribution was studied in the brain, liver and kidney of healthy rats. These organs were selected because they are frequently associated with chronic degenerative diseases. In addition, the liver represents the primary site of *trans*-resveratrol metabolism, and the kidney is important because of excretion through urine. Firstly, a daily dose of *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol was intraperitoneally administered for 14 days, 5 mg/kg body weight. One hour after the last administration, the organs were excised, and the *trans*-resveratrol content was determined by HPLC. The results showed that animals treated with *trans*-resveratrol-loaded lipid-core nanocapsules presented 2.5-, 6.6-, and 3.4-fold higher *trans*-resveratrol concentrations in the brain, liver and kidney, respectively, than those treated with free *trans*-resveratrol. Some studies showed a peak in plasma *trans*-resveratrol 1 h after its administration.[28,29] Thus, we assessed if *trans*-resveratrol might be accumulated in these tissues after 1 h of a single dose i.p. (5 mg/kg) of *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol. No *trans*-resveratrol was observed in the cerebral tissue, and a very low concentration was observed in the liver and kidney in both formulations. An experiment was performed using another group of animals that had received an identical treatment, but the tissues were analyzed 3 h after i.p. administration. The results presented a similar profile to those observed after 14 days of the treatment. As expected, a minor quantity of the drug was present. The *trans*-resveratrol concentration was higher in the animals treated with *trans*-resveratrol-loaded lipid-core nanocapsules in all organs evaluated when compared to free *trans*-resveratrol treatment. The results indicated that 3 h after the administration of both formulations, the concentrations of *trans*-resveratrol were very similar to those observed after 14 days of treatment. Considering the peak of *trans*-resveratrol in the organs, it is suggested that some *trans*-resveratrol was accumulated in the analyzed organs, primarily in the cerebral tissue.

Although free *trans*-resveratrol may cross the blood-brain barrier (BBB), our results showed that lipid-core nanocapsules were able to increase the amount of *trans*-resveratrol in the cerebral tissue. After administration, nanocapsules are taken up by the liver, spleen and, to a lesser extent, the bone marrow.[30] Within these tissues, nanoparticles are mainly taken up by cells of the mononuclear phagocyte system,[31] in which they are degraded to low molecular weight soluble compounds that are eliminated from the body by renal excretion.[32] In previous years, significant work has been performed in developing the so-called "stealth" particles that are "invisible" to macrophages.[11] Therefore, to avoid the intensive trapping of nanoparticles, *trans*-resveratrol-loaded lipid-core nanocapsules were coated with polysorbate 80. This strategy may result in a lower uptake of nanocapsules by the mononuclear phagocyte system and in a longer circulation time.[24] Several studies showed that a polysorbate 80 coating is a strategy used for the delivery of drugs into the brain.[17,33–35] Although no experiments were performed that provided physical evidence of lipid-core nanocapsule becoming lodged in the brain, we have observed a higher amount of *trans*-resveratrol in the brain of animals treated with *trans*-resveratrol-loaded lipid-core nanocapsules in comparison with animals treated with free *trans*-resveratrol. Lipid-core nanocapsules crossing the BBB remain to be determined. Although the polysorbate 80 coating decreased the uptake of lipid-core nanocapsules by the liver, it was still present and the metabolism might explain the increase of *trans*-resveratrol concentration in the liver and kidney.

When the concentration of *trans*-resveratrol in the same organs was evaluated after daily gavage administration of *trans*-resveratrol-loaded lipid-core nanocapsules or free *trans*-resveratrol for 14 days, a similar profile was observed to those by i.p. administration; however, in a lower proportion. It might be due to intense metabolism of *trans*-resveratrol by the enterocytes.[4,5] It has been reported that nanoparticles administered by the oral route could be trapped by Peyer's patches and circulate through the lymphatic system.[36,37] Therefore, the trapping of *trans*-resveratrol-loaded lipid-core nanocapsules by Peyer's patches could be occurring, which would, at least in part, explain the results described here.

Despite numerous studies presenting the beneficial effects of *trans*-resveratrol, the impairment of cyclooxygenase activity induced by this polyphenol has been reported.[38,39] Therefore, the treatment with *trans*-resveratrol might be associated with the development of adverse gastrointestinal effects. Another important topic addressed was the gastrointestinal safety of *trans*-resveratrol-loaded lipid-core nanocapsules in comparison with free *trans*-resveratrol, when dosed in a chronic schedule of administration. The data clearly demonstrate that the animals that received *trans*-resveratrol-loaded lipid-core nanocapsules showed a significant decrease in intestinal lesion indices when compared to animals treated with free *trans*-resveratrol. It is important to note that neither the 30% DMSO vehicle nor the load off lipid-core nanocapsules exhibited a significant increase in the intestinal lesion indices when compared to the control group. This suggests that *trans*-resveratrol-loaded lipid-core nanocapsule formulations displayed fewer adverse

effects than those observed after free *trans*-resveratrol administration, presenting a desirable increased gastrointestinal tolerance. The higher damage observed after gavage administration of free *trans*-resveratrol indicated that the mucosal side effects were local rather than systemic. This protective effects could be attributed to a slow release of the polyphenol in the acidic gastric environment or to the reduced damage of the acidic form of *trans*-resveratrol-loaded lipid-core nanocapsules compared with free *trans*-resveratrol. These results are in accordance with the previous work performed by our group, wherein treatment with diclofenac-loaded nanocapsules and nanospheres or treatment with indomethacin-loaded nanocapsules did not present a significant gastrointestinal damage.[15, 18] In the work presented, the side effects of both drugs were completely modified and reduced by the encapsulation in nanocapsules. Lipid-core nanocapsules induced a marked protective effect on the gastrointestinal mucosa compared with the ulcerative effect observed with the drug solution. Additionally, we analyzed the effects of both formulations upon the activities of hepatic enzymes γ-glutamyltransferase, alanine aminotransferase and aspartate aminotransferase, which might indicate hepatic lesions. As reported in prior work,[2, 25] the treatments did not present alterations in the activity of any enzymes, discarding the hepatic lesions. It is important to note that load off lipid-core nanocapsules did not cause any toxic effects, indicating the safe usage of this formulation.

Finally, the study indicated that lipid-core nanocapsules are highly stable and are able to improve the biodistribution of *trans*-resveratrol without causing any toxic effect, even if administered for long periods of time.

5. CONCLUSIONS

To our knowledge, this is the first study assessing the new formulation of lipid-core nanocapsules containing *trans*-resveratrol and their distribution in rat tissues. The most powerful evidence generated from this report shows that significantly higher *trans*-resveratrol concentrations were transported by polymeric lipid-core nanocapsules to all organs analyzed in comparison with free*trans*-resveratrol. The study also demonstrated the improvement of gastrointestinal safety of *trans*-resveratrol-loaded lipid-core nanocapsules. Considering the promising biological properties of *trans*-resveratrol, the present findings suggest that *trans*-resveratrol-loaded lipid-core nanocapsules may be a relevant therapeutic alternative in future studies of several diseases.

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CAPÍTULO II

Lipid-core nanocapsules improve the effects of resveratrol against Aβ-induced neuroinflammation

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Abstract: Resveratrol has attracted considerable interest for its beneficial effects for human health. However, the biological effects of resveratrol appear strongly limited by its low bioavailability. In the current study, we compare the effectiveness of free resveratrol (RSV) to that of resveratrol-loaded lipid-core nanocapsules (RSV-LNC) at improving the neuroprotective effects against the toxicity induced by Aβ. We found that pre- and co-treatments with RSV-LNC, possibly through sustained blocking of neuroinflammation, were able to protect organotypic hippocampal cultures against toxic effects triggered by Aβ1-42. Furthermore, RSV-LNC was able to increase IL-10 release even in the presence of Aβ and prevent/decrease reactive oxygen species formation (ROS), as well as the glial and JNK activation. On the other hand, pre- and co-treatment with RSV exhibited a lower ability to decrease neuroinflammation and cell death, and failed to increase IL-10 release. Taken together, our findings provide evidences that resveratrol, by using of the lipid-core nanocapsules, might represent a promising alternative for treating neurodegenerative processes related to the aging, including Alzheimer's disease.

Lipid-core nanocapsules improve the effects of resveratrol against Aβ-induced neuroinflammation

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Abstract

Resveratrol has attracted considerable interest for its beneficial effects for human health. However, the biological effects of resveratrol appear strongly limited by its low bioavailability. In the current study, we compare the effectiveness of free resveratrol (RSV) to that of resveratrol-loaded lipid-core nanocapsules (RSV-LNC) at improving the neuroprotective effects against the toxicity induced by Aβ. We found that pre- and co-treatments with RSV-LNC, possibly through sustained blocking of neuroinflammation, were able to protect organotypic hippocampal cultures against toxic effects triggered by Aβ1-42. Furthermore, RSV-LNC was able to increase IL-10 release even in the presence of Aβ and prevent/decrease reactive oxygen species formation (ROS), as well as the glial and JNK activation. On the other hand, pre- and co-treatment with RSV exhibited a lower ability to decrease neuroinflammation and cell death, and failed to increase IL-10 release. Taken together, our findings provide evidence that resveratrol, by using of the lipid-core nanocapsules, might represent a promising alternative for treating neurodegenerative processes related to the aging, including Alzheimer's disease.

Keywords

Alzheimer's disease, Amyloid-β; Neuroprotection; Neuroinflammation; Organotypic culture; Hippocampus; Resveratrol; Lipid-core nanocapsules

1. Introduction

Alzheimer's disease (AD) is the main chronic neurodegenerative disorder related to aging whose basic changes are manifested by the marked deterioration of memory and cognition. The neuropathologic hallmarks of AD are the extracellular deposits of amyloid-β peptide (Aβ), intracellular hyperphosphorylated tau protein, abnormalities in neuronal and synaptic function, and neuronal death. The presence of hyperphosphorylated tau protein and Aβ accumulation in the brain leads to the deposition of neurofibrillary tangles and senile plaques, respectively, which modulates inflammatory responses. This inflammatory response in neurons includes activation of microglia and astrocytes, resulting in the release of inflammatory mediators such as cytokines, chemokines, neurotrasmitters, and reactive oxygen species (ROS) (Tuppo and Arias, 2005). In this context, it is now widely recognized that neuroinflammation responses play a significant role in modulating disease progression (McGeer and McGeer, 2010).

Prolonged and widespread activation of microglia and astrocytes are apparent in AD brain. Microglia activated by Aβ shows an increased secretion of pro-inflammatory cytokines such as the interleukins (ILs) IL-1β, IL-6, and TNF-α, IL-8, macrophage inflammatory protein-1α (MIP-1α), and monocyte chemoattractan protein-1 (MCP-1) (Akiyama et al., 2000; Rogers and Lue, 2001; Tuppo and Arias, 2005). Although microglia has neuroprotective functions, neurotoxic mechanisms involving continuous activation of microglia and toxic factors released by microglia may lead to neuroinflammation. Subsequently, the continuous activation of microglia may act as trigger for the progression of AD pathology. Similar to microglia, astrocytes also secrete various pro-inflammatory molecules such as interleukins, prostaglandins, leukotrienes, thromboxanes, coagulation factors, complement factors, proteases, and protease inhibitors. Astrocytes activated by Aβ produce chemokines, cytokines, and ROS that may result in neuronal damage (Johnstone et

al., 1999; Akiyama et al., 2000; Smits et al., 2002). Receptor binding of cytokines stimulates a variety of intracellular signaling pathways that have been implicated in AD, including the activation of protein kinase C (PKC), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38/MAPK), PI3 kinase, extracellular signaling-related kinase (ERK), as well as activation of caspase-1 and -3 (Anisman, 2009; Van Eldik et al., 2007).

Despite progress in symptomatic therapy, an effective therapeutic approach that interferes with the neurodegenerative process involved in AD is still unavailable. Numerous studies have suggested that a wide range of polyphenols may have neuroprotective effects. Among these polyphenols, resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) was identified as a biologically active compound found in grapes, red wine, peanuts, and various berries. Resveratrol has attracted considerable interest for its beneficial potential effects for human health, presenting a wide range of pharmacological properties (Baur and Sinclair, 2006; Juan et al., 2002). Furthermore, several studies have recently shown that this polyphenol is associated with anti-amyloidogenic and neuroprotective properties *in vitro* and *in vivo* (Jang and Surh, 2003; Han et al., 2004; Marambaud et al., 2005; Vingtdeux et al., 2008; Karuppagounder et al., 2009; Vingtdeux et al., 2010).

On the other hand, the use of resveratrol is limited because of its poor solubility, its photosensitivity, being an easily oxidized substance (Piñeiro et al., 2006). Besides, it presents a short half-life of only 30-45 min (Bertelli et al., 1996; Baur and Sinclair, 2006), and a low bioavailability (Wenzel and Somoza, 2005). These drawbacks limit the development of medicines for therapeutic applications. In this context, an increasing number of recent studies have aimed at designing novel resveratrol formulations to overcome these limitations (reviewed in Amri et al., 2011). Among these, nanotechnology-based strategies have gained tremendous importance. Recently, we developed a lipid-core nanocapsule formulation containing resveratrol in an attempt to stabilize it, preserve its biological activities, and

improve its bioavailability. Additionally, we found that healthy rats treated with these lipid-core nanocapsules showed significantly higher concentrations of resveratrol in the brain when compared to rats treated with free resveratrol (Frozza et al., 2010). Previous studies of our group have already showed that the same lipid-core nanocapsules were able to carry increased concentrations of indomethacin into the brain decreasing tumor growth and improving the efficacy of indomethacin in experimental models of peripheral inflammation and neuroinflammation induced by oxygen-glucose deprivation (Bernardi et al., 2009a; Bernardi et al., 2009b; Bernardi et al., 2010). In this way, lipid-core nanocapsules might be considered promising candidates to overcome the limitations of resveratrol, since nanoparticles can be used to alter the kinetic of drugs, leading to sustained release, with a reduced requirement for frequent dosing, as well as to reduce drug adverse effects (Faraji and Wipf, 2009).

Although resveratrol has been shown protective effect against Aβ, the mechanisms underlying these neuroprotective effects still remains unclear. Hence, in the present study, in order to provide new evidence on the effects of resveratrol on Aβ-induced neurotoxicity, we sought to analyze, by using lipid-core nanocapsules, the contribution of resveratrol on the neuroinflammation induced by Aβ.

2. Methods

2.1. Chemicals and antibodies

Poly(ε-caprolactone) (PCL) (MW=65,000) was supplied by Aldrich (Strasbourg, France). Caprylic/capric triglyceride and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). *trans*-Resveratrol (>98% pure) was purchased from Gerbras (Anápolis, GO, Brazil). Span 60® (sorbitan monostearate), Propidium iodide (PI), 2',7'-dichlorofluorescin diacetate (DCFH2-DA), acrylamide, bisacrylamide, SDS and β-

mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aβ1-42 and Aβ42-1 peptides were purchased from American Peptide Co. (Sunnyvale, CA, USA). Culture medium, HBSS, Fungizone® (Amphotericin B) and horse serum heat inactivated were purchased from Gibco-Invitrogen (Grand Island, NY, USA). Gentamicin was purchased from Schering-Plough (Rio de Janeiro, RJ, Brazil). Millicell culture inserts (Millicell®-CM, 0.4 μm) were purchased from Millipore (Millipore®, Bedford, MA, USA) and 6-well culture plate were from Tissue culture test plates TPP (Tissue culture test plates TPP®, Switzerland). Ploy clonal primary antibodies to phosphorylated and total *c*-jun N-terminal kinase (JNK) were purchased from Invitrogen (Grand Island, NY, USA), and to GFAP and β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-rabbit IgG peroxidase-conjugated was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hybond-C nitrocellulose membranes were from Hybond™ ECL™ (Hybond™ ECL™ nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany). Substrate to detect enhanced chemiluminescence (ECL) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and X-ray films were purchased from Kodak (Kodak X-Omat, Rochester, NY, USA). All other chemicals and solvents used were of analytical or chromatographic grade.

2.2. Organotypic hippocampal culture

Hippocampal slice cultures were prepared from 6- to 8-day-old male *Wistar* rats obtained from in-house breeding colonies at the "Departamento de Bioquímica", "Universidade Federal do Rio Grande do Sul" (UFRGS- Porto Alegre, Brazil). All procedures used in the present study followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23 and were approved by the local Ethics Committee on the Use of Animals (CEP-UFRGS, protocol number 2007977). All efforts were made to minimize the number of animals and their suffering. Slice cultures were prepared as interphase cultures

according to a protocol of Stoppini and collaborators (1991), with some modifications (Frozza et al., 2009; Hoppe et al., 2010). Briefly, the animals were killed, the brains were removed, the hippocampi were isolated, and transverse hippocampal slices (400 μm thickness) were prepared by using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guildford, UK). The slices were placed on membrane inserts (0.4 mM Millicell®-CM culture plate inserts) in six-well plates. Each well contained 1 mL of culture medium consisting of 50% minimum essential medium (MEM), 25% Hank' balanced salt solution (HBSS), 25% horse serum, supplemented with (mM, final concentration): glucose 36, HEPES 25, and $NaHCO_3$; Fungizone® 1%, and gentamicin 0.100 mg/mL, pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with a 5%CO_2/95%O_2 atmosphere at 37 ºC for 4 weeks. Culture medium was changed three times a week.

2.3. Preparation of lipid-core nanocapsules

Lipid-core nanocapsule suspensions were prepared by interfacial deposition of the polymer as previously described (Jagër et al., 2009; Frozza et al., 2010). At 40 °C, *trans*-resveratrol (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 mL) and sorbitan monostearate (0.038 g) were dissolved in acetone (27 mL). In a separate flask, polysorbate 80 (0.038 g) was added to 53 mL of water (MilliQ®). The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 minutes, the acetone was evaporated and the suspensions were concentrated under reduced pressure at 40 °C. The final volume was adjusted to 10 mL. A control formulation (load off lipid-core nanocapsules - LNC) was prepared without *trans*-resveratrol.

2.4. Physicochemical characterization of the formulations

The pH values of the suspensions were determined using a potentiometer B-474 (Micronal, Brazil). Mean diameters (*z*-average), polydispersity index and zeta potentials were measured at 25 °C using a Zetasizer®nano-ZS ZEN 3600 model (Nanoseries, Malvern, UK),

after diluting the samples with MilliQ® water or with 0.01 mol/L NaCl aqueous solution, respectively. To avoid any sample selection, only the dilution media was filtered (Millipore 0.45 μm) prior to analysis. Measurements were carried out using 3 different batches for each formulation in triplicate. Te size and polydispersity indexes were calculated using the software (Dispersion Technology Software -DTS Nano - Version 5.02, Malvern) provided by the manufacturer (Malvern Instruments Ltd).

2.5. Analytical procedure

Resveratrol was analyzed by high-performance liquid chromatography (HPLC) at 306 nm. The content (total concentration) of resveratrol in the formulations (100 μL) was determined after dissolving the lipid-core nanocapsules containing resveratrol into acetonitrile (10 mL) and filtering (Millipore 0.45 μm) for analysis. The system consisted of a UV-Vis detector, pump and auto-injector S200 Perkin-Elmer (PerkinElmer Instruments, Norwalk, CT) and a Shim-pack CLC-C8 (M) column (150 mm, 4.6 mm, 5 μm, Shimadzu Corporation, Japan) with a guard-column. The mobile phase was prepared by using Milli-Q® water and HPLC grade acetonitrile, and consisted of acetonitrile/water (40:60 v/v) with a pH of 3.0 ± 0.5 corrected with 10% (v/v) orthophosphoric acid. The isocratic flow rate of the mobile phase was 1.2 mL/minute and the retention time of resveratrol was 3.45 minute. The HPLC method was validated presenting linearity between 2.5 and 17.5 μg/mL, ($r > 0.9999$), inter- and intraday variability lower than 2.0%, and accuracy from 96.19% to 97.21%. Resveratrol content was 0.964 ± 0.037 mg/mL and the encapsulation efficiency was 99.89 ± 1.3% for all batches (Frozza et al., 2010).

2.6. Peptide preparation and culture exposure

The Aβ1-42 peptide or the nonamyloidogenic reverse peptide Aβ42-1 was dissolved in sterilized bi-distilled water with 0.1% ammonium hydroxide at a concentration of 1mg/mL. The Aβ peptides were aggregated at 37 ºC for 72 hours. To establish the Aβ-induced

neurotoxicity, on the 28th day in *vitro*, either Aβ1-42 or Aβ42-1 (2 μM - final concentration) were directly added to the medium and incubated for 48 hours. Control slices received no Aβ peptides.

2.7. Resveratrol treatment

In an attempt to evaluate whether the lipid-core nanocapsules containing resveratrol could improve the effectiveness of resveratrol in protecting against toxicity triggered by Aβ1-42, we compared the effect of the treatment with these nanocapsules with those of free resveratrol. A stock concentration of 100 mM free resveratrol in ethanol (EtOH) was made up each time and nanocapsules containing resveratrol were made as described above. Both, free resveratrol (RSV) and resveratrol-loaded lipid-core nanocapsules (RSV-LNC) were directly diluted in culture medium to the desired concentration. Here, we used two schedules of cultures treatment. Firstly, we sought that previous treatments to exposure to Aβ with resveratrol could be more efficient in the neuroprotective effects. In this way, on 24th day *in vitro* the cultures were treated with RSV (5 and 10 μM) or RSV-LNC (5 and 10 μM) added to the culture medium and replaced at every exchange of culture medium, totaling 96 hours of treatment before Aβ exposure. The controls were treated with identical amount of vehicles (EtOH 0.05% or LNC). Next, in another set of experiments, RSV (5 and 10 μM) or RSV-LNC (5 and 10 μM) was added to the culture medium simultaneously to Aβ exposure. In the same way as described previously, the controls were treated with identical amount of vehicles (EtOH 0.05% or LNC).

2.8. Quantification of cellular death

Cell damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake. PI is a polar compound that is impermeable to an intact cell membrane, but it penetrates damaged cell membranes of dying cells and binds to nuclear DNA to generate a bright red fluorescence. Forty-six hours after the Aβ peptides exposure, organotypic cultures

were stained with PI (5 μM) for 2 hours. PI fluorescence was observed by an inverted fluorescence microscope (Nikon Eclipse TE 300). Images were captured using a CCD camera (DXM1200C Nikon Instruments Inc., USA), stored and subsequently analyzed, by using image analysis software (Scion Image software - http://www.scioncorp.com). The amount of PI fluorescence was determined densitometrically after transforming the red values into grey values. For quantification of neural damage, the percentage of area expressing PI fluorescence above background level was calculated in relation to the total area of each slice. PI intensity, meaning cell death, was expressed as a percentage of cell damage:

$$\text{Cell death (\%)} = F_d/F_0 \text{ X } 100$$

Where F_d is the PI uptake fluorescence of dead area of hippocampal slices and F_0 is the total area of each hippocampal slice.

2.9. Evaluation of intracellular ROS formation

Formation of intracellular peroxides was detected using an oxidant sensing fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH2-DA), which is de-esterified within cells by endogenous esterases to the ionized free acid, 2',7'-dichlorofluorescin; 2',7'-dichlorofluorescin is then oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) by hydroperoxides (Hempel et al., 1999). A 10 mM DCFH2–DA stock solution was prepared in dimethylsulfoxide (DMSO). Slices cultures were treated with a variety of stimuli as indicated above. Thereafter the capture of PI images, the culture medium was collected for cytokines determination, and the cultures were washed with phosphate-buffered saline (PBS, pH 7.4). Then, the cultures were incubated with 10 μM DCFH2-DA in PBS at 37 ºC for 30 minutes. The fluorescent DCF images were observed under a fluorescence microscope (Nikon Eclipse TE 300), captured by a digital camera, stored and subsequently analyzed by using the Scion Image software (http://www.scioncorp.com). For quantification of ROS formation, the percentage of area expressing DCF fluorescence above background level was calculated in

relation to the total area of each slice (Liu et al., 2003). DCF intensity, meaning ROS formation, was expressed as a percentage of fluorescence:

$$\text{Fluorescence (\%)} = F_d/F_0 \text{ X } 100$$

Where F_d is the DCF fluorescence of ROS formation area of hippocampal slices and F_0 is the total area of each hippocampal slice.

2.10. Determination of cytokine levels in the culture medium of organotypic cultures

To asses the contribution of resveratrol on the neuroinflammation responses triggered by Aβ, pro- and anti-inflammatory cytokines release were analyzed. After 3, 6, 12, 24 or 48 hours of Aβ1-42 (2 μM) exposure, as well as resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) treatments, the culture medium was collected, rapidly frozen, and stored at -20 ºC for later measurement of TNF-α, IL-1β, IL-6, and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's recommendations. Standard curves were obtained using recombinant rat TNF-α, IL-1β, IL-6, and IL-10. The values of cytokines were expressed as pg/mL medium.

2.11. Western blotting assay

After obtaining the fluorescent images (48 hours of Aβ1-42 exposure), slices were homogenized in lyses buffer (4% sodium dodecylsulfate -SDS-, 2 mM EDTA, 50 mM Tris). Aliquots were taken for protein determination (Peterson, 1979) and β-mercaptoethanol was added to a final concentration of 5%. Proteins were resolved (20 μg per lane) on 12% SDS-PAGE. After electrophoresis, proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad, Trans-Blot SD, Hercules, CA, USA). Membranes were incubated for 60 minutes at 4 ºC in blocking solution (Tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4 ºC. The

primary antibodies against the following proteins were used: anti-phospho JNK1/2 [pTpY$^{183/185}$] (pJNK, 1:1000), anti-JNK1/2 (1:500), anti-GFAP (1:3000), and anti-β-actin (1:1000). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1000). The immunocomplexes were visualized by using the ECL chemiluminescence detection system. Band density measurements were performed by using Optiquant software (Packard Instrument). For each experiment, the test groups were compared to control cultures not exposed to Aβ.

2.12. Data analysis

The results are presented as the mean ± SD of six to nine animals per group. The statistical comparisons of the data were performed by Two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using GraphPad Prism software version 5.01 (GraphPad Software Inc. La Jolla, CA, USA). *p*-values lower than 0.05 ($p < 0.05$) were considered significant.

3. Results

3.1. Physicochemical characterization of lipid-core nanocapsules

The lipid-core nanocapsule formulations were prepared by interfacial deposition of polymer without purification. Resveratrol-loaded lipid-core nanocapsules (RSV-LNC) and load off lipid-core nanocapsules (LNC) presented a macroscopic homogeneous aspect, such as white bluish opalescent liquids. After preparation, the RSV-LNC showed particle mean size of 241 ± 7 nm, polydispersity index of 0.16 ± 0.03, zeta potential of -14.1 ± 2.3 mV, and pH of 5.2 ± 0.3. Similar results were obtained for LNC: 238 ± 6 nm, 0.14 ± 0.01, -15.8 ± 3.0 mV, and 5.4 ± 0.4, for particle size, polydispersity index, zeta potential, and pH values, respectively. The suspensions showed stable monomodal size distributions and polydispersity indexes lower than 0.17, indicating narrow size distributions.

3.2. Lipid-core nanocapsules improve the neuroprotective effect of resveratrol against Aβ-induced cell death in rat organotypic hippocampal culture

To establish that Aβ causes cell death, we performed cytotoxicity experiments using rat organotypic hippocampal cultures exposed to Aβ1-42 peptide. Aggregated Aβ1–42 caused around 28% of cell death at concentrations of 2 μM (Figs. 1 and 2). Cultures exposed to the same concentration of scramble sequence of Aβ (Aβ42-1) showed no differences in cell survival compared to untreated control cultures (data not shown). Given that resveratrol presents a wide range of pharmacological properties (Baur and Sinclair, 2006; Juan et al., 2002) we thought whether resveratrol would be neuroprotective against Aβ-induced toxicity. Furthermore, here we used lipid-core nanocapsules to overcome the intrinsic features that lead to low bioavailability of resveratrol. In this way, we compared the effects of treatment with lipid-core nanocapsule formulation containing resveratrol (RSV-LNC) with those of treatment with free resveratrol (RSV). Therefore, to examine the neuroprotective effects of resveratrol against Aβ-induced toxicity, rat organotypic hippocampal cultures were pre- (96 hours) or co-treated with RSV (5 and 10 μM) or RSV-LNC (5 and 10 μM) in the presence or absence of Aβ1-42 (2 μM) for 48 hours. As shown in Figs. 1 and 2 (A-D), cell death in cultures exposed to Aβ1-42 was significantly increased when compared to control cultures. When organotypic hippocampal cultures were pretreated with RSV, only 10 μM of resveratrol was able to protect cells from Aβ-induced cell death (Fig. 1A and B). Interestingly, in the pretreatment of organotypic hippocampal cultures with the same concentrations of RSV-LNC (5 and 10 μM), both concentrations exhibited neuroprotective effects against Aβ-induced cell death (Fig. 1C and D). It is important to note that the treatment with RSV-LNC exhibited higher neuroprotective effect than those observed for RSV treatment. Next, we also compared the effects of co-treatment of cultures with the same concentrations of RSV or RSV-LNC. Similarly to that observed in the pretreatment, lipid-core nanocapsules improve the

neuroprotective/neurorescuing effects of resveratrol against Aβ toxicity in organotypic hippocampal culture (Fig. 2 A-D). Pre- and co-treatment of cultures in absence of Aβ with RSV and RSV-LNC, as well as vehicles (EtOH or LNC, respectively) were tested for possible intrinsic toxicity activity and showed no significant differences in cell survival compared to untreated control cultures.

3.3. Resveratrol inhibition of reactive species formation induced by Aβ is improved by lipid-core nanocapsules

In order to determine whether the increased vulnerability of organotypic hippocampal cultures to Aβ was associated with an increase in reactive species formation, we measured the ROS formation by using the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH2-DA). Upon oxidation by intracellular oxidants it forms the highly fluorescent 2',7'-dichlorofluorescein (DCF). As can be seen in Figs. 3 and 4, Aβ caused increased DCF staining in all regions of hippocampal slices. Cultures exposed to the same concentration of scramble sequence of Aβ (Aβ42-1) showed no differences in DCF fluorescence compared to untreated control cultures (data not shown). Considering the antioxidant effects of resveratrol, we evaluated these effects against reactive species formation triggered by Aβ. Likewise in cell death assay, organotypic hippocampal cultures were pre- (96 hours) or co-treated with RSV (5 and 10 μM) or RSV-LNC (5 and 10 μM) in the presence or absence of Aβ1-42 (2 μM) for 48 hours. DCF fluorescence was significantly decreased by both treatments at all concentrations tested under pretreatment of cultures (Fig. 3 A-D). It is important to note that treatment of cultures with RSV-LNC has noticeable enhanced antioxidant effect when compared to the same concentration of RSV. In an attempt to evaluate whether these antioxidant effects would be maintained simultaneously to Aβ exposure, the cultures were co-treated with RSV or RSV-LNC at the same concentrations. As can be seen in Fig. 4 (A-D), only the treatment with RSV-LNC was able to significantly decrease DCF fluorescence.

Cultures pre- or co-treated with RSV and RSV-LNC, as well as vehicle (EtOH or LNC, respectively), in absence of Aβ showed no significant differences in DCF fluorescence compared to untreated control cultures.

3.4. Resveratrol-loaded lipid-core nanocapsules present higher potency than free resveratrol on blocking pro-inflammatory cytokines release

Considering the involvement of neuroinflammation in the physiopathology of AD, the anti-inflammatory properties of resveratrol were assayed in organotypic hippocampal cultures exposed to Aβ. Therefore, the release of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 from Aβ- and resveratrol-treated organotypic hippocampal cultures was measured using specific cytokines kits. Aβ exposure of cultures had a significant overall effect on the release of inflammatory cytokines (Fig. 5 A-C), appearing to elevate the medium amounts of the all cytokines studied. In order to clarify the effects of resveratrol on pro-inflammatory cytokines release, firstly organotypic hippocampal cultures were pretreated (96 hours) with RSV or RSV-LNC at 5 and 10 μM, and culture medium was collected 3, 6, 12, 24, and 48 hours after Aβ exposure. As can be seen in Fig. 5 (A-C), RSV pretreatment of cultures was able to decrease the cytokines release only in the earlier times after Aβ exposure, while RSV-LNC pretreatment had an overall effect on decreasing of cytokines release. Additionally, no significant differences were observed between 5 and 10 μM of RSV-LNC treatment. Likewise, we thought whether co-treatment of cultures with resveratrol would be neuroprotective by decreasing the cytokines release induced by Aβ. In this way, cultures were treated with RSV or RSV-LNC at 5 and 10 μM simultaneously to Aβ exposure and culture medium was collected after 3, 6, 12, 24, and 48 hours. Similarly to that observed in the pretreatment, RSV-LNC had an overall effect in decreasing cytokines release, while RSV treatment only decreased the release in the earlier time after Aβ exposure (Fig. 6 A-C). Cultures pre- or co-treated with RSV and RSV-LNC, as well as vehicle (EtOH or LNC,

respectively), in absence of Aβ showed no significant differences in cytokines release compared to untreated control cultures (data not shown).

