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LIPOSSOMAS E NANOPARTÍCULAS NO TRATAMENTO DE PATOLOGIAS DO SISTEMA NERVOSO CENTRAL

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Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal de Pernambuco como pré-requisito para obtenção do título de doutor.

Área de concentração: Fármacos e medicamentos

Orientadora: Prof.ª Dr.ª Nereide Stela Santos Magalhães
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Prof. Dr. Rafael Matos Ximenes

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“Nada é tão nosso quanto nossos sonhos.”

Friedrich Nietzsche

RESUMO

Neste trabalho, a atividade dos dois fármacos (nimodipina e quercetina), com interesse no tratamento de patologias que afetam o sistema nervoso central (SNC), foram avaliados após a sua veiculação em diferentes formas farmacêuticas. No primeiro caso, a nimodipina foi encapsulada em nanocarreadores adaptados à via parenteral (lipossomas, NMD-Lipo) ou oral (nanopartículas mucoadesivas [NMD-NP] e nanopartículas mucopenetrantes [NMD-NP/PEG]). Posteriormente, a quercetina foi encapsulada em nanopartículas bioadesivas de zeina (NPQ). Para NMD-Lipo, a atividade ansiolítica do bloqueador dos canais de cálcio foi avaliada em camundongos usando os testes do campo aberto, claro e escuro e labirinto em cruz elevado. A toxicidade aguda de NMD-Lipo também foi medida. O efeito anticonvulsivante de NMD-Lipo foi estudado através do modelo de convulsão induzido por pilocarpina e a atividade antidepressiva através dos testes de suspensão pela cauda e nado forçado, além da inibição da enzima MAO_B. Para NMD-NP, a biodisponibilidade oral de nimodipina foi avaliada em ratos e a capacidade cognitiva em modelos de estresse induzido por corticosterona foi medida utilizando o teste do labirinto aquático de Morris. Finalmente, o efeito de NPQ em um modelo murino de Alzheimer (Samp8) foi avaliado usando o teste do labirinto aquático de Morris e a expressão de marcadores de neuroinflamação (GFAP e CD11b) no cérebro dos camundongos. Os lipossomas contendo nimodipina mostraram um tamanho médio de 107 nm, um potencial zeta negativo de -5 mV e uma eficiência de encapsulação (EE) de 99%. O carreamento da nimodipina em lipossomas melhorou significativamente o seu efeito ansiolítico, sem causar sedação ou relaxamento muscular. O tratamento com NMD-Lipo não mostrou toxicidade e preveniu as convulsões e a morte em 100% dos animais desafiados com pilocarpina. Além disso, NMD-Lipo mostrou atividade antidepressiva e um efeito de inibição importante sobre a atividade da enzima da MAO_B. Esta última observação evidenciou que o efeito antidepressivo de nimodipina lipossomal é mediado por um aumento dos níveis de catecolaminas. NMD-NP/PEG exibiu um tamanho de 191 nm e um potencial zeta de -23 mV, com uma carga útil de 69 µg/mg. A biodisponibilidade oral da nimodipina, encapsulada nas referidas nanopartículas, foi de 62%, em comparação com apenas 9% para o fármaco livre. Administrada por via oral, NP-NMD/PEG foi capaz de prevenir os danos de memória causados por estresse nos animais, enquanto que o fármaco livre não foi capaz de produzir esse efeito. O tratamento com NPQ promoveu uma redução de 36% na latência de chegada da plataforma, o que refletiu uma melhora na capacidade cognitiva dos camundongos SAMP8. Além disso, NPQ induziu uma diminuição na expressão GFAP dos camundongos. Em resumo, nimodipina lipossomal tem um potencial promissor no tratamento do SNC por via parenteral. Por outro lado, nanopartículas de polianidrido revestidas com PEG 2000 se mostram uma opção interessante para terapia oral, dado que o polímero é bioadesivo e PEG 2000 fornece propriedades mucopenetrantes necessárias para a chegada do fármaco na superfície absortiva dos enterócitos. Finalmente, a quercetina encapsulada em nanopartículas parece ser uma estratégia adequada para ser explorada no tratamento da doença de Alzheimer.

Palavras-chave: Nanotecnologia. Ansiedade. Eplepsia. Depressão. Memória.

ABSTRACT

In this work, the activity of two drugs (nimodipine and quercetin) with interest in the treatment of different diseases affecting the central nervous system (CNS), after their formulation in different pharmaceutical dosage forms, have been evaluated. In the former, nimodipine was encapsulated in different nanocarriers adapted to their parenteral (liposomes, NMD-Lipo) or oral (mucoadhesive [NMD-NP] and mucus-permeating nanoparticles [NMD-NP/PEG]). In the latter, quercetin was encapsulated in bioadhesive zein nanoparticles (NPQ). For NMD-Lipo, the anxyolitic activity of the calcium channel blocker was evaluated in mice using the open field, the light and dark and the elevated plus maze tests. The acute toxicity of NMD-Lipo was also measured using Hippocratic screening and biochemical and hematological analysis. The anticonvulsant effect of NMD-Lipo has been studied in pilocarpine seizure model and the antidepressant activity in the forced swim and tail suspension tests, and inhibition of MAO_B enzyme. For NMD-NP, the oral bioavailability of nimodipine was evaluated in rats and the cognitive ability in stress-induced corticosterone models was measured using the Morris water maze test. Finally, the effect of NPQ in a murine model of Alzheimer's (Samp8) was evaluated using the Morris water maze test and the expression of neuroinflammatory markers (GFAP and CD11b) in the brains of mice. Liposomes containing nimodipine displayed a mean size of 107 nm, a negative charge of -5 mV and an encapsulation efficiency (EE) of 99%. The loading of nimodipine in liposomes significantly improved its anxiolytic effect, without causing sedation or muscle relaxation. Treatment with NMD-Lipo showed no toxicity and prevented seizures and death in 100% of animals challenged with pilocarpine. Moreover, NMD-Lipo showed antidepressant activity and an important inhibition effect on the activity of the MAO_B enzyme. This last observation evidenced that the antidepressant effect of liposomal nimodipine is mediated by an increase on the levels of catecholamines. NMD-NP/PEG displayed a size of 191 nm and a zeta potential of -23 mV, with a payload of 69 µg/mg. The oral bioavailability of nimodipine, when encapsulated in these nanoparticles, was 62%, compared with only 9% for the free drug. Orally administered, NP-NMD/PEG was able to prevent the memory damage caused by stress in the animals, while the free drug was unable to produce that effect. Finally, treatment with NPQ promoted a 36% reduction in latency arrival platform by animals, which reflected an improvement in cognitive ability of SAMP8 mice. In addition, NPQ induced an important decrease in the GFAP expression of mice. In summary, liposomal nimodipine have promising potential for the treatment of CNS by injection route. On the other hand, poly(anhydride) nanoparticles coated with PEG 2000 may be an interesting option for oral therapy, since the polymer is bioadhesive and PEG 2000 provides mucus-permeating properties required for the arrival of the loaded drug to the absorptive surface of enterocytes. Finally, quercetin encapsulated in nanoparticles appears to be an adequate strategy to be explored in the treatment of Alzheimer's disease.

Keywords: Nanotecnology. Anxiety. Epilepsy. Depression. Memory

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nanoparticles containing nimodipine coated with PEG 2000 (NMP-NP/PEG).

LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise da variância
BBB	Barreira hematoencefálica
BCS	Sistema de classificação biofarmacêutica
BHE	Barreira hematoencefálica
BFCS	Barreira fluido cerebroespinal-sangue
Ca^{++}	Cálcio
CD11b	Aglomerado de diferenciação de granulócitos
CEEA	Comitê de ética em experimentação animal
Chol	Colesterol
CNS	Sistema nervoso central
Da	Daltons
DZP	Diazepam
Flu	Flumazenil
G	Gravidade
G	Grama
GFAP	Proteína ácida fibrilar glial
GR	Receptor de glucocorticoides
H	Hora
HPLC	Cromatografia líquida de alta eficiência
KCl	Cloreto de potássio
LSOA	Tempo de permanência nos braços abertos
LUV	Vesículas unilamelares grandes
MAO_B	Monoamina oxidase B
MAOIs	Inibidor das monoaminas oxidases
MG	Miligramas
mL	Mililitros
MLV	Vesículas multilamelares
mV	MiliVolt
NEOA	Número de entradas nos braços abertos
Nm	Nanômetros
NMD	Nimodipina

NP	Nanopartículas
P400	Cloridrato de pilocarpina a uma dose de 400 mg/kg
NMD-Lipo	Formulação lipossomal de nimodipina
PC	Fosfatidilcolina de soja
PCS	Espectroscopia de correlação de fótons
PDI	Índice de polidispersão
PEG	Polietilenoglicol
pH	Potencial hidrogeniônico
PVM/MA	Poli(metal vinil éter-co-anidrido maleico)
NMD-NP	Nanopartículas de poli(anidrido) contendo nimodipina
NMD-NP/PEG	Nanopartículas de poli(anidrido) carregadas com nimodipina recobertas com PEG 2000
NPQ	Nanoparticulas carregadas com quercetina
Q	Quercetina
ºC	Graus Celsius
µg	Microgramas
µl	Microlitros
Samp8	Camundongo de senescência acelerada
SD	Desvio padão
SEM	Erro padrão da média
SNC	Sistema Nervoso Central
SUV	Vesículas unilamelares pequenas
TEM	Microscopia eletrônica de transmissão
UNAV	Universidad de Navarra
UFPE	Universidade Federal de Pernambuco
UFPI	Universidade Federal do Piauí
UV	Ultravioleta

SUMÁRIO

1. INTRODUÇÃO

A busca por terapias para patologias que atingem o Sistema Nervoso Central (SNC) como tumores cerebrais, epilepsia, transtornos afetivos e doenças neurodegenerativas, vêm ocupando cada vez mais espaço nas pesquisas científicas. A importância das referidas disfunções tem se evidenciado pelo fato de que elas se tornam cada dia mais frequentes, além de representarem um grave empecilho à manutenção da saúde humana (Qin et al, 2011).

Tratamentos efetivos e não invasivos para os distúrbios neurológicos são limitados pelo difícil acesso dos fármacos ao SNC. A redução da permeabilidade dos supracitados agentes terapêuticos ao parênquima cerebral se deve principalmente à presença de duas barreiras anatômicas e bioquímicas: a barreira hematoencefálica (BHE) e a barreira fluido cerebroespinal-sangue (BFCS) (Wong, Wu, Bendayan, 2012). A importância desses obstáculos para a terapia das doenças neurológicas é ressaltada quando observamos que aproximadamente 100% dos fármacos de alto peso molecular (> 400 Da) e 98% dos copostos de baixo peso molecular (≤ 400 Da) não são capazes de atravessar a BHE (Paolino et al, 2011).

Nesse contexto, um dos maiores desafios para a obtenção de êxito nos tratamentos de distúrbios neurológicos constitui a liberação adequada do fármaco administrado, promovendo o direcionamento do princípio ativo ao sítio específico do SNC, no momento oportuno e em quantidades suficientes para a realização da terapia.

