UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Trabalho de Conclusão de Curso de Farmácia

INVESTIGAÇÃO DO POTENCIAL EFEITO NEUROPROTETOR DE NANOCÁPSULAS POLIMÉRICAS CONTENDO PHLORETIN EM UM MODELO *EX VIVO* DA DOENÇA DE ALZHEIMER

Felippo Bifi

Porto Alegre, junho de 2019

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Trabalho de Conclusão de Curso apresentado como requisito parcial para obtenção do grau de farmacêutico pelo curso de Farmácia da Universidade Federal do Rio Grande do Sul.

Felippo Bifi

Orientadora: Dra. Juliana Bender Hoppe

Porto Alegre, junho de 2019

“Mas eu não posso deixar de dizer, meu amigo
Que uma nova mudança, em breve, vai acontecer
E o que algum tempo era novo, jovem, hoje é antigo
E precisamos todos rejuvenescer.”

Belchior

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DESTAQUES

- O peptídeo βA_{1-42} induz morte celular em fatias organotípicas hipocampais.
- Nanocápsulas de phloretin previnem a morte celular causada pelo peptídeo βA_{1-42}.
- Phloretin nanoencapsulado diminui a ativação da enzima GSK-3β.
- O tratamento com as nanocápsulas de phloretin reduz o imunoconteúdo de GFAP.

RESUMO

A Doença de Alzheimer (DA) é a desordem neurodegenerativa mais prevalente relacionada ao envelhecimento. Quanto à fisiopatologia, a DA está, principalmente, relacionada ao aparecimento de placas senis compostas por agregados do peptídeo β-amiloide (βA) e de emaranhados neurofibrilares compostos pela proteína tau hiperfosforilada. Tais achados parecem promover neuroinflamação crônica através da ativação das células gliais e prejudicar diferentes vias de sinalização celular levando, consequentemente, à morte celular. Na tentativa de melhor compreender os mecanismos celulares e moleculares desencadeados pelo peptídeo βA, a cultura organotípica combina a preservação da multiplicidade celular original do tecido cerebral e de suas conexões interneurais, permitindo assim, sua utilização para estudo da toxicidade do peptídeo βA, bem como do potencial efeito neuroprotetor de compostos como o phloretin, um polifenol presente em diversos vegetais e frutas. Por outro lado, uma limitação para sua administração é a baixa solubilidade, que prejudica, assim, sua absorção. Visto isso, uma estratégia para o carreamento de fármacos para o cérebro e outros órgãos é o emprego de nanopartículas. Assim, o objetivo deste trabalho foi investigar o potencial efeito neuroprotetor de nanocápsulas de phloretin sobre a toxicidade do peptídeo βA_{1-42} em cultura organotípica de hipocampo de ratos Wistar. O presente estudo demonstrou que as nanocápsulas de phloretin (NC PHL) reduziram a morte celular induzida pelo peptídeo βA_{1-42} através da redução da incorporação de iodeto de propídeo (IP). Após investigar possíveis vias de sinalização envolvidas, foi verificado que as NC PHL impediram a ativação da proteína GSK-3β, reduziram o imunoconteúdo da proteína GFAP, marcadora de astrócitos reativos, e atenuaram a diminuição do imunoconteúdo da proteína pré-sináptica sinaptofisina induzida pelo peptídeo βA_{1-42}. Com isso, diante dos resultados promissores de neuroproteção do flavonoide phloretin em sua forma nanoencapsulada neste modelo de toxicidade do peptídeo βA_{1-42}, mais estudos serão realizados para esclarecer estes e outros mecanismos envolvidos.

Palavras-chave: Alzheimer, amiloide, organotípica, nanocápsula, phloretin, neuroproteção

LISTA DE ABREVIAÇÕES

βA	β-Amiloide
BDNF	Fator Neurotrófico Derivado do Cérebro
BHE	Barreira hematoencefálica
DA	Doença de Alzheimer
ERK	Cinase Regulada por Sinais Extracelulares
GFAP	Proteína Ácida Fibrilar Glial
GSK-3β	Glicogênio sintase cinase 3β
IL-1β	Interleucina 1β
IP	Iodeto de propídeo
JNK	Cinase c-Jun N-terminal
MAPK	Proteína Cinase Ativada por Mitógeno
NB	Nanocápsula branca
NC PHL	Nanocápsula de phloretin
PPA	Proteína Precursora Amiloide
SNC	Sistema nervoso central
TNF-α	Fator de Necrose Tumoral α

1. INTRODUÇÃO

A Doença de Alzheimer (DA) é a desordem neurodegenerativa mais prevalente relacionada ao envelhecimento, cuja incidência tem crescido com o aumento da expectativa de vida populacional. Clinicamente, a DA é caracterizada por prejuízo ou perda da memória, déficit cognitivo progressivo, entre outros danos comportamentais e sociais (Parihar and Hemnani, 2004).

Quanto à fisiopatologia, a DA está, principalmente, relacionada ao acúmulo do peptídeo beta-amiloide (βA) devido ao processamento anormal da proteína precursora amiloide (PPA) através das enzimas beta e gama secretases (Scharfenberg et al., 2019). Como resultado, poderá haver agregação dos monômeros em oligômeros que podem ser internalizados nas células sendo tóxicos a estas ou mesmo se agregar na forma de fibrilas extracelulares levando ao surgimento das placas senis, desencadeando um processo inflamatório crônico, comprometendo a transmissão sináptica e culminando na morte neuronal em regiões encefálicas importantes para cognição e memória, como o hipocampo e córtex cerebral (Querfurth and Laferla, 2010).

Outros importantes achados são os emaranhados neurofibrilares compostos majoritariamente pela proteína tau hiperfosforilada, que desta forma reduz a afinidade pelos microtúbulos dos axônios levando a sua desestabilização. O que determina o estado de fosforilação da proteína tau é a atividade de diversas enzimas cinases e fosfatases e, entre as cinases mais conhecidas e estudadas, está a glicogênio sintase cinase 3β (GSK-3β). A GSK-3β é conhecida, por este motivo, por ser a principal enzima responsável pela estabilização da proteína tau (Crespo-Biel et al., 2007; Fuentealba et al., 2004). Outra importante enzima que possui alta atividade na fisiopatologia da DA é a cinase c-Jun N-terminal (JNK), pertencente à família das proteínas cinases ativadas por mitógeno (MAPK). Esta é uma importante enzima mediadora que propaga sinais extracelulares relacionados com parâmetros de estresse oxidativo e inflamação para o interior da célula (Wang et al., 2014).

Estudos vêm demonstrando que a neuroinflamação é fortemente associada à fisiopatologia da DA, onde células como os astrócitos reativos e a

microglia ativada se acumulam próximos das placas fibrilares e, em uma situação crônica, liberam fatores neurotóxicos, como por exemplo, as citocinas pró-inflamatórias, que consequentemente auxiliarão no processo de degenaração neuronal. Este dano causado aos neurônios, por sua vez, liberará ativadores das células gliais, perpetuando assim o processo inflamatório (Guo et al., 2002; Meraz-Ríos et al., 2013).

Na tentativa de melhor compreender os mecanismos tóxicos desencadeados pelo peptídeo βA, que parece iniciar o processo neurodegenerativo na DA, diversos modelos experimentais têm sido utilizados. Dentre eles, a cultura organotípica combina a preservação da multiplicidade celular original do tecido cerebral e das conexões interneurais. Dessa forma, a utilização desse modelo permite o estudo das vias de sinalização envolvidas com a neurotoxicidade induzida pelo peptídeo βA, bem como o estudo do potencial efeito neuroprotetor de novas drogas (Frozza et al., 2013a; Hoppe et al., 2010; Nassif et al., 2007).

Apesar do progresso na terapia sintomática para a DA, abordagens terapêuticas eficazes que interferem nos processos neurodegenerativos subjacentes à DA ainda não estão disponíveis. Contudo, nos últimos anos, tem aumentado o interesse em estudos com novas moléculas extraídas de fontes naturais que possuem potencial efeito neuroprotetor (Naoi et al., 2019) e, entre elas, está o phloretin, um flavonoide da classe das dihidrochalconas. Este polifenol é muito encontrado em frutas e legumes, principalmente na maçã. Sua utilização atualmente se dá, majoritariamente, em dermocosméticos, onde Shin e colaboradores (2014) observaram que o efeito fotoprotetor do phloretin está estritamente correlacionado com o aumento da expressão de genes responsáveis pelo reparo por excisão de nucleotídeos. Além disso, alguns trabalhos recentes têm demonstrado seu efeito anti-inflamatório (Chang et al., 2012), antioxidante (Yang et al., 2011) e anticâncer (Duan et al., 2017; Wu et al., 2009; Xu, 2018). Desta forma, nos últimos anos, o phloretin tem sido estudado quanto ao seu envolvimento com o sistema nervoso central (SNC) em modelos de doenças como Parkinson (Barreca et al., 2017) e Alzheimer (Ghumatkar et al., 2018, 2019, 2015). LoPachin e colaboradores (2011) atribuem, pelo menos em parte, suas propriedades neuroprotetoras à formação de grupos enolatos em

sua estrutura molecular e, assim, esta função química pode complexar com cátions e atacar nucleofilicamente eletrófilos tóxicos que estão envolvidos na fisiopatologia de diversas doenças neurodegenerativas.