3.5. Resveratrol-loaded lipid-core nanocapsules present higher efficacy than free resveratrol on increasing anti-inflammatory cytokine release

Since resveratrol treatments decreased the pro-inflammatory cytokines release induced by Aβ in organotypic cultures, next we thought that anti-inflammatory effects of resveratrol could modulate the release of IL-10, a powerful anti-inflammatory cytokine. In an attempt to evaluate this hypothesis, similarly to the pro-inflammatory cytokines study, organotypic hippocampal cultures were firstly pretreated (96 hours) with RSV or RSV-LNC at 5 and 10 μM before Aβ exposure and the medium collected after 3, 6, 12, 24, and 48 hours for IL-10 analysis. Here it is important to note that the RSV treatment elevate the amounts of IL-10 in the medium only in control cultures (non-exposed to Aβ). Furthermore, this effect was observed only up to 6 hours. Interestingly, RSV-LNC treatment had an overall effect in significantly increase the amounts of IL-10 in the medium up to 48 hours in both cultures (control and exposed to Aβ) (Fig. 7A). This effect was more pronounced after Aβ-exposure, indicating that, in support to the decreasing in the pro-inflammatory cytokines release (Figs. 5 and 6), lipid-core nanocapsules improve the anti-inflammatory effects of resveratrol. Similar results were obtained when cultures were treated with the same concentrations of RSV or RSV-LNC simultaneously to Aβ exposure (Fig. 7B). Cultures pre- or co-treated with vehicle (EtOH or LNC), in presence or absence of Aβ showed no significant differences in IL-10 release compared to untreated control cultures (Fig. 7).

3.6. The decreasing of glial and JNK activation is associated with neuroprotective/neurorescuing effect of resveratrol in rat organotypic hippocampal culture exposed to Aβ

To reinforce the role of inflammation in our Aβ toxicity paradigm, we tested the possible requirement of astrocytes and JNK activation. For this, we evaluated whether resveratrol could be able to promote a decreasing of GFAP immunocontent and JNK phosphorylation using specific antibodies. First, we determined the effect of pretreatment (96 hours) of cultures with RSV or RSV-LNC at 5 and 10 μM in decreasing GFAP expression and JNK activation. As shown in Fig. 8 (A and B), while only 10 μM of RSV was able to decrease astrocyte activation lower concentration of RSV-LNC exerted this effect. In addition, it is important to note that RSV pretreatment failed to prevent JNK activation triggered by 48 hours of Aβ exposure (Fig. 8C). On the other hand, RSV-LNC pretreatment even at lower concentration prevents this event (Fig. 8D). Next, we evaluated whether co-treatment of cultures with resveratrol would be rescuing the cultures from glial and JNK activation triggered by Aβ. In this way, cultures were treated with RSV or RSV-LNC at 5 and 10 μM simultaneously to Aβ exposure and GFAP immunocontent a JNK phosphorylation were analyzed after 48 hours. Once again, RSV treatment failed to rescue cultures from Aβ-induced toxicity (Fig. 9 A and C), while RSV-LNC treatment was able to decrease glial and JNK activation (Fig. 9 B and D). Cultures pre- or co-treated with RSV and RSV-LNC, as well as vehicle (EtOH or LNC, respectively), in absence of Aβ showed no significant differences in GFAP immunocontent and JNK phosphorylation compared to untreated control cultures (Figs. 8 and 9).

4. Discussion

Basic and clinical research advances over the past decades have provided progresses about understanding the course of AD. Advances in therapeutic strategies for AD, that lead to even small delays in onset and progression of the condition, would significantly reduce the global burden of the disease. Here, we have used organotypic hippocampal culture to show

that neuroinflammation plays a prominent role in neurodegeneration events triggered by Aβ, and provide convincing evidence implicating the neuroprotective properties of resveratrol against the Aβ-induced toxicity.

Given that previous studies have shown that astrocytes are important mediators of Aβ-induced neurotoxicity (Bales et al., 2000; Paradisi et al., 2004; Garwood et al., 2011), organotypic hippocampal cultures are advantageous over isolated cell cultures, since the cellular interactions are preserved allowing studies about the responses of microglial, neurons, and glial cells to Aβ-induced toxicity. In this system, neurons and glial cells survive during long-term culture and physiologically mature over this period, allowing an extended survival study (Frotscher et al., 1995). For this reason, organotypic hippocampal culture provides a good experimental access when evaluating mechanisms of neurodegeneration and designing therapeutic agents (Stoppini et al., 1991; Holopainen, 2005). We found a significantly increase of cell death and reactive species formation when cultures were exposed to Aβ, which may have been driven by a pronounced disturbance on cytokines releasing, and glial and cell signaling alterations. Inhibiting the inflammatory responses with resveratrol decreased these effects triggered by Aβ, suggesting that resveratrol might be considered a promising anti-amyloidogenic candidate for AD treatment. However, despite resveratrol has been linked to many beneficial effects, its therapeutic applications remains very limited due to combination of several limiting factors including its poor water solubility, labile properties, and short biological half-life, meaning that resveratrol demonstrates very poor bioavailability. In view of the limitations such as these and those related to cross blood-brain barrier (BBB), nanoparticulated systems represent a promising alternative, since that during the past decade numerous attempts have focused on designing different strategies that aid to stabilize and protect several drugs in order to improve their bioavailability and achieve sustained release, as well as improve their passage across the BBB (Brambilla et al., 2011).

Among these, nanotechnology-based strategies, as used in this study, have gained tremendous importance. The application of these technological advances in neurological research, mainly in AD, is expected to have a major impact leading to the development of newer therapeutic modalities (Modi et al., 2009).

In previous work, we have already shown that the treatment with 25 μM of free resveratrol was able to protect organotypic cultures from cell death induced by oxygen-glucose deprivation (Zamin et al., 2006). In this way, we sought that using resveratrol-loaded lipid-core nanocapsules lower concentration of resveratrol could be neuroprotective against Aβ-induced toxicity, since that treatment of organotypic cultures with indomethacin-loaded lipid-core nanocapsules allowed the use of sub-therapeutic concentration of indomethacin with effective neuroprotective effects against oxygen-glucose deprivation lesion (Bernardi et al., 2010). Our results shown that both pre- and co-treatments were able to decrease the Aβ-induced cell death in hippocampal organotypic culture. However, while only the higher concentration of free resveratrol was effective against Aβ-induced cell death, lower concentrations of resveratrol-loaded lipid-core nanocapsules showed neuroprotective effects, confirming our initial hypothesis. Taking into account that resveratrol has a powerful antioxidant activity and that Aβ may induce oxidative stress, we next investigated whether the cell death induced by Aβ in our model was accompanied by increased formation of reactive species. As reveled by fluorescence of the DCF, Aβ-induced intracellular formation of reactive species was reduced by pretreatment with both RSV and RSV-LNC, but co-treatment of cultures showed antioxidant effects only with RSV-LNC. It is important to note that even in the pretreatment RSV-LNC presented higher efficacy in the antioxidant effect when compared to the same concentrations of RSV. These differences in the effects of resveratrol, free or into lipid-core loaded nanocapsules, may be explained either by sustained release of resveratrol or decreased binding to albumin present in the culture medium, since

this is an important limitation of bioavailability of resveratrol using *in vitro* models (Jannin et al., 2004). Additionally, possible mechanism underlying the superior efficacy of RSV-LNC against Aβ at lower concentration observed in the current study may include the enhanced intracellular resveratrol accumulation by lipid-core nanocapsule uptake. However, intracellular uptake of lipid-core nanocapsules by cells still has its maximum, this way presenting a potential mechanism to explain why both concentration of RSV-LNC caused similar neuroprotective effects. In agreement with earlier findings, our first set of results clearly implicated the potential neuroprotective effects of resveratrol against the Aβ-induced toxicity. Several studies have already shown that resveratrol prevents AD by reducing the cell death induced by Aβ, inhibiting the Aβ fibrillation and oligomers formation, and promoting clearance of Aβ (Han et al., 2004; Marambaud et al., 2005; Rivière et al., 2007; Ladiwala, et al., 2010). These and the results reported in the current study are encouraging considering that the development of therapies for AD represents a major challenge to academic, biotechnology, and pharmaceutical scientists.

Our next step was to verify the contribution of neuroinflammation to the genesis of Aβ-induced toxicity, as well as the effect of resveratrol in this event. The results presented here permitted us to suggest that neuroinflammation is strongly involved in Aβ toxicity. A pronounced release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 appears to have an important role in the Aβ-induced toxicity, which can lead to disturbances in glial homeostasis, cell signaling, ROS formation, and cell death (Van Eldik et al., 2007; Anisman, 2009; Hensley, 2010; Garwood et al., 2011). Our results are in accordance with those reported in the literature. Here, we showed that the kinetic of cytokines release TNF-α, IL-1β and IL-6 from organotypic hippocampal cultures exposed to Aβ presented a similar profile. All cytokines evaluated exhibited high release levels that persisted at least 48 hours after Aβ exposure. Our results clearly shown that even RSV treatment was able to decrease the pro-

inflammatory cytokines release only up to 6 hours, the treatment with RSV-LNC produced a significant and sustained decrease of TNF-α, IL-1β, and IL-6, persisting for long periods of time as 48 hours. Furthermore, both pre- and co-treatment with RSV-LNC significantly increased the levels of IL-10 in the presence or absence of Aβ, reaching maximal effects at 6 hours and remaining at least to 48 hours contributing for its anti-inflammatory effects, whereas treatments with RSV only increased the IL-10 release of cultures in absence of Aβ and only for up to 6 hours. In this way, it is thought that the RSV was able to partially break the inflammatory response induced by Aβ culminating in delayed cell death and reactive species formation as observed by PI and DCF fluorescence. Again, one plausible explanation for the difference effects found between RSV and RSV-LNC treatments is that the lipid-core nanocapsules can allow a sustained release of resveratrol from nanocapsules, preventing the oxidation of resveratrol as well as its binding to the albumin present in the culture medium, meaning higher bioavailability of resveratrol. Thus, the lipid-core nanocapsules permitted that resveratrol was delivered to the cell in a controlled way, providing a sustained blockage of neuroinflammation signaling propagation and prevent delay cell death. Furthermore, previous studies have already shown the enhanced cellular uptake of resveratrol by nanoparticulate delivery systems resulting in higher drug concentration into the cells (Shao et al., 2009; Teskac and Kristl, 2010). In accordance with intracellular drug accumulation, the high efficiency in the neuroprotective effects observed here may have been achieved by delivering resveratrol with lipid-core nanocapsules.

Astrocytes and microglia play central roles in host defense during brain infection and inflammation. These cells normally provide tissue maintenance and immune surveillance of the brain, but as potent source of proinflammatory cytokines and chemokines, astrocytes and microglia are pivotal in the progression of neurodegenerative process including AD. Considering that hippocampal culture preserves the interactions among glial, neuronal, and

microglial cells and given that previous studies have shown that astrocytes and microglial cells are important mediators of Aβ-induced neurotoxicity (Giulian et al., 1996; Garwood et al., 2011, Capiralla et al., 2011), in order to clarify the effect of resveratrol, we evaluated the astrocytes activation through increasing expression of glial fibrillary acidic protein (GFAP). Furthermore, previous evidence suggests that the activation by phosphorylation of stress-activated protein kinase (JNK) is closely associated with neural dysfunction in AD (Mehan et al., 2011). The activation of JNK has been described in cultured neurons after Aβ exposure, and its inhibition attenuates Aβ toxicity (Bozyczko-Coyne at al., 2001; Morishima et al., 2001). Additionally, JNKs are reported to be involved in the enlargement of microglia, as well as in the induction of proinflammatory cytokine genes coding for TFN-α, IL-6, or MCP-1 in addition to COX-2 (Waetzig and Herdegen, 2004), suggesting that JNKs are relevant co-mediators of the activation of microglia. Activation of JNKs in brain triggers the inflammatory process and enhances the expression of iNOS in microglia (Mehan et al., 2011). In the present study, we demonstrate that Aβ triggered astrocytes and likely microglial activation. The activation of these glial cells may have potentiated the neuroinflammation cascades leading to sustained JNK activation. In accordance with results found in cell death, reactive species formation, and cytokines release, pretreatment with RSV was able to reduce astrocytes activation only at higher concentration without any effect upon JNK phosphorylation, while pretreatment with RSV-LNC even in lower concentration was able to decrease astrocytes and JNK activation triggered by 48 hours of exposure to Aβ. Interestingly, resveratrol was able to block astrocytes and JNK activation following co-treatment only when loaded into lipid-core nanocapsules. These set of results permit us conclude that the release of proinflammatory cytokines led to a sustained activation of astrocytes and microglial cells with disturbances in the JNK signaling, which may have accounted to cell death observed 48 hours after Aβ exposure. It is interesting to note that the

use of lipid-core nanocapsules allowed resveratrol to be able to block this glial loop, independently of pre- or co-treatment, explaining the results here observed. Taken together, lipid-core nanocapsules delivery system shows advantages over free resveratrol in all assessment of in vitro Aβ-induced toxicity.

The mechanisms involved downstream Aβ remain to be determined. In the current study, we provide strong evidence that Aβ can trigger inflammatory responses that culminate with reactive species formation and cell death. Alterations in astrocytes activation observed herein are in agreement with those found by Garwood and co-workers (2011), indicating that this altered status occurs when astrocytes enter an active cytokine-secreting state. Moreover, the presence of microglial cells in our model may potentiates the toxic effects of Aβ. Both astrocytes and microglia have ability to produce a variety of cytokines, including IL-1, IL-6, IL-10, INF-α, INF-β, TNF-α, TNF-β; chemokines, including RANTES (CCL5), IL-8 (CXCL8), and MCP-1; nitric oxide (NO); and reactive oxygen species (Wyss-Coray, 2006; Cameron and Landreth, 2010; Lu et al., 2010; Garwood et al., 2011). Over-activation of glial cells and release of proinflammatory cytokines may lead to neuronal death. The binding of these factors to plasma membrane receptors, such as Toll-like receptors 2 (TLR2) and 4 (TLR4), induces various cell signaling pathways, including JNK, iNOS, and NF-κB activation, which may trigger a loop of sustained proinflammatory cytokines release (Buchanan et al., 2010; Cameron and Landreth, 2010; Capiralla et al., 2011). Resveratrol has been proposed to be an anti-inflammatory molecule and several studies have reported that resveratrol differentially modulates inflammatory responses of microglia and astrocytes induced by LPS (Candelario-Jalil et al., 2007; Lu et al., 2010) and Aβ (Capiralla et al., 2011). These effects have often been attributed to its actions on MAP kinases and NF-κB and/or AP-1 pathways. Resveratrol inhibition of MAP kinases, NF-κB and AP-1 may decrease the production of inflammatory cytokines and iNOS in response to Aβ. Furthermore, resveratrol

is an activator of SIRT1, which has been reported to inhibit NF-κB pathway activation (Yeung et al., 2004; Chen et al., 2005). The inhibition of SIRT1signaling by Aβ can be responsible for the activation of NF-κB and subsequent generation of proinflammatory cytokines. Therefore, it should be quite interesting to investigate whether activation of SIRT1 signaling also contributes to the inhibitory effect of resveratrol on proinflammatory cytokines secretion triggered by Aβ in hippocampal organotypic cultures. These pathways alterations may explain the results found in this work. Although further studies are needed, our data suggest that resveratrol may act in these signaling disturbances, and by this way prevent the cell death observed in our experimental model.

In summary, the results presented by us indicate a close association between cytokine release and the neurotoxic events that occur downstream of Aβ, and suggest that suppression of these inflammatory responses may represent a valid therapeutic strategy for the treatment of AD and related neurodegenerative disorders. Additionally, the present study provides strong evidence that resveratrol negatively controlled the neuroinflammation and the consequent cell death triggered by Aβ. Therefore, data from this study not only confirm the potential of resveratrol in treating neurodegenerative processes but also offer an effective way to improve the neuroprotective efficiency of resveratrol by nanocarrier delivery system. These findings provide further support for future studies aiming at precisely understanding of mechanisms involved in the neuroprotective effects of resveratrol. Furthermore, the combination of resveratrol and lipid-core nanocapsules-based delivery system may open new avenues for the treatment of Alzheimer's disease.

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Disclosure statement

The authors declare that they have no actual or potential conflicts of interest.

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Figure legends

Fig. 1. Effects of free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) pretreatments on cell damage triggered by Aβ in organotypic hippocampal cultures. (A and C) representative photomicrographs of hippocampal slices stained with PI 48 hours after Aβ exposure. (B and D) Quantitative analysis of hippocampal damage 48 hours after Aβ exposure. RSV or RSV-LNC (5 and 10 μM) were added 96 h before the Aβ exposure and maintained during next 48 hours. Bars represent the mean ± SD, n= 9 animals per group. $^{\#}p <$ 0.001 significantly different from the respective control culture; $^{**}p < 0.01$ significantly different from the Aβ and AβEtOH groups; $^{***}p < 0.001$ significantly different from the Aβ and AβLNC groups, (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 2. Effects of free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) co-treatments on cell damage induced by Aβ in organotypic hippocampal cultures. (A and C) representative photomicrographs of hippocampal slices stained with PI 48 hours after Aβ exposure. (B and D) Quantitative analysis of hippocampal damage 48 hours after Aβ exposure. Cultures were treated simultaneously to Aβ exposure with RSV or RSV-LNC (5 and10 μM) and the treatments were maintained during next 48 hours. Bars represent the mean ± SD, n= 9 animals per group. $^{\#}p < 0.001$ significantly different from the respective control culture; $^{**}p < 0.01$ significantly different from the Aβ and AβEtOH groups; $^{***}p < 0.001$ significantly different from the Aβ and AβLNC groups, (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 3. Effects of free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) pretreatments on ROS formation triggered by Aβ in organotypic hippocampal cultures. (A and C) representative photomicrographs of hippocampal slices fluorescence 48 hours after Aβ exposure. (B and D) Quantitative analysis of hippocampal DCF fluorescence 48 hours after Aβ exposure. Cultures were pretreated by 96 hours before to Aβ exposure with RSV or RSV-LNC (5 and 10 μM) and the treatments were maintained during next 48 hours. Bars represent the mean ± SD, n= 9 animals per group. $^{\#}p < 0.001$ significantly different from the respective control culture; $^{***}p < 0.001$ significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC), (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 4. Effects of free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) co-treatments on ROS formation triggered by Aβ in organotypic hippocampal cultures. (A and C) representative photomicrographs of hippocampal slices fluorescence 48 hours after Aβ exposure. (B and D) Quantitative analysis of hippocampal DCF fluorescence 48 hours after Aβ exposure. Cultures were treated with RSV or RSV-LNC (5 and 10 μM) simultaneously to Aβ exposure and the treatments were maintained during next 48 hours. Bars represent the mean ± SD, n= 9 animals per group. $^{\#}p < 0.001$ significantly different from the respective control culture; $^{***}p < 0.001$ significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC) (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 5. Resveratrol prevents the pro-inflammatory cytokines release triggered by Aβ in organotypic hippocampal slice cultures. Cultures were pretreated with RSV or RSV-LNC (5 and 10 μM) as described in Methods section. Levels of TNF-α (A), IL-1β (B), and IL-6 (C) were measured 3, 6, 12, 24, and 48 hours after Aβ exposure. Bars represent the mean ± SD, n= 4 animals per group. $^{\#}p < 0.001$ significantly different from control groups, $^{*}p < 0.05$ significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC); $^{**}p < 0.01$ significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC); $^{***}p < 0.001$ significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC); $^{\Delta}p < 0.05$ significantly different between respective RSV and RSV-LNC concentrations; $^{\$}p < 0.01$ significantly different between respective RSV and RSV-LNC concentrations; $^{\&}p < 0.001$ significantly different between respective RSV and RSV-LNC concentrations, (two-way ANOVA followed by Bonferroni post hoc test).

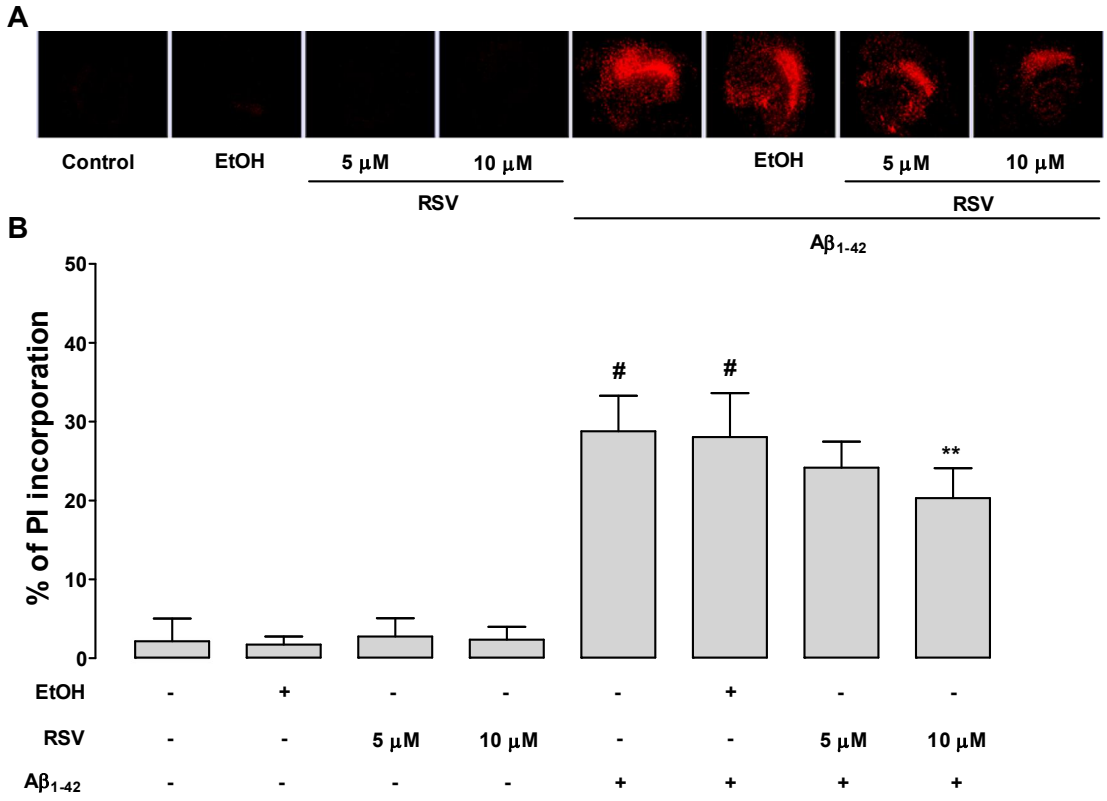
Fig. 6. Effects of co-treatment with resveratrol upon pro-inflammatory cytokines release triggered by Aβ in organotypic hippocampal slice cultures. Cultures were treated with RSV or RSV-LNC (5 and 10 μM) simultaneously to Aβ exposure as described in Methods section. Levels of TNF-α (A), IL-1β (B), and IL-6 (C) were measured 3, 6, 12, 24, and 48 hours after Aβ exposure. Bars represent the mean ± SD, n= 4 animals per group. $^{\#}p < 0.001$ significantly different from control groups, *p < 0.05 significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC); **p < 0.01 significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC); ***$p < 0.001$ significantly different from the Aβ and Aβ vehicle groups (AβEtOH or AβLNC); $^{\Delta}p < 0.05$ significantly different between respective RSV and RSV-LNC concentrations; $^{\$}p < 0.01$ significantly different between respective RSV and RSV-LNC concentrations; $^{\&}p < 0.001$ significantly different between respective RSV and RSV-LNC concentrations, (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 7. Resveratrol increases the anti-inflammatory cytokine IL-10 release in organotypic hippocampal slice cultures. (A) Cultures were pretreated with RSV or RSV-LNC (5 and 10 μM) as described in Methods section. Levels of IL-10 were measured 3, 6, 12, 24, and 48 hours after Aβ exposure. (B) Cultures were treated with RSV or RSV-LNC (5 and 10 μM) simultaneously to Aβ exposure as described in Methods section. Levels of IL-10 were measured 3, 6, 12, 24, and 48 hours after Aβ exposure. Bars represent the mean ± SD, n= 4 animals per group. ***$p < 0.001$ significantly different from the control or Aβ cultures treated with vehicle (EtOH or LNC); $^{\&}p < 0.001$ significantly different between respective RSV and RSV-LNC concentrations (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 8. Pretreatment with free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) modulates Aβ-induced glial activation and JNK phosphorylation. Organotypic hippocampal slices cultures were pretreated with RSV or RSV-LNC (5 and 10 μM) by 96 hours before Aβ exposure. Representative Western blot and graph showing quantification of GFAP immunocontent normalized by β-actin protein (loading control) (A and B, respectively). Representative Western blot and graph showing quantification ratio pJNK/JNK immunocontent normalized by β-actin protein (loading control) (C and D, respectively) of cultures pretreated with RSV or RSV-LNC, respectively. Bars represent the mean ± SD, n= 6 animals per group. $^{\#}p < 0.001$ significantly different from control groups; $*p < 0.05$ significantly different of cultures exposed to Aβ and treated with EtOH; $***p < 0.001$ significantly different of cultures exposed to Aβ and treated with LNC, (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 9. Effects of co-treatment with resveratrol over glial activation and JNK phosphorylation triggered by Aβ. The immunocontent of GFAP and the phosphorylation of JNK were detected in cell lysates by Western blot using specific antibodies. Organotypic hippocampal slices cultures were treated with RSV or RSV-LNC (5 and 10 μM) simultaneously to Aβ exposure. Representative Western blot and graph showing quantification of GFAP (A) and the ratio pJNK/JNK (C) immunocontent normalized by β-actin protein (loading control) of cultures co-treated with RSV. Representative Western blot and graph showing quantification of GFAP (B) and the ratio pJNK/JNK (D) immunocontent normalized by β-actin protein (loading control) of cultures co-treated with RSV-LNC. Bars represent the mean ± SD, n= 6 animals per group. $^{\#}p < 0.001$ significantly different from control groups, $^{***}p < 0.001$ significantly different of cultures exposed to Aβ and treated with LNC, (two-way ANOVA followed by Bonferroni post hoc test).

Fig. 1


A
Control
EtOH
5 μM
10 μM
RSV
EtOH
5 μM
10 μM
RSV
Aβ1-42
B
% of PI incorporation
50
40
30
20
10
0
#
#
**
EtOH - + - - - + - -
RSV - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

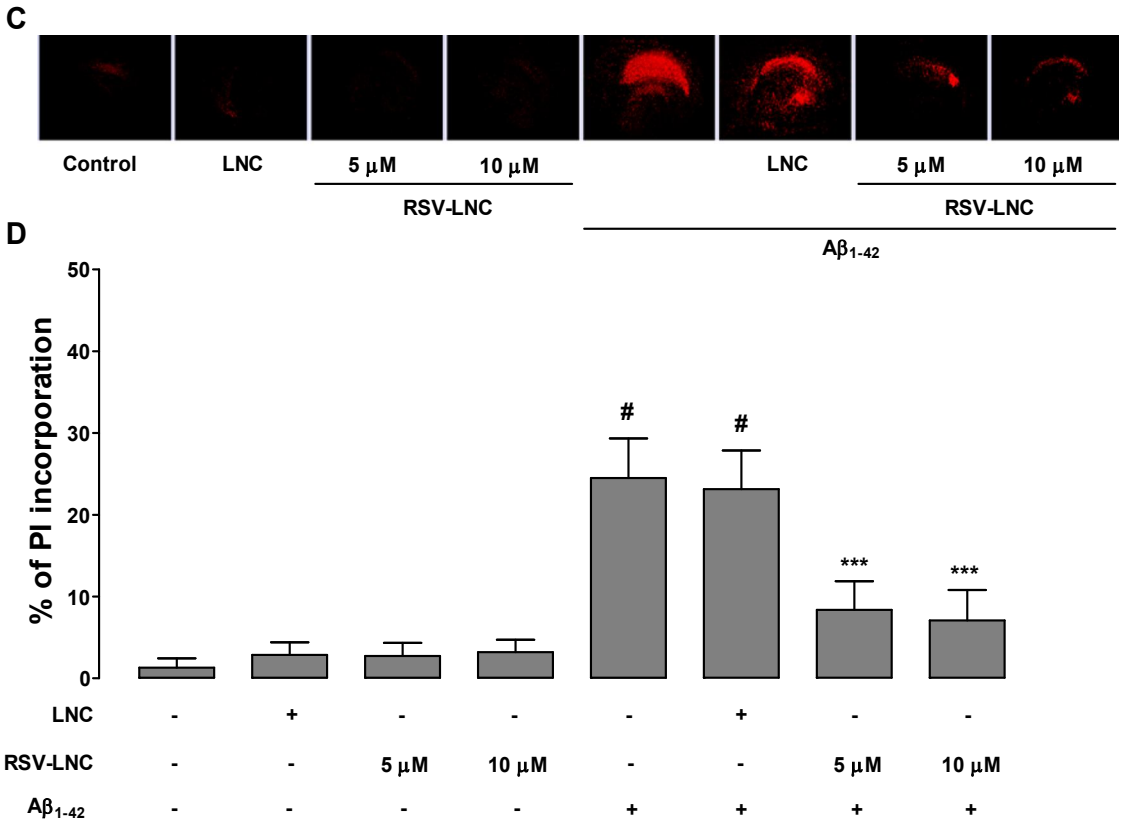
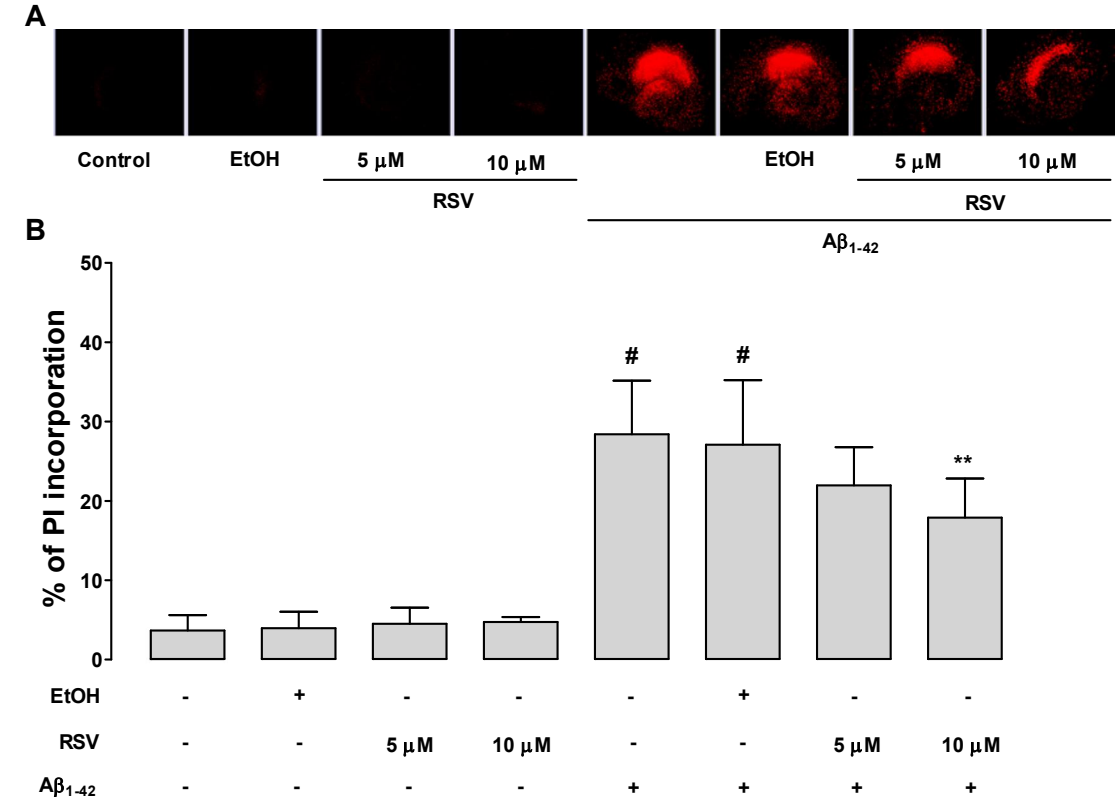

C
Control
LNC
5 μM
10 μM
RSV-LNC
LNC
5 μM
10 μM
RSV-LNC
Aβ1-42
D
% of PI incorporation
50
40
30
20
10
0
#
#
***
***
LNC - + - - - + - -
RSV-LNC - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

Fig. 2


A
Control
EtOH
5 μM
10 μM
RSV
EtOH
5 μM
10 μM
RSV
Aβ1-42
B
% of PI incorporation
50
40
30
20
10
0
#
#
**
EtOH - + - - - + - -
RSV - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