A causa exata da patogênese das enfermidades do SNC muitas vezes permanece desconhecida, todavia alguns ensaios nos dão indícios de alterações morfofisiológicas que podem estar relacionadas com o surgimento dessas patologias**.** É fato conhecido que o fluxo excessivo de cálcio (Ca^{++}) através das membranas, que resulta em concentrações intracelulares aumentadas do íon, pode desempenhar um papel importante na fisiopatologia dos transtornos afetivos (Maigaard et al, 2012), na atividade epileptiforme (N'Gouemo, 2013) e na indução de ansiedade (Kumar et al., 2012). Além disso, foi demostrado que a degeneração neuronal presente no envelhecimento é mediada por um aumento nos níves de Ca^{++} intracelular (Shen et al., 2016; Thibault, Gant, Landfield, 2007).

Como outro exemplo de agentes causadores de patologias do SNC podemos citar as atividades deletérias dos radicais livres e metabólitos oxidados que provocam oxidação de proteínas, peroxidação lipídica, oxidação do DNA e morte neuronal, e constituem um dos principais mecanismos patológicos envolvidos em doenças neurodegenerativas (Duyckaerts et

al., 2009; Iqbal and Grundke-Iqbal, 2008; Ittner and Gotz, 2011; Querfurth and LaFerla, 2010; Liu et al, 2013a).

A partir desta perspectiva podemos afirmar que a utilização de fármacos que sejam bloqueadores dos canais de Ca^{++}, bem como compostos que apresentem atividade antioxidante, constituem alternativas promissoras para o desenvolvimento de terapias para as enfermidades do SNC.

A nimodipina (NMD) um antagonista seletivo dos canais de Ca^{++} tipo L, foi investigada no tratamento de numerosas desordens neurológicas (Heffren, Mcintosh, Reiter, 2015). O referido fármaco atravessa facilmente a BHE, além de se ligar com alta afinidade e especificidade aos receptores dos canais de Ca^{++} cerebrais (Bailey, Hutsell, Newlan, 2013).

Estudos concluíram que a nimodipina tem a capacidade de aumentar o fluxo sanguíneo cerebral e é usado no tratamento da isquemia presente em inúmeras patologias que afetam o cérebro (Aslan et al, 2009), além de ser útil na terapia de desordens do humor (Pazzaglia et al, 1995; Frye et al, 2003), no tratamento da demências senis como a doença de Alzheimer (Koskinen et al, 2012) e apresentar propriedades anticonvulsivantes (Thomas, 1990; Paczynki, Meyer, Anderson, 1990; Marinho et al, 1997; Chakrabarti, Kaur, Garg, 1998; Zapater et al, 1998; Mikati et al, 2004; Nascimento et al, 2005; Hocht et al, 2007).

De maneira semelhante, a quercetina é um flavonoide natural encontrado em uma grande variedade de frutas e verduras e regularmente consumido por humanos (Petersen et al., 2016). Esse composto apresenta um elevado potencial nutracêutico e farmacêutico, uma vez que apresenta atividade antioxidante (Fiorani et al., 2010), anti-inflamatória (Garcia-Mediavilla et al., 2007), neuroprotetora contra a toxicidade induzida por agente (Kanter et al., 2016) e anti-isquêmica (Yao et al., 2012). Além das atividades supracitadas, a quercetina apresenta a capacidade de atravessar a BHE (Ishisaka et al., 2011) e tem mostrado potencial contra o déficit cognitivo observado nas doenças neurodegenerativas (Sharma et al., 2016).

Apesar das muitas opções de aplicabilidade, a quercetina e a nimodipina apresentam algumas características que limitam a sua eficácia clínica quando administrada por via oral. Esses fármacos apresentam baixa solubilidade nos fluidos gastrointestinais e alto metabolismo de primeira passagem hepático, o que causa baixa biodisponibilidade e atividade diminuída (Sun et al, 2016; Sun et al, 2013).

Uma alternativa viável para superar os supracitados inconvenientes é a veiculação desses fármacos em carreadores farmacêuticos de escala nanométrica. A nanotecnologia constitui uma ferramenta muito útil para melhorar o desempenho de compostos com baixa estabilidade físico-química, baixa solubilidade aquosa e biodisponibilidade insuficiente

(Sosnik, Carcaboso, 2014). Além disso, a veiculação de fármacos em nanossistemas que interagem com as células endoteliais dos microvasos da BHE auxilia a passagem dos compostos por essa barreira e consequentemente, proporcionam um aumento das concentrações do composto no parênquima cerebral (Wong, Wu, Bendayan, 2012).

Estudos mostram que a encapsulação de fármacos em lipossomas e nanopartículas podem facilitar a passagem do composto pela BHE e, por conseguinte, promover a entrega do fármaco no cérebro (Kuo, Liu, 2014; Zhang et al 2013). Com base nisso, o objetivo desse trabalho foi avaliar a atividade da nimodipina e da quercetina após a sua veiculação em diferentes formas farmacêuticas. A nimodipina foi encapsulada em nanocarreadores adaptados à via parenteral (lipossomas, NMD-Lipo) ou oral (nanopartículas mucoadesivas [NMD-NP] e nanopartículas mucopenetrantes [NMD-NP/PEG]). Por outro lado, a quercetina foi encapsulada em nanopartículas bioadesivas de zeina (NPQ). Posteriormente o efeito dos fármacos encapsulados foi testado em modelos murinos de ansiedade, depressão, eplepsia, Alzheimer e estresse.

2 REVISÃO BIBLIOGRÁFICA

2.1 Patologias do SNC

Patologias que afetam o SNC, a exemplo da ansiedade, depressão, fobias, esquizofrenia, epilepsia e Alzheimer, apresentam uma alta prevalência na sociedade atual. Estima-se que aproximadamente um terço da população da Europa sofra de algum tipo de distúrbio mental (Doria et al, 2015). Esses transtornos estão entre as patologias de maior incidência mundial e afetam pessoas de diversas faixas etárias e de ambos os sexos (Wittchen et al., 2011).

Inúmeros fatores como o estresse da vida cotidiana, o sedentarismo, hábitos alimentares pouco saudáveis, além do envelhecimento da população, podem ser os responsáveis pelo aumento na incidência de distúrbios neurológicos (Costa, 2014). Esses distúrbios afetam praticamente todos os aspectos da vida do paciente. Isso se comprova quando observamos que as funções pessoais, sociais, profissionais, bem como a saúde física do indivíduo acometido, podem ser drasticamente prejudicadas por estas patologias (Balasubramaniam, Telles, Doraiswamy, 2012).

Desta forma, pode-se dizer que as doenças que atingem o SNC produzem um impacto negativo não só sobre o paciente e suas famílias, mas também sobre toda a sociedade, uma vez que os tratamentos para essas enfermidades apresentam custos elevados e os distúrbios do SNC provocam redução da produtividade do trabalhador (Wittchen, Jacobi, 2005; Frye et al., 2006; Wang et al., 2009).

A ansiedade é um estado emocional que faz parte da existência humana, pois circunstâncias normais na vida das pessoas, como o desenvolvimento de algum tipo de sofrimento físico ou mental, bem como mudanças na vida cotidiana, podem ser associadas ao seu início (Richey et al, 2010). É um tipo de emoção que foi moldada pela seleção natural e sua função é manter os seres humanos alertas a perigos iminentes (Wong et al, 2016).

No entanto, a ansiedade pode passar de uma ocorrência normal para um quadro patológico quando é desproporcional a circunstância que a causa ou quando não existe um motivo aparente para a sua instalação (Salomons et al., 2010). A ansiedade patológica é caracterizada por uma preocupação excessiva e incontrolável sobre um número de fatores, nos quais os indivíduos envolvidos experimentaram pelo menos três dos seguintes sintomas: distúrbio do sono, tensão muscular, fadiga, dificuldade de concentração, irritabilidade, mente em branco e sentir-se dominado ou a beira de um abismo (Maack, Tull, Gratz, 2012). A forma patológica é debilitante e está associada a um risco aumentado de suicídio (Zou et al., 2012).

O tratamento farmacológico da ansiedade consiste no uso dos ansiolíticos benzodiazepínicos, buspirona e medicamentos antidepressivos (Bartley, Hay, Bloch, 2013). Apesar dos fármacos mostrarem grande eficácia, a sua administração tem alguns inconvenientes provocados por uma vasta gama de efeitos colaterais. Os mais comuns são: amnésia, indução de dependência e sedação (Raupp et al, 2008).

Como outro exemplo de distúrbio mental que afeta todos os aspectos da vida podemos citar a depressão. Os sentimentos de alegria e tristeza são de ocorrência natural na existência humana (Del Porto, 1999). A tristeza é uma resposta esperada em situações de perda, derrota, desapontamento e outras adversidades, que tem valor adaptativo do ponto de vista evolucionário, já que leva o indivíduo afetado a permanecer em retraimento e assim economizar energia e recursos para empreitadas futuras e, ao mesmo tempo, serve de alerta para as pessoas do seu convívio de que se está precisando de ajuda e atenção (Wittman, 2014). Todavia, quando a tristeza ultrapassa o limiar da normalidade observamos o desenvolvimento da depressão.

Além da tristeza sem motivo aparente, os sintomas observados nessa patologia são: irritabilidade, desânimo, retraimento social, baixa autoestima, humor disfórico, tendência autodepreciativa, alteração do sono e do apetite, ideação paranóide e pensamento recorrente de suicídio (Petrovic et al, 2016; Stella, 2002). Os motivos desencadeadores da depressão ainda não foram bem elucidados, mas acredita-se que eles possam envolver uma predisposição genética e influencias do sócio-ambientais (Liu et al., 2016).

É importante ressaltar que, de forma semelhante à ansiedade, a depressão é uma doença psiquiátrica grave e onerosa que frequentemente se transforma em uma condição clínica crônica (Verster et al., 2009). Estudos afirmam que 15% da população mundial sofre ou sofrerá de depressão em algum momento da sua vida (WHO, 2008).

Existem pelo menos 35 antidepressivos disponíveis no mercado com diferentes mecanismos de ação: antidepressivos tricíclicos, inibidores seletivos da captação da serotonina, inibidores seletivos da recaptação de norepinefrina, inibidores da recaptação de serotonina-norepinefrina, antidepressivos atípicos, inibidores da enzima monoamino-oxidase (MAO), inibidores seletivos da MAO_A e inibidores seletivos da MAO_B (Willner, Scheel-Krüger, Belzung, 2013). Toda vida, a depressão resistente ao tratamento é uma condição freqüente em pacientes. As estatísticas mostram que 50 a 60% das pessoas tratadas com antidepressivos obtêm resultados clinicamente insignificantes (Fornaro et al., 2014).