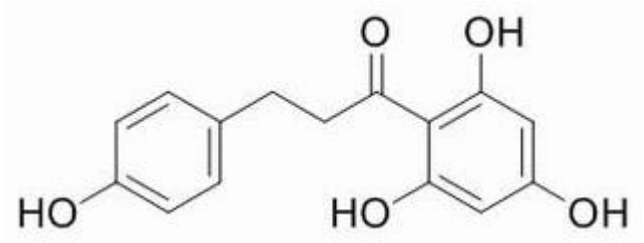

Figura 1. Estrutura química do phloretin.

Por outro lado, uma das grandes limitações do phloretin é sua baixa solubilidade em água (Wei et al., 2017) que prejudica sua absorção através da barreira hematoencefálica (BHE), sendo um impeditivo para o tratamento de doenças que acometem o SNC. Para superar essa imposição, uma estratégia para o carreamento de fármacos para o cérebro é o emprego de nanopartículas, sendo que estas podem se subdividir em nanocápsulas ou nanoesferas. As nanocápsulas, como objeto deste trabalho, são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica (Rezende et al., 2003). Em comparação com outros carreadores coloidais, as nanopartículas poliméricas apresentam maior estabilidade quando em contato com os fluidos biológicos. Além disso, sua natureza polimérica permite a obtenção das propriedades desejadas como liberação controlada e sustentada de drogas (Bernardi et al., 2013; Frozza et al., 2010; Roney et al., 2005).

Considerando que os tratamentos atuais não impedem a progressão da DA, o estudo de nanopartículas poliméricas contendo potenciais drogas neuroprotetoras são promissores candidatos para investigação de terapias inovadoras para o tratamento desta desordem do SNC, por serem capazes de atravessar a BHE e carrear grande quantidade destas substâncias ao encéfalo (Bernardi et al., 2013; Frozza et al., 2013a; Frozza et al., 2013b; Hoppe et al., 2013a). Assim sendo, o objetivo do presente trabalho foi investigar o potencial

efeito neuroprotetor de nanocápsulas de phloretin sobre a neurotoxicidade do peptídeo βA_{1-42} em cultura organotípica de hipocampo de ratos Wistar sobre parâmetros de neuroinflamação, integridade sináptica e dano celular.

2. MATERIAL E MÉTODOS

2.1. Materiais

O peptídeo βA_{1-42} foi adquirido da American Peptide Co. (Sunnyvale, CA, EUA). O phloretin foi adquirido da Enzo Life Sciences (Farmingdale, NY, EUA). O Meio Essencial Mínimo (MEM), Solução Salina Balanceada de Hanks (HBSS), fungizona, penicilina/estreptomicina e soro equino inativado foram adquiridos da Gibco (Grand Island, NY, EUA). Membranas semipermeáveis de cultura Millicell® de 0,4 µm foram adquiridas da Millipore® (Bedford, MA, EUA) e placas para cultura de tecido de 6 poços foram provenientes da TPP® (Trasadingen, Suíça). Iodeto de propídeo (IP) bem como acrilamida, bisacrilamida, dodecil sulfato de sódio (SDS) e β-mercaptoetanol usados em eletroforese em gel de poliacrilamida na presença de SDS (SDS-PAGE) foram adquiridos da Sigma-Aldrich Co. (St. Louis, MO, EUA). Os anticorpos foram adquiridos da Cell Signaling Technology (Beverly, MA, EUA), Dako Chemicals (Carpinteria, CA, EUA), Sigma-Aldrich Co. (St. Louis, MO, EUA) e Millipore® (Bedford, MA, EUA). Anticorpo anti-mouse conjugado com peroxidase foi obtido da Cell Signaling Technology. Anticorpo anti-rabbit conjugado com peroxidase, os reagentes que detectam quimioluminescência (ECL) e as membranas de nitrocelulose Hybond™ ECL™ foram adquiridas da Amersham Biosciences (Little Chalfont, Reino Unido). Todos os outros reagentes químicos utilizados neste estudo foram de grau analítico.

2.2. Cultura organotípica de fatias hipocampais

As culturas organotípicas de fatias hipocampais foram realizadas segundo o método descrito por Stoppini e colaboradores (1991), com algumas modificações (Frozza et al., 2009). Fatias de hipocampo de 400 µm foram obtidas

de ratos machos Wistar de 6 a 8 dias utilizando um fatiador de tecidos McIlwain e separadas em Solução Salina Balanceada de Hanks (HBSS) composta de: 36 mM de glicose, 1,26 mM de $CaCl_2$, 5,36 mM de KCl, 136,89 mM de NaCl, 0,44 mM de KH_2HPO_4, 0,34 mM de Na_2HPO_4, 0,49 mM de $MgCl_2$, 0,44 mM de $MgSO_4$, 25 mM de HEPES, 1% de fungizona 250 µg/mL e 1% de penicilina 10.000 U/mL/estreptomicina 10.000 µg/mL, pH 7,2. As fatias foram alocadas em membranas semipermeáveis de cultura Millicell® e estas membranas foram, então, inseridas em placas de cultura de tecido de 6 poços TPP®. Cada poço continha 1 mL de meio de cultura composto por 50% de Meio Essencial Mínimo (MEM), 25% de Solução Salina Balanceada de Hanks (HBSS) e 25% de soro equino inativado por calor, sendo este meio suplementado com (concentrações finais): 36 mM de glicose, 25 mM de HEPES e 4 mM de $NaHCO_3$, contendo 1% de fungizona 250 µg/mL e 1% de penicilina 10.000 U/mL/estreptomicina 10.000 µg/mL, pH 7,3. As culturas organotípicas foram mantidas em uma incubadora umidificada com uma atmosfera de 5% de CO_2/95% O_2 e temperatura de 37 ºC por 3 semanas. O meio de cutivo foi trocado duas vezes por semana. Todos os procedimentos com animais foram aprovados pelo Comitê de Ética de Uso de Animais local (CEUA/UFRGS nº 35.489) e estavam de acordo com o Guia do *National Institutes of Health* (NIH) para o Cuidado e Uso de Animais de Laboratório (publicação nº 8023, revisado em 1978). Todos esforços foram feitos para minimizar o sofrimento dos animais, bem como de utilizar apenas o número necessário.

2.3. Preparação de nanocápsulas de núcleo lipídico contendo phloretin

As suspensões de nanocápsulas foram preparadas pelo método de deposição interfacial de polímero pré-formado conforme proposto por Fessi e colaboradores (1989). 0,1 g do polímero poli-Ɛ-caprolactona (PCL, PM= 80.000), (Aldrich, Lesquin, França), 0,038 g de monoestearato de sorbitano (Delaware, Porto Alegre, Brasil), 330 µL de óleo de copaíba (Delaware) e 0,002 g do flavonoide phloretin (Enzo Life Sciences, Farmingdale, EUA) foram dissolvidos em 24 mL de acetona (Merck, Darmstadt, Alemanha) e 3 mL de etanol (Merck) sob agitação magnética constante a 40 ºC. Em béquer separado, 0,077 g do

polissorbato 80 (Delaware) foi adicionado a 54 mL de água MilliQ® (Millipore® Corporation, Bedford, EUA). A fase orgânica, totalmente solubilizada, foi vertida sobre a fase aquosa, sob agitação magnética constante, à temperatura ambiente, com auxílio de um funil. A suspensão resultante foi mantida sob agitação e, após 10 minutos, a acetona e o etanol foram removidos e a suspensão foi concentrada sob pressão reduzida a fim de ajustar o volume final para 10 mL. A concentração final de phloretin na suspensão foi de 0,2 mg/mL. Como controle, foram realizadas as nanocápsulas brancas, onde estas não possuíam phloretin, tendo todos os outros constituintes da formulação iguais e submetidas ao mesmo processo de preparo.

2.4. Preparação do peptídeo βA_{1-42}

1 mg de peptídeo βA_{1-42} (American Peptide Co.) foi dissolvido em água esterilizada bi-destilada com 0,1% de NH_4OH (Sigma-Aldrich Co.) a uma concentração de 1 mg/mL, sendo esta solução estoque armazenada a -20 ºC. Previamente a utilização, o peptídeo βA_{1-42} foi submetido ao processo de fibrilação à temperatura de 37 ºC por 72 horas (Nassif et al., 2007).