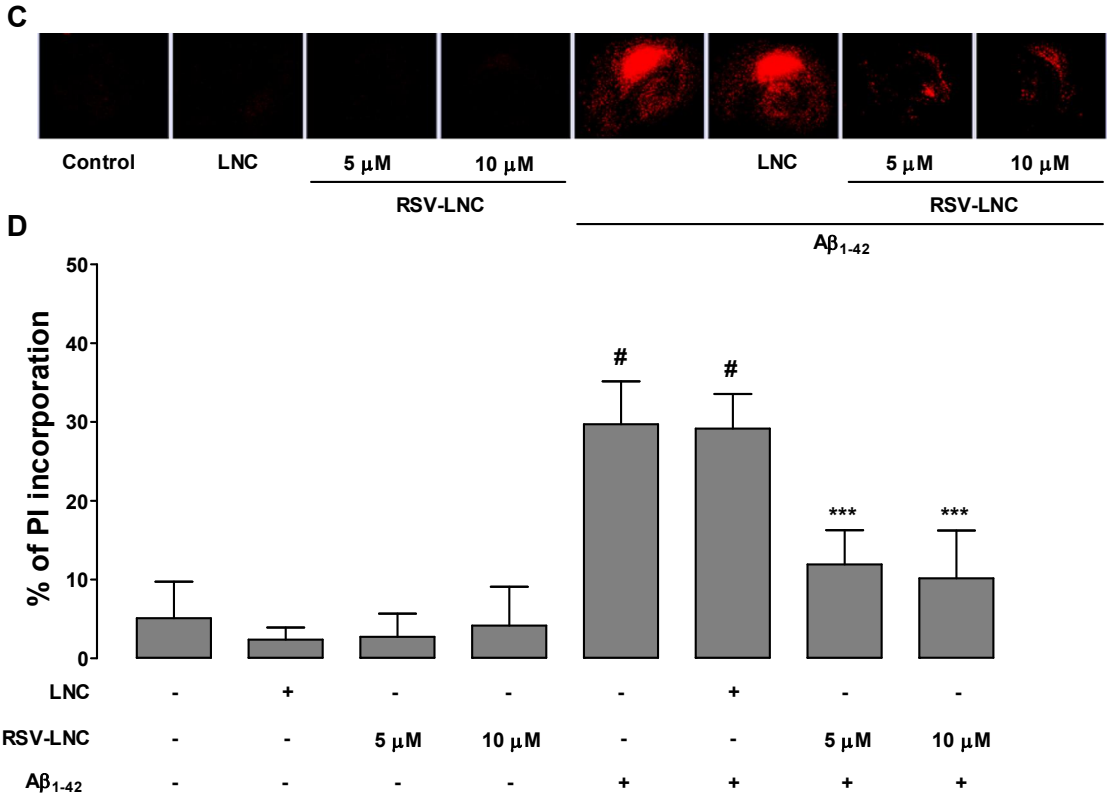
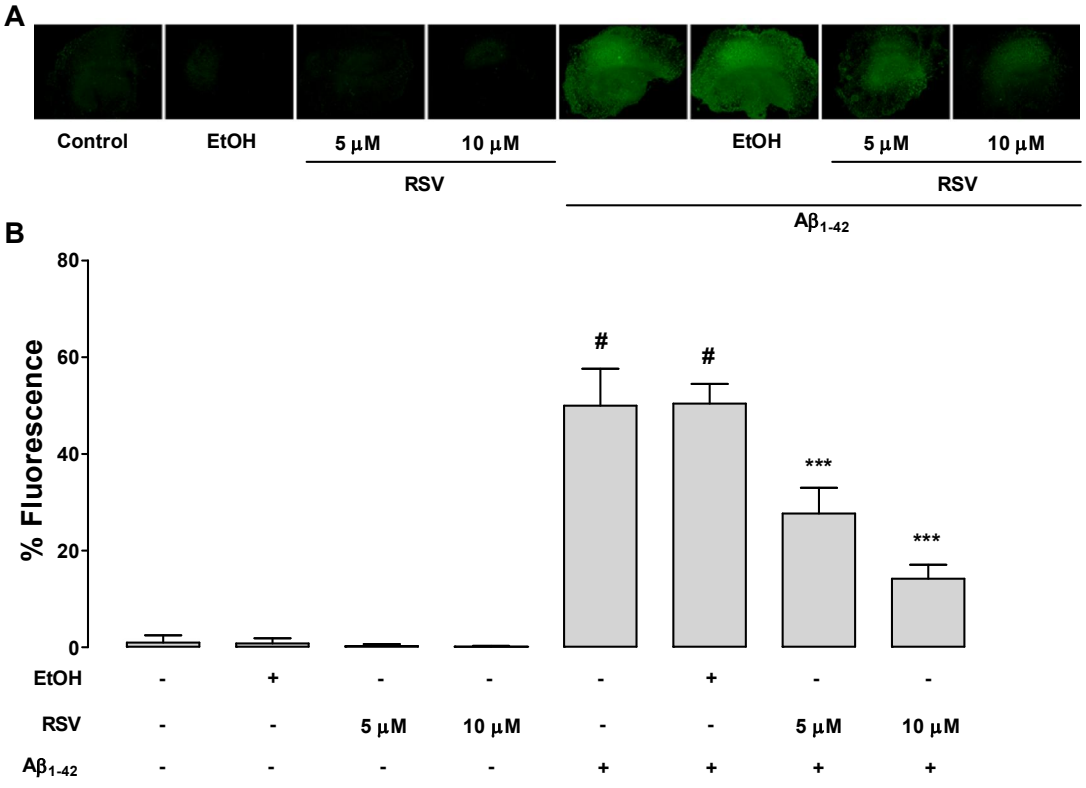

C
Control
LNC
5 μM
10 μM
RSV-LNC
LNC
5 μM
10 μM
RSV-LNC
Aβ1-42
D
% of PI incorporation
50
40
30
20
10
0
#
#
***
***
LNC - + - - - + - -
RSV-LNC - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

Fig. 3


A
Control
EtOH
5 μM
10 μM
RSV
EtOH
5 μM
10 μM
RSV
Aβ1-42
B
% Fluorescence
80
60
40
20
0
#
#
***
***
EtOH - + - - - + - -
RSV - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

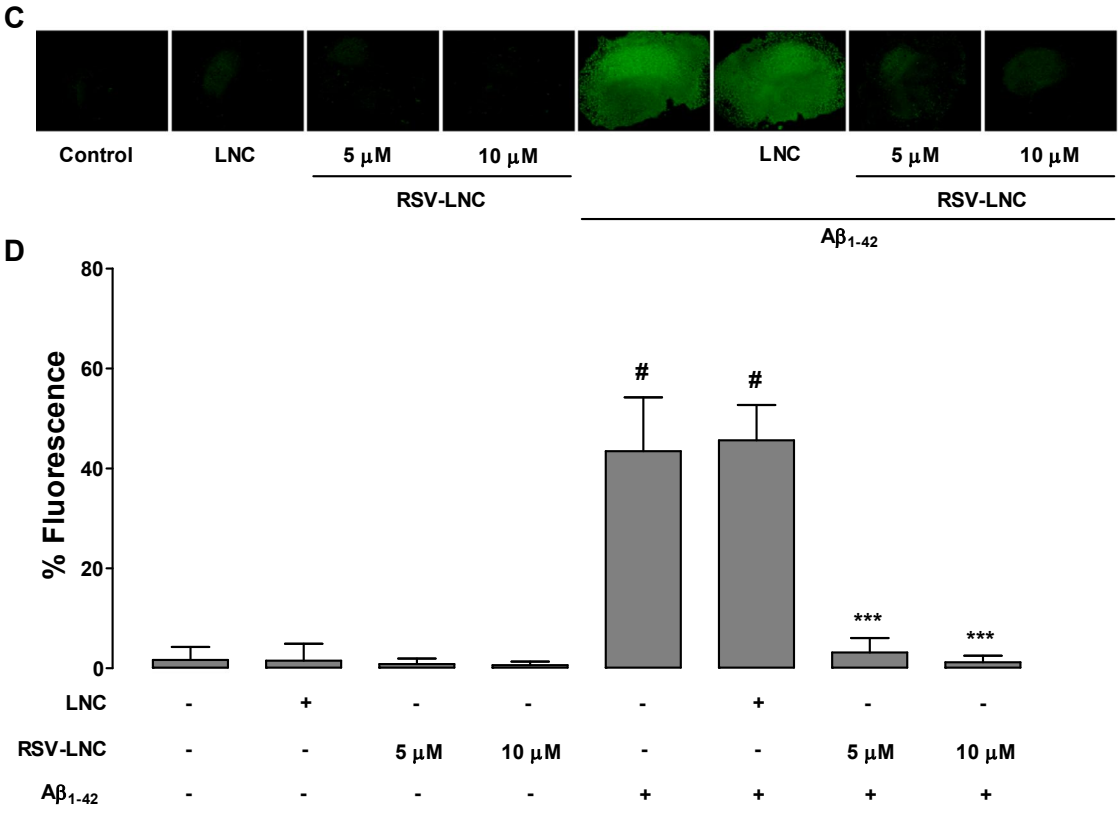
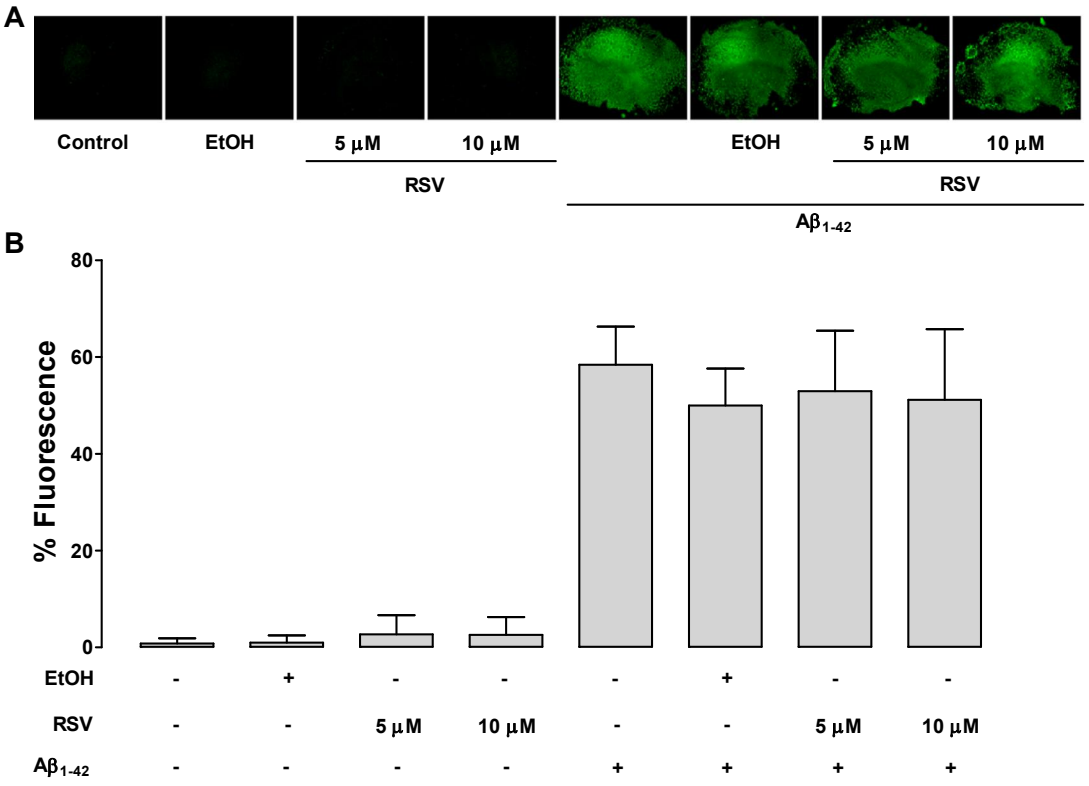

C
Control
LNC
5 μM
10 μM
RSV-LNC
LNC
5 μM
10 μM
RSV-LNC
Aβ1-42
D
% Fluorescence
80
60
40
20
0
#
#
***
***
LNC - + - - - + - -
RSV-LNC - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

Fig. 4


A
Control
EtOH
5 μM
10 μM
RSV
EtOH
5 μM
10 μM
RSV
Aβ1-42
B
% Fluorescence
80
60
40
20
0
EtOH - + - - - + - -
RSV - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

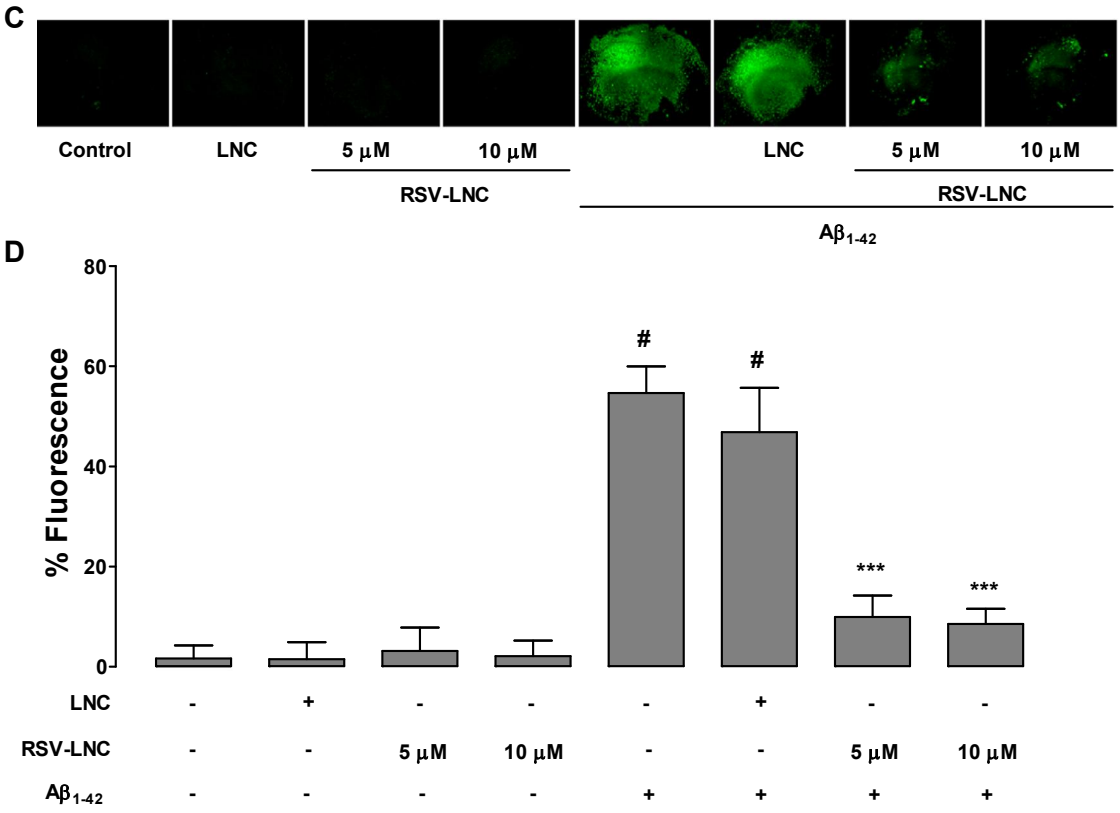
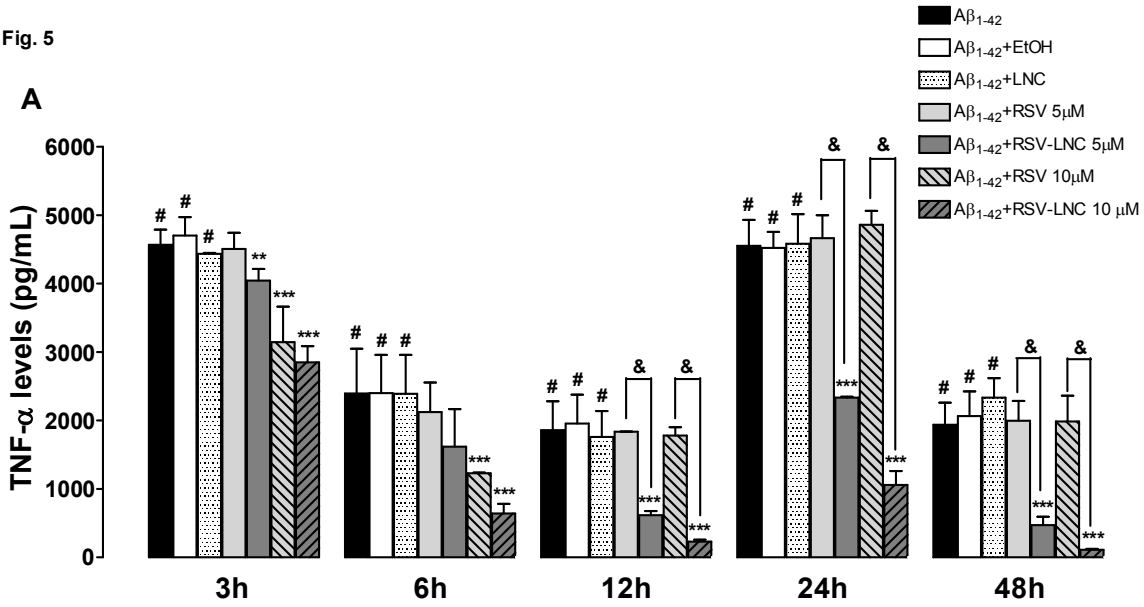
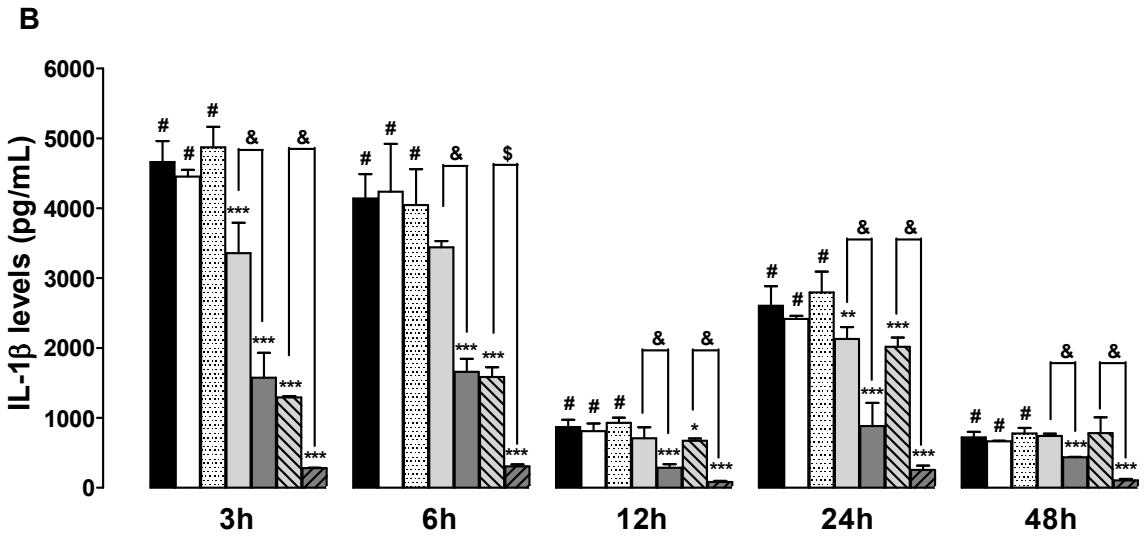

C
Control
LNC
5 μM
10 μM
LNC
5 μM
10 μM
RSV-LNC
RSV-LNC
Aβ1-42
D
% Fluorescence
80
60
40
20
0
#
#
***
***
LNC - + - - - + - -
RSV-LNC - - 5 μM 10 μM - - 5 μM 10 μM
Aβ1-42 - - - - + + + +

Fig. 5


A
TNF-α levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
Aβ1-42
Aβ1-42+EtOH
Aβ1-42+LNC
Aβ1-42+RSV 5μM
Aβ1-42+RSV-LNC 5μM
Aβ1-42+RSV 10μM
Aβ1-42+RSV-LNC 10 μM


B
IL-1β levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h

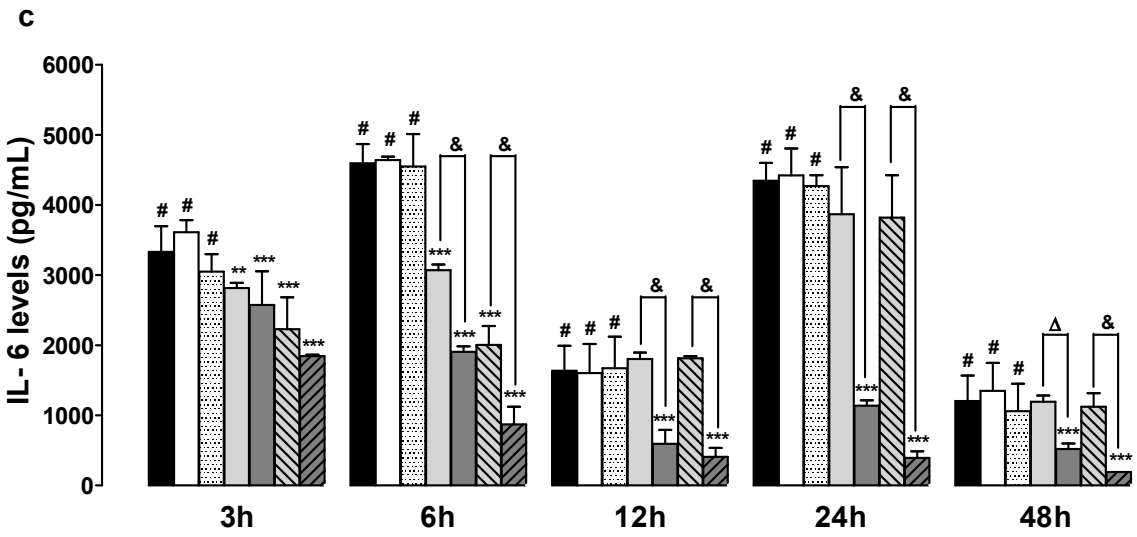
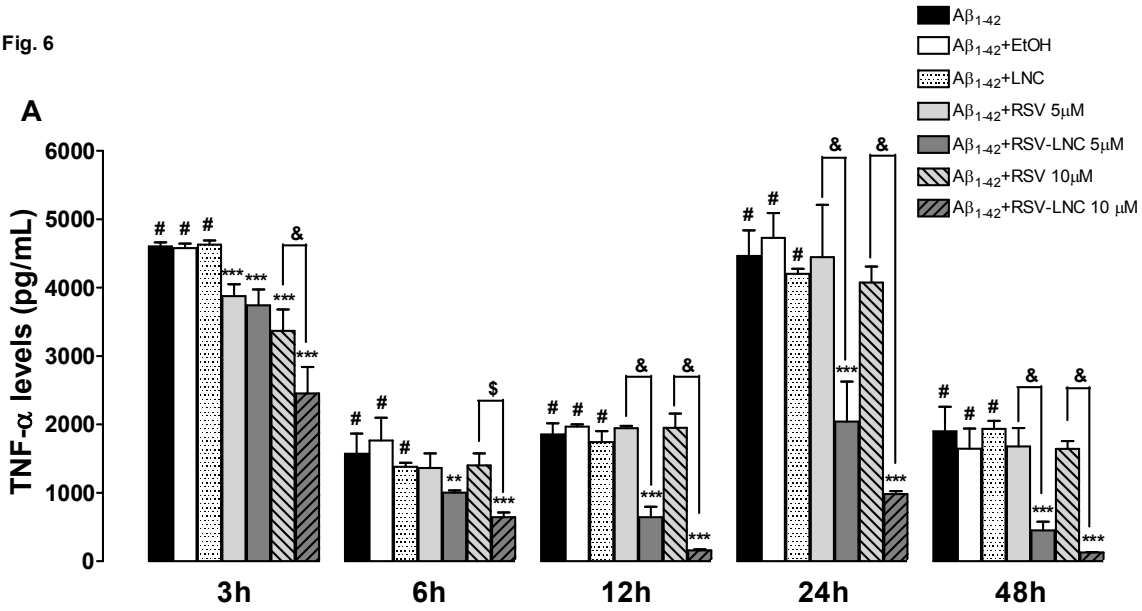
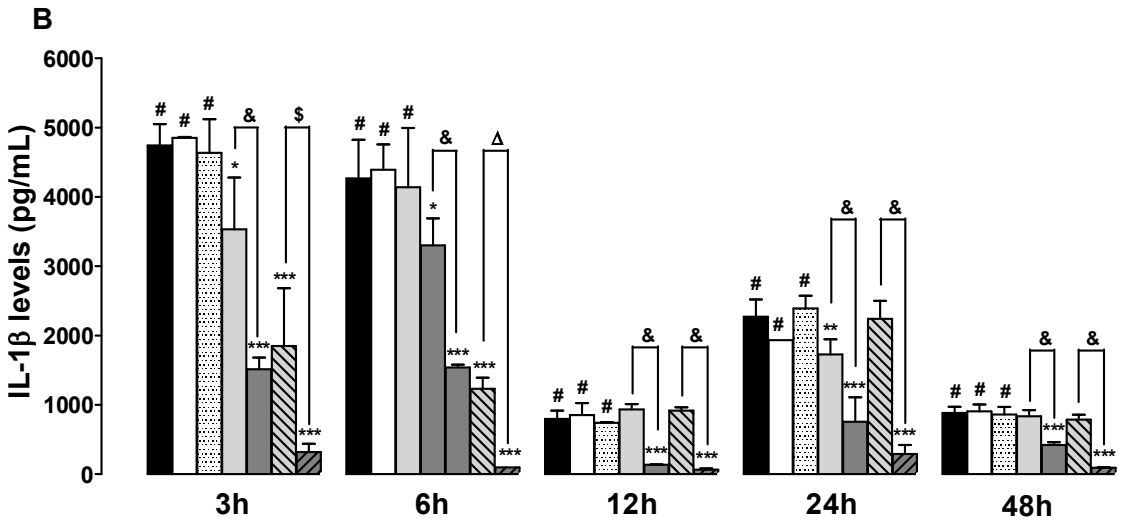

c
IL- 6 levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h

Fig. 6


A
TNF-α levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
Aβ1-42
Aβ1-42+EtOH
Aβ1-42+LNC
Aβ1-42+RSV 5μM
Aβ1-42+RSV-LNC 5μM
Aβ1-42+RSV 10μM
Aβ1-42+RSV-LNC 10 μM


B
IL-1β levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h

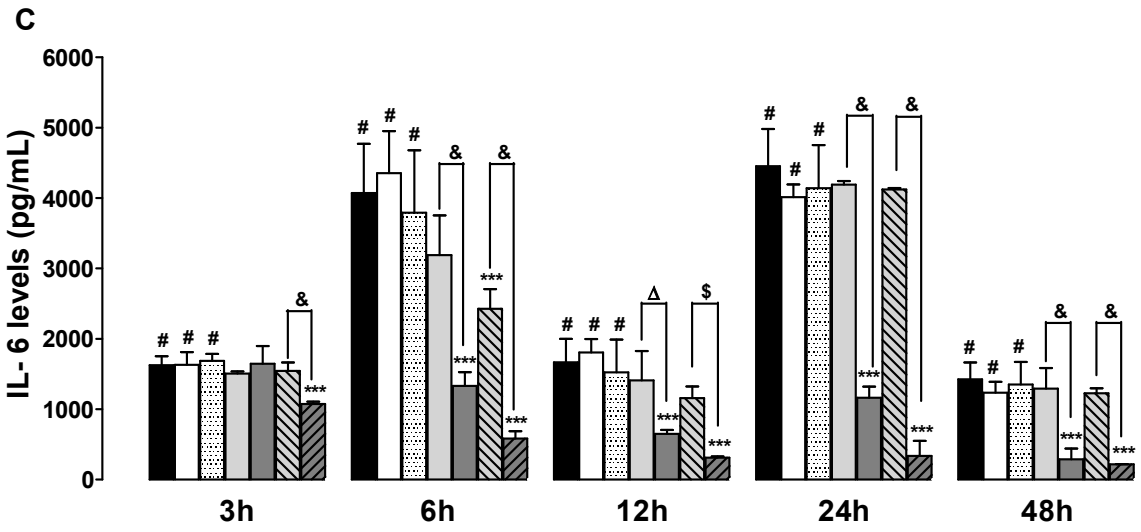
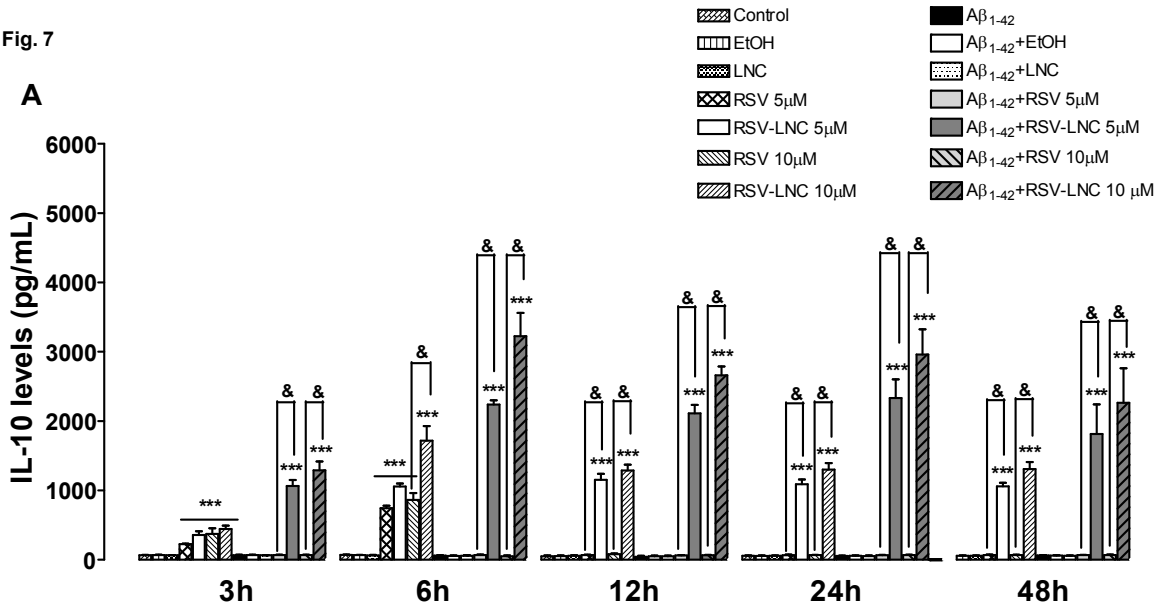

C
IL- 6 levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h

Fig. 7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IL-10 levels (pg/mL)
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5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
Control
EtOH
LNC
RSV 5μM
RSV-LNC 5μM
RSV 10μM
RSV-LNC 10μM
Aβ1-42
Aβ1-42+EtOH
Aβ1-42+LNC
Aβ1-42+RSV 5μM
Aβ1-42+RSV-LNC 5μM
Aβ1-42+RSV 10μM
Aβ1-42+RSV-LNC 10 μM