Outra patologia do SNC que merece atenção é a epilepsia, patologia caracterizada pela ocorrência de crises convulsivas (Roller, Gauau, 2016). Convulsões são alterações no

funcionamento do SNC caracterizadas por crises recorrentes originadas por descargas excessivas dos neurônios cerebrais que ocorrem pela alterações de equilíbrio entre os mecanismos de neurotransmissão inibitórios e excitatórios, resultando em mudanças persistentes do funcionamento cerebral normal e do estado cognitivo (Adeyemi et al, 2010; Zouhar et al, 1989). Podem apresentar manifestações motoras, sensitivas, sensoriais, psíquicas ou neurodegenerativas e são classificadas como tônicas (contrações mantidas durante algum tempo), clônicas (contrações intermitentes, quando os músculos são contraídos e relaxados de forma alternada) ou tônico-clônicas (Fortini el al, 2013**;** Loscher, 1998). Aproximadamente 50 milhões de pessoas apresentam crises convulsivas em todo o mundo e, apesar do distúrbio responder ao tratamento em cerca de 70% dos casos, apenas três quartos dos afetados recebe a terapia adequada (Bhutada et al, 2010).

O tratamento inicial da convulsão é realizado utilizando-se medicamentos que irão suprimir as crises. Os fármacos administrados atuam na estabilização das membranas celulares (reduzindo o fluxo de íons), aumentando a concentração de neurotransmissores inibitórios ou diminuindo a ação de neurotransmissores excitatórios. Os compostos aplicados na terapia da patologia são: fenobarbital, fenitoína, carbamazepina, benzodiazepínicos, oxcarbazepina, topiramato, gabapentina, lamotrigina e ácido valpróico (valproato) (Fontinelle, 2001). Contudo, as terapias apresentam algumas reações adversas como sonolência, lentificação na realização de tarefas e desconcentração, o que faz com que alguns pacientes abandonem o tratamento (Hadizadeh et al, 2013).

A doença de Alzheimer é um distúrbio cerebral neurodegenerativo letal, caracterizado por um comprometimento cognitivo progressivo e perda de memória, que é considerado a causa mais comum de demência em idosos (Orejana et al., 2013). A enfermidade envolve o acúmulo de placas β-amilóides extracelulares no cérebro dos indivíduos acometidos, o aparecimento progressivo da patologia tau intracelular, a perda de conexões sinápticas de regiões cerebrais específicas e estresse oxidativo extenso (Sabogal-Guáqueta et al, 2015). As atividades deletérias de metabolitos e radicais livres oxidados incluem oxidação de proteínas, peroxidação de lípidos, oxidação de DNA e, em última instância, morte neuronal (Querfurth and LaFerla, 2010).

A prevalência mundial da doença de Alzheimer foi estimada em 36 milhões no ano de 2010 e, com o envelhecimento da população, a previsão é que atinja 66 milhões de pessoas em 2030 e 115 milhões em 2050 (Prince et al., 2011). Apesar da sua grande incidência, poucos medicamentos foram aprovados pela “Food and Drug Administration” dos EUA para o tratamento do Alzheimer. Estes fármacos melhoram os sintomas todavía não alteram o

curso da progressão da doença e sua administração provoca inúmeros efeitos colaterais (Bassil, Grossberg, 2009).

Com base no exposto e devido a crescente ocorrência das chamadas “doenças do mundo moderno” vêm se observando um aumento na busca por tratamentos medicamentosos que curem ou pelo menos minimizem os sintomas e a progressão das patologias do SNC. Também é importante que, além do efeito desejado, os novos fármacos não apresentem efeitos colaterais que causem um impacto negativo na qualidade de vida do paciente (Neutel, Skurtveit, Berg, 2012; Nóbrega et al, 2012; Onocko-campos et al, 2013; Oyebode et al., 2012).

Deste modo, faze-se necessário a pesquisa de novos agentes terapêuticos para as enfermidades dos SNC que sejam efetivos, seguros e que sejam capazes de atravessar a barreira hematoencefálica (BHE).

2.2 Barreira Hematoencefálica

A BHE é uma interface dinâmica entre o sangue e o SNC que controla estritamente as trocas de substâncias entre o fluido sanguíneo e o cérebro, desempenhando um papel fundamental na homeostase cerebral e proporcionando proteção contra compostos tóxicos e agentes patogênicos (Cardoso, Brites, Brito, 2010).

A referida barreira é constituída por sistema cerebrovascular altamente complexo composto por aproximadamente 100 bilhões de capilares, cujas células endoteliais estão unidas por junções celulares do tipo junção de oclusão. Esse conjunto de capilares está envolto por um agrupamento celular constituído de astrócitos, pericitos e macrófagos e juntos formam uma estrutura densa (Figura 1) (Brightman et al, 1969) .

Como dito anteriormente, a função da BHE é proteger o cérebro da entrada de agentes potencialmente lesivos, no entanto ela também limita a chegada de agentes terapêuticos ao SNC. Mais de 98% dos medicamentos com um peso molecular inferior de 400 Da e todos aqueles com peso molecular superior a isso não são capazes de atravessar a BHE e consequentemente chegar ao cérebro (Soddu et al, 2015).

A maioria dos novos medicamentos desenvolvidos para tratar doenças neurológicas é abandonada na fase I ou II de ensaios clínicos por causa da dificuldade de acesso ao tecido nervoso (Khrestchatisky, Tokay, 2014). Nesse contexto, é importante atentar para o fato de que fármacos direcionados ao tratamento de doenças neurológicas devem ser concebidos de maneira a atravessar a BHE e devem ser desenhados anatômica e funcionalmente para isso.

As principais características que favorecem a passagem de uma molécula através da BHE são: natureza lipofílica, tamanho menor ou igual a 400 Da e afinidade pelo sistema de transporte endógeno (por exemplo, afinidade pelo transporte mediado por proteínas carreadoras, transporte mediado por receptores ou transcitose absortiva) (VanGilder et al, 2011). Dessa forma, o desenvolvimento de novas moléculas com atividade no SNC deve estar direciondo para moléculas pequenas, lipofílicas e com radicais que possuam afinidade com os sistemas de transporte endógeno.

Assim, a veiculação de compostos de interesse para o SNC em carreadores de escala nanométrica de natureza hidrofóbica parece uma opção inovadora e útil para facilitar a transposição das moléculas à BHE e à entrega dos referidos produtos ao cérebro (Wong, Wu, Bendayan, 2012).

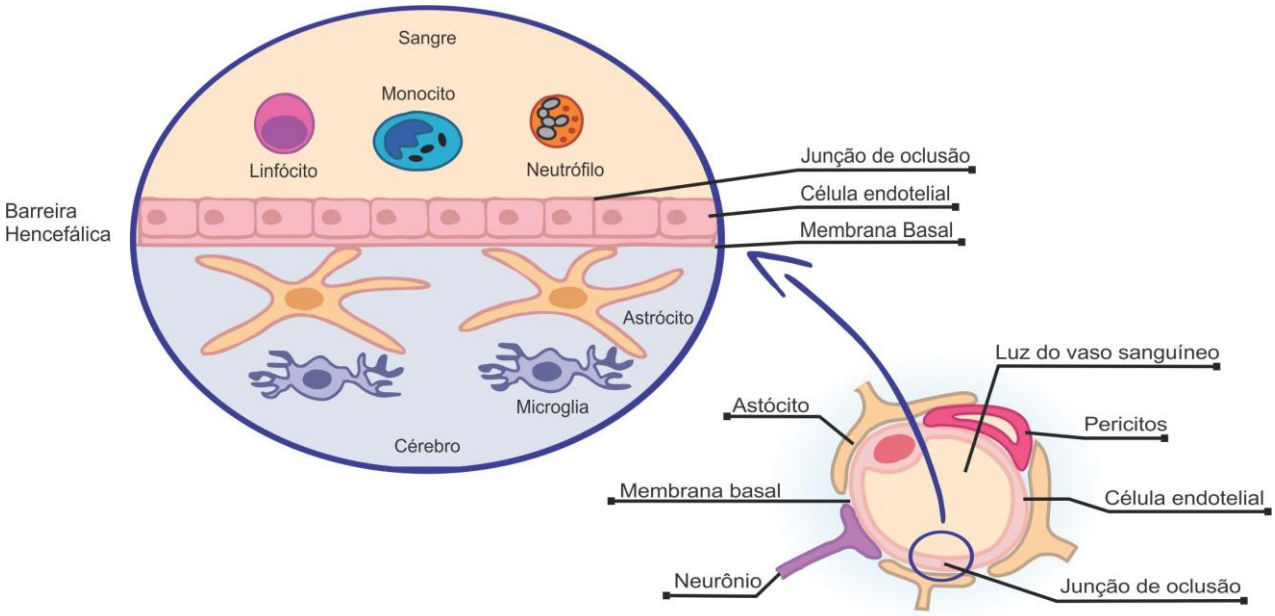


Figura 1: Estrutura da barreira hematoencefálica.

2.3 Nimodipina

Nimodipina ou 2,6-dimetil-4-(3-nitofenil)-1,4-di-hidropiridina-3-5-dicarboxilato de 2-metoxietilo-1-metileno (Tabela 1) é um bloqueador dos canais de cálcio tipo L do grupo das diidropiridinas desenvolvido originalmente pela Bayer e vendido comercialmente com e nome de Nimotop® (Zu et al, 2014).

Apresenta fórmula molecular $C_{21}H_{26}N_2O_7$ e peso molecular 418,4. Apresenta-se na forma de pó cristalino com coloração variando entre amarelo e amarelo claro. É praticamente insolúvel em água, facilmente solúvel em acetato de etila e ligeiramente solúvel em metanol. Apresenta polimorfismo e se degrada na presença de luz ultravioleta formando um derivado de nitrofenilpiridina (Real farmacopea española, 2015).

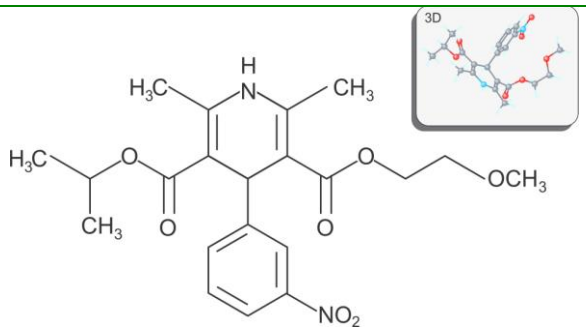
O fármaco é rapidamente absorvido após a administração oral, com pico de concentração plasmática de 1 hora. A taxa de ligação com proteínas plasmáticas é superior a 95% e sua eliminação é quase exclusivamente como os seus metabolitos. Menos de 1% é recuperado a partir de urina como NMD integra (Salgado-Figueroa, Gutiérrez, Squella, 2015).

Nimodipina inibe a entrada dos íons Ca^{++} na célula, diminuindo as contrações da musculatura vascular e consequentemente provocando vasodilatação (Bege el al, 2013). Essa diidropiridina pertence à classe II do sistema de classificação biofarmacêutico (baixa solubilidade e alta permeabilidade) e, devido a sua elevada lipofilicidade, atravessa facilmente a BHE. Essa característica, somada ao fato de que NMD apresenta seletividade para os vasos sanguíneos cerebrais, faz com que o referido fármaco tenha um efeito muito pronunciado no cérebro (Zhao et al, 2014).