2.5. Exposição das fatias ao peptídeo βA_{1-42} e tratamento com as nanocápsulas de phloretin

No 21º dia da cultura organotípica, as fatias foram mantidas em meio de cultivo reduzido em soro equino (5%). Para induzir neurotoxicidade, as fatias organotípicas foram expostas a 2 µM (concentração final) do peptídeo βA_{1-42} por 48 horas. Junto ao peptídeo, as fatias foram incubadas com as nanocápsulas de phloretin nas concentrações de 0,5, 1 e 5 µM (concentrações finais) ou às nanocápsulas brancas (veículo do phloretin) (volume igual ao utilizado na concentração de 5 µM) por 48 horas. Como controle, as fatias foram incubadas somente com meio de cultivo reduzido em soro equino.

2.6. Quantificação da morte celular

O dano celular foi avaliado por análise de imagem fluorescente de captação do iodeto de propídeo (IP) (Frozza et al., 2009; Noraberg et al., 1999). IP é um composto polar que é impermeável a membranas celulares íntegras, mas é capaz de penetrar em células danificadas e se intercalar ao DNA para gerar uma fluorescência vermelha brilhante. Após 48 de horas de exposição ao peptídeo βA e tratamento com as nanocápsulas de phloretin, 5 µM de IP foi adicionado ao meio de cultivo e mantido durante 1 hora. A captação de IP é um indicativo de lesão significativa de membranas celulares (Macklis and Madison, 1990). As fatias foram observadas com um microscópio invertido Eclipse TE 300 (Nikon Instruments Inc., Melville, NY, EUA) e capturadas utilizando uma câmera CCD (DXM1200C, Nikon Instruments Inc.). Posteriormente, as imagens foram analisadas usando o Scion Image Software (http://www.scioncorp.com) e, para fins de quantificação, a área da fluorescência de IP (transformado em pixels) detectada acima do plano de fundo foi dividida pelo total da área da fatia (Valentim et al., 2003). A intensidade de IP, que significa morte celular, foi expressa em percentagem de fluorescência:

$$\text{Morte celular (\%)} = (Fd \,/\, Fo) \times 100$$

Onde *Fd* é a fluorescência de captação da área morta das fatias de hipocampo e *Fo* é a área total de cada fatia de hipocampo.

2.7. Análise de *western blotting*

Após obtenção das imagens fluorescentes, as fatias organotípicas hipocampais foram homogeneizadas em tampão de lise composto por (concentrações finais): SDS 4%, EDTA 2 mM, Tris 50 mM e água. Finalizado o processamento, alíquotas foram utilizadas para determinação da concentração de proteínas presente (Peterson, 1979) e β-mercaptoetanol foi adicionado a uma concentração final de 5%. As proteínas presentes na amostra foram separadas (30 µg por poço) em gel de SDS-PAGE 12%. Após o término da migração eletroforética, as proteínas foram transferidas para membranas de nitrocelulose utilizando um aparato de transferência semi-seco Trans-Blot® SD (Bio-Rad). As membranas foram então incubadas por 60 minutos a 4 ºC em solução de bloqueio (solução tampão de Tris contendo 5% de leite em pó desnatado e 0,1%

de polissorbato 20, pH 7,4) e posteriormente incubadas com o anticorpo primário apropriado dissolvido em em solução de bloqueio *overnight* a 4 ºC. Os anticorpos primários utilizados foram: anti-fosfo-GSK-3β (Ser9) (1:1000; Cell Signaling), anti-GSK-3β (1:1000; Cell Signaling), anti-fosfo-JNK 1/2 (Thr183/Tyr185) (1:1000; Cell Signaling), anti-JNK 1/2 (1:1000; Cell Signaling), anti-fosfo-tau (Ser396) (1:1000; Cell Signaling), anti-tau (1:1000; Cell Signaling), anti-GFAP (1:3000; Dako Chemicals), anti-CD11b (1:1000; Millipore), anti-sinaptofisina (1:3000; Millipore) e anti-β-actina (1:1000; Sigma-Aldrich). Após, as membranas foram incubadas com o anticorpo secundário, sendo este anti-rabbit conjugado com peroxidase (1:1000; Amersham Biosciences) ou anti-mouse conjugado com peroxidase (1:1000; Cell Signaling) por 1 hora a temperatura ambiente. A quimioluminescência foi detectada em fotodocumentador ImageQuant™ LAS 4000 (GE Healthcare Life Sciences) após exposição das membranas ao reagente de ECL (Amersham Biosciences). Os valores foram expressos em porcentagem em relação ao controle.

2.8. Análise estatística

Análise de variância (ANOVA) de uma via foi aplicada para determinar as diferenças estatísticas entre os grupos experimentais. Comparações post-hoc foram realizadas pelo teste de Tukey. A análise foi realizada com Prism Graph Pad (v6.0, Graph Pad Software Inc., San Diego, CA, EUA). Os valores estão expressos como média + EP. Diferenças entre os valores médios foram consideradas estatisticamente significativas quando $p \leq 0,05$.

3. RESULTADOS

3.1. Phloretin nanoencapsulado previne a morte celular induzida pelo peptídeo βA_{1-42}

A exposição das culturas organotípicas de hipocampo ao peptídeo βA_{1-42} por 48 horas induziu um aumento significativo da fluorescência nas fatias verificada pela incorporação de IP de 16 ± 2,5 % no grupo βA_{1-42} (n=15) em

relação ao grupo controle que foi de 0,5 ± 0,12 % (n=12) ($p \leq 0,001$). Por outro lado, todas as concentrações testadas de nanocápsulas de phloretin: 0,5, 1 e 5 µM foram capazes de prevenir o dano causado pelo peptídeo βA_{1-42} observado através da incorporação de IP de 2,2 ± 0,5 % (n=12), 0,9 ± 0,2 % (n=12) e 1,0 ± 0,15 % (n=12), respectivamente ($p \leq 0,001$). Um aumento significativo na fluorescência também foi verificado no grupo no qual as fatias foram coincubadas com o peptídeo βA_{1-42} e as nanocápsulas brancas (17 ± 3,6 %, n=6, $p \leq 0,001$), indicando que nenhum outro componente da nanocápsula possui efeito neuroprotetor além do phloretin (Fig. 2). Visto isso, a concentração determinada das nanocápsulas de phloretin para o seguimento dos experimentos foi de 1 µM.

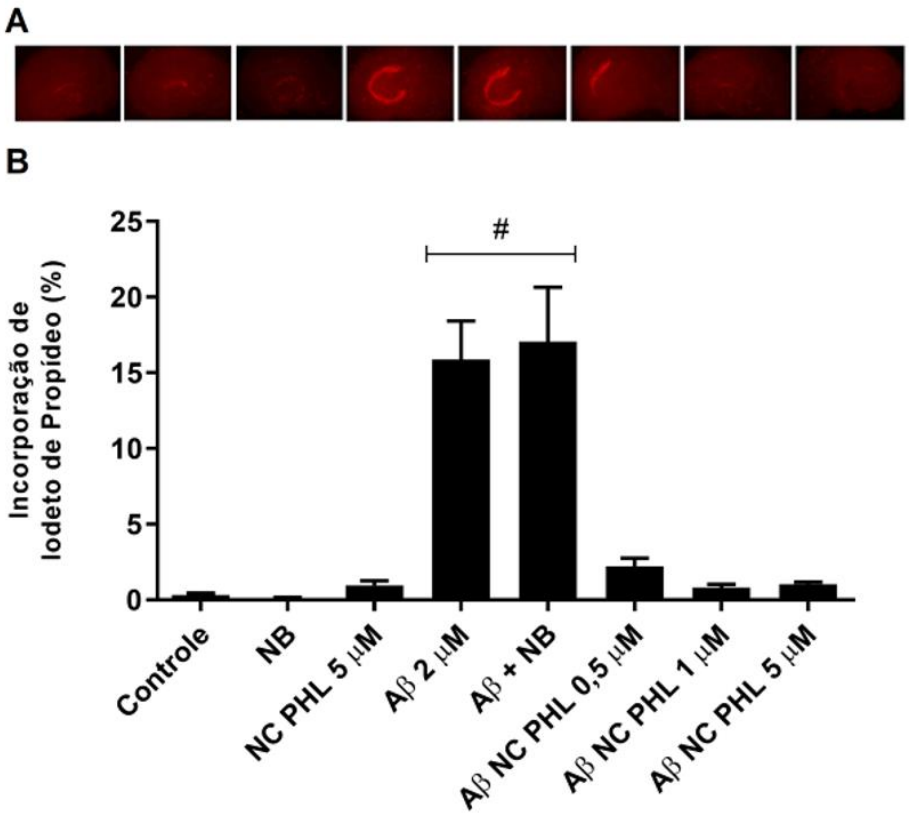

Figura 2. Nanocápsulas de phloretin reduzem a morte celular induzida pelo peptídeo βA_{1-42} em fatias organotípicas hipocampais. (A) Fotomicrografias representativas da captação de iodeto de propídeo em fatias organotípicas de hipocampo. (B) Quantificação da captação de iodeto de propídeo, expresso como percentual de fluorescência pela área total da fatia. As barras representam média + EP, n=6-15. # Significativamente diferente dos outros grupos experimentais (ANOVA de uma via seguida pelo teste de Tukey, $p < 0,001$).