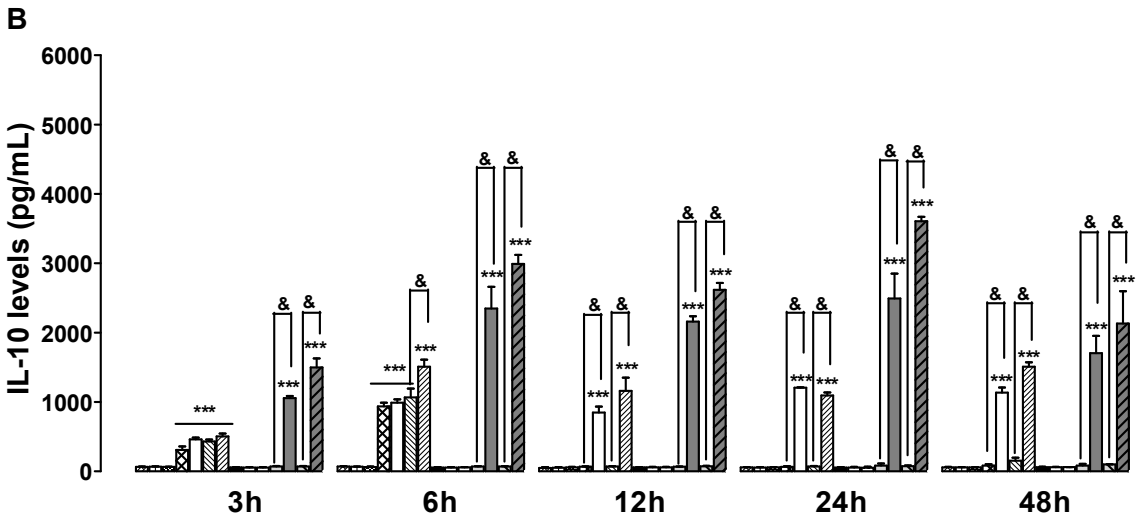
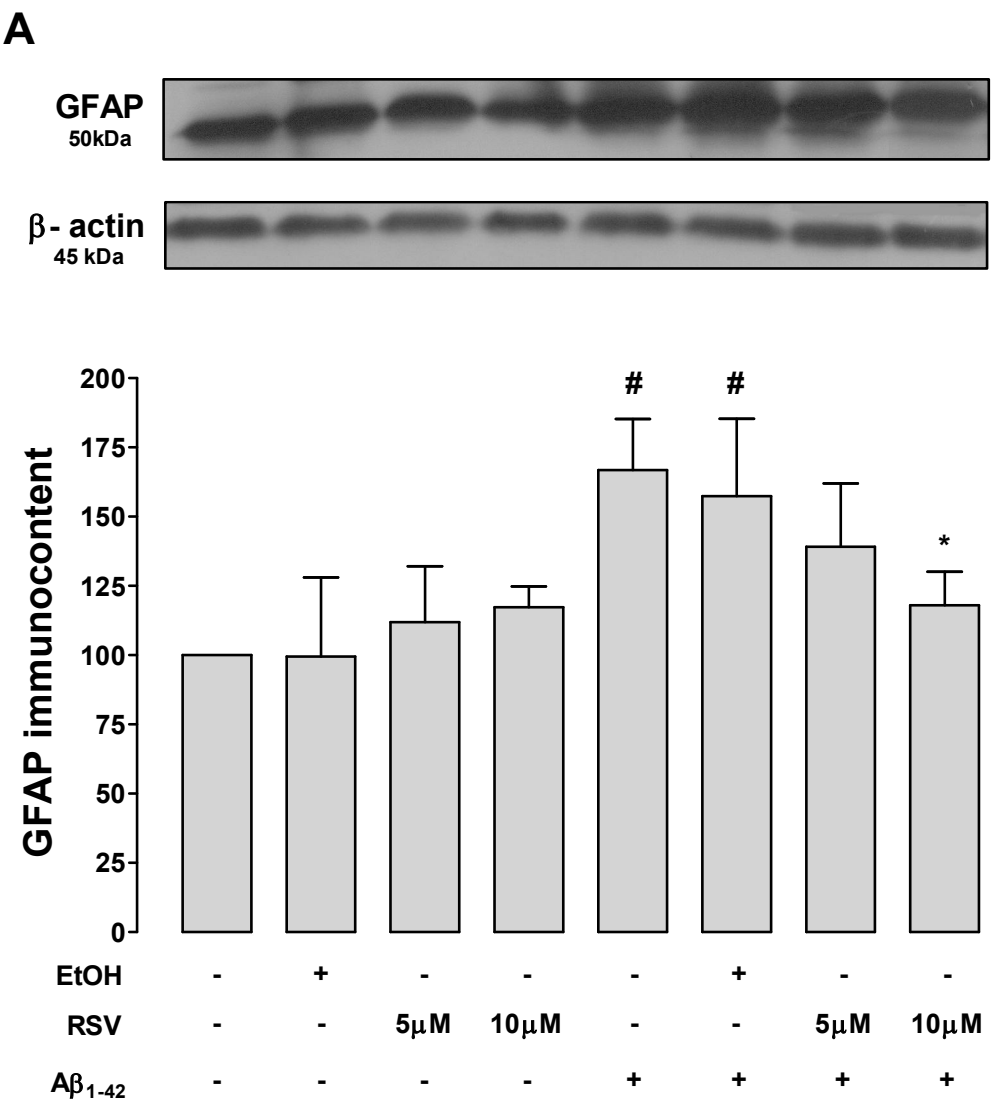
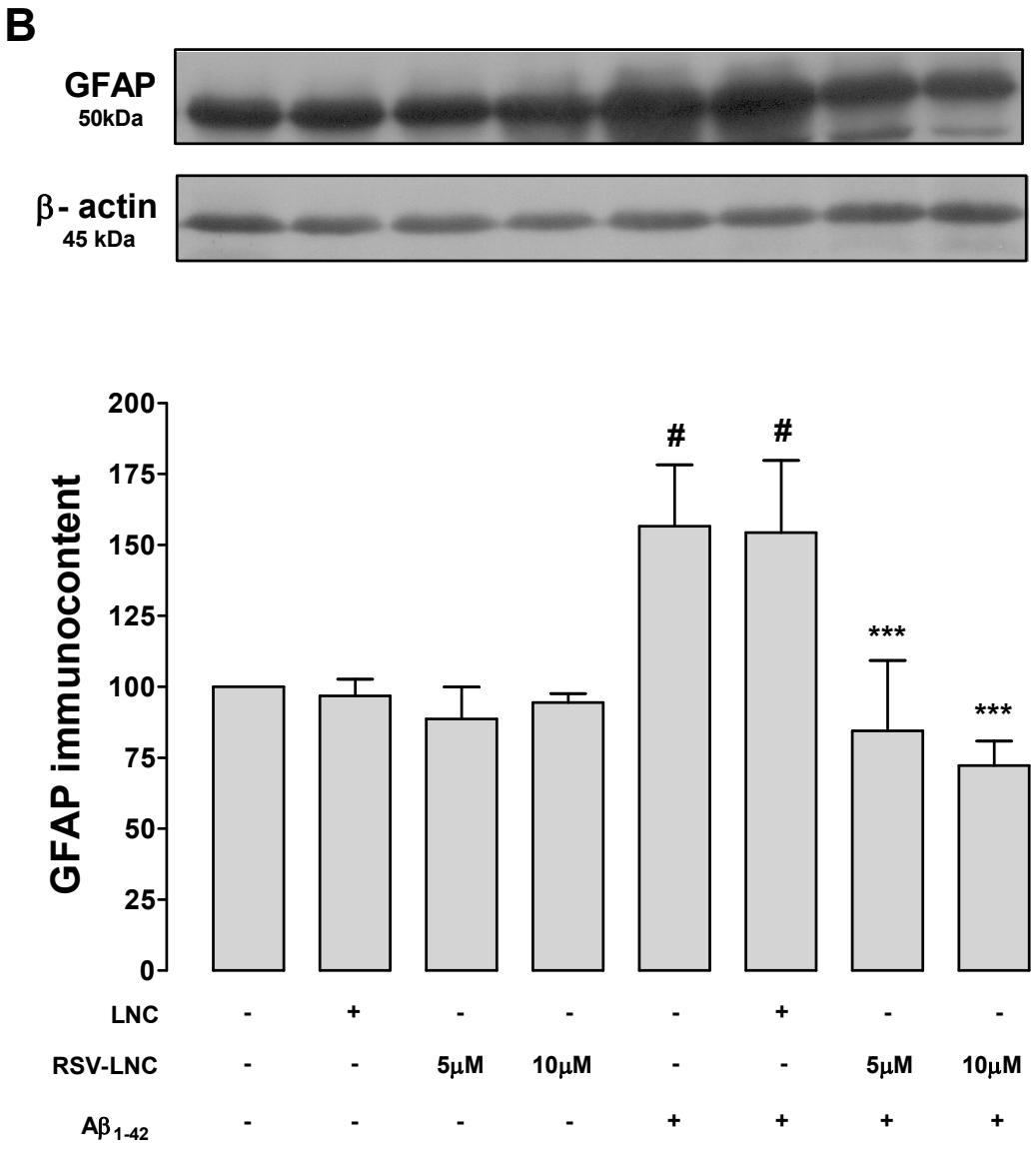
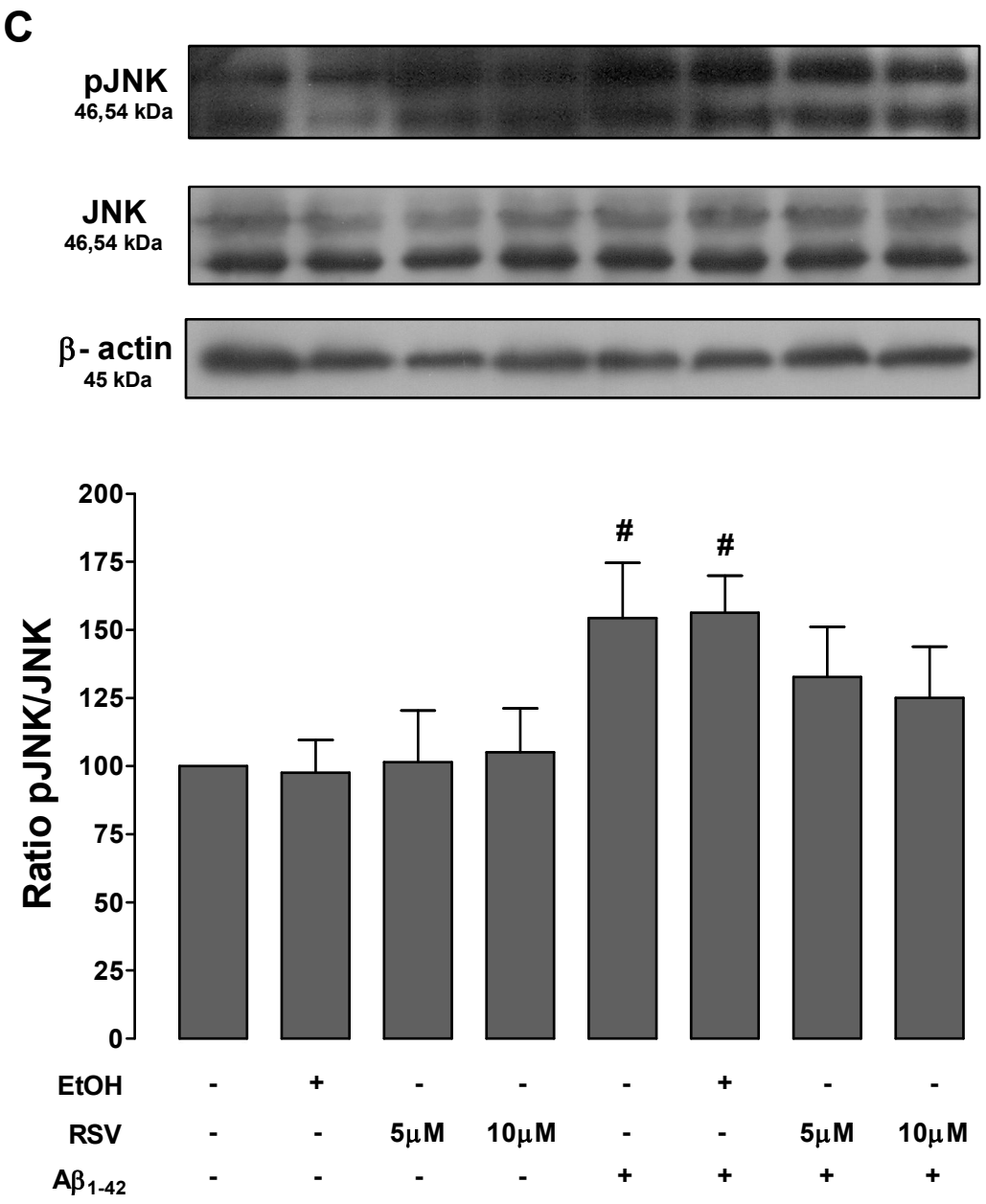

B
IL-10 levels (pg/mL)
6000
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
***
&

Fig. 8


A
GFAP
50kDa
β- actin
45 kDa
GFAP immunocontent
200
175
150
125
100
75
50
25
0
#
#
*
EtOH - + - - - + - -
RSV - - 5μM 10μM - - 5μM 10μM
Aβ1-42 - - - - + + + +


B
GFAP
50kDa
β- actin
45 kDa
GFAP immunocontent
200
175
150
125
100
75
50
25
0
#
#
***
***
LNC - + - - - + - -
RSV-LNC - - 5μM 10μM - - 5μM 10μM
Aβ1-42 - - - - + + + +


C
pJNK
46,54 kDa
JNK
46,54 kDa
β- actin
45 kDa
Ratio pJNK/JNK
200
175
150
125
100
75
50
25
0
#
#
EtOH - + - - - + - -
RSV - - 5μM 10μM - - 5μM 10μM
Aβ1-42 - - - - + + + +

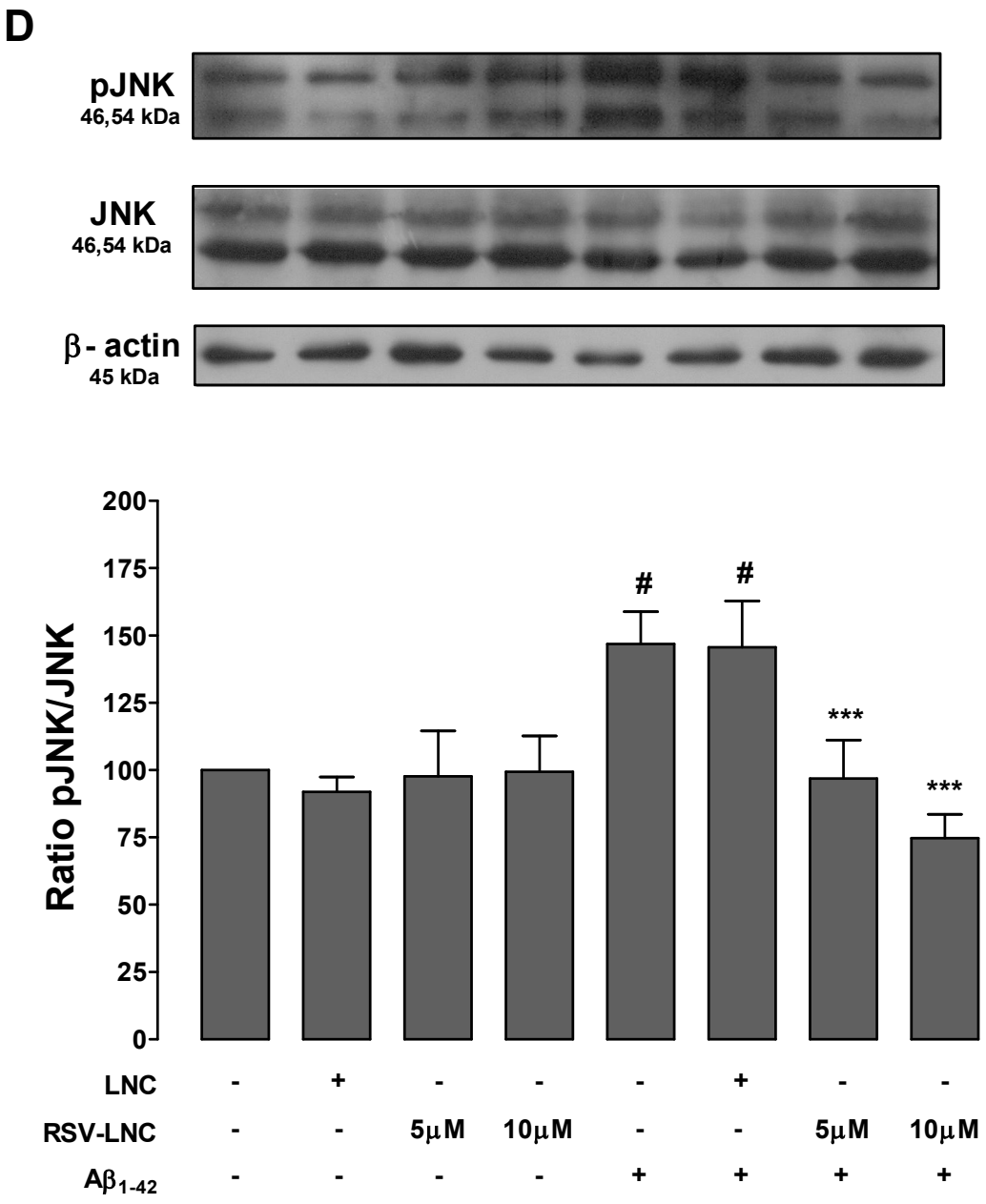
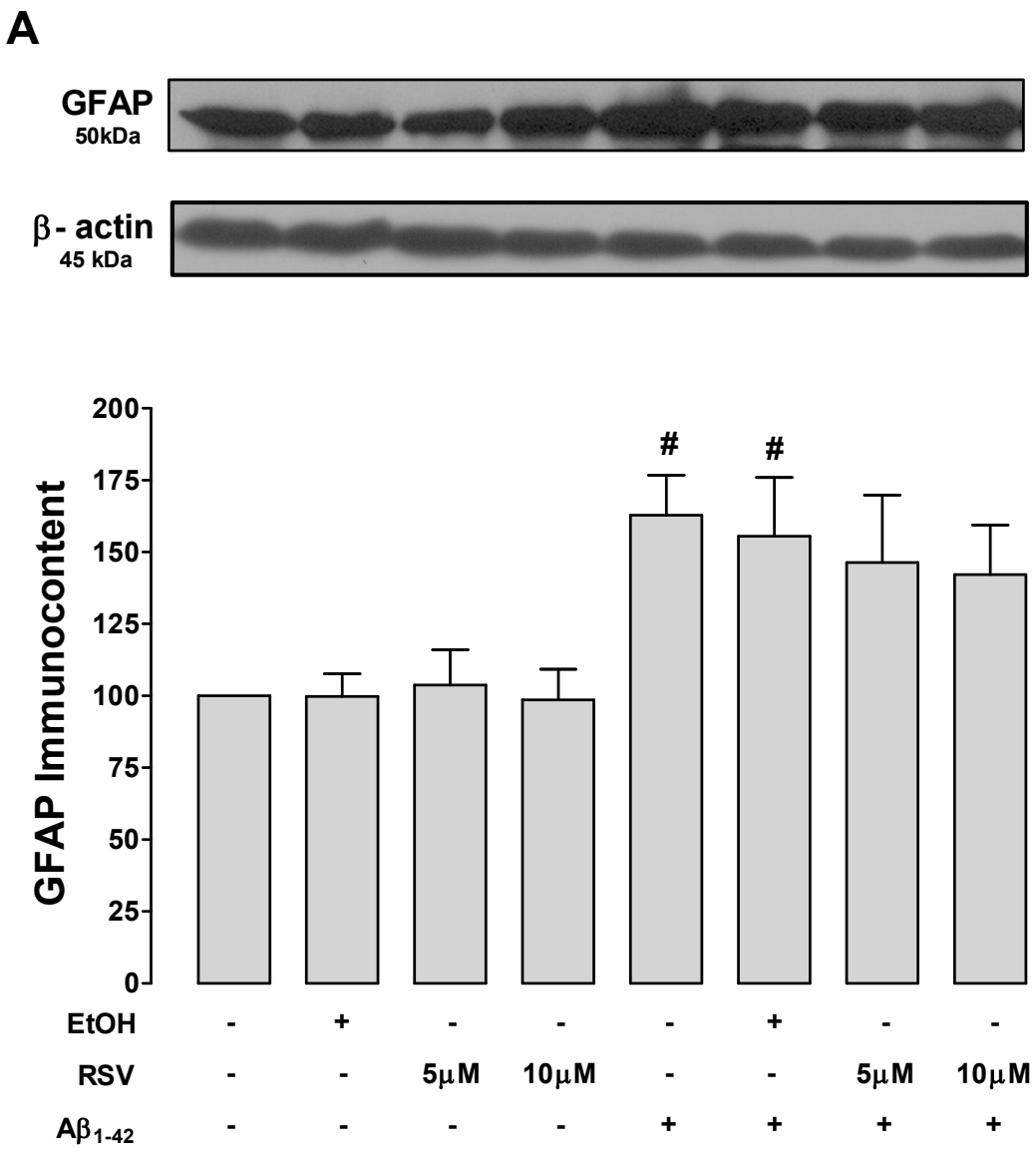
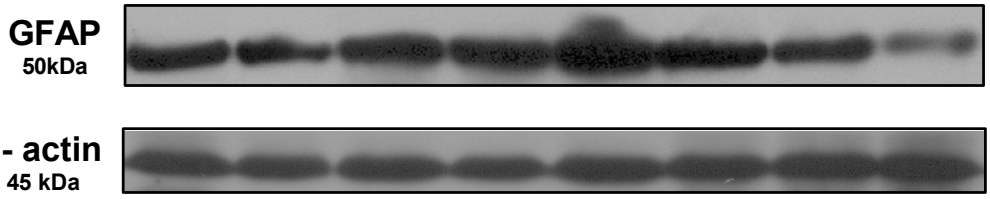
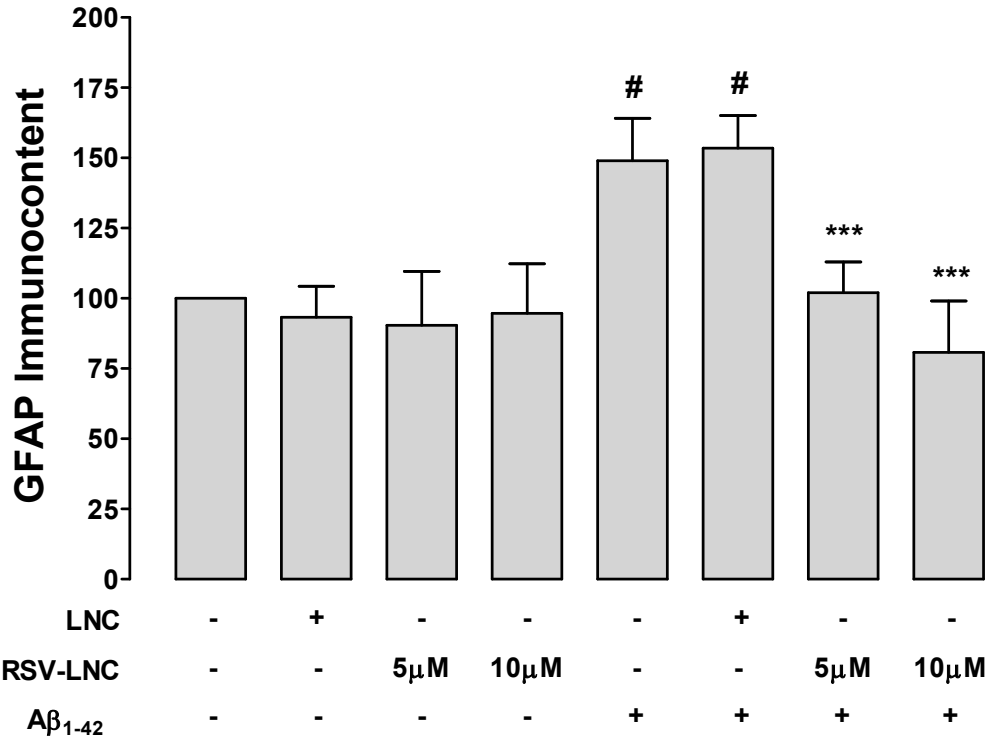

D
pJNK
46,54 kDa
JNK
46,54 kDa
β- actin
45 kDa
Ratio pJNK/JNK
200
175
150
125
100
75
50
25
0
#
#
***
***
LNC - + - - - + - -
RSV-LNC - - 5μM 10μM - - 5μM 10μM
Aβ1-42 - - - - + + + +

Fig. 9


A
GFAP
50kDa
β- actin
45 kDa
GFAP Immunocontent
200
175
150
125
100
75
50
25
0
#
#
EtOH - + - - - + - -
RSV - - 5μM 10μM - - 5μM 10μM
Aβ1-42 - - - - + + + +

B


GFAP
50kDa
β- actin
45 kDa


GFAP Immunocontent
200
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LNC - + - - - + - -
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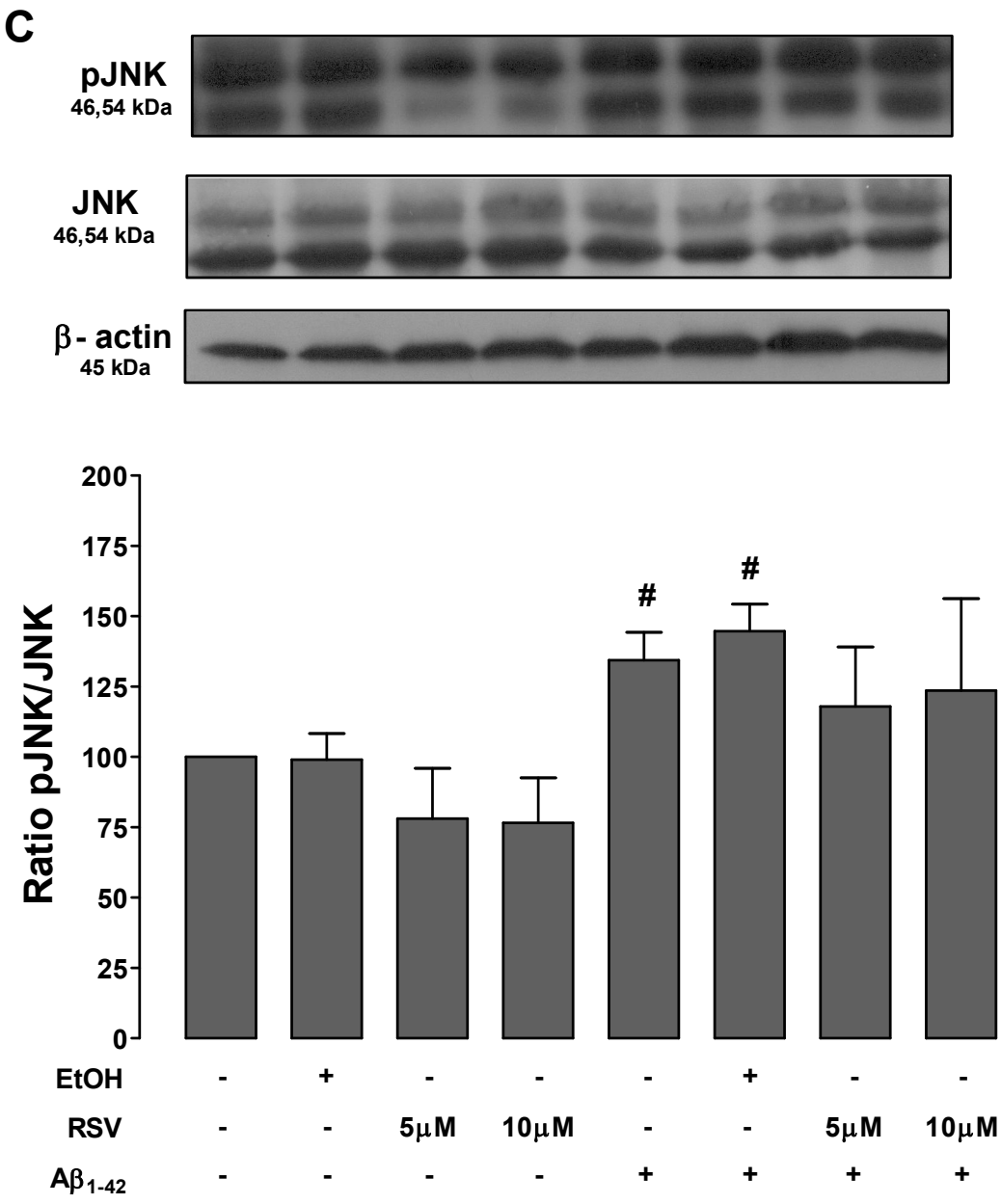
Aβ1-42 - - - - + + + +


C
pJNK
46,54 kDa
JNK
46,54 kDa
β- actin
45 kDa
Ratio pJNK/JNK
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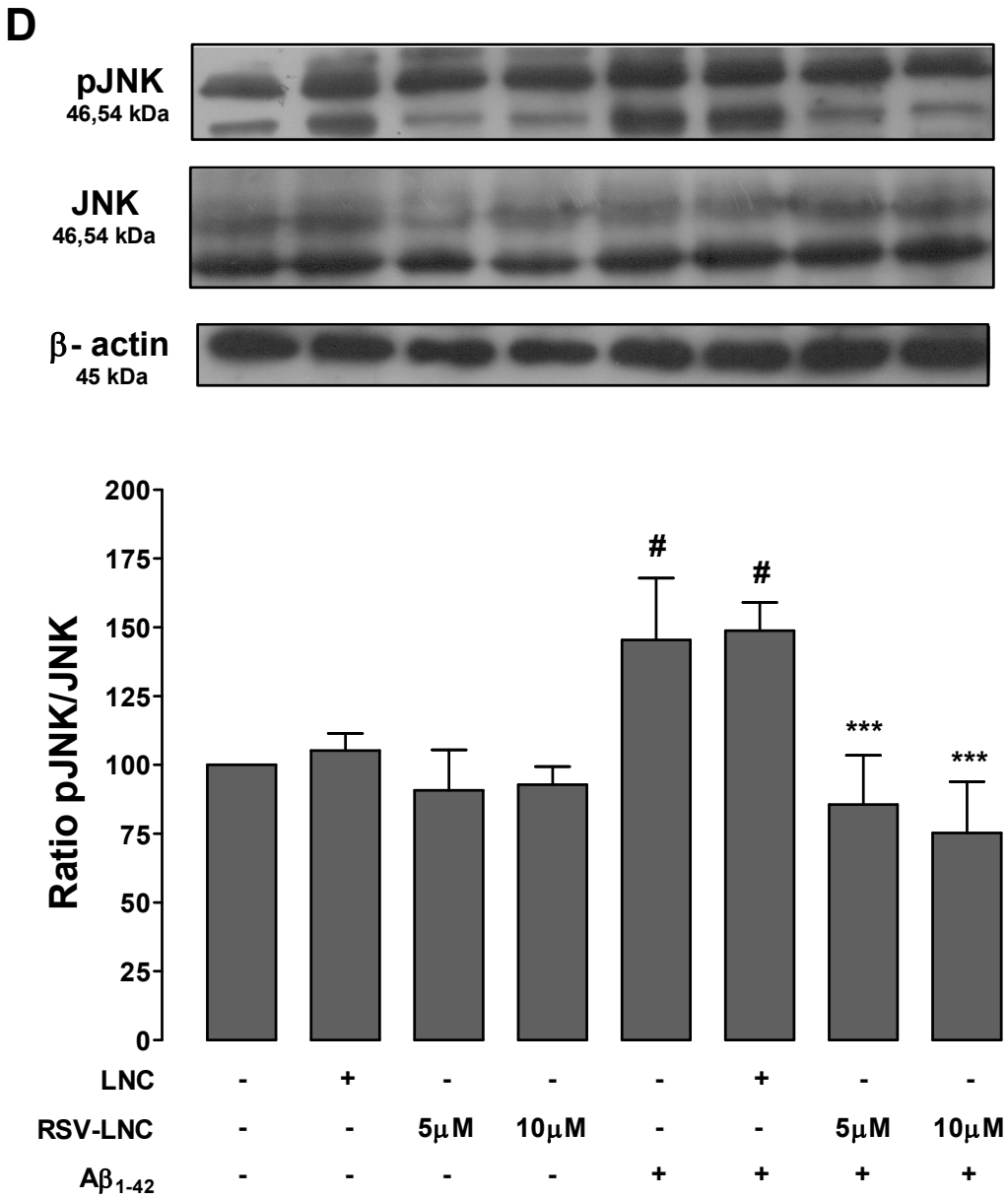
EtOH - + - - - + - -
RSV - - 5μM 10μM - - 5μM 10μM
Aβ1-42 - - - - + + + +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CAPÍTULO III

Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-core Nanocapsules

Rudimar L Frozza, Andressa Bernardi, Juliana B Hoppe, André B Meneghetti, Aline Matté, Ana M O Battastini, Adriana R Pohlmann, Sílvia S Guterres and Christianne Salbego

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Neuroprotective Effects of Resveratrol Against Aβ Administration in Rats are Improved by Lipid-core Nanocapsules

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Running title: *Resveratrol protects against Aβ-induced toxicity*

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Abstract

Alzheimer's disease (AD), a neurodegenerative disorder exhibiting a gradual decline in cognitive function, is characterized by the presence of neuritic plaques composed of neurofibrillary tangles and amyloid-β (Aβ) peptide. Available drugs for AD therapy have small effect sizes and do not alter disease progression. Several studies have been shown that resveratrol is associated with anti-amyloidogenic properties, but therapeutic application of its beneficial effects is limited. Here we compared the neuroprotective effects of resveratrol-loaded lipid-core nanocapsules with the effects displayed by free resveratrol treatment against intracerebroventricular injection of Aβ1-42 in rats. Animals received a single intracerebroventricular injection of aggregated Aβ1-42 (1 nmol/site) and, one day after Aβ infusion, they were administered with free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) (5 mg/Kg, each 12 h, intraperitoneally – i.p.), during 14 days. At the end of treatments, Aβ1-42-injected animals showed a significant impairment on learning-memory ability, which was paralleled by a significant decrease in hippocampal synaptophysin levels. Furthermore, animals exhibited activated astrocytes and microglial cells, as well as disturbance in JNK and GSK-3β activation, and destabilization of β-catenin levels. Noteworthy, by using lipid-core nanocapsules, resveratrol was able to rescue all deleterious effects of Aβ1-42 while treatment with RSV presented only partial beneficial effects, which can be explained by robust increase of brain bioavailability of resveratrol achieved by lipid-core nanocapsules. These findings not only confirm the potential of resveratrol in treating AD but also offer an effective way to improve the efficiency of resveratrol by nano-drug delivery systems.

Keywords: Alzheimer's disease, amyloid-β peptide, drug delivery, lipid-core nanocapsules, hippocampus, neuroprotection, resveratrol

INTRODUCTION

Alzheimer's disease (AD) is an aged-related neurodegenerative disease clinically characterized by progressive cognitive decline associated with progressive memory loss, spatial disorientation, and impairment in activities of daily living throughout the disease course. The major neuropathological lesions of AD include intracellular neurofibrillary tangles, which are paired helical filaments of hyperphosphorylated tau proteins, extracellular deposits of fibrillar amyloid-β (Aβ) peptide in senile plaques, and the build-up of soluble Aβ oligomers [1,2]. The deposition of Aβ in brain areas involved in cognitive functions is assumed to initiate an array of molecular and cellular cascades that eventually lead to synaptic dysfunction, synaptic loss, and neuronal death [3]. However, mechanistic molecular processes that link Aβ and neurodegeneration remain to be firmly established. Genetic factors which present as dominant mutations account for the few cases of family oriented early-onset AD.