Originalmente desenvolvida para tratar hipertensão, NMD é usada atualmente para tratar quadros de hemorragia subaracnoidea aguda e suas complicações (Riekes et al, 2015). A nimodipina também tem mostrado aplicabilidade na terapêutica da isquemia presente em diversas patologias que afetam o cérebro (Aslan et al, 2009), na terapia de desordens do humor e ansiedade (Frye et al, 2003; Pazzaglia et al, 1995), além de se mostrar promissora no tratamento das demências senís (Chalikwar et al, 2012), nos déficites de memória causados pelo estresse (Kumar, Singh and Jaggy 2012) e apresentar propriedades anticonvulsivantes (Marinho et al, 1997; Mikati et al, 2004; Nascimento et al, 2005).

Todavia, apesar da sua grande versatilidade, a administração da referida diidropiridina por via oral apresenta algumas limitações. NMD é lipofílica e por isso apresenta baixa solubilidade nos fluidos gastrointestinais, o que somado a um extenso metabolismo de primeira passagem hepático, resulta em uma biodisponibilidade que varia de 4 a 13% (Lei et

al, 2015). Además, a nimidipina é um substrato da p-glicoproteína que funciona como uma bomba de efluxo ATP-dependente que promove ativamente a saída da substância do interior da célula (Pathak et al, 2014). Por isso o fármaco deve ser administrado em uma dose de 60 mg a cada 4 horas, o que constitui uma frequência de administração muito alta e inconveniente ao pacientes (Chalikwar et al, 2012).

Nome comum	Nimodipina
Estrutura química	
Fórmula molecular	$C_{21}H_{26}N_2O_7$
Nomenclatura (IUPAC)	2,6-dimetil-4-(3-nitofenil)-1,4-di-hidropiridina-3-5-dicarboxilato de 2-metoxietilo-1-metileno
Peso molecular	418,4
PKa	5,4
Ph	7,3 (suspensão aquosa 1%)
Ponto de fusão	150°C
Solubilidade	Metanol: 62,5 mg/mL Acetato de etila: Solúvel Água: 2,3 µg/Ml
Toxicologia e Farmacocinética	
DL_{50}	Oral, rato: 2738 mg/kg Subcutânea, rato: 4234 mg/kg
Dose (mg/Kg)	5 mg/Kg (oral)
$AUC\infty$ ($\mu g \cdot min \cdot mL^{-1}$)	61,7
Clearence mL/h	81,7
$t_{1/2}$ (h)	1,28

Uma possível solução para esses problemas seria a administração endovenosa de NMD, todavia as injeções do fármaco contêm aproximadamente 40% de etanol e por isso

podem causar dor e processos inflamatórios como flebite (Huang et al, 2014). Visando superar esta limitação, nanocarreadores contendo nimodipina podem ser uteis para aumentar sua solubilidade, reduzindo o número de administrações, promovendo a melhora do paciente.

Tabela 1: Parâmetros físico-químicos, toxicológicos e farmacológicos da nimodipina.

2.4 Quercetina

Quercetina ou 3,3',4',5-7-penta-hidroxiflavona (Tabela 2) é um flavonoide pertencente a classe de compostos polifenólicos encontrado em chás, vinho tinto, frutas (ex:. maçã e uva) e alguns vegetais (ex:. alcaparra, cebola, tomate e alface) (Buchweitz et al, 2016). Em plantas, a quercetina geralmente é encontarada como sua forma conjugada por ligação covalente com uma unidade de açúcar (glucose, rutinose, ramnose ou xilose), formando O-β-glicosídeos (Erlund, 2004).

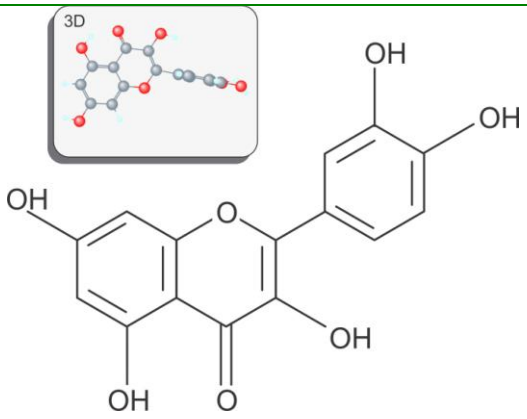
Apresenta fórmula molecular $C_{15}H_{10}O_7$ e peso molecular 302,2. Apresenta-se na forma de um pó amarelo intenso. É praticamente insolúvel em água, facilmente solúvel em ácido acético glacial e ligeiramente solúvel em etanol (Real farmacopea española, 2015). O flavonoide apresenta pico de concentração plasmática de 1,4 hora, meia-vida de 3,5 hora e biodisponibilidade que varia entre 2 a 10% após administração oral (Penalva et al, 2016).

Quercetina apresenta itensa atividade antioxidante devido à capacidade de sequestro dos radicais livres de oxigênio, além da capacidade de inibição da enzima xantina oxidase e da peroxidação lipídica (Fiorani et al, 2010). Além disso, a quercetina exerce efeitos neuroprotectores contra a toxicidade induzida por agente (Kanter et al, 2013) e aumenta a resistência dos neurônios ao estresse oxidativo e excitotoxicidade por modulação dos mecanismos de morte celular (Choi et al, 2014;. Liu et al, 2013b).

Estudos têm mostrado que quercetina também produz um efeito anti-inflamatório (Garcia-Mediavilla et al, 2007) através da inibição iNOS (Martinez-Florez et al, 2005) e da regulação da expressão da COX-2 (Banerjee, Van Der Vliet, Ziboh, 2002; de Pascual-Teresa et al, 2004), bem como tem um efeito anti-proliferativo em alguns tipos de câncer (Park et al, 2005; Russo et al, 2014), por meio de mecanismos que ativam a apoptose (Russo et al., 2012) e autofagia (Psahoulia et al., 2007).

Somadas as atividades supracitadas, o referido flavonóide mostra efeito protector contra isquemia (Yao et al, 2012) e aterosclerose (Lara-Guzman et al, 2012), além de melhorar a capacidade cognitiva de camundongos transgênicos para a doença de Alzheimer (Sabogal-Guáqueta et al, 2015).

Tabela 2: Parâmetros físico-químicos, toxicológicos e farmacológicos da quercetina.

Nome comum	Quercetina
Estrutura química	
Fórmula molecular	$C_{15}H_{10}O_7$
Nomenclatura (IUPAC)	2-(3,4-dihidroxifenil)-3,5,7-trihidroxicromen-4-ona
Peso molecular	302,2
PKa	6,7
Ph	4.50 – 6.50 (suspensão aquosa 1%)
Ponto de fusão	314°C
Solubilidade	Álcool: 3,45 mg/mL Ácido acetico Glacial: Solúvel Água: 10 µg/Ml
Toxicologia e Farmacocinética	
DL_{50}	160mg/Kg (oral) – Camundongo 620µM (*in vitro*)- Hepatócito (2h)
Dose (mg/Kg)	25 mg/kg (oral)
$AUC\infty$ ($\mu g \cdot min \cdot mL^{-1}$)	6,7
Clearence mL/h	29,2
$t_{1/2}$ (h)	3,5

Todavia, diferentes formas de derivados de quercetina parecem influenciar a sua taxa de absorção no intestino delgado e estômago (Mullen et al, 2008). O conteúdo e a forma de seus derivados têm um papel chave na sua absorção (Rahman, Biswas, Kirkham, 2006). As fomas glicosiladas, como as encontradas nos alimentos, de maneira geral apresentam melhor biodisponibilidade após absorção oral, contudo quando estudadas mostram menor atividade em relação às formas agliconas (sem a ligação com um grupo açúcar) (Ghanbarzadeh, et al,

2007). Isso resalta a necesidade de encontrar um veículo para a administração da quercetina aglicona por via oral.

2.5 Lipossomas

Lipossomas são vesículas esféricas de escala nanométrica, compostas por uma ou várias bicamadas fosfolipídicas concêntricas circundando uma fase aquosa interna (Figura 2) (Menon, Yin, Misran, 2015). Podem desempenhar a função de carreadores de fármacos, biomoléculas ou agentes de diagnóstico (Cadena et al, 2013). Apresentam-se como veículos de moléculas hidrofílicas (no interior da vesícula aquosa) e/ou moléculas lipofílicas (na bicamada de fosfolipídios), protegendo os compostos encapsulados contra uma variedade de ameaças que podem levar a liberação imediata ou degradação (Kulkarn, Yadav, Vaidya, 2011).

Esses nanocarreadores constituem um método alternativo para a administração de compostos tóxicos, fotossensíveis ou que apresentem baixa solubilidade na forma livre e têm mostrado a capacidade de melhorar à farmacocinética e farmacodinâmica das moléculas encapsuladas (Cavalcanti et al, 2011; Santos-Magalhães, Mosqueira, 2010). Além disso, são biodegradáveis, não-tóxicos, não-imunogênicos e biocompatíveis (Sebaaly et al 2015).

Essas vesículas são constituídas por fosfolipídios de natureza sintética ou natural, esteróis e um antioxidante (Vemuri, Rhodes, 1995). Os lipídeos mais empregados na produção de lipossomas são os que apresentam forma cilíndrica, a exemplo das fosfatidilcolinas, fosfatidilserina, fosfatidilglicerol e esfingomielina, que naturalmente formam uma bicamada estável em solução aquosa. As fosfatidilcolinas são muito usadas, uma vez que apresentam grande estabilidade frente a variações no meio (Batista, Carvalho, Santos-Magalhães, 2007).