3.2. Nanocápsulas de phloretin previnem a ativação da proteína GSK-3β induzida pelo peptídeo βA_{1-42}

Para verificar a expressão da enzima GSK-3β, foi realizada a análise através da técnica de *western blotting* utilizando anticorpos para sua forma fosforilada em Ser9 bem como de seu imunoconteúdo total. Foi observado que as fatias organotípicas que foram coincubadas com o peptídeo βA_{1-42} e nanocápsulas de phloretin 1 µM aumentaram a razão p-GSK-3β^{Ser9}/GSK3β (médias em relação ao controle de 122 ± 19 % do grupo βA + NC PHL contra 70 ± 9 % do grupo βA_{1-42}) (Fig. 3), o que indica um aumento significativo de sua forma inativa quando comparado ao grupo βA_{1-42} (n=8, $p \leq 0,05$). Quanto a relação p-JNK$^{Thr183/Tyr185}$ 1/2/JNK 1/2 não foi observada diferença estatística significativa entre os grupos analisados (média em relação ao controle do grupo βA_{1-42} 184 ± 55 % e do grupo βA + NC PHL 124 ± 21 %, n=5, p= 0,2951) (Fig. 4). Quanto à relação p-tau^{Ser396}/tau foi observado uma tendência ao aumento da forma fosforilada em Ser 396 no grupo βA_{1-42} (128 ± 7 %) quando comparada ao grupo controle (100 ± 0,15 %) (n=5, p=0,0834) (Fig. 5). As fatias hipocampais tratadas com as nanocápsulas brancas (NB) e com βA_{1-42} + NB, não foram diferentes do grupo controle e do grupo βA_{1-42}, respectivamente, em todos os parâmetros avaliados neste trabalho utilizando a técnica de *western blotting* (dados não mostrados).

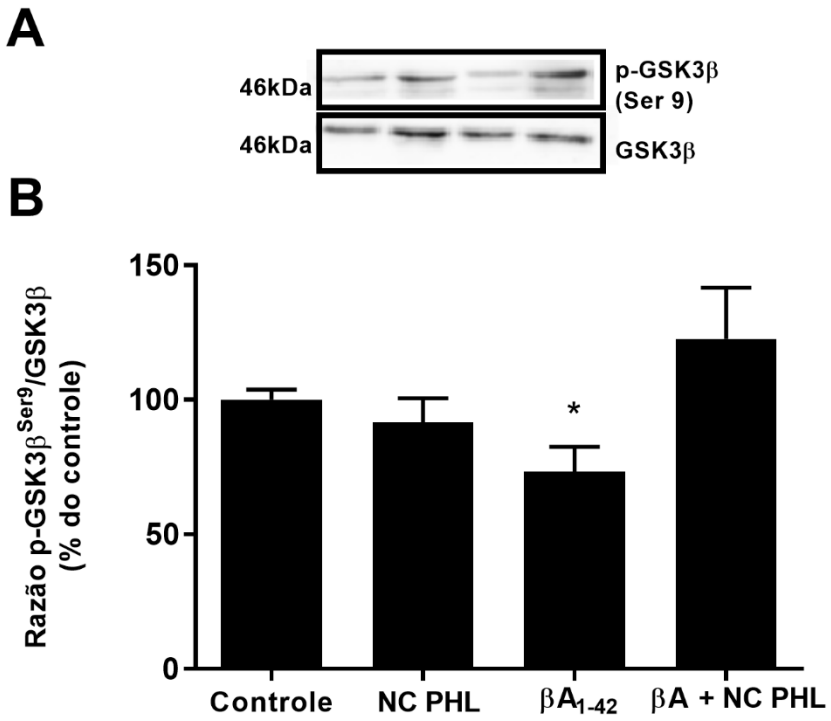

Figura 3. Phloretin nanoencapsulado previne a ativação de GSK-3β induzida pelo peptídeo βA_{1-42} em fatias organotípicas de hipocampo. (A) Imagem representativa de *western blotting* da proteína GSK-3β e de sua forma fosforilada (Ser9). (B) Quantificação do imunoconteúdo de p-GSK-$3\beta^{Ser9}$/GSK3β. A proteína β-actina foi utilizada como controle. As barras representam média + EP, n=7-8. * Significativamente diferente do grupo βA + NC PHL (ANOVA de uma via seguida pelo teste de Tukey, $p < 0,05$).

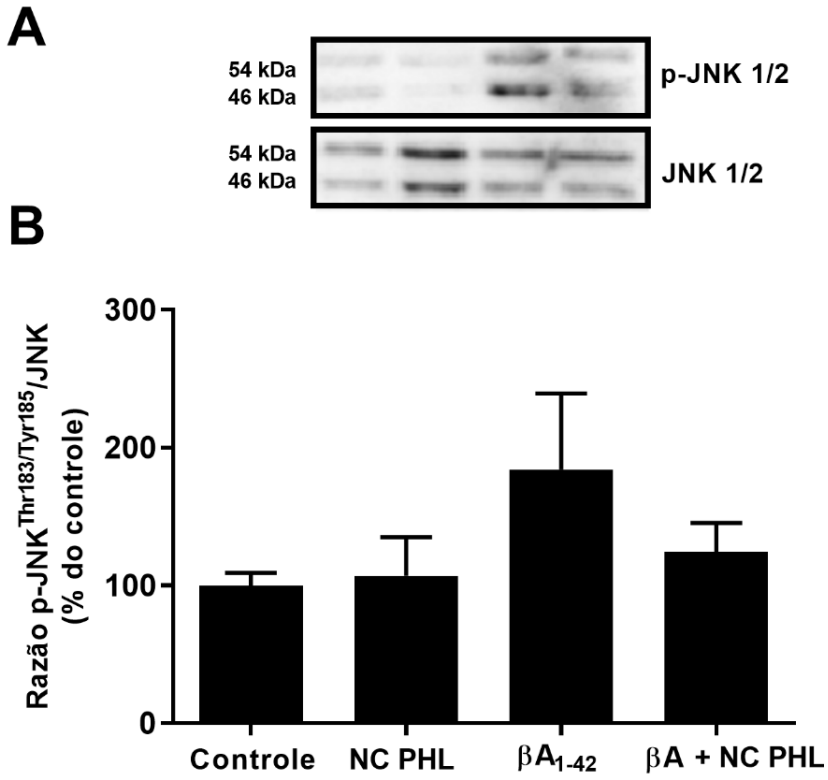

Figura 4. Avaliação dos níveis de fosforilação da proteína JNK 1/2 em fatias expostas ao βA_{1-42} e cotratadas com NC PHL por 48 horas. (A) Imagem representativa de *western blotting* da proteína JNK 1/2 e de sua forma fosforilada (Thr183/Tyr185). (B) Quantificação do imunoconteúdo de p-$JNK^{Thr183/Tyr185}$ 1/2/JNK 1/2. A proteína β-actina foi utilizada como controle. As barras representam média + EP, n=5.

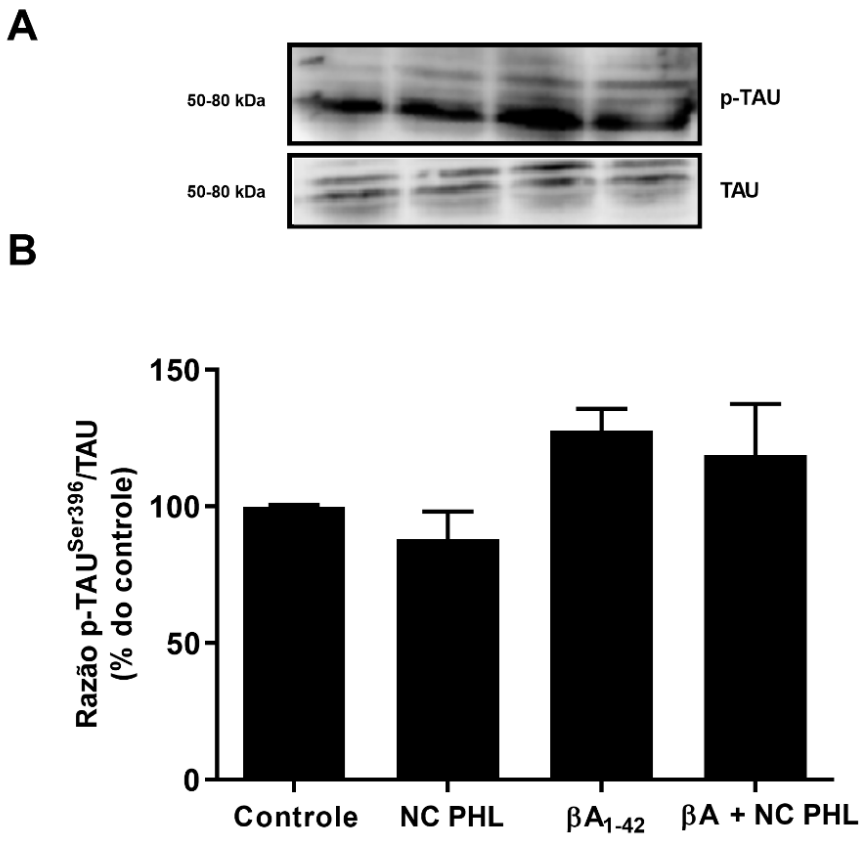

Figura 5. Avaliação dos níveis de fosforilação da proteína tau em fatias expostas ao βA_{1-42} e cotratadas com NC PHL por 48 horas. (A) Imagem representativa de *western blotting* da proteína tau e de sua forma fosforilada (Ser396). (B) Quantificação do imunoconteúdo de p-tau^{Ser396}/tau. A proteína β-actina foi utilizada como controle. As barras representam média + EP, n=6-7.