Available drugs for AD therapy have small effect sizes and do not alter disease progression. Given the current absence of disease-modifying treatments, there has been growing interest in identification of effective strategies for prevention of AD. Furthermore, the presence of a blood-brain barrier (BBB) presents a huge challenge for effective entry of the majority of drugs, and thus severely restricts the therapy of many diseases affecting the central nervous systems (CNS), including AD. Since the majority of drugs and large molecular weight particulate agents do not readily permeate into brain parenchyma, one of the most significant challenges facing CNS drug development is the availability of effective brain drug targeting technology [4]. Therefore, the application of technological advances in neurological research is expected to have a major impact leading to the development of newer therapeutic modalities [5]. Nanotechnology is at the core of biotechnology and it is likely to play a significant role in this regard. In this way, nanoparticles have become an important area

of research in the field of drug delivery because they have the ability to deliver a wide range of drugs to varying areas of the body for a sustained period of time [6].

Nanoparticles are polymeric particles made of natural or synthetic polymers ranging size from 1 to 1000 nm [7]. The drugs may be dissolved into the nanoparticles, entrapped, encapsulated, and/or adsorbed or attached. Several authors have delivered a variety of drugs using nanoparticles as carriers (reviewed by [6]). Furthermore, the development of new practical treatment modalities for the therapy of neurodegenerative diseases is currently a highly active area of research [5], and the literature suggests that novel nanoparticles could be used as potential drug carriers across the BBB [5,8-11]. The mechanisms of encapsulation and release of several drugs from nanoparticulated systems have been extensively studied by our group [12-15]. Moreover, we have already shown that lipid-core nanocapsules were able to delivery drugs into the brain in rats [16,17].

Epidemiological studies have shown that moderate wine intake reduces the risk of developing AD [18-20]. Resveratrol (3,5,4'-trihydroxystilbene) is a non-flavonoid polyphenolic compound that occurs in abundance in grapes, red wine, and other foods that are commonly consumed as part of human diet, and it is suspect to afford antioxidant and neuroprotective properties and therefore to contribute to the beneficial effect of wine consumption on the neurodegenerative process [21,22]. Resveratrol presents a wide range of pharmacological properties including inhibition of oxidation of human low-density lipoprotein, suppression of cyclooxygenase-2 and inducible nitric oxide syntase (iNOS) activities, and anti-inflammatory and antioxidant effects [23]. Recently, it has been shown that this polyphenol is associated with anti-amyloidogenic properties *in vitro* and *in vivo* [20,24-26]. Furthermore, resveratrol presents chemopreventive effects [27], and increases the activity of SIRT, a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases [28,29], resulting in improved cellular stress resistance and longevity [30].

However, therapeutic application of these beneficial effects of resveratrol remains very limited due to its poor solubility, photosensitivity, short biological half-life, and rapid metabolism and elimination, meaning very low bioavailability [23,31]. The extensive metabolism of resveratrol lead to formation of various glucuronide and sulfate conjugates of unknown potential biological activities. In this way, the development of the therapeutic potential of resveratrol can only be applied *in vivo* if the limitations tied to its bioavailability can be overcome. In this context, an increasing number of recent studies have aimed at designing novel resveratrol formulations to overcome these limitations (reviewed in [32]). Recently, we developed a resveratrol-loaded lipid-core nanocapsules formulation in an attempt to stabilize the drug, preserve its biological activities, and improve its bioavailability. Additionally, we found that healthy rats treated with these lipid-core nanocapsules showed significantly higher concentrations of resveratrol in the brain when compared to rats treated with free resveratrol even after gavage administration [17].

Although many studies have reported the protective effects of resveratrol against Aβ, the mechanisms underlying these neuroprotective effects still remain to be determinate. Thereby, the present study evaluated the ability of resveratrol to protect against Aβ1-42-induced synaptotoxicity and memory impairment and investigated the some underlying mechanisms. Furthermore, we compared the neuroprotective effects of resveratrol-loaded lipid-core nanocapsules to the effects displayed by free resveratrol treatment against intracerebroventricular injection of Aβ1-42 in rats.

MATERIALS AND METHODS

Chemicals

Poly(ε-caprolactone) (PCL) (MW=85,000) was supplied by Aldrich (Strasbourg, France). Caprylic/capric triglyceride and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). *trans*-Resveratrol (>98% pure) was purchased from Gerbras (Anápolis, GO, Brazil). Span 60® (sorbitan monostearate), acrylamide, bisacrylamide, SDS and β-mercaptoethanol were purchase from Sigma Chemical Co. (St. Louis, MO, USA). Aβ1-42 was purchased from Bachem Americas Inc. (Torrance, CA, USA) and Aβ42-1 peptide was from American Peptide Co. (Sunnyvale, CA, USA). Primary antibodies to phosphorylated and total GSK-3β and secondary anti-mouse IgG peroxidase-conjugated were purchased from Cell Signal Technology (Beverly, MA, USA). Primary antibodies to phosphorylated and total *c*-jun N-terminal kinase (JNK) were acquired from Invitrogen (Grand Island, NY, USA); synaptophysin was obtained from Millipore® (Bradford, MA, USA); and to GFAP, phospho-β-catenin, β-catenin and β-actin were purchased from Sigma Chemical Co. Isolectin B4 was purchased from Sigma Chemical Co. Anti-rabbit IgG peroxidase-conjugated were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hybond-C nitrocellulose membranes were from Hybond™ ECL™ (Hybond™ ECL™ nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany). Substrate to detect enhanced chemiluminescence (ECL) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and X-ray films were purchased from Kodak (Kodak X-Omat, Rochester, NY, USA). All other chemicals and solvents used were of analytical or chromatographic grade. All reagents were used as they were received.

Preparation of lipid-core nanocapsules

Lipid-core nanocapsule suspensions were prepared by interfacial deposition of the polymer as previously described [17,33]. At 40 °C, *trans*-resveratrol (0.05 g), poly(ε-

caprolactone) (0.50 g), capric/caprylic triglyceride (1.650 mL) and sorbitan monostearate (0.19 g) were dissolved in acetone (135 mL). In a separate flask, polysorbate 80 (0.19 g) was added to 265 mL of water (MilliQ®). The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was evaporated and the suspensions were concentrated under reduced pressure at 40 °C. The final volume was adjusted to 50 mL. A control formulation (load off lipid-core nanocapsules - LNC) was prepared without *trans*-resveratrol.

Physicochemical characterization of the formulations

The pH values of the suspensions were determined using a potentiometer B-474 (Micronal, Brazil). Mean diameters (*z*-average), polydispersity index and zeta potential were measured at 25 °C using a Zetasizer®nano-ZS ZEN 3600 model (Nanoseries, Malvern, UK), after diluting the samples with MilliQ® water or with 0.01 mol/L NaCl aqueous solution, respectively. To avoid any sample selection, only the dilution media was filtered (Millipore® 0.45 μm) prior to analysis. Measurements were carried out using 3 different batches for each formulation in triplicate. The size and polydispersity indexes were calculated using the software (Dispersion Technology Software -DTS Nano - Version 5.02, Malvern) provided by the manufacturer (Malvern Instruments Ltd).

Analytical procedure

Resveratrol was analyzed by high-performance liquid chromatography (HPLC) at 306 nm. The content (total concentration) of resveratrol in the formulations (100 μL) was determined after dissolving the lipid-core nanocapsules containing resveratrol into acetonitrile

(10 mL) and filtering (Millipore® 0.45 μm) for analysis. The system consisted of a UV-Vis detector, pump and auto-injector S200 Perkin-Elmer (PerkinElmer Instruments, Norwalk, CT) and a Shim-pack CLC-C8 (M) column (150 mm, 4.6 mm, 5 μm, Shimadzu Corporation, Japan) with a guard-column. The mobile phase was prepared by using Milli-Q® water and HPLC grade acetonitrile, and consisted of acetonitrile/water (40:60 v/v) with a pH of 3.0 ± 0.5 corrected with 10% (v/v) orthophosphoric acid. The isocratic flow rate of the mobile phase was 1.2 mL/min and the retention time of resveratrol was 3.45 min. The HPLC method was validated presenting linearity between 2.5 and 17.5 μg/mL, ($r > 0.9999$), inter- and intraday variability lower than 2.0%, and accuracy from 96.19% to 97.21%. Resveratrol content was 0.964 ± 0.037 mg/mL and the encapsulation efficiency was 99.89 ± 1.3% for all batches [17].

Peptide preparation

The Aβ1-42 peptide or the nonamyloidogenic reverse peptide Aβ42-1 were dissolved in sterilized bi-distilled water with 0.1% ammonium hydroxide at concentration of 1mg/mL and stored to -20 °C. Before use, the Aβ peptides allowed aggregating by incubation at 37 ºC for 72h.

Animals

Male adult *Wistar* rats (280–330 g) were obtained from in-house breeding colonies at the "Departamento de Bioquímica", "Universidade Federal do Rio Grande do Sul" (UFRGS-Porto Alegre, Brazil). Animals were housed in cages under optimum light conditions (12:12 h light–dark cycle), temperature (22 ± 1 °C), and humidity (50 to 60%), with food and water

provided *ad libitum*. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23 and were approved by the local Ethics Committee on the Use of Animals (CEP-UFRGS, protocol number 2007977). All efforts were made to minimize the number of animals and their suffering.

Surgical procedure

Animals were anesthetized with Equitesin (3.5 mL/Kg i.p.), placed in a stereotaxic frame, and a middle saggital incision was made in the scalp and sterilized conventionally. Holes were drilled in the skull with a dental drill on both sides over the lateral ventricles. Injection coordinates were chosen according to the atlas of Paxinos and Watson [34]: 0.8 mm posterior to bregma; 1.5 mm lateral do saggital suture; 3.5 mm beneath the surface of brain. Rats received a single infusion of 5 μL into each lateral ventricle of Aβ1-42 or Aβ42-1 (total of 2 nmol in 10 μL). Controls animals received bilateral intracerebroventricular (icv) injections of equal volume of bi-distilled water with 0.1% ammonium hydroxide. Microinjections were performed using a 10-μL Hamilton syringe fitted with a 26-gauge needle. All infusions were made at a rate of 1 μL/min during 5 min. At the end of infusion, the needle was left in place for an additional 3-5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution. After the injection, the scalp was sutured and the animals were allowed to recover from the anesthesia on a heating pad to maintain body temperature at 37.5 ± 0.5 °C. The animals were submitted to behavioral tasks 2 weeks after Aβ injection.

Drug administration and experimental design

The experimental schedule is shown in Figure 1. One day after surgical procedure, the animals were randomly divided into eight groups as follows: (i) control animals infused icv with water with 0.1% ammonium hydroxide: (1) untreated (Control group), (2) treated with free resveratrol (RSV group), and (3) treated with resveratrol-loaded lipid-core nanocapsules (RSV-LNC); (ii) animals infused icv with Aβ1-42: (4) untreated (Aβ group), (5) treated with ethanol 50% (Aβ EtOH group), (6) treated with load-off lipid-core nanocapsules (Aβ LNC group) (7) treated with free resveratrol (Aβ RSV group), and (8) treated with resveratrol-loaded lipid-core nanocapsules (Aβ RSV-LNC group). Animals injected with Aβ42-1 received no treatment and only submitted to behavioral tasks 2 weeks after Aβ injection.

Free resveratrol (RSV) was dissolved in ethanol 50% (EtOH) at a concentration of 1 mg/mL and it was made up each time, and lipid-core nanocapsules containing resveratrol (RSV-LNC) were prepared as described above. Daily doses reaching 10 mg/Kg/day of RSV and RSV-LNC, divided in two administrations of 5 mg/Kg each 12 h, were administered intraperitoneally (i.p.) to the animals for 14 consecutives days. In the same way, a vehicle treated group (EtOH 50% or LNC, respectively) with identical volumes those treated with RSV or RSV-LNC was run in parallel in rats infused with Aβ. The behavior tests were started on day 14 after Aβ infusion and were carried out sequentially.

Behavioral analysis

Spontaneous alternation. Hippocampal-dependent memory performance was assessed by measuring spontaneous alternation performance during 8 min in the Y-maze test, which allows evaluating cognitive searching behavior, although it does not allow isolating memory performance [reviewed in 35]. The experimental apparatus used in the present study consisted of three arms (40 cm long, 25 cm high and 10 cm wide, labeled A, B, and C) of black painted

made of plywood with an equilateral triangular central area in a testing room with a constant illumination. Each rat was placed at the end of one arm and allowed to move freely through the apparatus during 8 min. Behavior was recorded by a video camera mounted vertically above the test arena for later analysis using the video tracking program (ANY-maze^{TM}, Stoelting Co., USA). The number of arm choices and pattern of choices were recorded for each animal. An arm entry was counted when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices in overlapping triplet sets (i.e. ABC, BCA, CBA). The percentage of alternation was calculated as total of alternations/(total arm entries-2) x 100. Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory.

Novel object recognition task. The object recognition task was performed following the guidelines recently reviewed [36]. This task is based on the spontaneous tendency of rodents to explore novel objects [37]. The task was performed in an apparatus made of wood covered with impermeable Formica (dimensions: 40 x 50 x 50 cm), which had black floor and walls, in a testing room with a constant illumination. The objects used presented similar textures, colors and sizes, but different shapes, and were placed near the two corners at either end of one side of the apparatus. The objects chosen were two cuboids glass blocks, a cylindrical bottle filled with water, and a block-shaped dodecahedron; these objects were heavy enough to prevent rats from moving. A day before the testing, rats were submitted to habituation session, whereby they were allowed to explore the apparatus for 5 min without objects. On the following day, rats were acclimated in the testing room during 2 h before the beginning of the sessions. Firstly, rats were submitted to training session (24 h after habituation) that consisted in leaving the animals in the apparatus containing two identical objects (A and A1). After training, rats were placed in their home-cages for 3h. The testing session to evaluate the short-

term recognition memory was performed 3 h after the training session and rats were allowed to explore the apparatus with two dissimilar objects, a familiar and a novel one (A and B, respectively). Long-term recognition memory was evaluated 24 h after the training session and another two dissimilar objects, a familiar and a novel one (A and C, respectively) were presented. In all sessions each rat was always placed in the apparatus facing the wall and allowed to explore the objects for 5 min, and then the rat was put back in its home cage. Behavior was recorded by a video camera mounted vertically above the test arena and analyzed using the video tracking program (ANY-maze^{TM}, Stoelting Co., USA). The total number of trials per animals consisted of three trials, which comprised the training and two test sessions. Overall, the animals started to explore the objects 1 min after they had entered in the apparatus. A greater percentage of time spent exploring novel object as a function of the total amount of time spent exploring both objects during testing [time spent with novel object/(time spent with novel object + time spent with familiar object)] was considered an index of enhanced cognitive performance (Recognition Index). Among trials the objects were cleaned with 10% ethanol solution. Active exploration was defined by directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or forepaws. Sitting on the object was not considered exploratory behavior.

Western blotting assay

After the behavioral tasks, the animals were killed by lethal dosage of anesthesia, blood samples were collected by cardiac puncture, the brain was rapidly removed from the skull, and the hippocampus was dissected on dry ice. The hippocampus was homogenized in ice-cold lyses buffer (4% sodium dodecylsulfate -SDS-, 2 mM EDTA, 50 mM Tris) containing proteinase inhibitor cocktail. The homogenates were denatured for 5 min at 100

°C, and then centrifuged at 10,000 *X* g for 30 min. The supernatant containing cytosolic fraction was collected, protein concentration was determined [38], and β-mercaptoethanol was added to a final concentration of 5%. Equal amounts of proteins were resolved (50 µg per lane) on 10% SDS-PAGE. After electrophoresis, proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad, Trans-Blot SD, Hercules, CA, USA). Membranes were incubated for 60 min at 4º C in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4º C. The primary antibodies against the following proteins were used: anti-phospho-β-catenin [$pSer^{45}$] (1:1000), anti-β-catenin (1:4000), anti-GFAP (1:3000), anti-phospho GSK-3β [$pSer^{9}$] (1:1000), anti-GSK-3β (1:1000), anti-phospho JNK1/2 [$pTpY^{183/185}$] (pJNK, 1:1000), anti-JNK1/2 (1:500), anti-synaptophysin (1:3000), and anti-β-actin (1:1000). After washing, the membranes were incubated with adjusted secondary antibodies coupled to horseradish peroxidase (1:1000) for 2 h. The immunocomplexes were visualized by using the ECL chemiluminescence detection system. Band density measurements were performed by using Optiquant software (Packard Instrument). For each experiment, the test groups were compared to control cultures not exposed to Aβ.

Isolectin B4 (IB4) reactivity

In an attempt to evaluate the activation of microglial cells after Aβ icv injection, we analyzed the IB4 reactivity. Proteins (25 µg per line) were resolved on 8% SDS-PAGE, and electro-transferred to nitrocellulose membranes as described in Western blotting assay. Membranes were incubated overnight at 4 °C in albumin solution (5% albumin and 2% Tween 20 in PBS, pH 7.4). After washing, IB4 peroxidase conjugated was incubated in PBS

containing 0.05%Tween 20 overnight in a final concentration of 0.250 μg/mL. The chemioluminescence was detected using X-ray films.

HPLC analysis of resveratrol levels in the brain

In order to determine the levels of resveratrol in the cerebral tissue, HPLC analysis was performed as previously described [17]. Briefly, at 1 h after the last ip administration, animals were killed by decapitation. The brain was rapidly removed from the skull, weighed, washed in ice cold 0.9% NaCl, and the hemispheres were separated. 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 *X* g for 30 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 20 μL for all samples. The quantity of resveratrol was calculated by comparing the peak area ratio from tissue samples of treated animals with those of the corresponding concentration standards of *trans*-resveratrol in acetonitrile injected directly into the HPLC system.

Determination of hepatic enzymes in serum

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

markers of metabolic and tissue toxicity. These experiments were performed in a LabMax 240 analyzer (Labtest Diagnostica, Brazil).

Data analysis

All experiments were carried out at least in triplicate except for behavioral tests. The results are presented as the mean ± SD of seven to fifteen animals per group. The statistical comparisons of the data were performed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using GraphPad Prism software version 5.01 (GraphPad Software Inc. La Jolla, CA, USA). *p*-Values lower than 0.05 ($p<0.05$) were considered significant.

RESULTS

Physicochemical characterization of lipid-core nanocapsules

The lipid-core nanocapsule formulations were prepared by interfacial deposition of polymer without purification. Resveratrol-loaded lipid-core nanocapsules (RSV-LNC) and load off lipid-core nanocapsules (LNC) presented macroscopic homogeneous aspects, such as white bluish opalescent liquids. After preparation, the RSV-LNC showed particle mean size of 249 ± 5 nm, polydispersity index of 0.12 ± 0.05, zeta potential of -14 ± 1.8 mV, and pH of 5.5 ± 0.2. Similar results were obtained for LNC: 233 ± 4 nm, 0.15 ± 0.03, -13.6 ± 2.5 mV, and 5.2 ± 0.3, for particle size, polydispersity index, zeta potential, and pH values, respectively. The suspensions showed stable monomodal size distributions and polydispersity indexes lower than 0.15, indicating narrow size distributions.

Effects of resveratrol against Aβ1-42-induced memory impairment

AD is characterized clinically by a progressive decline in learning and memory processes. To investigate some of the mechanisms involved in Aβ-induced cognitive decline, the spontaneous alternation and novel object recognition tasks were used. Furthermore, we compared the effects of treatment with free resveratrol (RSV) to those of resveratrol-loaded lipid-core nanocapsules (RSV-LNC) against Aβ-induced toxicity. We observed that 2 weeks after a single icv administration of 2 nmol of Aβ1-42 rats displayed a decrease of spontaneous alternation in the Y-maze (Fig. 2). In order to evaluate the effects of resveratrol, animals were daily treated with 10 mg/Kg of RSV or RSV-LNC for 14 days. As shown in Fig. 2, there was no statistical difference in spontaneous alternation behavior between Aβ-infused and RSV treated Aβ-infused rats. However, it is important to note that the treatment with RSV-LNC, at the same dosage, significantly attenuated the impairment of this behavior triggered by Aβ1-42 (p<0.001). The number of arm entries did not differ among all the treatment groups of rats (data not shown), indicating that changes in alternation behavior were not due to generalized exploratory, locomotor or motivational effects. Next, we also evaluate the effects of Aβ1-42 infusion, as well as RSV and RSV-LNC treatments on recognition memory by submitting the animals to novel object recognition task. As it can be observed in the Figure 3B, when the animals were placed in the arena 3 h after first exploration period (training session, Fig. 3A), Aβ infused rats were not able to discriminate between the familiar and novel objects, as shown by a similar exploration time for both objects and worse discrimination index (Fig. 3B). Treatment with 10 mg/Kg of RSV was ineffective to improve short-term recognition memory, since animals spent more time exploring the familiar object and lost the ability to discriminate between the two objects. On the other hand, discrimination was restored by treating animals with the same dose of RSV-LNC (p<0.001) (Fig. 3B). Similar results were

found when long-term recognition memory was evaluated ($p<0.001$) (Fig. 3C), indicating that only rats treated with RSV-LNC were able to distinguish between familiar and new one objects. Animals icv infused with water and 0.1% ammonium hydroxide were treated with RSV or RSV-LNC, at the same schedule, for possible intrinsic toxicity and they showed no impairment of either spontaneous alternation or recognition memory when compared to control animals. Treatment with vehicle (EtOH 50% or LNC, respectively) had no effects against spontaneous alternation and recognition memory impairments triggered by Aβ (Fig 3C,D). Animals icv infused with Aβ42-1 had no alteration either spontaneous alternation or memory recognition (data not shown).

Resveratrol decrease synaptic dysfunction triggered by Aβ

To further evaluate the synaptic integrity, we performed Western blotting analysis for the presynaptic protein synaptophysin (a specific pre-synaptic marker). A significant reduction in the synaptophysin levels was found in Aβ1-42-infused rats 15 days after intracerebroventricular injection ($p<0.05$), suggesting a decrease in synaptic function (Fig. 4). Consistent with findings in the behavior analyzes, only the treatment with RSV-LNC was able to block the decreased synaptophysin levels induced by Aβ1-42 ($p<0.05$) (Fig. 4). Animals icv infused with water and 0.1% ammonium hydroxide were treated with RSV or RSV-LNC, at the same schedule, and they showed no alternation in the synaptophysin levels when compared to control animals, excluding possible intrinsic synaptotoxicity of resveratrol. Treatment with vehicle (EtOH 50% or LNC, respectively) had no effects upon the decreased synaptophysin levels triggered by Aβ (Fig. 4).

Effect of resveratrol on glial and microglial activation triggered by Aβ

Astrogliosis and microglial activation are some of the earliest pathological hallmarks of AD and may occur in response to the increasing number of degenerating neurons, or to the accumulation of Aβ. The reactivity for IB4 analysis for microglial activation obtained from hippocampus of Aβ1-42-infused rats was increased and RSV-LNC treatment allowed that resveratrol reduced activated microglia (Fig. 5A). Similarly, activated astrocytes (immunoblot with GFAP antibody) were significantly increased by Aβ1-42. In the same way, while RSV was ineffective to block astrocytes activation, RSV-LNC treatment significantly decrease the activated astrocyte immunoreactivity ($p<0.01$) (Fig. 5B). Animals icv infused with water and 0.1% ammonium hydroxide were treated with RSV or RSV-LNC, at the same schedule, and showed no alternation in astrocytes and microglial activation when compared to control animals. Treatment with vehicle (EtOH 50% or LNC, respectively) had no effects upon astrocytes and microglial activation triggered by Aβ (Fig 5A,B).

Signaling pathways involved in the neuroprotection afforded by resveratrol treatment against neurotoxicity triggered by Aβ

In order to evaluate the neuroprotective effects of resveratrol, we attempted to outline some intracellular events triggered after icv Aβ1-42 injection. Therefore, we next tested the possible requirement of JNK in our AD paradigm. As shown in Fig. 6A, basal level of activated JNK was detected in the hippocampus; however, an evident increase in JNK phosphorylation was found after Aβ1-42 injection ($p<0.001$). Again, only RSV-LNC was capable to blocking JNK activation ($p<0.001$), although RSV treatment showed a slight tendency to decrease JNK phosphorylation (Fig. 6A). Additionally, we previously reported that GSK-3β signaling pathway plays a key role in the neuroprotective effect or resveratrol against injuries induced by oxygen-glucose deprivation [39], and now, we tested whether

GSK-3β could be involved in the Aβ1-42-induced toxicity. As can be seen in Fig. 6B, while Aβ1-42 induced activation of GSK-3β, an increase in phosphorylation/inactivation of GSK-3β was noticed after both, RSV and RSV-LNC treatment (p<0.001). Several studies had implicated the involvement of Wnt/β-catenin pathway on toxicity triggered by Aβ [40-42]. In this way, because GSK-3β is a major player in Wnt/β-catenin signaling, and because it is well established that regulation of β-catenin stability is a crucial control mechanism in Wnt signaling, we investigated the effect of resveratrol in this cascade pathway. To determine whether Aβ1-42 altered the stability of β-catenin, we analyzed cytoplasm β-catenin levels, as well as phophorylated β-catenin levels by Western blotting. Rats infused with Aβ1-42 displayed increased phosphorylated β-catenin (p<0.01) and reduced cytoplasm β-catenin levels (p<0.01) (Fig. 6 C and D, respectively). However, both RSV and RSV-LNC treatments were able to decrease the β-catenin phosphorylation and rescue cytoplasm β-catenin levels (p<0.05) (Fig. 6 C and D, respectively). Animals icv infused with water and 0.1% ammonium hydroxide were treated with RSV or RSV-LNC, at the same schedule, and showed no alternation in cell signaling when compared to control animals. Treatment with vehicles (EtOH 50% or LNC, respectively) had no protective effects upon cell signaling disturbances triggered by Aβ (Fig 6A-D).

Lipid-core nanocapsules improve brain bioavailability of resveratrol

Since RSV-LNC treatment displayed enhanced effect of resveratrol against Aβ1-42-induced toxicity in rats, we hypothesized that the lipid-core nanocapsules could be improving the cerebral bioavailability of resveratrol. On this purpose, quantitative analyses by HPLC were performed to determine the cerebral bioavailability of resveratrol after treatment of rats infused with Aβ1-42 using the same doses (10 mg/Kg/day i.p.) of RSV or RSV-LNC. As can

be seen in Fig. 7, a significantly higher quantity of resveratrol in the brain was found in animals treated with RSV-LNC in comparison with animals treated with free resveratrol treatment (p<0.001).

Investigation of possible side effects of resveratrol treatment

The treatment with RSV or RSV-LNC (10 mg/Kg/day, ip), as well as vehicles EtOH 50% and LNC, respectively, did not induce mortality or alter the body weight within a 14-day of treatment (data not shown). The activities of hepatic enzymes γ-glutamyltransferase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were assessed in rat blood serum. None of the treated animals presented significant alterations in the investigated enzymes, suggesting no hepatic alterations or metabolic toxicity in the animals in the tested conditions (data not shown). Further, none of treated animals presented alterations in the hematological parameters (data not shown).

DISCUSSION

In the current study, we demonstrate that a single intracerebroventricular injection of Aβ1-42 induces memory dysfunction which may be related to synaptic degeneration, a cardinal feature of early phases of AD. This model has been a useful complement to transgenic approaches to AD neuropathology, beyond has been especially useful in development and evaluation of therapeutics approaches [43]. Noteworthy, the memory dysfunction found here appears to be related to the synaptic dysfunction. This support the hypothesis that synaptic dysfunction induced by Aβ is the primary marker of AD and precedes neuronal death [3,44]. Further, our results are suggestive of astroglial and microglial

activation after Aβ1-42 injection. In addition, our data show that resveratrol protects from behavioral impairments, astrocyte and microglial activation, as well as cell signaling disturbances triggered by Aβ *in vivo*. Of high interest, by using lipid-core nanocapsules as a resveratrol carrier, we found that resveratrol was significantly increased into brain tissue of rats injected with Aβ1-42, culminating in neuroprotective effect. This supports the notion that prevention of synaptic impairment may underlie the ability of resveratrol to prevent Aβ-induced memory dysfunction which can be a relevant strategy to the therapy of neurodegenerative process involved in AD.

Loss of memory and impairment of cognitive functions represent classical signs observed in AD patients [1]. Our experimental protocol (based on the icv injection of Aβ1-42 in rats) caused a significant decline in the spontaneous alternation and the object recognition memory, which are highly dependent of hippocampal system [45]. Additionally, the icv infusion of Aβ42-1 did not affect either spontaneous alternation or the ability of animals in recognizing new objects, indicating that the observed behavioral impairments in Aβ1-42-exposed animals were dependent on the peptide sequence/structure. In an attempt to reduce the toxicity induced by Aβ1-42, we evaluated the effects of resveratrol, a natural polyphenolic compound in grapes, peanuts, and a variety of other foods [23].

Neuroprotective effects of resveratrol against memory impairments induced by Aβ remain to be established. Here, we have noticed that treatment with 10 mg/Kg/day of free resveratrol failed to improve the cognitive damage induced by icv Aβ injection. On the other hand, by using lipid-core nanocapsules, treatment at same dosage robustly improved the Aβ-induced memory impairment, with treated rats displaying performances equivalent to their control counterparts. Because RSV-LNC treatment was initiated after Aβ infusion, these data suggest that resveratrol not only prevented memory loss, but also restored the cognitive deficit. Resveratrol significantly improved the recognition index performance, indicating that

RSV-LNC-treated rats were able to distinguish between the familiar and new objects. Thereby, we suppose that improvement in spontaneous alternation and recognition index performances of RSV-LNC treated animals reflects neuroprotective effect of resveratrol on hippocampal regions.

Synapse loss at specific encephalic structures of AD patients significantly correlates with the severity of their cognitive symptoms [46]. In our experimental model, icv Aβ1-42 injection significantly decreased the hippocampal levels of synaptophysin (a specific pre-synaptic marker). The decreased synaptophysin levels and the impaired spontaneous alternation and object recognition memory in Aβ1-42-exposed rats greatly suggest a link between both events. The synaptotoxic effects of Aβ1-42 might be crucial in causing the observed memory deficits. These results are in accordance with those relating synaptic loss to toxicity induced by icv injection of Aβ toxicity [47-49]. This hypothesis is reinforced by the effects of resveratrol against the synaptotoxicity triggered by Aβ1-42, since resveratrol rescued the synaptic changes as can be seen by the increase of synaptophysin levels, the major synaptic vesicle protein.

Despite resveratrol has attracted considerable interest for its beneficial potentials for human health, its use is strongly limited by its low bioavailability, which is a barrier to development of therapeutic applications. In recent years, an increasing number studies have focused on novel formulation approaches to stabilize and protect resveratrol from degradation, increase its solubility in water in order to improve its bioavailability, to achieve a sustained release, and ultimately to target resveratrol to specific locations via multiparticulate forms and colloidal carriers (reviewed by [32]). In this context, we recently developed lipid-core nanocapsules containing resveratrol and reported that this formulation improve the brain bioavailability of resveratrol [17]. These same lipid-core nanocapsules have already shown increased delivery of indomethacin into rat brain reducing tumor growth [16], improving the

efficacy of indomethacin in experimental models of peripheral inflammation [50,51], and decreasing neuroinflammation induced by oxygen-glucose deprivation [52]. Therefore, our next step was to evaluate the amount of resveratrol reaching the brain of rats after 14 days of treatment with equivalent doses of RSV or RSV-NC in an attempt to explain the differences observed in the improvement of memory and synaptic impairments induced by Aβ1-42. Thus, a possible mechanism underlying the superior efficacy of treatment with RSV-LNC against Aβ-induced toxicity may include the enhanced brain bioavailability of resveratrol allowed by using lipid-core nanocapsules, according with our initial findings [17]. However, whether nanocapsules are able to cross the BBB remain to be determined, although some authors have already shown that the coating of nanocapsules with polysorbate 80 is essential for the delivery of drugs into the brain [53-55].

The synaptic loss and the death of specific types of neurons in AD are provoked by a cascade of multiple deleterious molecular and cellular events rather than a single pathogenic factor. One of the features present in AD brain and analyzed in the present study is the presence of reactive astrocytes and activated microglial cells triggered by Aβ1-42, which plays a prominent role in the neurodegenerative process. These cells normally provide tissue maintenance and immune surveillance of the brain, but as potent source of proinflammatory cytokines and chemokines, astrocytes and microglia are pivotal in the progression of neurodegenerative process including AD [56]. In fact, previous studies have shown that astrocytes and microglial cells are important mediators of Aβ-induced neurotoxicity become a prominent source of inflammatory mediators and reactive oxygen or nitrogen species [57,58]. Our results clearly show that resveratrol was able to decrease both astrocyte and microglial activation induced by icv injection of Aβ1-42. Recently, using organotypic hippocampal culture exposed to Aβ1-42, we found that resveratrol exhibited potent anti-inflammatory effects decreasing cytokine release, reactive species formation, and glial activation,

culminating in the prevention of cell death (unpublished data). In the same way, previous evidence suggests that the activation by phosphorylation of stress-activated protein c-Jun N-terminal kinase (JNK) is closely associated with neural dysfunction in AD [47,59]. The activation of JNK has been described in cultured neurons after Aβ exposure, and its inhibition attenuates Aβ toxicity [60,61]. Additionally, JNKs are reported to be involved in the enlargement of microglia, as well as in the induction of proinflammatory cytokine genes coding for TFN-α, IL-6, or MCP-1 in addition to COX-2 [62], suggesting that JNKs are relevant co-mediators of the activation of microglia. Further, activation of JNKs in brain induced by Aβ leads to enhanced expression of iNOS, contributing for neurodegenerative process and cognitive damage [47]. Recently, it has also been established a fundamental role of JNK in the regulation of tau hyperphosphorylation, a hallmark of AD [63]. In the present study, we demonstrate that Aβ triggered astrocytes and microglial activation. The activation of these glial cells may have led to release of inflammation factors culminating in the sustained JNK activation. The association of these events is closely related to memory dysfunction as described by Medeiros and collaborators [47]. Our results show that by using lipid-core nanocapsules, resveratrol was able to decrease of reactive astrocytes and microglia, as well as JNK activation. Therefore, we suggest that blocking of this vicious cycle was correlated with the improvements of memory performance impaired by Aβ.