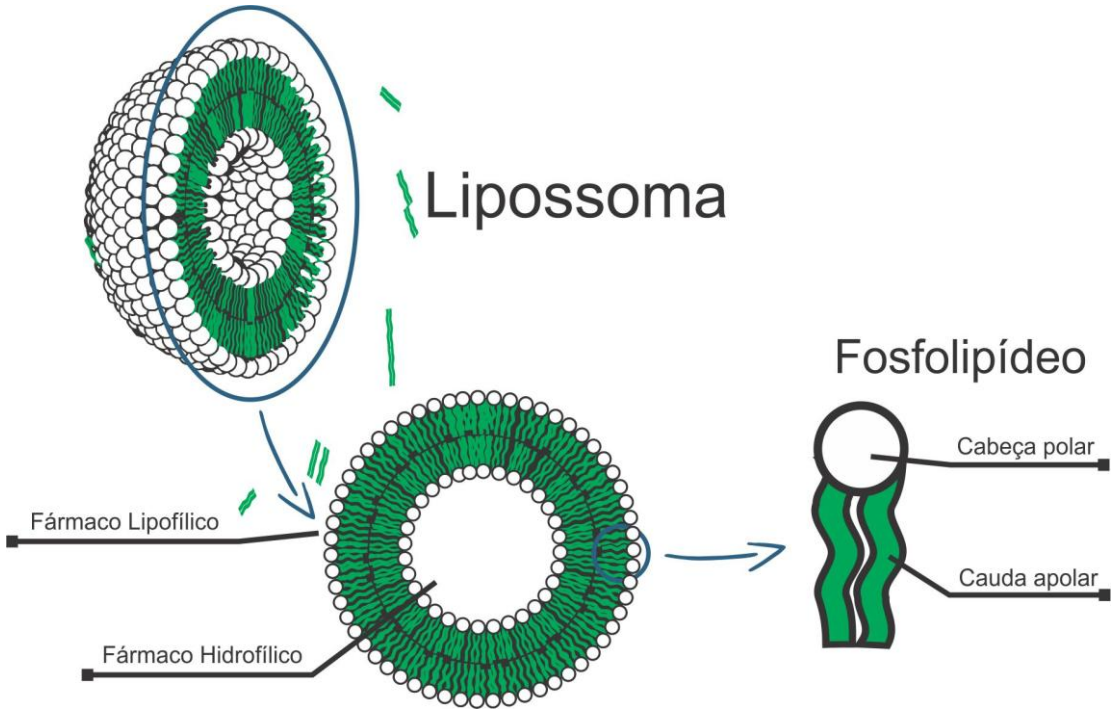


Figura 2: Estutura de um lipossoma covencional.

Os lipossomas são classificados de acordo com seu tamanho e número de bicamadas lipídicas. Os nanossistemas que apresentam uma bicamada lipídica única são denominados unilamelares e o que apresentam bicamadas múltiplas são denominados multilamelares (Šegota, Težak, 2006). Desta forma, existem as vesículas unilamelares pequenas (SUV – *small unilamellar vesicles*) que medem entre 20 a 100 nanômetros de diâmetro, as vesículas unilamelares grandes (LUV – *large unilamellar vesicles*) que apresentam diâmetro superior a 100 nm e as vesículas multilamelares (MLV- *multilamellar vesicles*) que apresentam diâmetros superiores a 0,5 µm (Figura 3). Cada bicamada lipídica apresenta espessura que varia entre 5 a 20 nm, dependendo das concentrações lipídicas e do método de produção dos lipossomas. (Gharib et al, 2015).

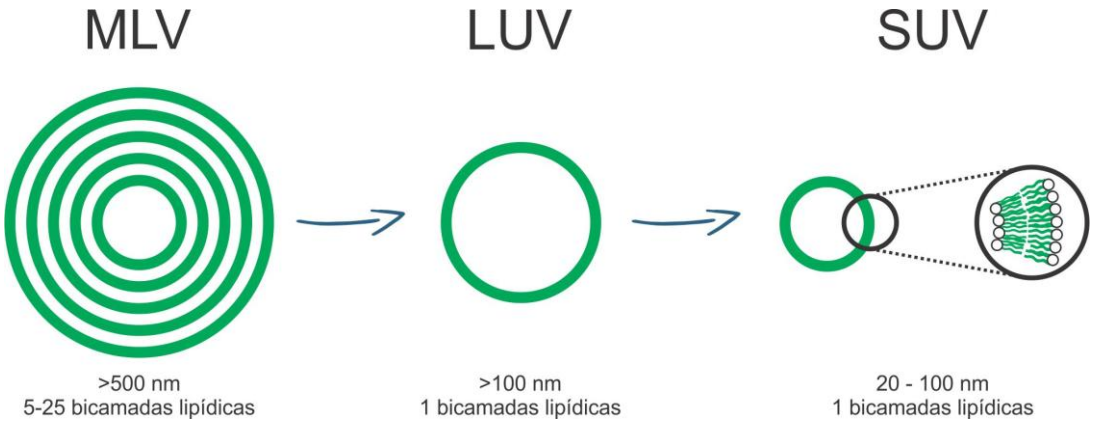


Figura 3: Classificação dos lipossomas de acordo com o tamanho e número das bicamadas lipídicas.

O uso de lipossomas como sistema de liberação controlada de fármacos teve início logo após sua invenção por Bangham e colaboradores em 1965 (Bangham, Standish, Watkins, 1965). Esses referidos nanossistemas entram nas células principalmente através de mecanismos de endocitose, onde os lipossomas são englobados pela a membrana celular e levados ao citoplasma (Ziello, Huang, Jovin, 2010). A liberação da molécula encapsulada nos nanocarreadores depende de inúmeros fatores, em especial da velocidade de difusão do fármaco no meio em que se encontra ou da degradação das bicamadas lipídicas (Ninomiya et al, 2016).

Os primeiros medicamentos à base de lipossomas (Myocet e Doxil) foram aprovados pela agência regulatória americana *Food and Drug Administration* (FDA) para o tratamento de câncer em 1995 (Pinheiro et al, 2011). Desde então, uma vasta gama de agentes terapêuticos, incluindo fármacos lipofílicos (a exemplo do paclitaxel) e hidrofílicos (a exemplo da hidroxiureia) foram encapsulados nesses nanossistemas com a finalidade de superar limitações apresentadas pelas moléculas e melhorar a sua eficácia. Hoje existem cerca de 8 formulações farmacêuticas à base de lipossomas no mercado e mais 53 formulações lipossomais em diferentes fases de ensaios clínicos (Movahedi et al, 2015).

Os lipossomas podem ter a sua estrutura básica alterada, possibilitando uma maior aplicação terapêutica. Modificações da superfície utilizando polímeros hidrofílicos, tais como o polietilenoglicol (PEG), prolongam o tempo de circulação dos lipossomas (Torchilin, 2009). Isso ocorre porque o recobrimento de PEG permite que os nanocarreadores escapem do sistema retículo-endotelial, permanecendo mais tempo na corrente sanguínea e chegando a maiores quantidades no sítio-ativo. Deste modo os lipossomas de longa circulação, também chamados furtivos, proporcionam uma maior biodisponibilidade ao fármaco encapsulado (Periyasamy et al, 2012).

Como exemplo de outra alteração estrutural de lipossomas, podemos citar a utilização de ligantes conjugados nos lipídeos de superfície dos nanocarreadores (Elsabahy, Wooley, 2012). Essa técnica pode ser aplicada para produção de lipossomas sítio-específicos, que conferem direcionamento do fármaco encapsulado a sítio-ativos de células e órgãos, aumentando a efetividade e diminuindo os efeitos colaterais dos tratamentos (Li, An, Yan, 2015).

Deste modo temos os lipossomas convencionais, que podem ou não apresentar carga de superfície, os lipossomas furtivos ou de longa circulação e os lipossomas sítios-específicos (Figura 4).

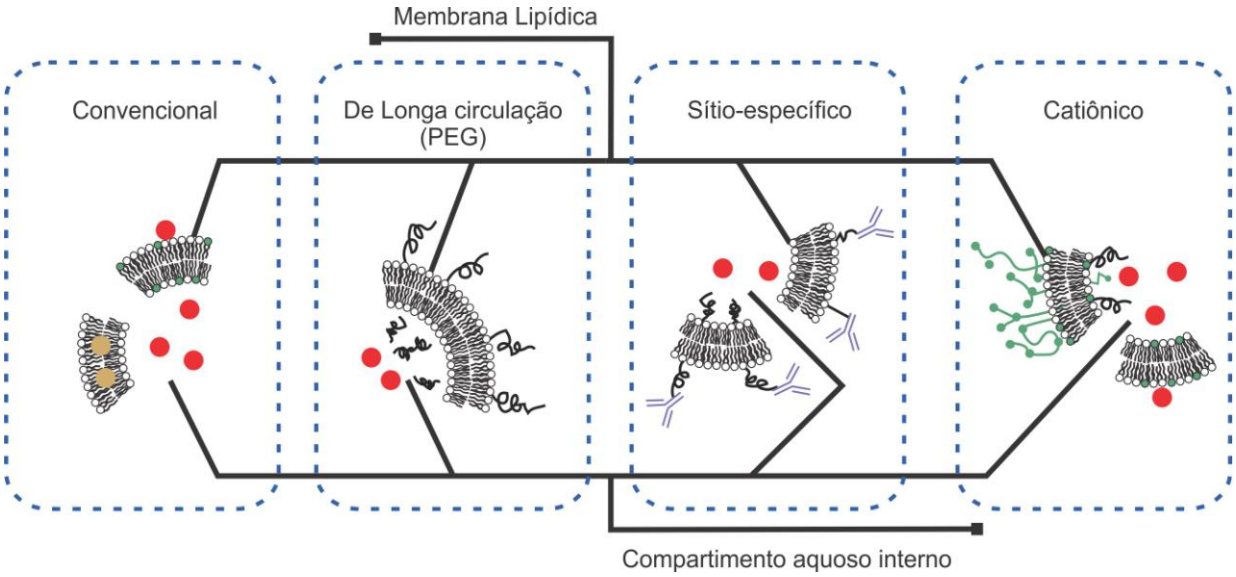


Figura 4: Tipos de lipossomas.

2.6 Nanopartículas

Nanopartículas são sistemas coloidais constituídos por carreadores de escala nanométrica que podem veicular fármacos ou agentes bioativos (Wong et al, 2007; Invernici et al, 2011). Apresentam tamanhos que variam de 10-1000 nm (Irache et al, 2011) e podem ser produzidas utilizando polímeros, lipídeos ou ambos os materiais (Alyaudtin et al, 2001; Kim, Martin, 2006).

Nanopartículas podem ser classificadas como nanoesferas ou nanocápsulas, de acordo com a metodologia empregada na sua produção. Nanocápsulas são sistemas nos quais o fármaco está confinado a uma cavidade oleosa rodeada por uma membrana polimérica única, enquanto nanoesferas são sistemas com uma estrutura compacta em que o agente terapêutico está retido dentro da matriz coloidal ou revestido na superfície da partícula por conjugação ou adsorção (Figura 5) (Couvreur et al, 2002; Couvreur e Vauthier, 2006) .

Nos últimos anos, as nanopartículas poliméricas têm se mostrado de grande interesse para as ciências farmacêuticas, uma vez que vem sendo demonstrado que esses carreadores oferecem proteção adequada contra a degradação, além de promover uma liberação controlada do composto encapsulado (Inchaurraga et al, 2014). As vantagens da utilização desses nanossistemas como carreadores de fármaco são: alta estabilidade quando em contato

com fluidos biológicos, elevada capacidade de encapsulação de moléculas, viabilidade de incorporação de compostos hidrofílicos e hidrofóbicos e propriedades de liberação sustentada (Irache et al, 2011).