3.3. Nanocápsulas de phloretin reduzem o aumento do imunoconteúdo de GFAP induzido pelo peptídeo βA_{1-42} em fatias hipocampais

Visto que a neuroinflamação é um dos principais achados na DA, foi analisada a imunoreatividade da proteína ácida fibrilar glial (GFAP), marcadora de astrócitos reativos, através da técnica de *western blotting*. Pudemos verificar que as fatias hipocampais coincubadas com βA_{1-42} e nanocápsulas de phloretin reduziram significativamente o imunoconteúdo de GFAP em relação às fatias que receberam apenas o peptídeo βA_{1-42} (médias em relação ao controle de 142 ± 15 % no grupo βA_{1-42} comparado a 73 ± 12 % no grupo βA + NC PHL, n=6, $p \leq 0,05$). Em nossas condições experimentais, CD11b, uma integrina marcadora de microglia ativada, não teve seu imunoconteúdo alterado entre os diferentes

grupos (médias em relação ao controle de 137 ± 15 % no grupo βA_{1-42} comparado a 91 ± 22 % no grupo βA + NC PHL, n=5, p = 0,2447) (Fig. 6).

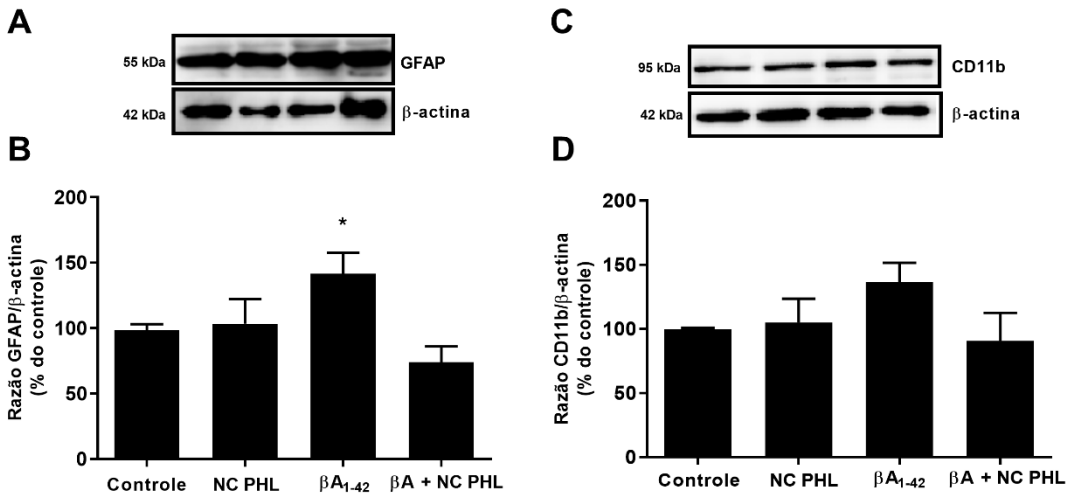

Figura 6. Imunoconteúdo de GFAP é reduzido após tratamento com as nanocápsulas de phloretin por 48 horas comparado ao grupo βA_{1-42}. (A) Imagem representativa de *western blotting* das proteínas GFAP e β-actina. (B) Quantificação do imunoconteúdo de GFAP/β-actina. (C) Imagem representativa de *western blotting* das proteínas CD11b e β-actina. (B) Quantificação do imunoconteúdo de CD11b/β-actina. As barras representam média + EP, n=5,6 (GFAP); 5 (CD11b). * Significativamente diferente do grupo βA + NC PHL (ANOVA de uma via seguida pelo teste de Tukey, p < 0,05).

3.4. Phloretin nanoencapsulado previne parcialmente a sinaptoxicidade induzida pelo peptídeo βA_{1-42} em fatias organotípicas hipocampais

A fim de verificar a densidade de proteínas relacionadas à transmissão sináptica, o imunoconteúdo de sinaptofisina, importante proteína localizada na membrana de vesículas pré-sinápticas, foi determinado. Após a quantificação, verificou-se que o tratamento das fatias hipocampais por 48 horas com peptídeo βA_{1-42} causou uma diminuição significativa do imunoconteúdo de sinaptofisina comparado ao grupo controle (44 ± 8 %, n=6, $p \leq 0{,}001$). Por outro lado, não houve uma prevenção significativa desta redução de imunoconteúdo quando as fatias foram coincubadas com o peptídeo βA_{1-42} e nanocápsulas de phloretin (média em relação ao controle de 62 ± 11 % para o grupo βA + NC PHL) (Fig. 7).

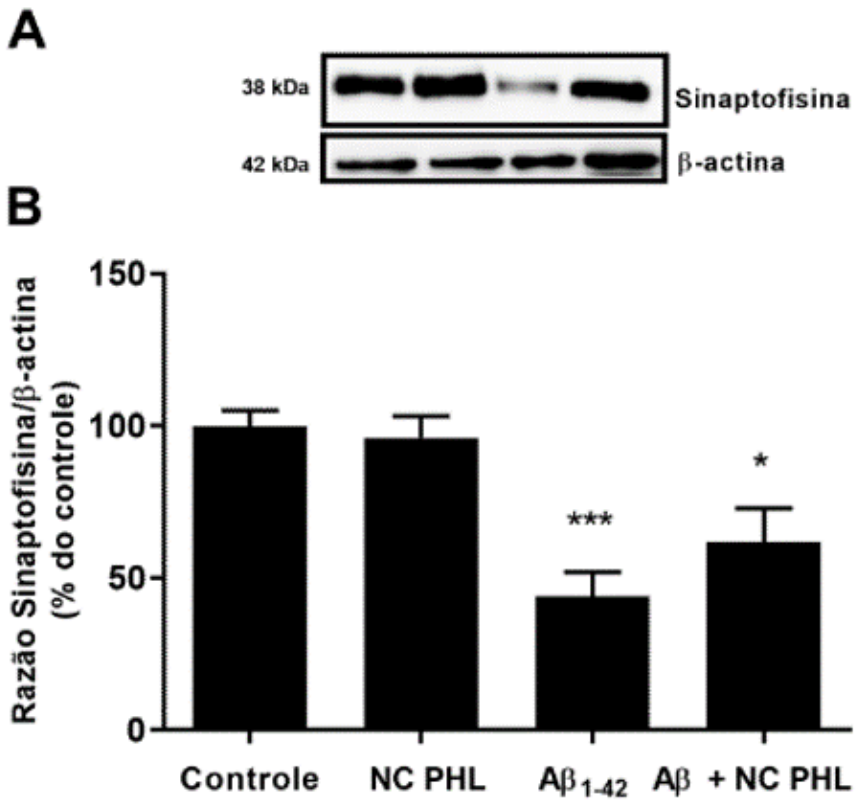

Figura 7. Phloretin nanoencapsulado atenua a sinaptoxicidade induzida pelo peptídeo βA_{1-42} em fatias organotípicas de hipocampo. (A) Imagem representativa do imunoconteúdo das proteínas sinaptofisina e β-actina. (B) Quantificação do imunoconteúdo de sinaptofisina/β-actina. As barras representam média + EP, n=6. *** Significativamente diferente dos grupos controle e NC PHL (ANOVA seguida pelo teste de Tukey, $p < 0,001$). * Significativamente diferente dos grupos controle e NC PHL (ANOVA seguida pelo teste de Tukey, $p < 0,05$).