Activation of glycogen synthase kinase-3β (GSK-3β) has been shown to be a key component in signaling pathways that underlie neurodegeneration [64]. GSK-3β hyperactivation is implicated in AD because it can increase Aβ production and toxicity, and the neuroinflammation. In neurons, GSK-3β directly phosphorylates several microtubule-associated proteins (MAPs) such as tau, MAP-1B, and MAP-2B [65]. Another substrate of GSK-3β *in vitro* is β-catenin, a protein implicated in cell adhesion and in the Wnt signaling pathway, which is known to be involved in the control of tissue patterning, cell fate, cell

proliferation, and development of the vertebrate CNS [66-68], beyond be closely related in the degenerative process involved in Aβ-induced toxicity [40-42]. Free cytoplasmic β-catenin levels are strictly controlled by phosphorylation of the NH_2-terminal region. This reaction, which targets β-catenin to the proteosome for ubiquitin-mediated degradation, requires association with several kinases [69]. Consistent with the amyloid cascade hypothesis, we found that Aβ induced a strongly activation of GSK-3β, which may be involved in the increased levels of β-catenin phosphorylation and consequent β-catenin destabilization. Given that we have previously shown that resveratrol was able to increase the phosphorylation/inactivation of GSK-3β in an in vitro model of oxygen-glucose deprivation [39], we sought evaluate the effect of resveratrol against the GSK-3β-activation induced by Aβ. Although only RSV-LNC treatment was able to improve memory and synaptic damage, as well as astrocytes, microglial, and JNK activation, to our surprise both, RSV and RSV-LNC treatments were able to cause an increase in the inhibition of GSK-3β activity by phosphorylation of serine 9. This effect in turn led to decreasing of phosphorylation levels of β-catenin induced by Aβ, contributing for stabilization of β-catenin as evidenced by increased cytoplasmic levels of β-catenin, which may cause TCF-induced transcriptional activation and preventing Aβ toxicity. Whereas the amount of resveratrol in the brain tissue was three times higher in animals treated with nanocapsules decreasing the behavioral impairment and the changes in the synaptic function and glial activation triggered by Aβ, likely lower concentration of resveratrol may be effective in the modulation of both proteins, GSK-3β and β-catenin, explaining these similarity in the results after RSV or RSV-LNC treatments.

Degradation of β-catenin is initiated upon amino-terminal serine/threonine phosphorylation, and GSK-3β appears to be a central player for ubiquitination-dependent proteolysis of β-catenin. Although GSK-3β phosphorylates β-catenin at serine 33/37, and this phosphorylation is critical for β-catenin recognition by ubiquitination apparatus, it has been

recognized that β-catenin phosphorylation *in vivo* requires casein kinase Iα (CKIα), which phosphorylates β-catenin at serine 45 and whose phosphorylation of β-catenin precedes and is obligatory for subsequent GSK-3β phosphorylation of β-catenin [69]. Thereby, our results show that resveratrol treatment was able to inhibit not only GSK-3β activation induced by Aβ1-42 but also the phosphorylation of β-catenin at serine 45 therefore stabilizing β-catenin levels, which might be related to the improvement of memory performance. In fact, recent studies concentrated on establishing the molecular complexity of Wnt/β-catenin signaling in neurons propose that inhibition of GSK-3β decrease Aβ neurotoxicity by reducing *tau* phosphorylation, increase β-catenin stabilization and improve memory performance suggesting that restoring β-catenin signaling may alleviate the underlying neuronal deficits in AD [70,71].

In conclusion, the present findings reinforce and extend the notion of the hazardous effects of Aβ1-42 toward hippocampal synaptic homeostasis and cognitive performance. Furthermore, we show that resveratrol is able to counteract the behavioral and cell signaling impairments triggered by icv administration of Aβ1-42. Moreover, this is the first report relating the ability of resveratrol in protect against synaptic degeneration induced by Aβ. The most powerful evidence from this report is the significant difference in the amount of resveratrol in cerebral tissue achieved by using lipid-core nanocapsules. Therefore, data from this study not only confirm the potential of resveratrol in treating AD but also offer an effective way to improve the efficiency of resveratrol by nano-drug delivery systems. Our results open the possibility that resveratrol could be a promising molecule for further pharmacological studies on the search for therapeutic strategies to treat or prevent AD.

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FIGURES LEGENDS

Figure 1. Experimental schedule

Figure 2. Effect of free resveratrol (RSV) and resveratrol-loaded lipid-core nanocapsules (RSV-LNC) on spontaneous alternation behavior. Rats were injected (2 nmol, icv) with Aβ1-42 or bi-distilled water and daily administered with RSV or RSV-LNC (10 mg/Kg, ip), starting 1 day after Aβ1-42 injection, and maintained for 14 days. Spontaneous alternation behavior during 8 min session in the Y-maze task was measured after 14 days of treatment. Columns indicate mean ± SD, n= 8-12 animals in each experimental group. *Significant differences between the indicated columns ($p<0.05$) (two-way ANOVA followed by Bonferroni post hoc test).

Figure 3. Effect of free resveratrol (RSV) and resveratrol-loaded lipid-core nanocapsules (RSV-LNC) on the novel object recognition memory. Rats were injected (2 nmol, icv) with Aβ1-42 or bi-distilled water and daily administered with RSV or RSV-LNC (10 mg/Kg, ip), starting 1 day after Aβ1-42 injection, and maintained for 14 days. Graphics show object recognition index during 5 min in the training session **(A)**; short-term memory test session performed 3 h after training **(B)**; and long-term memory test session performed 24 h after training session **(C)**. Columns indicate mean ± SD, n= 8-12 animals in each experimental group. ***Significant differences between familiar and new object for each group ($p<0.001$) (two-way ANOVA followed by Bonferroni post hoc test).

Figure 4. Aβ1-42 injection causes synaptotoxicity which is reduced by resveratrol-loaded lipid-core nanocapsules treatment. Western blot analysis for synaptophysin and β-actin

protein (loading control) was performed in the hippocampus of animals after injected with Aβ1-42 (2 nmol, icv) and treated by 14 days with free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) (10 mg/Kg, ip), starting 1 day after Aβ injection. Graphic shows representative quantification of synaptophysin immunocontent normalized by β-actin protein (loading control). The values represent synaptophysin level expressed as the average percentage increase (mean ± SD) over basal levels, n= 5-8 animals in each experimental group. #Significant differences between the indicated columns (p<0.01) (two-way ANOVA followed by Bonferroni post hoc test).

Figure 5. Resveratrol-loaded lipid-core nanocapsules treatment reduces astrocyte and microglial activation in the hippocampus after icv injection of Aβ1-42. Representative bands of Isolectin B4 (IB4) **(A)**, and GFAP **(B)** in the hippocampus 15 days after icv injection of Aβ1-42 (2 nmol, icv) and treatment with free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) (10 mg/Kg, ip). Graphic shows representative quantification of GFAP immunocontent normalized by β-actin protein (loading control) **(B)**. The values represent GFAP level expressed as the average percentage increase (mean ± SD) over basal levels, n= 5-8 animals in each experimental group. #Significantly different from all control groups (p<0.05); **Significantly different from Aβ1-42 and Aβ1-42 treated with vehicles groups (p<0.01) (two-way ANOVA followed by Bonferroni post hoc test).

Figure 6. Effect of resveratrol on the JNK and GSK-3β activation, and β-catenin destabilization triggered by Aβ1-42. Western blot analysis were performed in the hippocampus of animals after injected with Aβ1-42 (2 nmol, icv) and treated by 14 days with free resveratrol (RSV) or resveratrol-loaded lipid-core nanocapsules (RSV-LNC) (10 mg/Kg, ip). **(A)** Representative Western blot and graphic showing quantification of ratio pJNK/JNK

immunocontent normalized by β-actin protein (loading control). The values represent pJNK/JNK level expressed as the average percentage increase (mean ± SD) over basal levels, n= 5-8 animals in each experimental group. #Significantly different from all control groups (p<0.001); ***Significantly different from Aβ1-42 and Aβ1-42 treated with vehicles groups (p<0.001). **(B)** Representative Western blot and graphic showing quantification of ratio pGSK-3β/GSK-3β immunocontent normalized by β-actin protein (loading control). The values represent pGSK-3β/GSK-3β level expressed as the average percentage increase (mean ± SD) over basal levels, n= 5-8 animals in each experimental group. ***Significant differences between the indicated columns (p<0.001). **(C)** Representative Western blot and graphic showing quantification of β-catenin phosphorylated at Serine 45 immunocontent normalized by β-actin protein (loading control). The values are expressed as the average percentage increase (mean ± SD) over basal levels, n= 5-8 animals in each experimental group. #Significantly different from all groups (p<0.01); **Significantly different from Aβ1-42 and Aβ1-42 treated with vehicles groups (p<0.01). **(D)** Representative Western blot and graphic showing quantification of β-catenin immunocontent normalized by β-actin protein (loading control). The values are expressed as the average percentage increase (mean ± SD) over basal levels, n= 5-8 animals in each experimental group. *Significant differences between the indicated columns (p<0.05). Two-way ANOVA followed by Bonferroni post hoc test.

Figure 7. Lipid-core nanocapsules improve the brain bioavailability of resveratrol. Brain amount of resveratrol was analyzed by HPLC in rats injected (2 nmol, icv) with Aβ1-42 or bi-distilled water and daily administered with RSV or RSV-LNC (10 mg/Kg, ip) for 14 days, as described in Material and Methods session. The values are depicted as mean ± SD of five animals per group. ***Significantly different between the indicated columns (p<0.001). Two-way ANOVA followed by Bonferroni post hoc test.

Figure 1

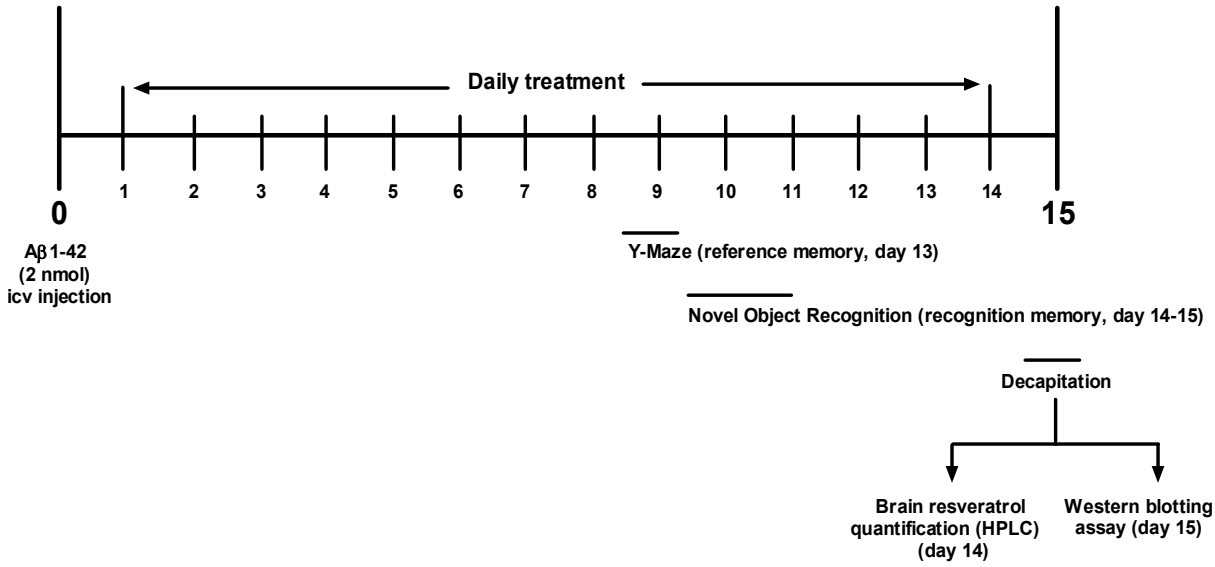

Daily treatment
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
15
Aβ 1-42
(2 nmol)
icv injection
Y-Maze (reference memory, day 13)
Novel Object Recognition (recognition memory, day 14-15)
Decapitation
Brain resveratrol
quantification (HPLC)
(day 14)
Western blotting
assay (day 15)

Figure 2

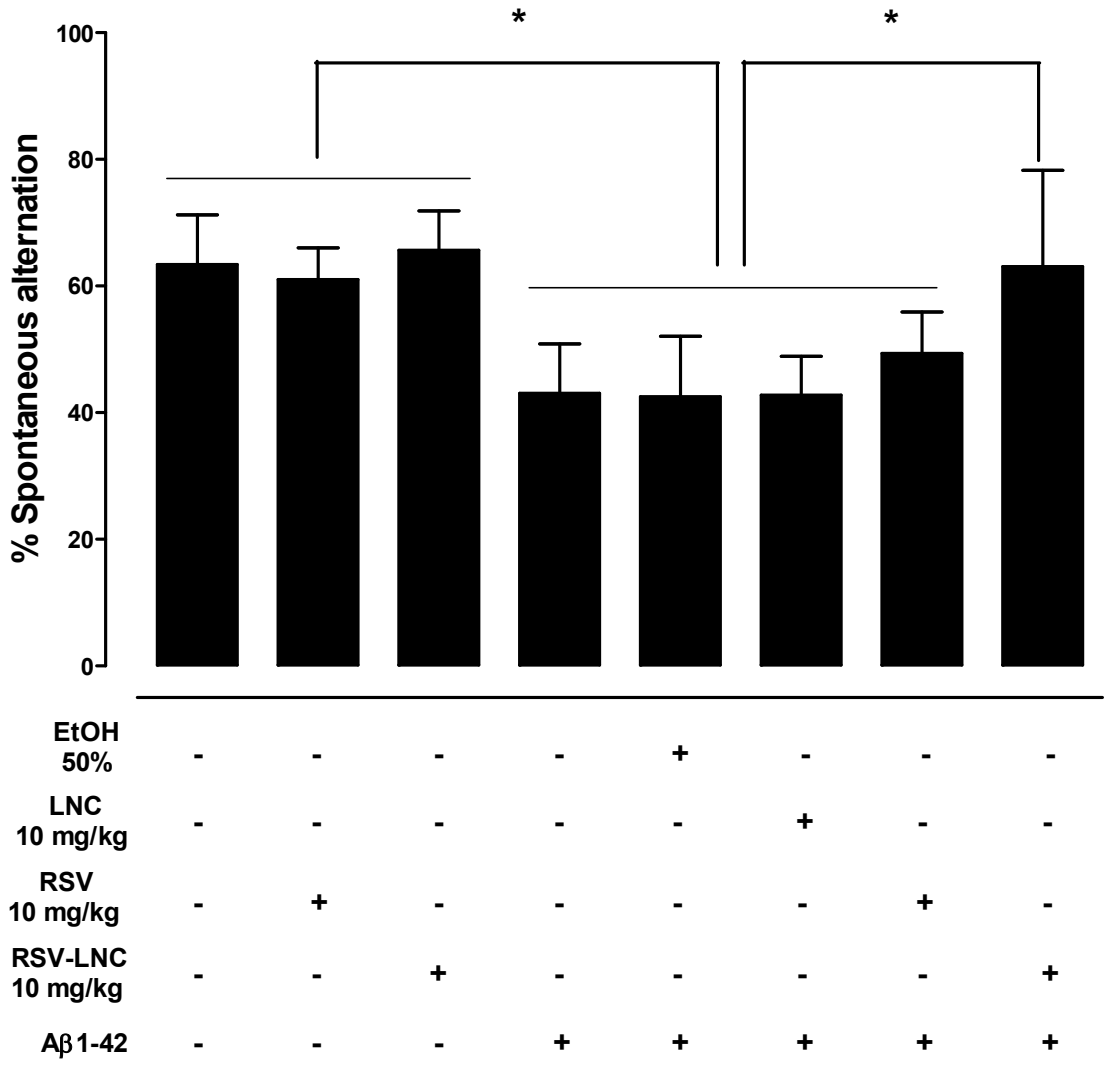

*
*
100
80
60
40
20
0
% Spontaneous alternation
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42
- - - - + - - -
- - - - - + - -
- + - - - - + -
- - + - - - - +
- - - + + + + +

Figure 3

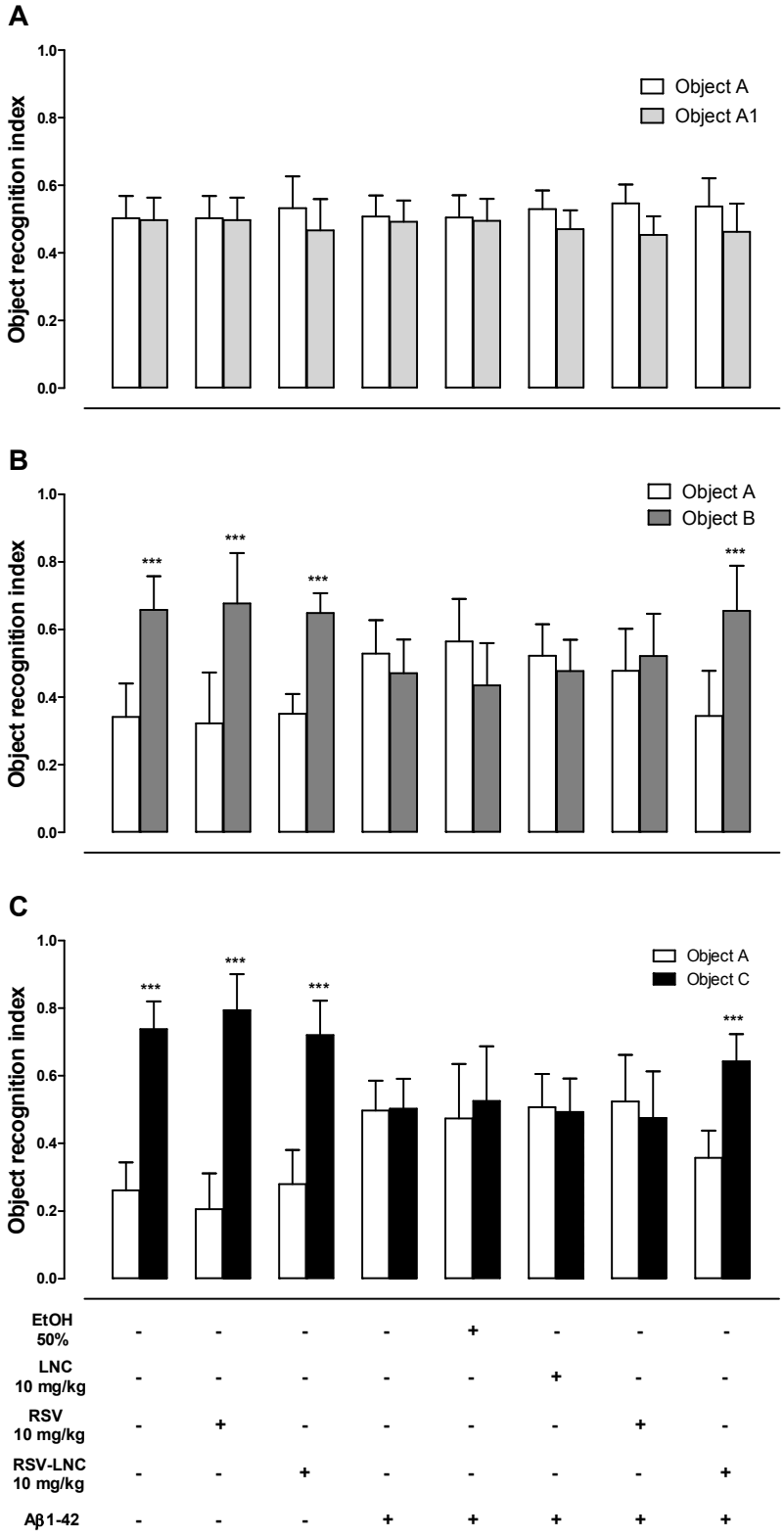

A
Object recognition index
1.0
0.8
0.6
0.4
0.2
0.0
Object A
Object A1
B
Object recognition index
Object A
Object B
***
C
Object recognition index
Object A
Object C
***
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42

Figure 4

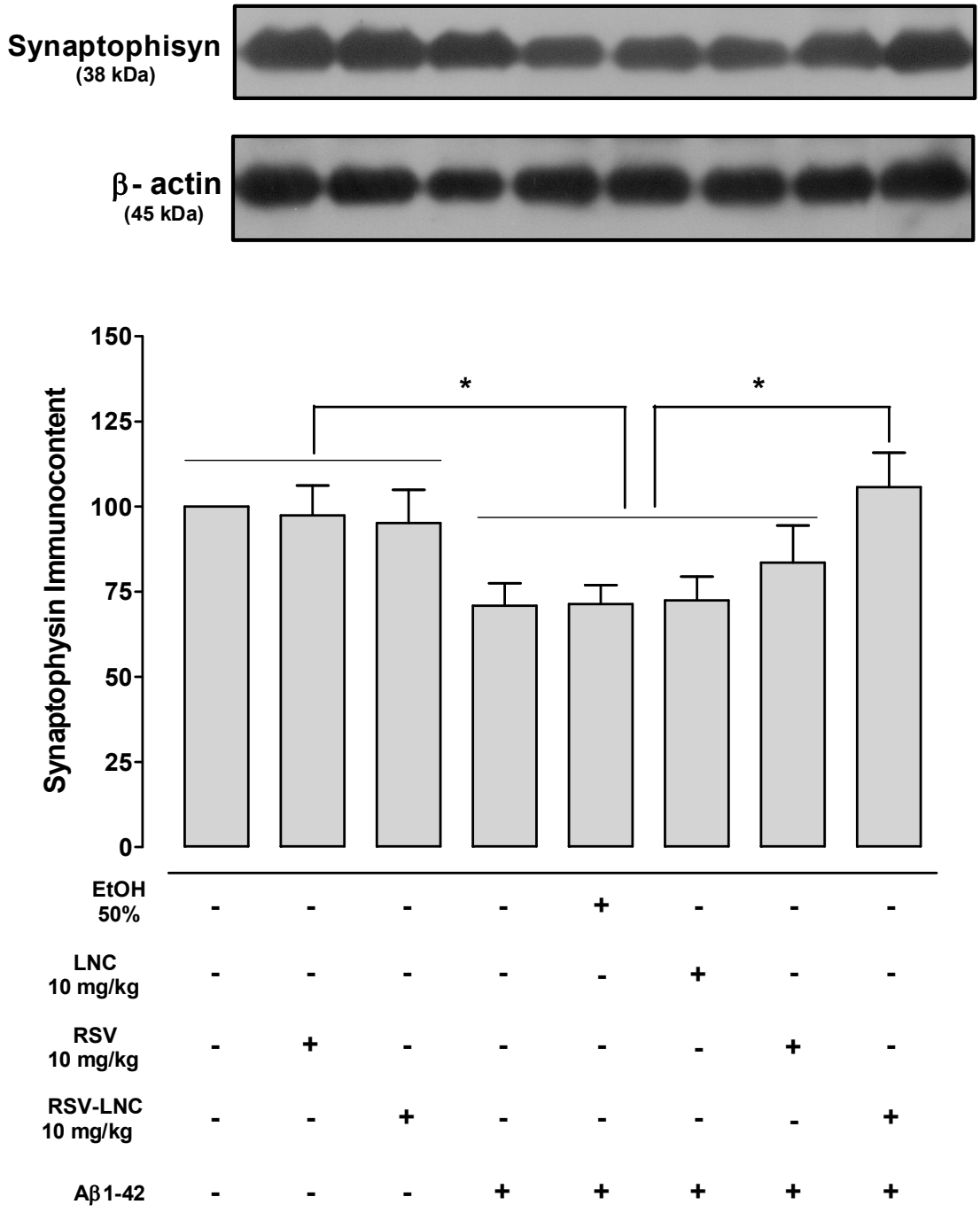
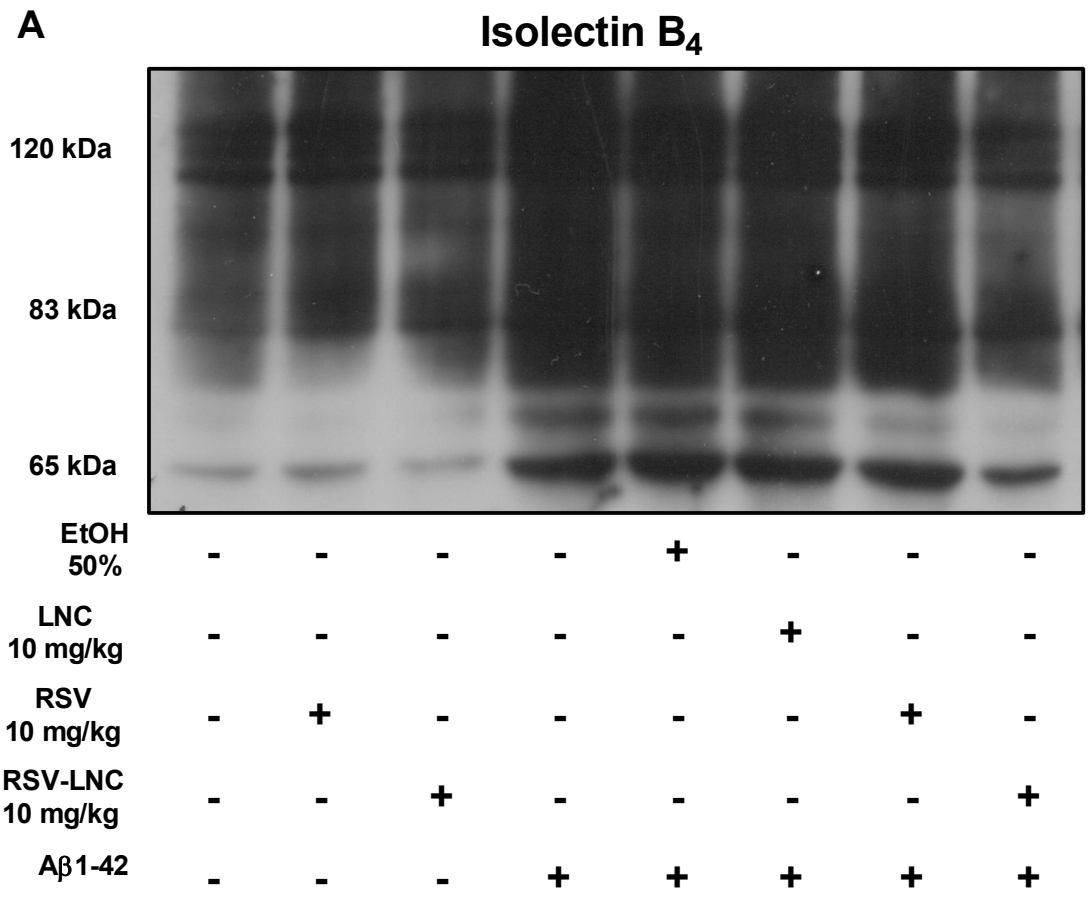

Synaptophisyn
(38 kDa)
β- actin
(45 kDa)
Synaptophysin Immunocontent
150
125
100
75
50
25
0
*
*
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42

Figure 5


A
Isolectin B4
120 kDa
83 kDa
65 kDa
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42
- - - - + - - -
- - - - - + - -
- + - - - - + -
- - + - - - - +
- - - + + + + +