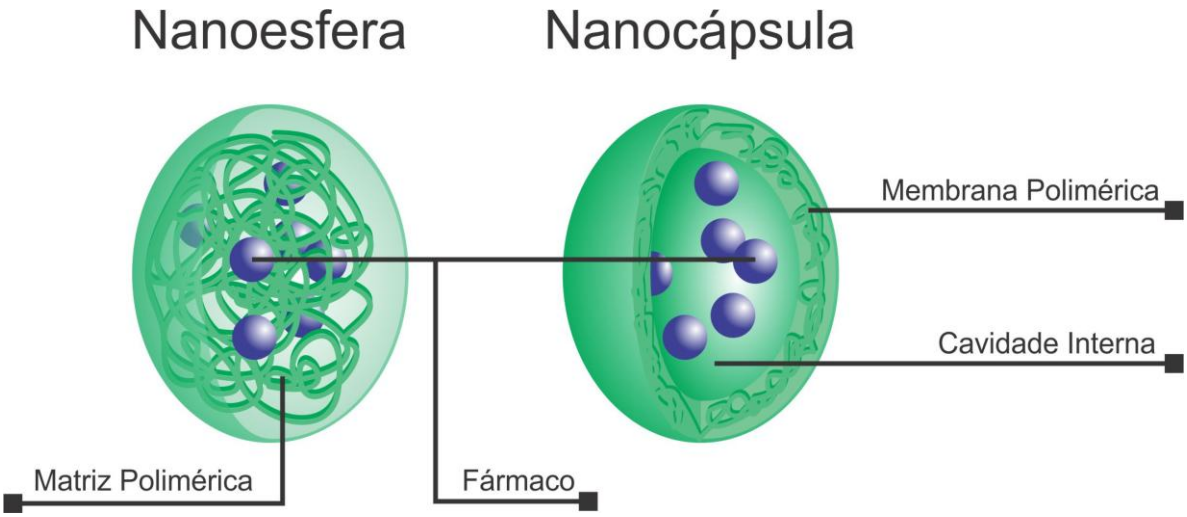


Figura 5: Estrutura das nanopartículas (nanoesfera e nanocápsula).

Vários estudos comprovaram a capacidade das nanopartículas poliméricas de melhorar a absorção e a biodisponibilidade de moléculas fracamente absorvidas após administração via oral. Esses nanocarreadores apresentaram bons resultados como sistemas de liberação controlada de insulina (Damge et al, 1988; Babu et al, 2008), ciclosporina (El-Shabouri, 2002; Italia et al, 2007), 5-fluorouridina (Arbos et al., 2004) e gencitabina (Reddy, Couvreur, 2008).

Os resultados supracitados parecem estar relacionados a propriedades físico-químicas e biofarmacêuticas das nanopartículas. Entre outros fatores, a capacidade desses nanocareadores de desenvolver interações bioadesivas com o intestino seria um dos aspectos mais importantes na promoção da absorção oral do medicamento encapsulado (Agüeros et al, 2009). As nanopartículas administradas por via oral podem interagir com a superfície gastrointestinal e desenvolver fenômenos adesivos (bioadesão ou mucoadesão), aumentando o seu tempo de permanência em contato estreito com a mucosa ou proporcionando um direcionamento a uma zona específica do intestino (Porfire et al, 2010).

No entanto, quando as nanopartículas são administradas por via oral apenas uma pequena parte do fármaco encapsulado parece atingir a mucosa gastrointestinal. Isso se deve ao fato de que para chegar à mucosa intestinal, as nanopartículas devem evitar eventuais interações com proteínas ou restos de alimentos presentes no lúmen de intestino, para então ultrapassar a barreira de muco. Além disso, os carreadores que interagem com os compostos

de muco podem ser aprisionados no gel de secreção mucosa e então removidos (Ponchel, Irache, 1998; Yoncheva, Lizarraga, Irache, 2005).

Para a produção de nanopartículas de administração via oral, faz-se então necessária a utilização de um polímero que, além de não-tóxico e biocompatível não interaja com os componentes do muco intestinal, sendo mucopenetrantes.

Vários estudos vêm sendo realizados utilizando diferentes tipos de polímeros e copolímeros na fabricação de nanopartículas. Entre eles, o copolímero poli (metil vinil éter-co-anidrido maleico), disponível comercialmente com o nome de Gantrez®, é um polianidrido que permite uma preparação eficiente e fácil de nanopartículas sob condições suaves, utilizando um método de deslocamento de solvente (Rebouças et al, 2012).

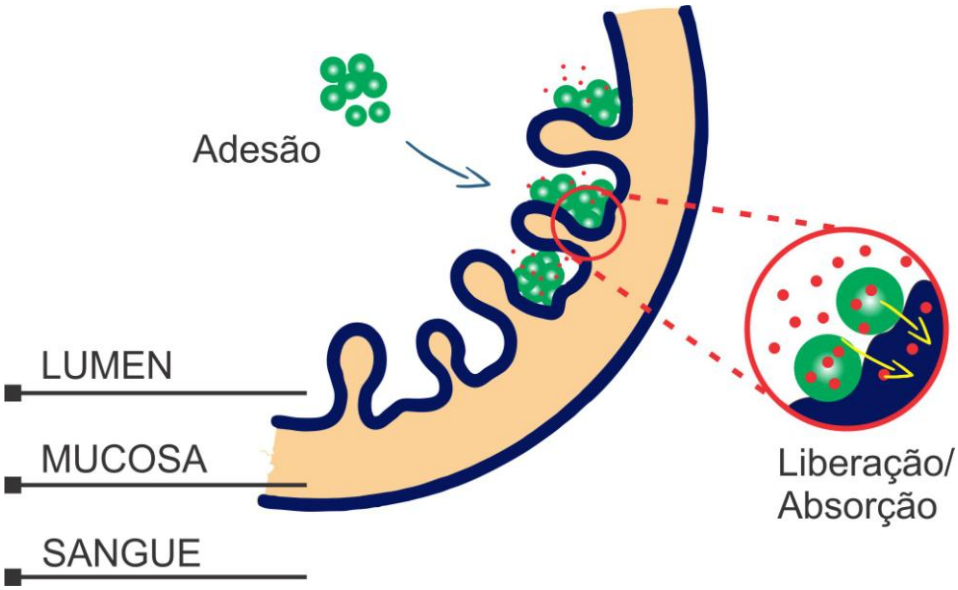


Figura 6: Representação da interação das nanopartículas bioadesivas com a mucosa intestinal.

As nanopartículas Gantrez®-PEG têm demonstrado uma elevada capacidade para desenvolver interações mucopenetrantes dentro do intestino, o que facilita o contato estreito do fármaco encapsulado com a superfície das células de absorção intestinais. Além disso, a presença do PEG pode adicionar um benefício suplementar uma vez que tem sido descrito que o referido polímero pode afetar negativamente a atividade da glicoproteína-P e da isoenzima citocromo P450 3A4, aumentando a presença do fármaco nos seus sítios-ativos (Zabaleta el al, 2012).

Outro polímero que vem sendo testado com êxito na produção de nanopartículas é a zeína. A zeína é a maior proteína de armazenamento de milho com um caráter hidrofóbico

como resultado de sua composição de aminoácidos (altos teores de leucina, prolina e alanina) (Padua, Wang 2009). O referido polímero é insolúvel em água, mas é solúvel em uma solução aquosa de álcool e pode ser processado para formar películas, fibras, micropartículas ou nanopartículas (Peñalva et al, 2015). Além disso, a zeína é conhecida pela sua elevada resistência térmica e por formar uma barreira protetora contra a oxidação, que pode ser de interesse para a encapsulação ou incorporação de compostos sensíveis à oxidação ou à temperatura (Penãlva et al, 2017).

A nanomedicina constitui uma ferramenta muito útil no desenvolvimento de formulações para a terapia das patologias que atingem o SNC (Zhao, 2016). Como exposto anteriormente, a nimodipina e a quercetina são fármacos que apresentam a capacidade de atravessar a barreira hematoencefálica, todavia apresentram baixa biodisponibilidade, e consequentemente baixa atividade, quando administrados por via oral. Nesse contexto, a nanotecnologia na presente investigação não seria aplicada como promotora ou facilitadora da passagem do fármaco pela BHE, mas como meio usado para aumentar a concentração sanguínea do composto administrado.

Dessa forma a encapsulação da nimodipina em nanocarreadores adaptados à via parenteral (lipossomas) ou oral (nanopartículas de Gantrez® sem recobrimento e de Gantrez® recobertas com PEG), assim como a encapsulação da quercetina em nanopartículas bioadesivas de zeína, apresentam-se como estratergias promissoras para incrementar a biodisponilidade da nimodipina e da quercetina.

3. OBJETIVOS

3.1. Objetivo Geral

- Avaliar a atividade terapêutica de fármacos (nimodipina e quercetina) veiculados em nanocarreadores (lipossomas e nanopartículas) para aplicação no tratamento de patologias que afetam o sistema nervoso central (SNC).

3.2. Objetivos Específicos

- Produzir sistemas de liberação controlada para nimodipina (lipossomas e nanoparticulas mucopenetrantes) ou quercetina (nanopartículas mucoadesivas) com tamanho, homogeneidade, carga de superfície e alto teor de fármaco encapsulado;

- Avaliar a atividade ansiolítica da nimodipina encapsulada em lipossomas (NMD-Lipo) através dos modelos animais de campo aberto, claro e escuro e labirinto em cruz elevado;

- Avaliar o mecanismo de ação ansiolítico da nimodipina encapsulada em lipossomas (NMD-Lipo);

- Avaliar a toxicidade sistêmica e a neurotoxicidade dos lipossomas contendo nimodipina (NMD-Lipo) em camundongos;

- Avaliar a atividade anticonvulsivante da nimodipina encapsulada em lipossomas (NMD-Lipo) através do modelo animal de convulsão induzido por pilocarpina;

- Avaliar a atividade antidepressiva da nimodipina encapsulada em lipossomas (NMD-Lipo) através dos modelos animais de suspensão pela cauda, nado forçado e atividade da enzima Monoamina oxidase B;

- Avaliar a biodisponibilidade da nimodipina encapsulada em nanopartículas mucoadesivas (NMD-NP), bem como em nanopartículas mucopenetrantes (NMD-NP/PEG) após administração oral em ratos;

- Avaliar a atividade da nimodipina encapsulada em nanopartículas mucopenetrantes (NMD-NP/PEG) na atividade ansiolítica e na proteção contra os déficits de memória causados pelo estresse induzido por costicosterona em camundongos.

- Avaliar a atividade da quercetina encapsulada em nanopartículas de zeína em um modelo murino da Doença de Alzheimer (Samp8).