4. DISCUSSÃO

A Doença de Alzheimer é a desordem de maior prevalência entre pessoas idosas e seus principais achados no cérebro de pessoas acometidas são os agregados do peptídeo βA (Querfurth and Laferla, 2010). Diversos estudos vêm sendo realizados a fim de investigar possíveis alvos moleculares e celulares que possam previnir ou desacelerar a progressão da neurodegeneração que culminará nos déficits cognitivos observados em pacientes com a DA. Visto que muitas moléculas em sua forma livre possuem limitações para o uso na farmacoterapia, devido, principalmente, a sua baixa biodisponibilidade, o uso da

nanotecnologia vem sendo amplamente abordado (Li et al., 2015). Como modelo de estudo, a técnica de cultura organotípica foi escolhida por sua praticidade no estudo de mecanismos de neurotoxicidade e neuroproteção, onde as células neuronais e gliais do tecido utilizado, em nosso caso o hipocampo, estrutura cerebral crítica para os processos de aprendizado e memória sendo uma das primeiras afetadas pela DA, amadurem fisiologicamente durante o período cultivado e permanecem viáveis por longos períodos experimentais (Nassif et al., 2007).

O presente trabalho mostrou que os agregados do peptídeo βA_{1-42} causaram morte celular na concentração de 2 µM após 48 horas de exposição às fatias organotípicas hipocampais, o que corrobora com o encontrado por Frozza e colaboradores (2013a). Por outro lado foi verificado que no grupo experimental onde as fatias foram coincubadas com o peptídeo βA_{1-42} e nanocápsulas de phloretin, em todas as concentrações testadas, houve redução do dano causado às celulas presentes no tecido. Até o presente momento há poucos trabalhos que estudam o possível efeito neuroprotetor de phloretin em modelos experimentais da Doença de Alzheimer, além de não haver nenhum estudo que verifique sua forma nanoencapsulada em modelos experimentais da DA ou qualquer outro modelo de doença que acomete o SNC. Quanto ao phloretin em sua forma livre, foi verificado por Ghumatkar e colaboradores (2019) que ratos Wistar que receberam phloretin via oral por 21 dias nas doses de 2,5 e 5 mg/kg, após injeção intracerebroventricular do peptídeo βA_{25-35}, apresentaram redução do acúmulo do peptídeo βA no hipocampo, uma menor incidência de núcleos apoptóticos na região do giro denteado hipocampal e uma diminuição na neuroinflamação mediada pela citocina pró-inflamatória TNF-α. Em outro trabalho, os autores sugerem que um possível mecanismo de neuroproteção do pré-tratamento com phloretin em um modelo de isquemia cerebral em ratos seja através da regulação da homeostase do estado redox em regiões do córtex cerebral, visualizada pelo aumento de enzimas antioxidantes através da ativação da via do Nrf2 (fator nuclear eritroide 2 relacionado ao fator 2), que atua regulando a expressão de genes de diversas proteínas com ação antioxidante (Liu et al., 2015).

Na tentativa de determinar possíveis mecanismos celulares pelos quais o phloretin possui seu efeito neuroprotetor contra a neurotoxicidade do peptídeo βA_{1-42} em nosso modelo experimental, determinamos o nível de fosforilação da proteína tau, envolvida na formação dos emaranhados neurofibrilares intracelulares compostos por agregados hiperfosforilados desta proteína. Neste estudo observamos uma tendência ao aumento da razão p-tau^{Ser396}/tau nas fatias organotípicas hipocampais quando incubadas por 48 horas com o peptídeo βA_{1-42} 2 µM. Por sua vez, em nossas condições experimentais, o cotratamento com nanocápsulas de phloretin 1 µM não demonstrou prevenir esta tendência de aumento da fosforilação da proteína tau na Ser 396. Mais esforços deverão ser direcionados a estes experimentos para a conclusão deste resultado, visto que trabalhos anteriores desenvolvidos pelo nosso grupo utilizando este modelo experimental demonstraram um aumento significativo da fosforilação da proteína tau em Ser 199 e Ser 202 desencadeada pelo peptídeo βA_{25-35} (Hoppe et al., 2010).

Neste estudo também avaliamos o estado de fosforilação da enzima GSK-3β, considerada a principal cinase com função de manter a estabilidade da proteína tau. Neste estudo foi observado uma diferença significativa entre as fatias tratadas apenas com βA_{1-42} e as fatias cotratadas com βA_{1-42} e as nanocápsulas de phloretin 1 µM em relação à razão p-GSK-3β^{Ser9}/GSK-3β, em que, as nanocápsulas de phloretin previniram significativamente a ativação desta enzima, que desta forma não é capaz de fosforilar a proteína tau impedindo, a longo prazo, sua hiperfosforilação e, consequentemente, a formação dos emaranhados neurofibrilares. Além de sua função na modulação da fosforilação da proteína tau, a GSK-3β é considerada uma enzima pró-apoptótica por atuar inibindo uma variedade de fatores de transcrição importantes para a sobrevivência celular. Na via de sinalização da enzima fosfatidilinositol-3 cinase (PI3K), a GSK-3β encontra-se ativa quando não fosforilada, podendo ser inibida através do aumento da fosforilação no sítio específico Ser 9 pela enzima Akt (também conhecida como proteína cinase B) e, neste estado de inativação, contribuindo para a sobrevivência celular (Llorens-Martín et al., 2014). Desta maneira, o aumento na fosforilação da GSK-3β em Ser 9 observado nas fatias coincubadas com βA_{1-42} e as nanocápsulas de phloretin corrobora com a

prevenção da morte celular evidenciado pela significativa diminuição da incorporação de iodeto de propídeo neste grupo experimental.

Para melhor compreender os mecanismos pelos quais as nanocápsulas contendo phloretin possam atuar, alguns parâmetros de neuroinflamação foram avaliados. O imunoconteúdo da proteína GFAP foi determinado e pudemos verificar que as fatias hipocampais coincubadas com o peptídeo e o phloretin nanoencapsulado apresentaram uma redução significativa do imunoconteúdo de GFAP quando comparadas com as fatias incubadas apenas com peptídeo βA_{1-42}, indicando assim uma possível diminuição da astrogliose e, consequentemente, da neuroinflamação. Vários estudos encontraram níveis aumentados de GFAP em tecidos cerebrais, como no córtex frontal e no hipocampo, de pacientes com DA (Kamphuis et al., 2014; Korolainen et al., 2005). Um fator importante relacionado à sinalização celular induzida por eventos inflamatórios está ligado às proteínas cinases ativadas pelo estresse, como as MAPK, dentre elas a JNK, a ERK (cinase regulada por sinal extracelular) e a p38 (Mehan et al., 2011). Em seus resultados, Frozza e colaboradores (2013a) verificaram que a liberação de citocinas pró-inflamatórias, como IL1-β e TNF-α, levou a uma ativação sustentada das células astrocitárias e microgliais com um distúrbio na sinalização de JNK. Em nosso estudo, não foi possível concluir o efeito do βA_{1-42} bem como das nanocápsulas de phloretin, sobre a modulação da enzima JNK, sendo necessário um maior números de experimentos para a sua conclusão. Quanto à integrina CD11b, presente em células microgliais ativadas, não foi verificada diferença significativa em seu imunoconteúdo dentre os grupos experimentais, este resultado pode estar relacionado a um tempo insuficiente de exposição do peptídeo βA_{1-42} às fatias organotípicas hipocampais para causar uma ativação crônica da microglia no tecido hipocampal, visto que, inicialmente, a microglia fagocítica tem como função fagocitar e degradar o peptídeo βA gerado a partir do processamento anormal da PPA. (Tuppo and Arias, 2005).

Como uma forma de investigar parâmetros relacionados com a integridade sináptica nas fatias organotípicas hipocampais, foi verificado o imunoconteúdo da proteína pré-sináptica sinaptofisina. A sinaptofisina é a proteína mais abundante de vesículas sinápticas e, portanto, utilizada na

tentativa de quantificar as sinapses (Edelmann et al., 1995). Além disso, a perda progressiva da sinaptofisina é bem estabelecida em diversos modelos experimentais utilizados para o estudo da DA (Harwell and Coleman, 2016; Sze et al., 1997). Verificamos que a exposição das fatias hipocampais ao peptídeo βA_{1-42} reduziu significativamente os níveis de sinaptofisina. Este resultado corrobora com achados prévios do nosso grupo e demais grupos que estudam os mecanismos envolvidos na toxicidade do peptídeo βA (Frozza et al., 2013b; Ghumatkar et al., 2018; Hoppe et al., 2013b; Klein et al., 2019). Em nosso modelo experimental, o phloretin nanoencapsulado na concentração de 1 µM incubado concomitantemente com o peptídeo βA_{1-42} não foi capaz de prevenir totalmente a redução expressiva do imunoconteúdo de sinaptofisina induzida pelo peptídeo βA_{1-42} nas fatias organotípicas hipocampais. Esse resultado pode sugerir que para as nanocápsulas de phloretin exercerem seu efeito neuroprotetor contra a sinaptoxicidade induzida pelo peptídeo βA_{1-42} possa ser necessário mais experimentos utilizando uma prévia sensibilização do tecido ao phloretin ou experimentos utilizando uma concentração superior a 1 µM de nanocápsulas de phloretin em nosso modelo experimental. Em um estudo recente, Ghumatkar e colaboradores (2018) observaram que, em um modelo *in vivo* de injeção intrahipocampal de βA_{1-42} em ratos Wistar, o pré-tratamento com phloretin livre na dose de 5 mg/kg por 28 dias através de gavagem protegeu significativamente a diminuição da expressão da proteína sinaptofisina causada pela injeção do peptídeo βA_{1-42} no hipocampo. Neste trabalho, os autores discutem que diante das alterações observadas no imunoconteúdo de sinaptofisina, os níveis do fator neurotrófico derivado do cérebro (BDNF) possam estar sendo modulados pelo phloretin, visto que esta neurotrofina é amplamente expressa no cérebro de mamíferos e é responsável por regular a plasticidade cerebral e, por fim, sugerem a necessidade do estudo do envolvimento das neurotrofinas, principalmente do BDNF, na neuroproteção mediada pelo phloretin contra a toxicidade do peptídeo βA (Ghumatkar et al., 2018).