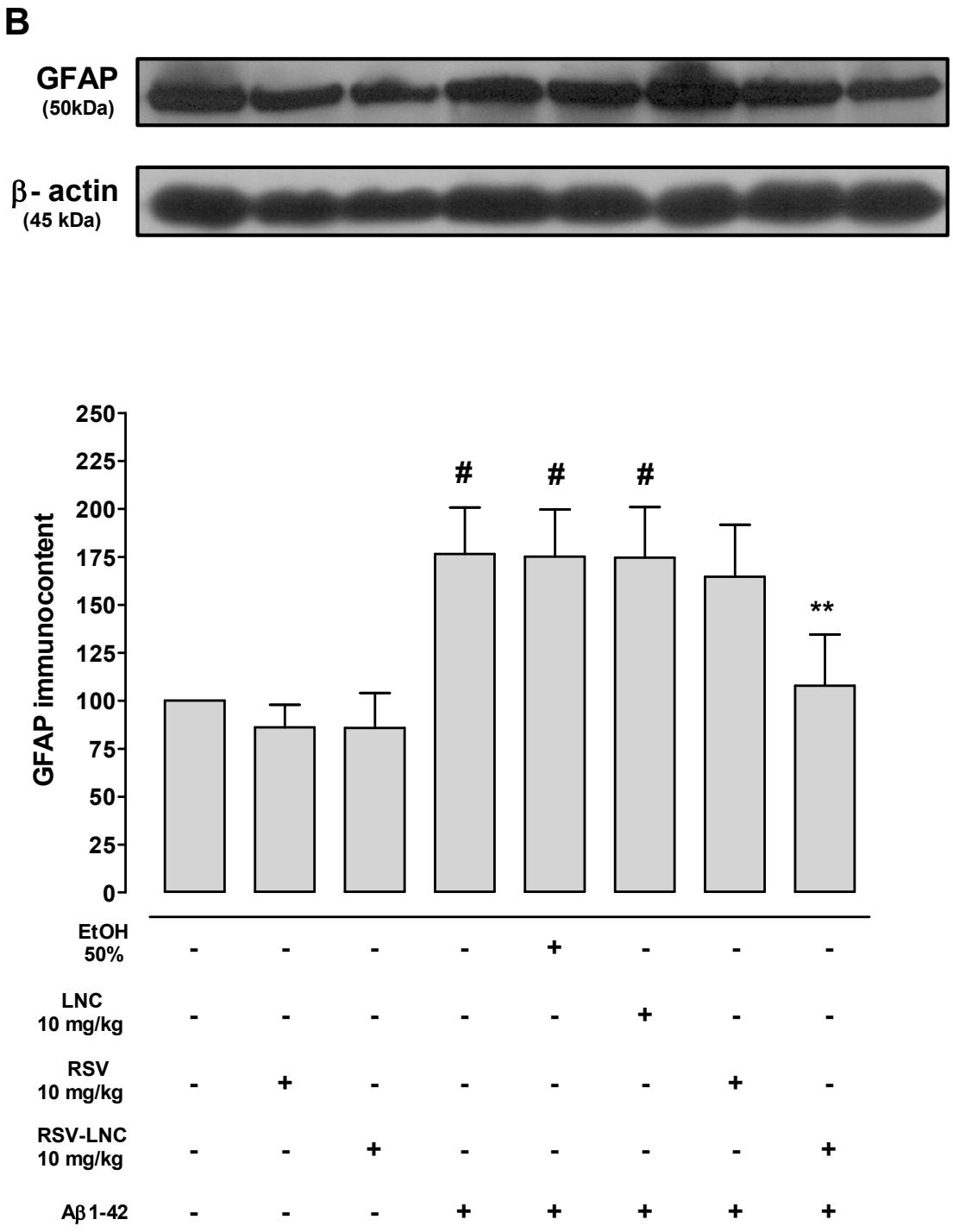
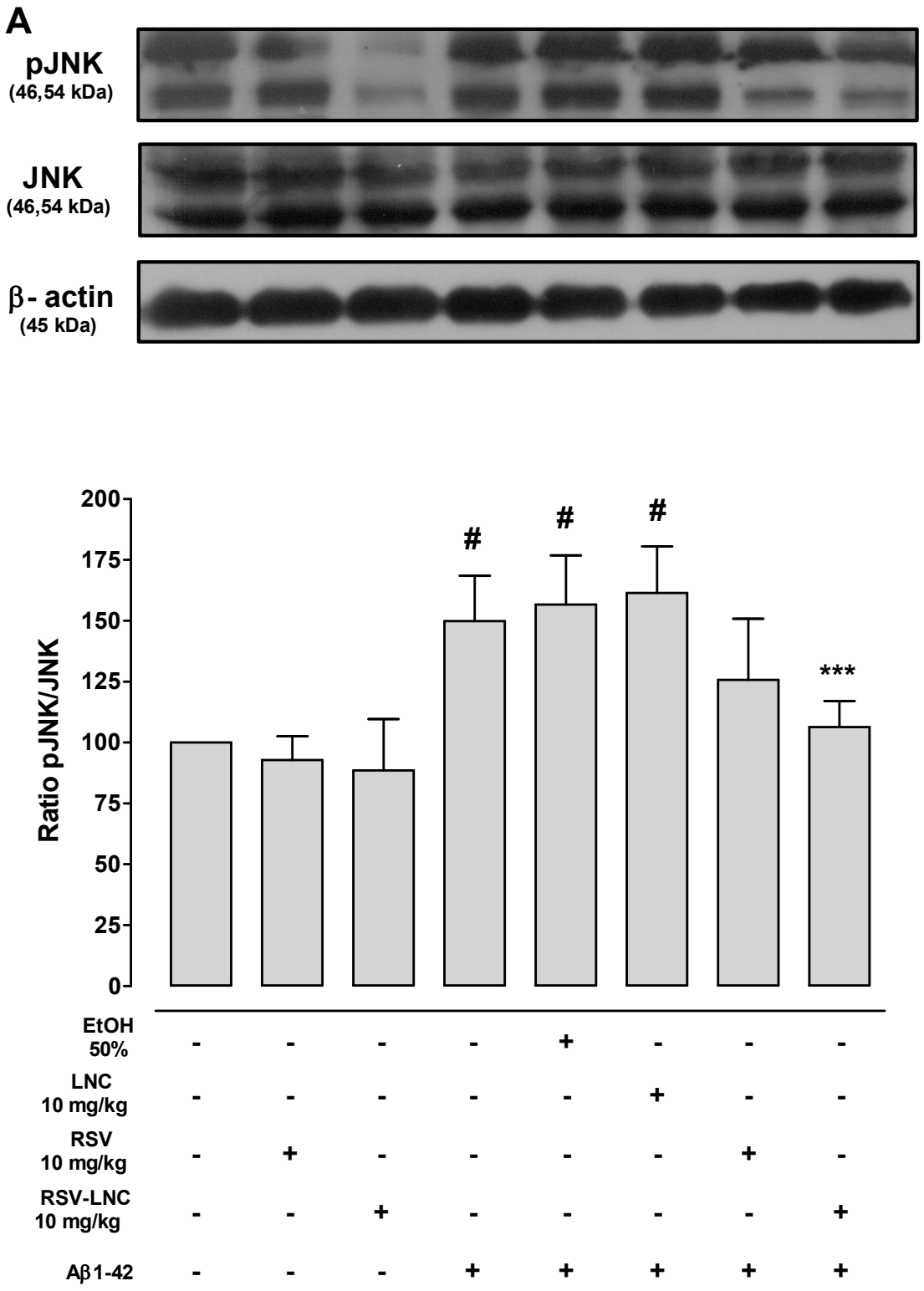
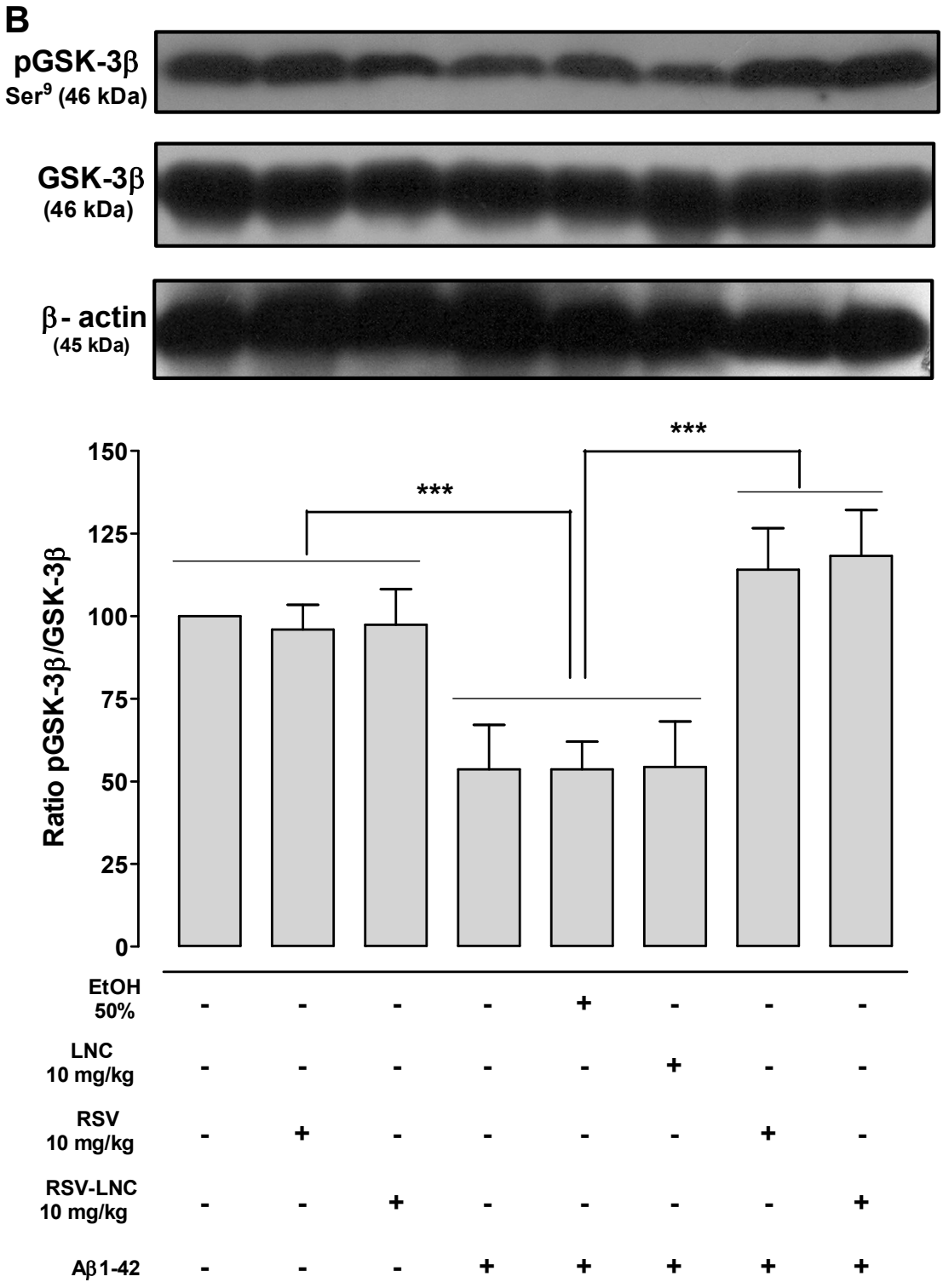
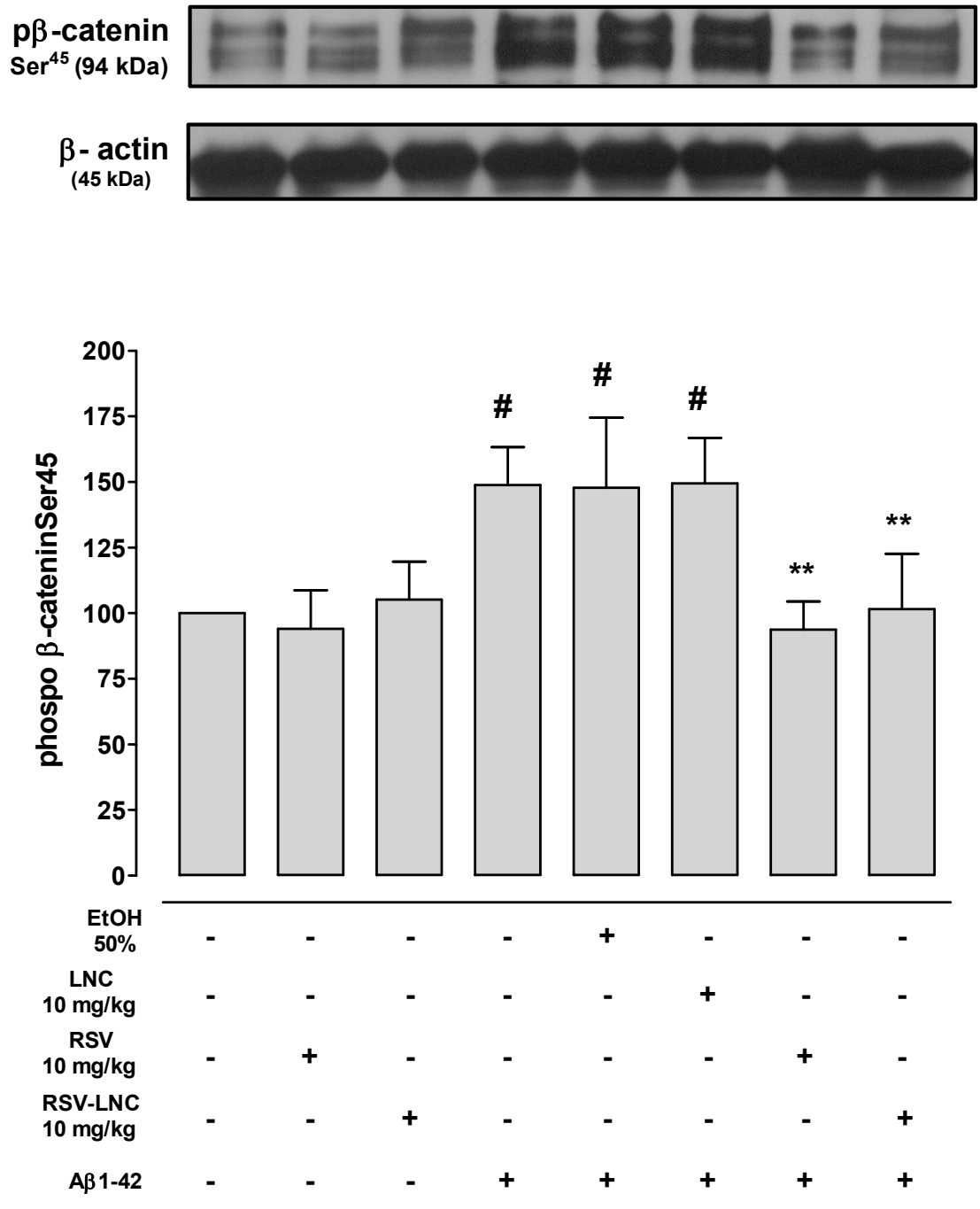

B
GFAP
(50kDa)
β- actin
(45 kDa)
GFAP immunocontent
250
225
200
175
150
125
100
75
50
25
0
#
#
#
**
EtOH
50%
LNC
10 mg/kg
RSV
10 mg/kg
RSV-LNC
10 mg/kg
Aβ1-42

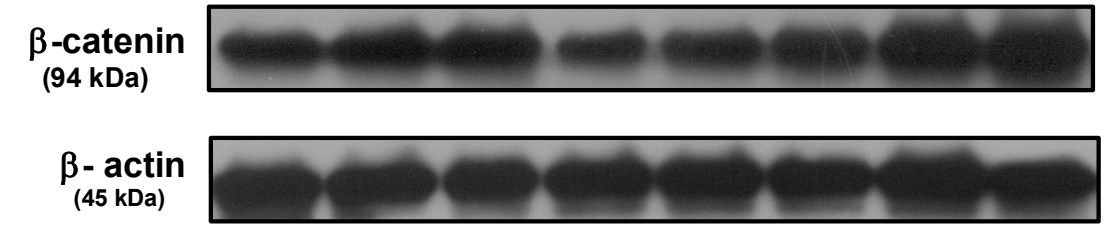
Figure 6


A
pJNK
(46,54 kDa)
JNK
(46,54 kDa)
β- actin
(45 kDa)
Ratio pJNK/JNK
200
175
150
125
100
75
50
25
0
#
#
#
***
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42


B
pGSK-3β
Ser9 (46 kDa)
GSK-3β
(46 kDa)
β- actin
(45 kDa)
***
***
Ratio pGSK-3β/GSK-3β
150
125
100
75
50
25
0
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42


C
pβ-catenin
Ser45 (94 kDa)
β- actin
(45 kDa)
phospo β-cateninSer45
200
175
150
125
100
75
50
25
0
#
#
#
**
**
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42

D


β-catenin
(94 kDa)
β- actin
(45 kDa)

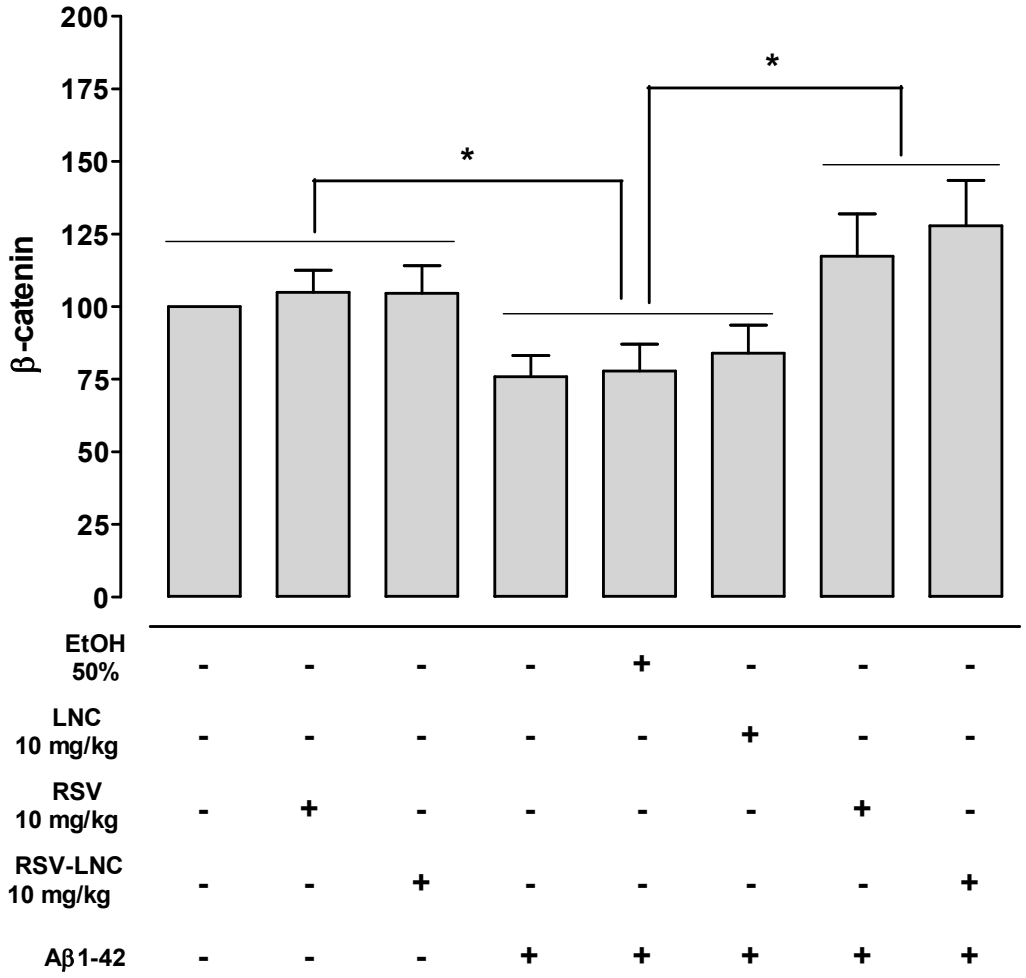
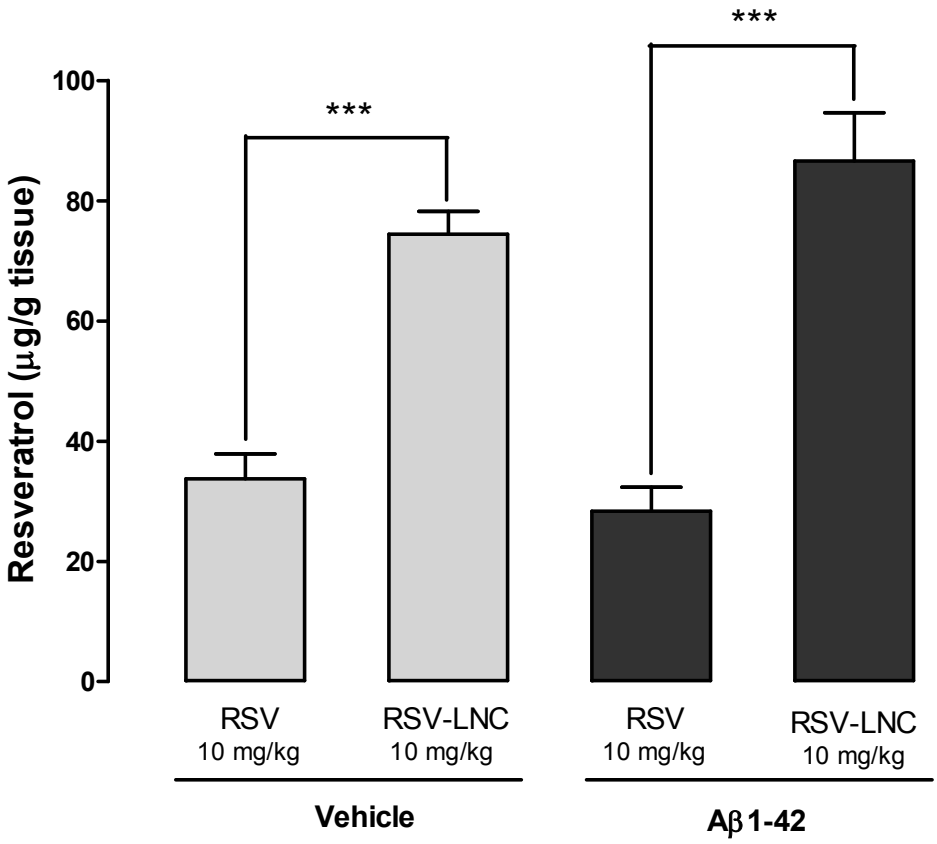

β-catenin
200
175
150
125
100
75
50
25
0
*
*
EtOH 50%
LNC 10 mg/kg
RSV 10 mg/kg
RSV-LNC 10 mg/kg
Aβ1-42

Figure 7


***
***
100
80
60
40
20
0
Resveratrol (μg/g tissue)
RSV
10 mg/kg
RSV-LNC
10 mg/kg
RSV
10 mg/kg
RSV-LNC
10 mg/kg
Vehicle
Aβ 1-42

DISCUSSÃO

A doença de Alzheimer (DA) é a principal forma de demência relacionada à idade, responsável por mais de 60% dos casos, e tende a se tornar um problema de saúde pública em razão do envelhecimento populacional. Mais de uma década após a aprovação do uso de inibidores da acetilcolinesterase para o tratamento da DA, tratamentos utilizando um único medicamento ou a combinação de terapias que possam efetivamente parar ou modificar o curso da doença ainda não se encontram disponíveis. Ensaios clínicos utilizando inibidores de secretases, agentes capazes de prevenir a agregação e consequentemente a formação de oligômeros e fibrilas do Aβ, imunoterapia para a remoção do Aβ, agentes que modulam a fosforilação da *tau*, as funções mitocondriais, os receptores de serotonina, os produtos finais de glicação avançada e fatores neurotróficos estão em andamento; porém, os resultados são pouco animadores.

Nas últimas décadas, produtos de origem vegetal, tais como polifenóis presentes no ginkgo biloba, quercetina, catequinas, curcumina e resveratrol têm recebido atenção especial na prevenção e, potencialmente, no tratamento de doenças neurodegenerativas. Considerando que o resveratrol tem sido relacionado aos efeitos benéficos do consumo prolongado de vinho tinto na prevenção de diversas doenças crônicas incluindo a DA, o objetivo central desta Tese de Doutorado foi avaliar o efeito do resveratrol sobre a toxicidade desencadeada pelo Aβ através da exposição de culturas organotípicas de hipocampo de ratos ao Aβ e através da injeção intracerebroventricular (icv) do Aβ em ratos. Entretanto, devido à baixa solubilidade em água, à alta instabilidade e à farmacocinética desfavorável do resveratrol, nós buscamos uma alternativa na nanotecnologia para superar estas limitações na tentativa de melhorar a biodisponibilidade do resveratrol. Os resultados obtidos nesta Tese não somente confirmam o potencial do resveratrol na prevenção e no tratamento da DA como também oferecem uma forma eficiente para melhorar a sua biodisponibilidade através dos inúmeros benefícios oferecidos pela nanobiotecnologia.

O resveratrol tem atraído considerável interesse por seus potenciais efeitos benéficos para a saúde humana, os quais incluem a quimioprevenção, as atividades anti-inflamatória, antioxidante, anti-diabética, anti-obesidadade, cardio e neuroprotetoras, além de aumentar a sobrevivência de diversas espécies (Baur e cols., 2006; Baur e Sinclair, 2006; Saiko e cols., 2008). Entretanto, diversas evidências têm questionado os efeitos atribuídos ao resveratrol, devido à sua alta taxa de metabolização e reduzida biodisponibilidade (Kuhnle e cols., 2000; Goldberg e cols., 2003; Walle e cols., 2004). Dessa forma, estabilizar a molécula de resveratrol preservando suas atividades biológicas e melhorando a sua biodisponibilidade tem se tornado objetivo de intenso estudo. Neste contexto, no Capítulo I desta Tese nós buscamos desenvolver um sistema nanocarreador a fim de melhorar a biodisponibilidade cerebral do resveratrol para posterior análise do seu efeito sobre a neurotoxicidade induzida pelo Aβ em modelos *in vitro* e *in vivo* (Capítulos II e III).

A nanotecnologia está no centro da biotecnologia e desempenha um importante papel no desenvolvimento de novas modalidades terapêuticas. Considerando que nanopartículas poliméricas têm sido amplamente empregadas para a entrega de compostos biologicamente ativos, nossa hipótese inicial, a qual constitui o Capítulo I, teve como foco principal desenvolver uma formulação capaz de superar algumas limitações apresentadas pelo resveratrol bem como aumentar a sua biodisponibilidade cerebral mesmo com a administração de baixas dosagens. Dessa forma, o isômero *trans* do resveratrol foi incorporado em uma nanocápsula polimérica com núcleo lipídico, através do método de deposição interfacial de polímeros pré-formados (Fessi e cols., 1989; Jäger e cols., 2009), o qual tem sido amplamente utilizado para a produção de nanocápsulas. A suspensão de nanocápsulas formadas apresentou uma distribuição homogênea com o tamanho médio das nanocápsulas menor que 300 nm e com elevada taxa de associação (99,9%). O tamanho das nanocápsulas contendo resveratrol está de acordo com o tamanho usualmente encontrado para nanocápsulas preparadas pelo

método de deposição interfacial (100-500 nm) (Couvreur e cols., 2002; Schaffazick e cols., 2005; Bernardi e cols., 2009a). As suspenões apresentaram uma distribuição de tamanho monomodal e índices de polidispersão menores que 0,3, indicando uma pequena variação do tamanho. Não foram observadas alterações significativas tanto nestas características quanto no conteúdo de resveratrol associado às nanocápsulas durante um período de três meses, sugerindo estabilidade da formulação. Além disso, resultados similares foram obtidos para formulações idênticas, porém não incorporadas de resveratrol, indicando compatibilidade entre o polímero utilizado na produção das nanocápsulas e o resveratrol.

Nanomateriais utilizados para a entrega de fármacos devem contemplar diversos requisitos, tais como biocompatibilidade, compatibilidade com o fármaco, cinética de biodegradação e propriedades mecânicas adequadas, bem como fácil processamento. Nos últimos 20 anos, polímeros biodegradáveis sintéticos têm sido altamente empregados em uma ampla variedade de metodologias para a produção de dispositivos para entrega de fármacos, genes e agentes para imagem devido à sua biocompatibilidade e biodegradabilidade. Poliamidas, poli aminoácidos, poli alquil-α-cianoacrilatos, poliésteres, poli ortoésteres, poliuretanos e poliacrilamidas têm sido usados para preparar vários dispositivos (Jain, 2000; Panyam e Labhasetwar, 2003). Dentre estes, a poli(ε-caprolactona), polímero de escolha para a produção das nanocápsulas empregadas nesta Tese, se destaca devido à sua biocompatibilidade, biodegradabilidade e propriedades mecânicas, pois é um polímero semi-cristalino que apresenta degradação mais lenta quando comparado aos polímeros amorfos (Guterres e cols., 2007). A poli(ε-caprolactona) é um dos mais importantes polímeros biodegradáveis utilizados na medicina, sendo amplamente empregado em suturas (Lu e Chen, 2004). Além disso, poliésteres como a poli(ε-caprolactona) apresentam propriedades bioadesivas, característica relevante pois a bioadesividade confere um acréscimo na deposição das partículas em regiões do trato gastrointestinal, aumentando assim a absorção

sistêmica de fármacos (Lamprecht e cols., 2000a). Vários trabalhos têm demonstrado a importância do emprego deste material polimérico no desenvolvimento de sistemas carreadores de fármacos nanoparticulados (Lamprecht e cols., 2000a; 2000b; Sinha e cols., 2004; Lu e Chen, 2004; Fattal e cols., 2007), sendo empregados inclusive para uso intravenoso (Ravi Kumar, 2000).

Outro fator importante que pode afetar a estabilidade das nanopartículas é o potencial zeta. O potencial zeta reflete o potencial de superfície das partículas, o qual é influenciado pelas mudanças na interface com o meio dispersante, em razão da dissociação de grupos funcionais na superfície da partícula ou da adsorção de espécies iônicas presentes no meio aquoso de dispersão (Magenhein e Benita; 1991; Mosqueira e cols., 2000). O potencial zeta pode influenciar a estabilidade das nanopartículas em suspensão através das repulsões eletrostáticas entre as partículas, as quais são maiores quando o potencial zeta aumenta, levando à formação de partículas mais estáveis com distribuição uniforme do tamanho (Couvreur e cols., 2002). Os resultados obtidos na caracterização das nanocápsulas contendo resveratrol mostram que o potencial zeta das nanocápsulas foi de aproximadamente -14 mV, suportando a observação da estabilidade da nossa formulação. Além disso, na análise de espalhamento de luz, realizada a fim de estudar a estabilidade física da formulação, foi observado uma leve tendência de cremagem, provavelmente devido a uma pequena porção de nanoemulsão presente na formulação. Entretanto, este fenômeno é reversível e típico para suspensões.

Uma vez que as nanocápsulas apresentaram alta estabilidade e eficiência no encapsulamento do resveratrol, dando sequência a nossa hipótese do uso de nanocarreadores como uma ferramenta para melhorar a biodisponibilidade do resveratrol, nós avaliamos a biodistribuição do resveratrol em nanocápsulas e comparamos com a biodistribuição do resveratrol livre (em solução). Dessa forma, ratos *Wistar* saudáveis foram tratatos com 5

mg/kg de resveratrol nanoencapsulado ou resveratrol livre, na mesma dose, por 14 dias. Além disso, nós avaliamos duas vias de administração: um grupo de animais foi administrado pela via intraperitoneal (i.p.), enquanto outro grupo foi administrado por gavagem. A análise por cromatografia líquida de alta eficiência (HPLC – *High-Performance Liquid Cromatography*) revelou que 1 h após a última administração, animais que receberam resveratrol em nanocápsulas intraperitonealmente apresentaram quantidades superiores de resveratrol em 2,5, 6,6 e 3,4 vezes no cérebro, fígado e rim, respectivamente, quando comparados aos animais que receberam doses idênticas de resveratrol livre. Considerando que o resveratrol pode ser detectado na circulação mesentérica pouco tempo após a sua administração, que apresenta um pico plasmático em aproximadamente 60 minutos, porém sua distribuição nos tecidos necessita de algumas horas (Andlauer e cols., 2000; Vitrac e cols., 2003; Somoza, 2008; Almeida e cols., 2009), nós hipotetizamos que os níveis de resveratrol encontrados nestes órgãos poderiam ser decorrentes do seu acúmulo, uma vez que o tratamento foi realizado diariamente pelo período de 14 dias. Para provar nossa hipótese, um novo esquema de administração foi realizado. Primeiramente, 1 h após uma única administração i.p. de ambos, resveratrol nanoencapsulado ou resveratrol livre em doses equivalentes, não foi detectado resveratrol no cérebro em nenhum dos grupos e apenas baixas quantidades foram observadas no fígado e no rim tanto nos animais tratados com resveratrol em nanocápsulas quanto nos tratados com resveratrol livre. Na sequência, na análise realizada 3h após uma única administração i.p. resultados similares àqueles encontrados após 14 dias de tratamento foram observados, embora em menores proporções. Estes resultados confirmam nossa hipótese de acúmulo tecidual do resveratrol.

Similar ao perfil de distribuição tecidual do resveratrol obtido com administração i.p., animais administrados com resveratrol em nanocápsulas por gavagem durante 14 dias apresentaram quantidades superiores de resveratrol em todos os órgãos analisados. Aqui é

importante ressaltar o aumento na biodistribuição do resveratrol proporcionado pelas nanocápulas através da administração por gavagem, dado que a camada de muco que protege a superfície do epitélio intestinal representa uma importante barreira à penetração de nanopartículas (Wang e cols., 2008; Cone, 2009), devido ao seu aprisionamento e remoção, reduzindo o tempo de permanência das nanopartículas no trato gastrointestinal bem como sua penetração nas células endoteliais (Ensign e cols., 2011). Além disso, as variações de pH e a presença de enzimas pancreáticas podem desestabilizar as nanopartículas ocasionando a liberação do fármaco. Entretanto, alguns estudos têm relatado que nanopartículas administradas pela via oral podem ser captadas pelas células M, as quais estão sobrepostas às placas de Peyer. Uma vez captadas e presentes nas placas de Peyer, as nanopartículas passam para a circulação linfática (Shakweh e cols., 2004; Prego e cols., 2005). Estes trabalhos são confirmados pelos resultados relatados por des Rieux e cols., onde um robusto aumento no transporte de nanopartículas de poliestireno com tamanho entre 200 e 500 nm foi observado *in vitro* quando células epiteliais de intestino foram co-cultivadas com células que haviam sido diferenciadas para atingir características de células M (des Rieux e cols., 2005). Dessa forma, a captação de sistemas nanoparticulados pelas placas de Peyer constitui uma importante via para a entrega de fármacos e de vacinas (Shakweh e cols., 2004). Além disso, como mencionado anteriormente, a poli(ε-caprolactona) apresenta propriedades bioadesivas, conferindo um acréscimo na deposição de partículas em regiões do trato gastrointestinal facilitando a captação mediada por tanscitose nos enterócitos (Shakweh e cols., 2004), explicando, pelo menos em parte, os nossos resultados que demonstram um aumento na biodisponibilidade do resveratrol proporcionado pelo uso de nanocápsulas, mesmo quando administradas por gavagem.

Embora o resveratrol permeie a BHE, nossos resultados mostram que nanocápsulas poliméricas com núcleo lipídico foram capazes de aumentar a quantidade de resveratrol no

cérebro de ratos saudáveis. Este aumento proporcionado pelas nanocápsulas pode estar relacionado à redução do metabolismo do resveratrol nos enterócitos e no fígado, além da prevenção da ligação do resveratrol às proteínas plasmáticas, a qual poderia reduzir a biodisponibilidade cerebral do resveratrol. Embora experimentos fornecendo evidências de que as nanocápsulas atravessem a BHE e se depositem no parênquima cerebral não tenham sido realizados, diversos estudos demonstram que o revestimento de sistemas nanoparticulados com polisorbato 80 é uma estratégia amplamemte utilizada para a vetorização de compostos ao cérebro (Kreuter e cols., 1995; Alyautdin e cols., 1997; Gulyaev e cols., 1999; Bernardi e cols., 2009a), uma vez que o polisorbato 80 poderia promover a adsorção de proteínas plasmáticas às nanocápsulas favorecendo o seu transporte através das células endoteliais da BHE por meio da endocitose receptor-mediada. Além disso, o polisorbato 80 permite que sistemas nanoparticulados permaneçam por um longo tempo na circulação sem serem fagocitados pelo sistema fagocitário mononuclear (Wilson e cols., 2008) o que poderia fornecer um mecanismo para a liberação sustentada do resveratrol permitindo uma concentração plasmática constante.

O grande número dos estudos realizados em animais revela que o resveratrol é bem tolerado, não exercendo efeitos tóxicos. Entretanto, considerando que o resveratrol pode diminuir a atividade da COX-1 (Szewczuck e cols., 2004) e, como consequência da exposição repetida ao resveratrol por longos períodos de tempo, diminuir a secreção do muco gástrico podendo levar ao desenvolvimento de efeitos adversos no trato gastrointestinal, nossos resultados mostram que o nanoencapsulamento do resveratrol foi capaz de prevenir a toxicidade no trato gastrointestinal quando comparado com o tratamento com resveratrol livre. Estes resultados corroboram com trabalhos prévios do nosso grupo, demonstrando que a incorporação de diclofenaco ou de indometacina em nanocápsulas poliméricas de núcleo lipídico resultou na diminuição dos efeitos adversos no trato gastrointestinal característicos

de ambos os fármacos (Guterres e cols., 2001; Bernardi e cols., 2009b). A necropsia dos animais ao término dos tratamentos não demonstrou alterações macro ou microscópicas no cérebro, coração, pulmão, estômago, fígado e rim. Além disso, o tratamento por 14 dias com nanocápsulas não alterou os níveis das enzimas γ-glutamil transferase, aspartato aminotransferase e alanina aminotransferase, indicando ausência de toxicidade hepática nas condições avaliadas.