4. DEVELOPMENT AND EVALUATION OF LIPOSOMAL FORMULATION CONTAINING NIMODIPINE ON ANXIOLYTIC ACTIVITY IN MICE

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Abstract

Nimodipine has been investigated in the treatment of anxiety. Its administration, however, presents a number of limitations, particularly by low bioavailability, low aqueous solubility and photosensitivity. These difficulties can be resolved by the use of nanometer-scale pharmaceutical carriers. The goal of the present study was thus to develop a liposomal formulation containing nimodipine (NMD-Lipo) and evaluate anxiolytic activity using models of anxiety (open-field, light and dark and elevated plus-maze test). The results suggest that administration of NMD-Lipo has no sedative or muscle relaxant effect in animals, since there was no reduction in the number of crossings, grooming and rearings. The increased residence time of the animals treated with NMD-Lipo in the bright field is a reflection of the anxiolytic-like activity of the formulation. Furthermore, the reduction in residence time of rodents treated with the combination of flumazenil and NMD-Lipo in the illuminated box suggest that NMD-Lipo act on benzodiazepine receptors. The increase in the number of entries and length of stay in the open arms of mice treated with NMD-Lipo suggest the anxiolytic activity of the formulation and the reduction in number of entries and length of stay in the open arms of rodents treated with a combination of flumazenil and NMD-Lipo suggest that NMD-Lipo act on benzodiazepine receptors.

Keywords: Anxiolytic; Liposome; Mice; Nimodipine

1. Introduction

Anxiety is an emotional state that is part of human existential, since normal circumstances in people's lives, such as the development of some kind of physical and mental suffering, as well as changes in everyday life, may be associated with its onset. It is a type of emotion that has been shaped by natural selection, since it makes people alert to impending dangers (Richey et al., 2010). However, anxiety may cease to be a natural occurrence and progress to pathological condition when it occurs disproportionately to the triggering event that the cause or when there is no apparent reason for its onset (Salomons et al, 2010).

Pathological anxiety is characterized by excessive and uncontrollable worry about a considerable number of factors, in which the individuals involved have experienced at least three of the following symptoms: feeling keyed up or on edge, sleep disturbance, muscle tension, being easily fatigued, difficulty concentrating or having one's mind go blank, and irritability (Maack et al., 2012). A pathological form is debilitating, reduces the quality of life of patients and is associated with an increased risk of death and suicide (Zou et al., 2012).

Treatments currently applied for anxiety disorders include pharmacotherapy and cognitive behavioral therapy (Bartley et al., 2013). The pharmacological treatment of pathological anxiety consists of the use of benzodiazepines, buspirone and antidepressants. Athough this drugs shows great efficacy in the therapy of pathology, its administration has many drawbacks. For example, benzodiazepines can cause some side effects such as amnesia, induction of dependence and sedation which cause inconveniences for the patients (Raupp et al., 2008). The search for new therapeutic agents with anxiolytic properties is therefore of paramount importance.

Research has shown that the excessive flow of calcium through the membrane, which results in increased levels of intracellular ion, may play a role in the pathophysiology of affective disorders (Maigaard et al., 2012), epileptiform activity (N'Gouemo, 2013) and in the

induction of anxiety (Kumar et al., 2012). From this perspective, the application of nimodipine, a selective antagonist of L-type calcium channels, has been investigated in the treatment of numerous neurological disorders (Yanpallewar et al., 2004).

Nimodipine has high lipophilicity and hence easily crosses the blood brain barrier. Studies have concluded that this drug has the ability to increase cerebral blood flow and its use in the treatment of ischemia present in numerous pathologies affecting the brain (Aslan et al., 2009), besides being useful in the therapy of mood disorders (Frye et al., 2003; Pazzaglia et al., 1995), in treatment of senile dementia (Chalikwar et al., 2012), and displaying anticonvulsant properties (Marinho et al, 1997; Mikati et al, 2004; Nascimento et al, 2005).

However, the administration of nimodipine has a number of limitations, owing chiefly to its high first-pass effect in liver, which results in decreased bioavailability, low aqueous solubility and photosensitivity (Sun et al., 2013). These difficulties can be overcome through the use of nanometer-scale pharmaceutical carriers. These nanosystems are useful tools to improve the pharmacokinetic profile of drugs that have limited pharmaceutical applicability (Santos-Magalhães and Mosqueira, 2010). Furthermore, nanotechnology is great to improve the therapy of diseases that affect the central nervous system because the drugs applied in those treatments normally cannot cross the blood-barrier brain and could substantially benefit from the use of nanocarriers (Wong et al, 2012).

Based on these findings, the goal of the present study was twofold: the design of a liposomal formulation containing nimodipine and the evaluation of the drug anxiolytic effects tested in three animal models of anxiety (open field, the light and dark and the elevated plus-maze test).

2. Material and methods

2.1. Material

Cholesterol (Chol), trehalose, nimodipine, diazepam and flumazenil were purchased from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Production of liposomal formulation derived from nimodipine (NMD-Lipo)

Liposomes containing nimodipine (NMD-Lipo) were prepared using the method of hydrating the lipid film (Lira et al., 2009). NMD-Lipo were produced using the lipids soybean phosphatidylcholine and cholesterol (117.6 mM) at 8:2 ratio and drug concentration of 1.0 mg/ml. These constituents were dissolved in a mixture of chloroform: methanol (3:1 v/v) under magnetic stirring. The solvents were removed in vacuum evaporation at 80 rpm for 60 min at 37 ± 1 °C, result in a thin lipid film. This film was then hydrated with 10 ml of pH 7.4 phosphate buffer solution resulting in the production of large multilamellar vesicles (MLV). This liposomal suspension was then subjected to sonication (Vibra Cell, Branson, USA) at 200 W and 300 Hz for 40 s to obtain small unilamellar liposomes (SUV).

2.3. Characterization of NMD-Lipo

After 24 hours of production, NMD-Lipo was characterize by evaluating the features: macroscopic aspects, pH, particle size, polydispersity index, zeta potential, drug content and encapsulation efficiency. The pH of the liposomes was measured using a digital pH meter (Bioblock Scientific 99, Prolabo, Paris, France) at room temperature. The particle size and polydispersity of the liposomes were determined using photon correlation spectroscopy (Particle Analyzer™ Delsa Nano S, Beckman-Coulter, USA). For this analysis 300 μL of the

liposomal suspension was diluted in 1 ml of dionized water (Milli Q Plus, Millipore, USA). The zeta potential of the liposomes, corresponding to the surface charge of the vesicles, was measured using a Zetatrac NC-148 apparatus (Microtrac, USA). A sample of the liposomes (50 µL) was diluted in 5 mL of dionized water before analysis.

The content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm. A standard curve of nimodipine was prepared at concentrations of 0.5, 1, 2, 3, 4, 5 and 6 µg/ml of nimodipine using methanol as solvent. Subsequently, an aliquot of liposomes (30 µl) was diluted in methanol to a final concentration of theoretical 3 µg/ml of nimodipine

The encapsulation efficiency of nimodipine into liposomes was determined by the technique of ultrafiltration/ultracentrifugation using the Ultrafree® units (Millipore, USA). A liposomal sample aliquot (400 µl) was transferred to filtering units and subjected to ultracentrifugation at 8776 g for 1 h. The amount of encapsulated nimodipine was obtained from the difference between the total quantity measured in the formulation and that of the filtrate obtained after centrifugation. The readings were performed at 237 nm.

2.4. Studies of anxiolytic activity of NMD-Lipo

2.4.1. The experimental units

Animal models of anxiety are applied for the evaluation of anxiolytic or anxiogenic compounds, as well as the identification of their mechanisms of action and study of the neurobiology of disease. We used male *Swiss* mice aged 2 months of age and weighing 25-30 g, from the Central Animal Facility of the Center for Agricultural Sciences, Federal University of Piauí. The animals used in the experiment remained on the premises of the Experimental Neurochemistry Laboratory Research, for seven days, for proper acclimatization. The experimental units received water and diet (Labina ®) *ad libitum* and were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) and temperature (25

± 1 °C). The experimental protocols and procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA / UFPI N° 014/11).

2.4.2. Treatments

The mice were divided into thirteen groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), diazepam at a dose of 2 mg/kg (positive control), nimodipine at doses of 0.1, 1 and 10 mg/kg (groups Free NMD 0.1, 1 and 10 respectively), NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10 respectively), flumazenil at a dose 2.5 mg/kg (group Flu), flumazenil in combination with diazepam (group Flu + DZP) and flumazenil in combination with NMD-Lipo (groups Flu + NMD-Lipo 0.1, Flu + NMD-Lipo 1, and Flu + NMD-Lipo 10). The behavioral assessments were carried out 30 minutes after drug administration.

2.4.3. Open Field Test

The motor activity of the animals was observed by means of an acrylic open field (transparent walls and black floor, 30 x 30 x 15 cm) and divided into nine equal quadrants based on the model described by Archer (1973). After 30 minutes of treatment, the animals were placed, one at a time, in the center of the field for quantification of the number of crossings with four legs (spontaneous locomotor activity), number of self-cleaning behavior (grooming) and the number of lifting (rearing) without abutting the wall during the period of 5 minutes.

2.4.4. The Light and Dark Test

The apparatus used is made of acrylic divided into two compartments (bright field and dark field) that communicate through a small door 5 by 5 cm (Crawley, 1985). The dark field

(black acrylic, 27 x 18 x 29 cm) is poorly lit. The bright field (acrylic, 27 x 18 x 29 cm) is illuminated by ambient light. Each animal was observed for 5 minutes. The parameter used is the dwell time in the bright field in seconds.

2.4.5. Elevated Plus-Maze Test

The elevated plus-maze for mice (Lister, 1987) consists of two opposing open arms (30 x 5 cm) and two closed (30 x 25 x 5 cm), likewise opposing cross-shaped arms. The open and closed arms are connected by a central platform (5 x 5 cm) high and 45 cm from the floor. The animals were placed in the center of the apparatus with the head turned to towards one of the closed arms and their behavior was observed for 5 minutes. The behavioral measures were recorded: number of entries in the open arms (NEOA) and length of stay of the animal in the open arms (LSOA).

2.5. Statistical Analyses

Values are expressed as mean ± standard error of mean (SEM) of the number of animals used in experiments. Differences between groups were determined by analysis of variance (ANOVA) followed, when a difference was detected, by the Student-Newman-Keuls t-test with post hoc test. The significance level for rejecting the null hypothesis was always $p \leq 5\%$.

3. Results

3.1. Characterization of NMD-Lipo

The data of NMD-Lipo properties indicates that the liposomes are small unilamellar vesicles (SUV) with size 107.17 ± 1.53 nm, homogeneity (PDI= 0.303), pH of 7.4, surface charge of -5.32 ± 1.29 mV and drug encapsulation efficiency of 99 ± 0.45%.

3.2. Open Field Test

The results are summarized in Table 1. In the open field test, there was a decrease in the number of crossings, rearings and groomings of diazepam-treated mice compared to the control group. The animals receiving both free NMD and NMD-lipo at doses of 0.1, 1 and 10 mg/kg showed no reduction in the number of crossings, rearings and groomings. The groups treated with flumazenil and flumazenil combinations with diazepam and NMD-lipo also showed no significant differences when compared to the control group.

Table 1: Effects of free NMD, NMD-Lipo, flumazenil, diazepam and their associations in mice using the open-field test.