5. CONCLUSÕES

A partir dos resultados obtidos com o presente trabalho, nosso estudo demonstrou que as nanocápsulas de phloretin possuem potencial efeito neuroprotetor contra a toxicidade do peptídeo βA_{1-42} em cultura organotípica hipocampal observado através da redução da incorporação de IP nas células, indicativo de morte celular, bem como impedir a ativação da proteína cinase GSK-3β, reduzir os níveis de astrócitos reativos verificado pela diminuição do imunoconteúdo de GFAP e prevenir parcialmente a diminuição da proteína pré-sináptica sinaptofisina induzida pelo peptídeo βA. Sendo assim, em virtude dos resultados promissores do flavonoide phloretin em sua forma nanoencapsulada neste trabalho, mais estudos serão realizados para esclarecer estes e outros mecanismos envolvidos na neuroproteção das nanocápsulas de phloretin contra a toxicidade do peptídeo beta-amiloide em culturas organotípicas de hipocampo.

FINANCIAMENTO

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CONFLITO DE INTERESSE

Nenhum conflito de interesse a declarar.

REFERÊNCIAS

Barreca, D., Curr, M., Tellone, E., Giunta, M.L., 2017. Neuroprotective effects of

phloretin and its glycosylated derivative on rotenone-induced toxicity in human SH-SY5Y neuronal-like cells. Res. Commun. 43, 549–557. https://doi.org/10.1002/biof.1358

Bernardi, A., Frozza, R.L., Hoppe, J.B., Salbego, C., Pohlmann, A.R., Battastini, A.M.O., Guterres, S.S., 2013. The antiproliferative effect of indomethacin-loaded lipid-core nanocapsules in glioma cells is mediated by cell cycle regulation, differentiation, and the inhibition of survival pathways. Int J Nanomedicine. 8, 711–729. https://doi.org/10.2147/IJN.S40284

Chang, W.T., Huang, W.C., Liou, C.J., 2012. Evaluation of the anti-inflammatory effects of phloretin and phlorizin in lipopolysaccharide-stimulated mouse macrophages. Food Chem. 134, 972–979. https://doi.org/10.1016/j.foodchem.2012.03.002

Crespo-Biel, N., Canudas, A.M., Camins, A., Palla, M., 2007. Kainate induces AKT,ERK and cdk5/ GSK3β pathway deregulation, phosphorylates tau protein in mouse hippocampus. Neurochem Int. 50, 435–442. https://doi.org/10.1016/j.neuint.2006.10.002

Duan, H., Wang, R., Yan, X., Liu, H., 2017. Phloretin induces apoptosis of human esophageal cancer via a mitochondria-dependent pathway. Oncol Lett. 14, 6763–6768. https://doi.org/10.3892/ol.2017.7037

Edelmann, L., Hanson, P.I., Chapman, E.R., Jahn, R., 1995. Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. EMBO J. 14, 224–31. https://doi.org/10.1002/j.1460-2075.1995.tb06995.x

Fessi, H., Puisieux, F., Devissaguet, J., Ammoury, N., Benita, S., 1989. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 55, 1–4. https://doi.org/10.1016/0378-5173(89)90281-0

Frozza, R.L., Bernardi, A., Hoppe, J.B., Meneghetti, A.B., Battastini, A.M.O., Pohlmann, A.R., Guterres, S.S., Salbego, C., 2013a. Lipid-core nanocapsules improve the effects of resveratrol against Aβ-induced neuroinflammation. J Biom Nanotech. 9, 2086–2104.

https://doi.org/10.1166/jbn.2013.1709

Frozza, R.L., Bernardi, A., Hoppe, J.B., Meneghetti, A.B., Matté, A., Battastini, A.M.O., Pohlmann, A.R., Guterres, S.S., Salbego, C., 2013b. Neuroprotective effects of resveratrol against Aβ administration in rats are improved by lipid-core nanocapsules. Mol Neurobiol. 47, 1066–1080. https://doi.org/10.1007/s12035-013-8401-2

Frozza, R.L., Bernardi, A., Paese, K., Hoppe, J.B., da Silva, T., Battastini, A.M., Pohlmann, A.R., Guterres, S.S., Salbego, C., 2010. Characterization of trans-resveratrol-loaded lipid-core nanocapsules and tissue distribution studies in rats. J Biomed Nanotechnol. 6, 694–703. https://doi.org/10.1166/jbn.2010.1161

Frozza, R.L., Horn, A.P., Hoppe, J.B., Simão, F., Gerhardt, D., Comiran, R.A., Salbego, C.G., 2009. A comparative study of β-amyloid peptides Aβ1-42 and Aβ25-35 toxicity in organotypic hippocampal slice cultures. Neurochem Res. 34, 295–303. https://doi.org/10.1007/s11064-008-9776-8

Fuentealba, R.A., Farias, G., Scheu, J., Bronfman, M., 2004. Signal transduction during amyloid-β-peptide neurotoxicity: role in Alzheimer disease. Brain Res Rev. 47, 275–289. https://doi.org/10.1016/j.brainresrev.2004.07.018

Ghumatkar, P., Peshattiwar, V., Patil, S., Muke, S., Whitfield, D., 2018. The effect of phloretin on synaptic proteins and adult hippocampal neurogenesis in Aβ(1-42)-injected male Wistar rats. J Pharm Pharmacol. 70, 1022–1030. https://doi.org/10.1111/jphp.12925

Ghumatkar, P.J., Patil, S.P., Jain, P.D., Tambe, R.M., Sathaye, S., 2015. Nootropic, neuroprotective and neurotrophic effects of phloretin in scopolamine induced amnesia in mice. Pharmacol Biochem Behav. 135, 182–191. https://doi.org/10.1016/j.pbb.2015.06.005

Ghumatkar, P.J., Patil, S.P., Peshattiwar, V., Vijaykumar, T., Dighe, V., Vanage, G., 2019. The modulatory role of phloretin in Aβ25-35 induced sporadic Alzheimer's disease in rat model. Naunyn Schmiedebergs Arch Pharmacol. 392, 327–339. https://doi.org/10.1007/s00210-018-1588-z

Guo, J., Yu, J., Grass, D., Beer, F.C. De, Kindy, M.S., 2002. Inflammation-

dependent cerebral deposition of serum amyloid A protein in a mouse model of amyloidosis. J Neurosci. 22, 5900–5909. https://doi.org/10.1523/JNEUROSCI.22-14-05900.2002

Harwell, C.S., Coleman, M.P., 2016. Synaptophysin depletion and intraneuronal Aβ in organotypic hippocampal slice cultures from huAPP transgenic mice. Mol Neurodegener. 11, 1–16. https://doi.org/10.1186/s13024-016-0110-7

Hoppe, J.B., Coradini, K., Frozza, R.L., Oliveira, C.M., Meneghetti, A.B., Bernardi, A., Pires, E.S., Beck, R.C.R., Salbego, C.G., 2013a. Free and nanoencapsulated curcumin suppress β-amyloid-induced cognitive impairments in rats: Involvement of BDNF and Akt/GSK-3β signaling pathway. Neurobiol Learn Mem. 106, 134–144. https://doi.org/10.1016/j.nlm.2013.08.001

Hoppe, J.B., Frozza, L., Horn, A.P., Comiran, R.A., Bernardi, A., Martha, M., Oliveira, A.M., 2010. Amyloid-β neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3β, tau and neuroinflammation. J Pineal Res. 48, 230–238. https://doi.org/10.1111/j.1600-079X.2010.00747.x

Hoppe, J.B., Frozza, R.L., Pires, E.N.S., Meneghetti, A.B., Salbego, C., 2013b. The curry spice curcumin attenuates beta-amyloid-induced toxicity through beta-catenin and PI3K signaling in rat organotypic hippocampal slice culture. Neurol Res. 35, 857–866. https://doi.org/10.1179/1743132813Y.0000000225