Dessa forma, os resultados obtidos no Capítulo I desta Tese forneceram evidências de que nanocápsulas poliméricas com núcleo lipídico contendo resveratrol são altamente estáveis e apresentam elevada eficiência de encapsulamento, constituíndo uma excelente ferramenta para superar as limitações intrínsecas ao resveratrol e aumentar a sua biodistribuição. Estes resultados forneceram suporte ao nosso objetivo de avaliar o efeito do resveratrol sobre a toxicidade induzida pelo peptídeo Aβ em modelos *in vitro* e *in vivo*.

Como apresentado na Introdução, recentes estudos sugerem que o resveratrol exerce um potente efeito neuroprotetor na DA (Marambaud e cols., 2005; Vingtdeux e cols., 2008; Karuppagounder e cols., 2009; Vingtdeux e cols., 2010) embora os mecanismos pelos quais sua ação é exercida são pouco compreendidos. Extensivas pesquisas na última década revelam que a maioria das doenças crônicas tais como o câncer, doenças cardiovasculares, pulmonares e neurológicas, bem como diabetes e doenças auto-imunes exibem desregulação de diversas vias de sinalização, as quais estão ligadas ao desenvolvimento e à progressão da inflamação. Em se tratando da DA, os mecanismos inflamatórios podem ser considerados como um terceiro componente da doença, os quais uma vez iniciados pela degeneração podem contribuir significativamente para a sua progressão e cronicidade. Apesar do cérebro ter sido considerado imunologicamente privilegiado por muito tempo devido à BHE impedir a passagem de células do sistema imunológico e pela inabilidade de suas células constituintes exercerem resposta imunológica, nas últimas décadas este dogma foi quebrado. Atualmente,

é amplamente reconhecido que a neuroinflamação está intimamente ligada à ativação dos astrócitos e da microglia levando a liberação sustentada de mediadores inflamatórios e citotóxicos que aceleram o processo neurodegenerativo, bem como secretam fatores que podem recrutar linfócitos e monócitos através da BHE acentuando o processo inflamatório. Considerando que o resveratrol apresenta atividade neuroprotetora e antiinflamatória, o Capítulo II desta Tese teve como objetivo avaliar o efeito do resveratrol sobre a neuroinflamação desencadeada pela exposição de culturas organotípicas de hipocampo de ratos ao Aβ.

Dado que alguns estudos utilizando culturas primárias mostram que os astrócitos são importantes mediadores da toxicidade desencadeada pelo Aβ (Paradisi e cols., 2004; Garwood e cols., 2011), a cultura organotípica apresenta algumas vantagens sobre a cultura de células isoladas. A cultura organotípica de hipocampo combina a acessibilidade e a manutenção *in vitro* de fatias de tecido preservando as conexões sinápticas e a anatomia do hipocampo (Stoppini e cols., 1991; Bruce e cols., 1996). Neste sistema, neurônios e células gliais sobrevivem por longo período de tempo em cultivo "amadurecendo" fisiologicamente durante sua sobrevivência, permitindo o estudo de fatores fisiológicos e de compostos farmacológicos que possam contribuir com a sobrevivência celular e com a plasticidade sináptica, bem como a biologia de proteínas específicas e cascatas de sinalização celular (Frotscher e cols., 1995; Holopainen, 2005). Dessa forma, a cultura organotípica fornece um excelente modelo para estudar a resposta dos neurônios, dos astrócitos e da microglia frente à toxicidade induzida pelo Aβ bem como avaliar compostos que possam reduzir os danos causados pelo Aβ.

Com o objetivo de compreender os mecanismos de toxicidade induzidos pelo Aβ culturas organotípicas de hipocampo de ratos foram mantidas em cultivo por 30 dias e expostas a 2 μM do Aβ1-42 no 28º dia por 48 h. Embora evidências sugiram que agregados

oligoméricos sejam os principais responsáveis pela toxicidade do Aβ, em nosso estudo o Aβ1-42 foi submetido aos processos de formação de fibrilas amplamente empregados pela literatura, entretanto nós não excluímos a presença de oligômeros em nossa preparação. Os resultados obtidos neste Capítulo mostram que o Aβ1-42 induziu um significativo aumento na morte celular, como pode ser evidenciado pelo aumento na incorporação do iodeto de propídeo. Tendo em mente que o nanoencapsulamento aumentou a biodisponibilidade do resveratrol, nós tratamos as culturas com o resveratrol em nanocápsulas e comparamos o efeito com o tratamento das culturas com resveratrol livre. Além disso, nós avaliamos o efeito do tratamento previamente à exposição ao Aβ (culturas tratadas com 5 ou 10 μM de resveratrol nanoecapsulado ou resveratrol livre a partir do 24º dia de cultivo) e o efeito do tratamento simultâneo à exposição do Aβ (culturas tratadas com com 5 ou 10 μM de resveratrol nanoencapsulado ou resveratrol livre simultaneamente à exposição do Aβ). Como descrito no Capítulo II, ambos os tratamentos, prévio e simultâneo, foram capazes de reduzir a morte celular induzida pelo Aβ. Entretanto, enquanto o tratamento com resveratrol livre apresentou efeito neuroprotetor somente na maior concentração, ambas as concentrações do resveratrol nanoencapsulado apresentaram este efeito, confirmando a hipótese de que as nanocápsulas melhoram a biodisponibilidade do resveratrol mesmo em um sistema *in vitro*. Evidências suportando a nossa hipótese também vêm dos resultados obtidos pela análise da formação de espécies reativas, como pode ser evidenciado pelo aumento na oxidação da diclorofluoresceína (DCF – *dichlorofluorescein*) induzido pelo Aβ. Ainda que no tratamento prévio o resveratrol livre tenha sido capaz de prevenir a produção de espécies reativas, este efeito não foi observado no tratamento simultâneo; enquanto tanto o tratamento prévio quanto o simultâneo com concentraçãoes equivalentes de resveratrol nanoencapsulado reduziram a produção de espécies reativas induzida pelo Aβ.

Levando em cosideração os resultados sobre a morte celular e sobre o estresse oxidativo induzio pelo Aβ, é possível perceber que o nanoencapsulamento do resveratrol conferiu uma acentuada ação neuroprotetora, visto que mesmo na menor concentração o efeito foi pronunciado. Esta acentuada atividade do resveratrol pode estar relacionada a dois importantes fatores mediados pelo uso das nanocápsulas: (i) elas podem ter atuado como um "reservatório" de resveratrol, permitindo a liberação sustentada e o mantendo disponível para a captação celular; e (ii) a prevenção da ligação do resveratrol à albumina presente no meio de cultivo, a qual pode reduzir a disponibilidade do resveratrol (Lancon e cols., 2004; Delmas e cols., 2011), bem como a prevenção da oxidação do resveratrol no meio de cultivo (Long e cols., 2010; Yang e cols., 2010). Além disso, o resveratrol pode estar sendo acumulado no interior das células através da captação das nanocápsulas mediada por endocitose. Isso explicaria porque não foi observada diferença entre as duas concentrações do resveratrol nanoencapsulado, uma vez que o processo de endocitose pode ser saturado.

A inflamação é uma resposta orquestrada envolvendo a regulação e a ativação de uma grande variedade de proteínas e genes. A neuroinflamação tem sido conhecida por exercer um papel central na patogenia da DA, uma vez que a liberação de citocinas pró-inflamatórias parece desempenhar um papel central na toxicidade induzida pelo Aβ, levando a distúrbios na homeostasia celular, geração de espécies reativas e morte celular. Nossos resultados estão de acordo com estas evidências, uma vez que a exposição da cultura organotípica ao Aβ levou a liberação sustentada das citocinas pró-inflamatórias TNF-α, IL-1β e IL-6 por até 48 h. Consistente com os resultados observados sobre a morte celular e o estresse oxidativo, tanto no tratamento prévio quanto no simultâneo, o resveratrol nanoencapsulado foi capaz de reduzir siginificativamente a liberação destes mediadores pró-inflamatórios em todos os tempos analizados (3, 6, 12, 24 e 48 h após a exposição ao Aβ). Entretanto, o tratamento com resveratrol livre, nas mesmas condições e concentrações, foi efetivo apenas nos tempos

iniciais (3 e 6 h) de exposição ao Aβ. Ainda nesta perspectiva, ambos os tratamentos com resveratrol nanoencapsulados elevaram a liberação da citocina aniinflamatória IL-10 atingindo seu efeito máximo em 6 h e mantendo-se pelo menos até 48 h. É importante ressaltar aqui que o tratamento com o resveratrol nanoencapsulado não apenas elevou a liberação da IL-10 nas culturas controle como também apresentou uma potencialização deste efeito nas culturas expostas ao Aβ, enquanto o aumento na liberação da IL-10 nos tratamentos com resveratrol livre foi observado apenas nos tempos de 3 e 6 h e, mais importante, somente na ausência do Aβ. Dessa maneira, nossos resultados sugerem que enquanto o tratamento com resveratrol livre bloqueou parcialmente a resposta inflamatória, o uso das nanocápsulas permitiu a potencialização da atividade antiinflamatória do resveratrol, culminando na redução da morte celular e do estrese oxidativo.

Considerando que as nanocápsulas podem estar conferindo a liberação sustentada do resveratrol como discutido acima favorecendo sua biodisponibilidade, esses eventos podem explicar as diferenças entre os tratamentos com resveratrol livre e nanoencapsulado. Além disso, tendo em mente que a inflamação leva à ativação de diversos genes e que estudos mostram que o resveratrol pode acumular-se no citoplasma e na região perinuclear (Lancon e cols., 2004; Delmas e cols., 2011), a captação celular das nanocápsulas pode permitir a entrega intracelular do resveratrol promovendo o bloqueio sustentado da neuroinflamação, prevenindo a propagação das alterações na sinalização celular e reduzindo a morte celular, explicando dessa forma a diferença entre os tratamentos com resveratrol livre e nanoencapsulado. Esta hipótese pode ser suportada pelos trabalhos mostrando que nanopartículas são endocitadas e concentradas próximo ao núcleo celular (Kristl e cols., 2003; Shenoy e cols., 2005; Shao e cols., 2009; Teskac e Kristl, 2010).

A ativação prolongada e amplamente difundida da microglia e dos astrócitos é aparente na DA. Ainda que inicialmente astrócitos e microglia atuem promovendo a remoção

do Aβ, os elevados níveis de citocinas e quimiocinas secretados levam ao agravamento da neurodegeneração. A geração de um microambiente inflamatório pode desencadear a ativação de diversas vias de sinalização celular, comprometento a sobrevivência neuronal e contribuindo para a geração de mediadores tóxicos. Entre estas vias de sinalização celular, destaca-se a das proteínas cinases ativadas por estresse como a JNK (*c-Jun N-terminal Kinase*). A ativação da JNK tem sido descrita em cultura de neurônios exposta ao Aβ e sua inibição pode contribuir significativamente para a redução da neurotoxicidade desencadeada pelo Aβ (Bozyczko-Coyne e cols., 2001; Morishima e cols., 2001). Além disso, a JNK pode estar envolvida nos processos de ativação microglial e na indução da expressão de genes responsáveis pela síntese de TFN-α, IL-6 e MCP-1, além do aumento na expressão da COX-2, iNOS e NFκB e do aumento na fosforilação da *tau* (Waetzig e Herdegen, 2004; Mehan e cols., 2011; Ploia et al 2011). A liberação destes fatores inflamatórios, bem como ativação da COX-2 e da iNOS, podem levar a geração de espécies reativas de oxigênio e nitrogênio. Neste ambiente inflamatório e altamente oxidativo, os astrócitos podem sofrer uma alteração no seu fenótipo metabólico reduzindo sua capacidade de controlar as alterações homeostáticas e diminuindo sua habilidade em proteger os neurônios (Gavillet e cols., 2008). Dessa forma, compostos que apresentem a capacidade de reduzir essas alterações podem ser considerados potentes candidatos para o tratamento das alterações celulares e moleculares desencadeadas na DA. Neste contexto, o resveratrol tem sido proposto como um potente composto anti-inflamatório e antioxidante modulando diferentemente a ativação dos astócitos e da microglia em resposta ao LPS e ao Aβ (Candelario-Jalil e cols., 2007; Lu e cols., 2010 Capiralla e cols., 2012).

Na sequência da nossa investigação, os resultados mostram que o Aβ desencadeou ativação astrocitária, evidenciada pelo aumento no imunoconteúdo da GFAP (*Glial Fibrillary Acidic Protein*) e, provavelmente, a ativação microglial, potencializando as cascatas

envolvidas na neuroinflamação com consequente ativação sustentada da JNK. Estas alterações podem ter contribuído para a geração de um ciclo vicioso levando à morte celular observada após 48 h de exposição das culturas ao Aβ. Enquanto o resveratrol livre reduziu a ativação astrocitária somente quando adicionado previamente à exposição do Aβ e na maior concentração sem alterar a ativação da JNK, ambos os tratamentos, prévio e simultâneo, com resveratrol nanoencapsulado foram capazes de bloquear o ciclo ativação astrocitária/microglial - liberação de citocinas pró-inflamatórias - ativação da JNK, provavelmente pelos processos descritos acima, reduzindo a toxicidade desencadeada pelo Aβ contribuindo para a redução na morte celular.

Como descrito na anteriormente, astrócitos e microglia expressam uma classe de receptores de reconhecimento como evidenciado pela classe receptores do tipo Toll capazes de detectar agentes infecciosos e danos teciduais no SNC. Assim, a ligação do Aβ a estes receptores pode ter desencadeado os processos neuroinflamatórios observados. Além disso, a ligação do Aβ aos RAGEs e aos receptores do tipo NOD (NLRs - *NOD-like receptors*) também pode ter contribuído para os efeitos tóxicos que levaram à morte celular. A ligação do Aβ a estas três classes de receptores de reconhecimento pode convergir para um evento comum através da formação do inflamassoma, caracterizado pelo envolvimento da caspase-1, do NFκB e pela liberação e citocinas pró-inflamatórias, principalmente IL-1β (Masters e O´Neill, 2011). Dessa forma, podemos sugerir que o resveratrol poderia estar atuando prevenindo a formação do inflamassoma, uma vez que através da ativação da SIRT1 o resveratrol pode inibir a via de sinalização mediada pelo NFκB e reduzir o processo inflamatório desencadeado pelo Aβ.

Os resultados obtidos neste Capítulo demonstram que a inflamação está intimamente ligada à toxicidade desencadeada pelo Aβ e sugerem que a redução da resposta inflamatória pode representar uma estratégia terapêutica para o tratamento da DA. Ainda neste Capítulo,

nossos resultados não somente fornecem fortes evidências sobre o potencial efeito do resveratrol no tratamento do processo neurodegenerativo observado na DA como também oferecem uma alternativa para melhorar a sua eficiência por meio do uso de um sistema nanocarreador.

Tendo em mente os resultados obtidos no Capítulo I, onde a incorporação do resveratrol em nanocápsulas poliméricas com núcleo lipídico proporcionou um aumento de 2,5 vezes na quantidade de resveratrol no cérebro de ratos saudáveis, e os resultados obtidos no Capítulo II, onde o uso destas nanocápsulas permitiu a potencialização da atividade antiinflamatória e neuroprotetora do resveratrol, nosso próximo passo foi avaliar o efeito do resveratrol em um modelo *in vivo* de toxicidade induzida pelo Aβ. Para tanto, utilizamos o modelo de injeção intracerebroventricular (icv) do Aβ em ratos *Wistar*. Apesar da maioria dos estudos *in vivo* abordando as alterações características da DA utilizarem roedores transgênicos, a utilização do modelo de administração icv do Aβ tem sido especialmente útil no desenvolvimento e na avaliação de novas modalidades terapêuticas. Embora variável em termos de tamanho e estado de agregação do Aβ utilizado, do procedimento e do local de injeção e dos testes comportamentais empregados, diversos estudos tem consistentemente demonstrado a ocorrência de déficits comportamentais relacionados à perda de memória após a injeção intracerebral do Aβ.

Da mesma forma como discutido no Capítulo II, o Aβ1-42 utilizado em nosso estudo foi submetido aos processos de formação de fibrilas amplamente empregados pela literatura, entretanto, nós não excluímos a presença de oligômeros em nossa preparação. Os resultados obtidos no Capítulo III desta Tese mostram que a injeção icv do Aβ1-42 (2 nmol) causou um significativo déficit comportamental, como pode ser evidenciado pela redução na alternação espontânea e pela alteração no teste de reconhecimento de objetos. Estes resultados corroboram com estudos prévios mostrando que a injecção icv de 2 nmol de oligômeros do

Aβ1-42 levou ao desenvolvimento de alterações comportamentais após um período de 15 dias (Cunha e cols., 2008; Canas e cols., 2009). O teste de alternação espontânea tem sido amplamente utilizado para estudar de memória espacial de curto prazo onde se pode analizar alterações nos processos colinérgicos (Hughes, 2004). O teste de reconhecimento de objetos constitui um teste de memória não-espacial utilizado para analizar memória de curta e longa duração a qual traduz alterações no córtex e no hipocampo. Alterações no desempenho do teste de reconhecimento de objetos são dependentes das regiões cerebrais que constituem os primeiros alvos do Aβ, dessa maneira fornecem uma medida sensível das alterações cognitivas iniciais (Dodart e cols., 2002; Sipos e cols., 2007). Alterações em intervalos longos de retenção da memória (maiores que 3 h) sugerem que ambos, córtex e hipocampo estejam funcionalmente danificados.

Considerando que os efeitos do resveratrol sobre o dano cognitivo induzido pelo Aβ não são conhecidos e os resultados obtidos no Capítulo II forneceram fortes evidências de que o resveratrol pode reduzir a inflamação e a morte celular, nós tentamos compreender o efeito do resveratrol sobre o dano cognitivo e as alterações moleculares induzidos pela injeção icv do Aβ1-42. Dessa maneira, 24 h após a infusão do Aβ1-42, iniciou-se o tratamento com resveratrol. Neste estudo, o efeito do tratamento com resveratrol nanoencapsulado foi novamente comparado com o tratamento com o resveratrol livre. Assim, os animais foram tratados i.p. durante 14 dias com 10 mg/Kg, dividido em duas aplicações diárias de 5 mg/Kg (12/12h), do resveratrol nanoencapsulado ou a mesma dose de resveratrol livre. Como está descrito no Capítulo III, enquanto o tratamento com resveratrol livre não foi eficaz em reduzir o dano cognitivo induzido pelo Aβ, os animais tratados com o resveratrol nanoencapsulado exibiram desempenho semelhantes aos animais controle nos testes comportamentais. É importante ressaltar que devido ao tratamento ter iniciado após a indução da lesão, o uso das

nanocápsulas não apenas proporcionaram ao resveratrol a capacidade de prevenir o dano cognitivo como também a capacidade de restaurar o desempenho comportamental.

A perda sináptica pode levar ao desenvolvimento de alterações no aprendizado e na memória e está envolvida em diversos processos neurodegenerativos. Segundo a nova versão da hipótese da cascata amilóide apresentada na Introdução, a disfunção sináptica constitui a melhor correlação com os distúrbios cognitivos associados com a DA e precede a morte neuronal. Nós observamos que o déficit cognitivo encontrado após a administração do Aβ1-42 parece estar relacionado à disfunção sináptica, como pode ser evidenciado pela redução no imunoconteúdo da sinaptofisina, uma proteína pré-sinaptica amplamente utilizada como marcador da integridade sináptica. Além disso, o tratamento dos animais com resveratrol nanoencapsulado foi capaz de restaurar os níveis da sinaptofisina, enquanto o tratamento com resveratrol livre não foi eficaz na redução da sinaptotoxicidade induzida pelo Aβ, fornecendo suporte à idéia da redução na densidade sináptica como principal alteração inicial da DA. Consistente com estes resultados, estudos prévios relatam que o Aβ induz alterações sinápticas em cultura de neurônios (Evans e cols., 2008) podendo se ligar a proteínas sinápticas causando sinaptotoxicidade, a qual precede a morte neuronal, como ocorre em diferentes modelos de animais transgênicos da DA (Oddo e cols., 2003; Lacor e cols., 2007; Jacobsen e cols., 2006).

Tendo em mente que os resultados obtidos no Capítulo I mostraram que animais saudáveis tratados com nanocápsulas apresentaram maiores quantidades de resveratrol no tecido cerebral, os resultados obtidos neste estudo, onde apenas os animais administrados com Aβ e tratados com resveratrol nanoencapsulado exibiram desempenho semelhante aos animais controles, podem ser explicados pelo aumento na biodistribuição do resveratrol mediado por esse sistema nanocarreador. Ainda consistente com os resultados do Capítulo I, a análise da quantidade de resveratrol por HPLC revelou que os animais tratados com nanocápsulas

superaram em três vezes a quantidade de resveratrol no tecido cerebral em comparação aos animais tratados com resveratrol livre. Embora nós não tenhamos evidências de que as nanocápsulas encontram-se no parênquima cerebral, como discutido anteriormente, o revestimento de sistemas nanoparticulados com polissorbato 80 é uma estratégia amplamemte utilizada para a entrega de compostos ao cérebro. Além disso, as nanocápsulas podem permanecer por maiores períodos de tempo na circulação, prevenindo a rápida metabolização e excreção do resveratrol além de favorecer a liberação controlada do resveratrol aumentando assim a sua translocação pela BHE. Resultados similares foram obtidos com o uso deste mesmo sistema nanocarreador em trabalhos prévios do nosso grupo de pesquisa, onde a incorporação da indometacina em nanocápsulas poliméricas com núcleo lipídico levou a uma maior concentração da indometacina no parênquima cerebral reduzindo o crescimento tumoral (Bernardi e cols., 2009a).

O resveratrol pode modular a função de diversas proteínas regulatórias envolvidas na transdução de sinal, no crescimento, na sobrevivência e na morte celular. Na DA, a perda sináptica e a morte de determinadas populações neuronais é desencadeada por múltiplas alterações celulares e moleculares. Como já mencionado anteriormente, astrócitos e microglia participam ativamente nas alterações desencadeadas pelo Aβ. Consistente com os resultados obtidos *in vitro* (Capítulo II), no estudo *in vivo* o tratamento com resveratrol nanoencapsulado foi capaz de reduzir a ativação dos astrócitos (evidenciada pela redução do imunoconteúdo da GFAP) e a ativação da microglia (evidenciada pela redução da reatividade da Isolectina B4) induzida pelo injecção icv do Aβ. Ainda nexte contexto, o resveratrol nanoencapsulado reduziu a ativação da JNK desencadeada pelo Aβ. Este conjunto de resultados nos permite concluir que as alterações celulares e moleculares desencadeadas pela administração icv do Aβ são semelhantes àquelas observadas pela exposição de culturas organotípicas de hipocampo ao Aβ, e que a resposta inflamatória pode estar desempenhando um papel central

no processo degenerativo, levando à disfunção sináptica e consequentemente à alteração comportamental.

A proteína cinase GSK-3β (*Glycogen Synthase Kinase-3β*) tem sido implicada em múltiplos processos celulares e está intimamente ligada à patogênese de diversas doenças, inclusive a DA. Recentemente, o uso de inibidores da GSK-3β tem emergido como uma potente alternativa terapêutica para o tratamento de doenças associadas a níveis anormalmente elevados de sua ativação, tais como diabetes tipo 2 e DA. A hiperativação da GSK-3β na DA pode estar associada ao aumento na produção e à toxicidade do Aβ, uma vez que ela parece estar envolvida no processamento da APP (Balaraman e cols., 2006). Além disso, a GSK-3β é uma das principais cinases envolvidas na hiperfosforilação da *tau* e formação dos emaranhados neurofibrilares (Maccioni e cols., 2001). Outro importante substrato da GSK-3β é a proteína β-catenina. A β-catenina desempenha um papel central na via de sinalização canônica mediada pela Wnt e está envolvida no controle da diferenciação tecidual, no destino celular e na proliferação celular e no desenvolvimento do SNC de vertebrados (Cadigan e cols., 1997; Lee e cols., 2000; Galceran e cols., 2000), além de estar intimamente relacionada aos processos degenerativos desencadeados pelo Aβ (Garrido e cols., 2002; De Ferrari e cols., 2003; Li e cols., 2011). Os níveis citoplasmáticos da β-catenina são regulados por fosforilação de tal maneira que livre a β-catenina pode migrar para o núcleo e induzir a expressão de diversos genes envolvidos na sobrevivência e na homeostase neuronal ou ser direcionada às sinapses para modular o fortalecimento sináptico em resposta à despolarização (Caricasole e cols., 2003). Entretanto, uma vez fosforilada a β-catenina é degradada via sistema ubiquitina-proteossoma (Aberle e cols., 1997).

Nossos resultados mostram que a administração icv do Aβ desencadeou um acentuado aumento na ativação da GSK-3β (evidenciado pela redução no imunoconteúdo da GSK-3β fosforilada em serina 9) acompanhado pelo aumento do imunoconteúdo da β-catenina

fosforilada em serina 45 e pela diminuição dos níveis citoplasmáticos da β-catenina livre. Embora a fosforilação da β-catenina mediada pela GSK-3β ocorra nos resíduos de serina 33 e 37, a fosforilação em serina 45, a qual é mediada pela atividade da caseína cinase-Iα (CKIα - *casein kinase Iα*), é um passo obrigatório e precede a subsequente fosforilação da β-catenina pela GSK-3β (Liu e cols., 2002). Ambos os tratamentos, com resveratrol nanoencapsulado ou com resveratrol livre foram eficazes em aumentar a fosforilação/inativação da GSK-3β, bem como em restaurar a estabilização da β-catenina. É importante ressaltar aqui que não foram observadas diferenças entre o tratamento com resveratrol nanoencapsulado e com resveratrol livre. Considerando que a quantidade de resveratrol no tecido cerebral foi elevada em três vezes nos animais tratados com nanocápsulas explicando as diferenças nos resultados encontrados nas alterações comportamentais, sináptica e na ativação glial, provavelmente nenores concentrações de resveratrol possam ser efetivas na modulação de ambas as proteínas, GSK-3β e β-catenina, explicando a similaridade nos resultados.

De forma geral, os resultados obtidos neste Capítulo são consistentes com aqueles observados no Capítulo II e suportam a hipótese do potencial efeito neuroprotetor do resveratrol sobre a toxicidade induzida pelo Aβ, além de fornecerem evidências para o desenvolvimento de novas modalidades terapêuticas para o tratamento da DA.

Em resumo, como esquematizado na Figura 1, os resultados obtidos nesta Tese demonstram que a incorporação do resveratrol em nanocápsulas poliméricas de núcleo lipídico permitiu uma melhor biodistribuição deste polifenol. Dessa forma, as maiores concentrações teciduais do resveratrol podem ter contribuído para a modulação no cenário inflamatório, resultando no maior efeito neuroprotetor frente à lesão induzida pelo Aβ.

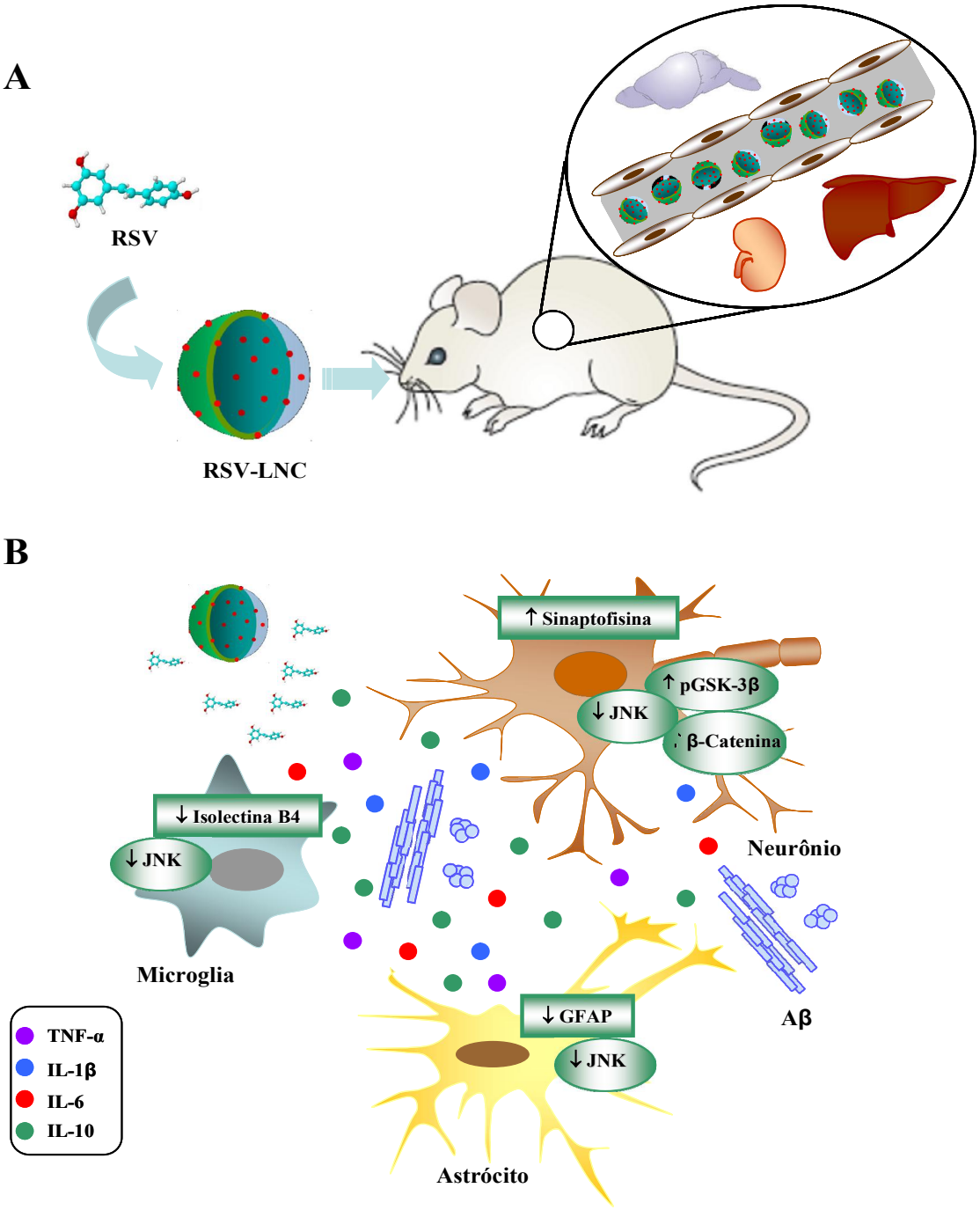


Figura 1. Esquema representativo dos principais resultados obtidos nesta Tese. (A) A incorporação do resveratrol (RSV) em nanocápsulas poliméricas de núcleo lipídico (RSV-LNC) permitiu uma melhor biodistribuição do resveratrol. **(B)** Principais mecanismos propostos para o efeito neuroprotetor do resveratrol frente à lesão induzida pelo Aβ.

CONCLUSÕES

1. Gerais

Os resultados obtidos nesta Tese demonstram que o desenvolvimento de um sistema nanocarreador possibilitou aumentar a biodisponibilidade cerebral do resveratrol permitindo um acentuado efeito neuroprotetor deste polifenol frente à toxicidade induzida pelo Aβ, fornecendo evidências para o desenvolvimento de uma nova alternativa terapêutica para a prevenção e para o tratamento da doença de Alzheimer.

2. Específicas

- ✓ Nanocápsulas poliméricas com núcleo lipídico apresentaram elevada eficiência de encapsulamento do resveratrol constituíndo uma eficiente ferramenta para superar as limitações intrínsecas ao resveratrol e aumentar a sua biodisponibilidade.

- ✓ A inflamação está intimamente ligada à toxicidade desencadeada pelo Aβ e a utilização de nanocápsulas permitiu ao resveratrol a inibição sustentada dos eventos neuroinflamatórios levando a prevenção/redução da morte celular.

- ✓ O tratamento com resveratrol nanoencapsulado apresentou maior eficácia sobre as alterações comportamentais induzidas pelo Aβ quando comparado com o tratamento com resveratrol livre. Este efeito pode estar sendo mediado pela redução da ativação astrocitária e microglial e pela restauração da atividade sináptica e da sinalização celular.

PERSPECTIVAS

Como continuação deste trabalho, pretende-se trabalhar com os seguintes objetivos:

- Avaliar o efeito do resveratrol sobre a agregação do Aβ;
- Determinar a participação dos receptores do tipo Toll, do tipo NOD e os RAGEs, bem como do NFκB nos processos neuroinflamatórios desencadeados pelo Aβ levando à formação do inflamassoma;
- Investigar o envolvimento da SIRT1 na neuroproteção exercida pelo resveratrol;
- Avaliar se o resveratrol altera a liberação das Wnts e a ativação das vias canônica e não-canônica;
- Investigar se oligômeros do Aβ induzem alterações comportamentais e alteram a expressão de proteínas pré e pós-sinápticas e se o tratamento com resveratrol é capaz de reduzir a toxicidade dos oligômeros;
- Avaliar o efeito do Aβ bem como do tratamento com resveratrol sobre a fosforilação da *tau*;
- Avaliar a participação da microglia sobre a toxicidade do Aβ através de técnicas de imunohistoquímica.

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