Kamphuis, W., Middeldorp, J., Kooijman, L., Sluijs, J.A., Kooi, E., Moeton, M., Freriks, M., Mizee, M.R., Hol, E.M., 2014. Neurobiology of aging glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. Neurobiol Aging. 35, 492–510. https://doi.org/10.1016/j.neurobiolaging.2013.09.035

Klein, C.P., Hoppe, J.B., Saccomori, A.B., Dos Santos, B.G., Sagini, J., Crestani, M.S., August, P.M., Hözer, R.M., Grings, M., Parmeggiani, B., Leipnitz, G., Navas, P., Salbego, C.G., Matté, C., 2019. Physical exercise during pregnancy prevents cognitive impairment induced by amyloid-β in adult

offspring rats. Mol. Neurobiol. 56, 1–17. https://doi.org/10.1007/s12035-018-1210-x

Korolainen, M.A., Auriola, S., Nyman, T.A., Alafuzoff, I., Pirttila, T., 2005. Proteomic analysis of glial fibrillary acidic protein in Alzheimer's disease and aging brain. Neurobiol Dis. 20, 858–870. https://doi.org/10.1016/j.nbd.2005.05.021

Li, Z., Jiang, H., Xu, C., Gu, L., 2015. A review: Using nanoparticles to enhance absorption and bioavailability of phenolic phytochemicals. Food hydrocoll. 43, 153–164. https://doi.org/10.1016/j.foodhyd.2014.05.010

Liu, Y., Zhang, L., Liang, J., 2015. Activation of the Nrf2 defense pathway contributes to neuroprotective effects of phloretin on oxidative stress injury after cerebral ischemia/reperfusion in rats. J Neurol Sci. 351, 88–92. https://doi.org/10.1016/j.jns.2015.02.045

Llorens-Martín, M., Jurado, J., Hernández, F., Avila, J., 2014. GSK-3β, a pivotal kinase in Alzheimer disease. Front Mol Neurosci. 7, 1–11. https://doi.org/10.3389/fnmol.2014.00046

LoPachin, R.M., Gavin, T., Geohageb, B.C., Zhang, L., Casper, D., Lekhraj, R., Barber, D.S., 2011. β-dicarbonyl enolates: a new class of neuroprotectants. J Neurochem. 116, 132–143. https://doi.org/10.1111/j.1471-4159.2010.07091.x.

Macklis, J.D., Madison, R.D., 1990. Progressive incorporation of propidium iodide in cultured mouse neurons correlates with declining electrophysiological status: a fluorescence scale of membrane integrity. J Neurosci Methods. 31, 43–46. https://doi.org/10.1016/0165-0270(90)90007-3

Mehan, S., Meena, H., Sharma, D., 2011. JNK: a stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. J Mol Neurosci. 43, 376–390. https://doi.org/10.1007/s12031-010-9454-6

Meraz-Ríos, M.A., Toral-Rios, D., Franco-Bocanegra, D., Villeda-Hernández, J., 2013. Inflammatory process in Alzheimer's disease. Front Integr Neurosci. 7, 1–15. https://doi.org/10.3389/fnint.2013.00059

Naoi, M., Wu, Y., Shamoto-nagai, M., Maruyama, W., 2019. Mitochondria in neuroprotection by phytochemicals: Bioactive polyphenols modulate mitochondrial apoptosis system, function and structure. Int J Mol Sci. 20. https://doi.org/10.3390/ijms20102451

Nassif, M., Hoppe, J., Santin, K., Frozza, R., Zamin, L.L., Horn, A.P., Salbego, C., 2007. β-Amyloid peptide toxicity in organotypic hippocampal slice culture involves Akt/PKB, GSK-3β, and PTEN. Neurochem Int. 50, 229–235. https://doi.org/10.1016/j.neuint.2006.08.008

Noraberg, J., Kristensen, B.W., Zimmer, J., 1999. Markers for neuronal degeneration in organotypic slice cultures. Brain Res Protoc. 3, 278–290. https://doi.org/10.1016/S1385-299X(98)00050-6

Parihar, M.S., Hemnani, T., 2004. Alzheimer's disease pathogenesis and therapeutic interventions. J Clin Neurosci. 11, 456–467. https://doi.org/10.1016/j.jocn.2003.12.007

Peterson, G.L., 1979. Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal Biochem. 220, 201–220. https://doi.org/10.1016/0003-2697(79)90222-7

Querfurth, H.W., Laferla, F.M., 2010. Alzheimer's Disease. N Engl J Med. 362, 329–344. https://doi.org/10.1056/NEJMra0909142

Rezende, S., Stanisçuaski, S., Lucca, L. De, Raffin, A., 2003. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. Quim Nova. 26, 726–737. https://doi.org/10.1590/S0100-40422003000500017

Roney, C., Kulkarni, P., Arora, V., Antich, P., Bonte, F., Wu, A., Mallikarjuana, N.N., Manohar, S., Liang, H., Kulkarni, A.R., Sung, H., Sairam, M., Aminabhavi, T.M., 2005. Targeted nanoparticles for drug delivery through the blood-brain barrier for Alzheimer's disease. J Control Release. 108, 193–214. https://doi.org/10.1016/j.jconrel.2005.07.024

Scharfenberg, F., Armbrust, F., Marengo, L., Pietrzik, C., Becker, C., 2019. Regulation of the alternative β-secretase meprin β by ADAM-mediated shedding. Cell Mol Life Sci. 1–14. https://doi.org/10.1007/s00018-019-

03179-1

Shin, S., Kum, H., Ryu, D., Kim, M., Jung, E., Park, D., 2014. Protective effects of a new phloretin derivative against UVB-induced damage in skin cell model and human volunteers. Int J Mol Sci. 15, 18919–18940. https://doi.org/10.3390/ijms151018919

Stoppini, L., 1991. A simple method for organotypic cultures of nervous tissue. J Neurosci Methods. 37, 173–182. https://doi.org/10.1016/0165-0270(91)90128-M

Sze, C.I., Troncoso, J.C., Kawas, C., Mouton, P., Price, D.L., Martin, L.J., 1997. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. J Neuropathol Exp Neurol. 56, 933–944. https://doi.org/10.1097/00005072-199708000-00011

Tuppo, E.E., Arias, H.R., 2005. The role of inflammation in Alzheimer's disease. Int J Biochem Cell Biol. 37, 289–305. https://doi.org/10.1016/j.biocel.2004.07.009

Valentim, L.M., Rodnight, R., Geyer, A.B., Horn, A.P., Tavares, A., Cimarosti, H., Netto, C.A., Salbego, C.G., 2003. Changes in heat shock protein 27 phosphorylation and immunocontent in response to preconditioning to oxygen and glucose deprivation in organotypic hippocampal cultures. Neuroscience. 118, 379–386. https://doi.org/10.1016/S0306-4522(02)00919-3

Wang, S., Zhang, C., Sheng, X., Zhang, X., Wang, B., Zhang, G., 2014. Peripheral expression of MAPK pathways in Alzheimer ' s and Parkinson ' s diseases. J Clin Neurosci. 21, 810–814. https://doi.org/10.1016/j.jocn.2013.08.017

Wei, Y., Zhang, J., Hussain, A., Liang, H., 2017. Molecular model and in vitro antioxidant activity of a water-soluble and stable phloretin/hydroxypropyl-β-cyclodextrin inclusion complex. J Mol Liq. 236, 68–75. https://doi.org/10.1016/j.molliq.2017.03.098

Wu, C., Ho, Y., Tsai, C., Wang, Y., Tseng, H., Wei, P., Lee, C., Liu, R., Lin, S., 2009. In vitro and in vivo study of phloretin-induced apoptosis in human liver

cancer cells involving inhibition of type II glucose transporter. Int J Cancer. 2219, 2210–2219. https://doi.org/10.1002/ijc.24189

Xu, M., 2018. Anticancer activity of phloretin against human gastric cancer cell lines involves apoptosis, cell cycle arrest, and inhibition of cell invasion and JNK Signalling Pathway. Med Sci Monit. 18, 6551–6558. https://doi.org/10.12659/MSM.910542

Yang, Y.C., Lii, C.K., Lin, A.H., Yeh, Y.W., Yao, H.T., Li, C.C., Liu, K.L., Chen, H.W., 2011. Induction of glutathione synthesis and heme oxygenase 1 by the flavonoids butein and phloretin is mediated through the ERK/Nrf2 pathway and protects against oxidative stress. Free Radic Biol Med. 51, 2073–2081. https://doi.org/10.1016/j.freeradbiomed.2011.09.007

ANEXO - Normas da revista *Neurochemistry International: The Journal of Cellular and Molecular Neuroscience*

Encontram-se a partir da próxima página.

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AUTHOR INFORMATION PACK

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