Groups	Number of Crossings	Number of Rearings	Number of Groomings
Control	63.14 ± 2.60	3.80 ± 0.75	25.60 ± 1.63
DZP	30.43 ± 1.21[a]	1.80 ± 0.24[a]	12.80 ± 0.97[a]
Free NMD 0,1	63.14 ± 2.36[b]	3.80 ± 0.37[b]	25.60 ± 1.69[b]
Free NMD 1	63.86 ± 3.04[b]	4.00 ± 0.44[b]	25.20 ± 1.77[b]
Free NMD 10	63.71 ± 3.38[b]	4.00 ± 0.32[b]	24.80 ± 1.35[b]
NMD-Lipo 0,1	66.71 ± 1.60[b]	4.00 ± 0.32[b]	24.20 ± 1.88[b]
NMD-Lipo 1	67.86 ± 2.44[b]	4.00 ± 0.32[b]	24.20 ± 2.31[b]
NMD-Lipo 10	66.57 ± 3.95[b]	4.00 ± 0.32[b]	25.20 ± 1.68[b]
Flu	67.29 ± 4.79[b]	4.20 ± 0.37[b]	26.60 ± 1.29[b]
Flu + DZP	74.29 ± 3.97[b]	4.20 ± 0.37[b]	27.00 ± 1.76[b]
Flu +NMD-Lipo 0,1	71.57 ± 3.43[b]	4.20 ± 0.37[b]	27.60 ± 1.07[b]
Flu + NMD-Lipo 1	72.00 ± 2.86[b]	4.20 ± 0.49[b]	27.40 ± 0.98[b]
Flu + NMD-Lipo 10	71.71 ± 4.97[b]	4.20 ± 0.37[b]	25.60 ± 1.88[b]

Values are mean ± S.E.M. of number of crossings, rearings and groomings of mice (n=8). [a]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from control group, [b]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from diazepam group.

3.3. Light and Dark Test

The results are summarized in Table 2. In the light and dark test, the animals treated with diazepam a dose of 2 mg/kg showed a significant increase in the time spent in the light field when compared to the control group. The mice treated with free NMD at the doses of 0.1, 1 and 10 mg/kg showed no change in time spent in the light field. For their part, rodents treated with NMD-Lipo at the doses of 0.1, 1 and 10 mg/kg were shown to spend a significantly longer time in the bright field than the control group. The mice pretreated with flumazenil and subsequently given with diazepam and NMD-lipo showed a reduction in the time spent in the light field, compared to animals that did not receive the antagonist.

Table 2: Effects of free NMD, NMD-Lipo, flumazenil, diazepam and their associations in mice in the light and dark test.

Groups	Time spent in the bright field (s)
Conrol	92.50 ± 2.59
DZP	124.5 ± 6.75[a]
Free NMD 0.1	94.83 ± 3.26
Free NMD 1	98.17 ± 3.20
Free NMD 10	109.2 ± 5.60
NMD-Lipo 0.1	124.3 ± 4.85[a,c]
NMD-Lipo 1	148.7 ± 8.36[a,b,d]
NMD-Lipo 10	149.0 ± 7.20[a,b,e]
Flu	92.50 ± 0.76
Flu + DZP	91.83 ± 2.69[b]
Flu + NMD-Lipo 0.1	95.00 ± 1.21[f]
Flu + NMD-Lipo 1	104.5 ± 7.20[g]
Flu + NMD-Lipo 10	102.8 ± 2.81[h]

Values are mean ± S.E.M. of the time spent in the bright field of 8 mice (by group) used in the experiments. [a]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from control. [b]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from diazepam group. [c]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test), significantly different from free NMD 0.1 group. [d]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from free NMD 1 group. [e]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from free NMD 10 group. [f]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from NMD-Lipo 0,1 group. [g]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from

NMD-Lipo 1 group. [h]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from NMD-Lipo 10 group.

3.4. Elevated Plus-Maze Test

The results are summarized in Table 3. In the elevated plus-maze test, the animals treated with diazepam a dose of 2 mg/kg showed a significant increase in the number of entries and length of stay in the open arms when compared to the control group. The rodents that received free NMD at the doses of 0.1, 1 and 10 mg/kg showed no change in the number of entries and a modest increase in length of stay in the open arms when compared to the control group. The mice treated with NMD-lipo at the doses of 0.1, 1 and 10 mg/kg exhibited a significant increase in the number of entries and length of stay when compared to the control group. The mice pretreated with flumazenil and subsequently given with diazepam and NMD-lipo showed a reduction in the number of entries and length of stay in the open arms, compared to animals that did not receive the antagonist.

Table 3: Effects of free NMD, NMD-Lipo, flumazenil, diazepam and their associations in mice in the elevated plus maze test.

Groups	Number of entries in the open arms	Length of stay in the open arms
Control	5.86 ± 0.40	40.43 ± 3.75
DZP	7.57 ± 0.65[a]	93.71 ± 1.51[a]
Free NMD 0.1	3.29 ± 0.36	68.71 ± 5.41[a]
Free NMD 1	3.71 ± 0.68	87.43 ± 3.68[a]
Free NMD 10	4.57 ± 0.43	83.29 ± 10.02[a]
NMD-Lipo 0.1	7.14 ± 0.88[a,c]	111.7 ± 2.05[a,b,c]
NMD-Lipo 1	7.86 ± 0.86 [a,d]	118.7 ± 5.74[a,b,d]
NMD-Lipo 10	8.29 ± 0.92[a,b,e]	128.0 ± 8.98[a,b,e]
Flu	5.86 ± 0.55	39.14 ± 3.54
Flu + DZP	5.86 ± 0.74[b]	41.00 ± 1.76[b]
Flu + NMD-Lipo 0.1	5.71 ± 0.68[f]	43.86 ± 1.10[f]
Flu + NMD-Lipo 1	6.29 ± 0.60[g]	42.43 ± 1.34[g]
Flu + NMD-Lipo 10	5.57 ± 0.37[h]	43.14 ± 1.30[h]

Values are mean ± S.E.M. of the number of entries and length of stay in the open arms of 8 mice (by group) used in the experiments. [a]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from control. [b]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from diazepam group. [c]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test), significantly different from NMD 0.1 group. [d]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from NMD 1 group. [e]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from nimo 10 group. [f]p <0.05 (ANOVA followed by t-test Student-Newman-Keuls with post hoc test) significantly different from NMD-Lipo 0,1 group. [g]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from NMD-Lipo 1 group. [h]p <0.05 (ANOVA followed by Student-Newman-Keuls t-test with post hoc test) significantly different from NMD-Lipo 10 group

4. Discussion

Anxiety is usually diagnosed based on reports by patients, which demonstrates the subjective nature of the disorder and represents a challenge for the development of animal models for this disease. However, since the introduction of anxiolytics, which enabled pharmacological validation tests and a better understanding of the pathophysiology of the disease, the conducting of experiments that mimic anxiety has received a considerable boost (Kumar et al, 2013).

Over the past 50 years, anxiolytic benzodiazepines have been the drugs of choice for treatment of anxiety. However, despite showing benefits well described and characterized, benzodiazepines can also cause numerous side effects, such as sedation, muscle relaxation, anterograde amnesia and physical dependence, which lead patients to seek alternative therapies (Kapan and Sadock, 2005; Sousa et al., 2008). In this context, the production of liposomes containing nimodipine has emerged as a possible alternative for the treatment of anxiety.

In the present study, diazepam was chosen as positive control because it is the benzodiazepine reference drug. Benzodiazepines are widely used in the treatment of anxiety, sleep disorders, seizures and other diseases, and are among the most commonly prescribed psychotropic medications worldwide, with a prevalence of use of around 2-7 %. Despite the well-described benefits, the misuse of benzodiazepines can cause a number of problems, such as deficits in learning, attention, memory and depression, as well as falls, car accidents and other hazards to the user (Quaglio et al., 2012). Furthermore, the risk of dependence after long-term use has been described, as reflected in the appearance of a series of symptoms when the drug is abruptly withdrawn (Galdino et al., 2012).

Flumazenil was used in the experiment because it is a nonselective antagonist of the benzodiazepine binding site of GABAA receptors (Divljakovi´c et al., 2013). Its

administration assists in elucidating the mechanism of action of the formulation, since the possible decrease in anxiolytic effects in animals treated with flumazenil before receiving NMD-Lipo, and the resulting channel antagonism of benzodiazepines, is indicative of the action of nanoencapuslated nimodipine in such receptors.

As sedation and muscle relaxation are major annoyances reported by patients undergoing treatments fort anxiety, it is vital that new anxiolytic formulations do not present these adverse reactions. Thus, the open field test is sorely needed in the search for new compounds to combat the aforementioned disorder, since it enables the assessment of the animal´s motor activity, detecting whether the administration of the test compound caused changes in the consciousness or muscle tone of mice.

The decrease in the number of crossings, rearings and groomings of diazepam-treated mice compared to the control group suggests that diazepam reduced the animals´ mobility. The absence of reduction in the number of crossings, rearings and groomings of animals that received both free NMD and NMD-Lipo suggests that the drug and the liposomal formulation which it contains do not alter the mobility of the animals, as occurs with diazepam.

The absence of changes in the mobility of rodents in the groups treated with NMD-Lipo constitutes a major advance in drug development against anxiety, as sedation and muscle relaxation are major complaints of patients that using the anxiolytic drugs currently available on the market.

The light and dark test anxiety is generated by conflict resulting from the tendency to explore both compartments and their innate aversion to bright environments. Thus, the increase in light-dark transitions and the increase in stay of the animal in the bright field are indicative of the anxiolytic activity of the formulation (Enkel et al., 2013).

The mice treated with free NMD showed no change in time spent in the light field, which suggests that the drug has no anxiolytic activity at the doses tested. On the other hand,

the increased residence time of the animals treated with NMD-Lipo in the bright field is evidence of the nimodipine anxiolytic-like activity when encapsulated into liposomes. The results of mice treated with the liposomal formulation were similar (NMD-Lipo 0.1) and better (NMD-Lipo 1 e 10) to the group treated with diazepam and much better than in the animals treated with non-encapsulated nimodipine, which suggests that the encapsulation of the drug into liposomes promoted its controlled release, increasing its bioavailability and improving its effect. Furthermore, the reduction in residence time in the illuminated box of rodents treated with the combination of flumazenil and NMD-Lipo, compared with the animals treated only with NMD-Lipo, suggests that NMD-Lipo act on benzodiazepine receptors (Table 2).

The elevated plus-maze test use as a measure of anxiety is based on the natural aversion of rodents for open spaces. The larger the exploratory capacity of the animals and the longer it remains in the open arms, the lower is their level of anxiety. The validity of the elevated plus-maze animal model of anxiety is based on the animal´s sensitivity to a number of anxiolytic and anxiogenic drugs and treatment for stressors (Casarrubea et al., 2013).

The increase in the number of entries and length of stay in the open arms of the animals treated with NMD-Lipo demonstrated the anxiolytic-like activity of the formulation. In addition, the reduction in the number of entries and length of stay in the open arms of rodents treated with the combination of flumazenil and NMD-Lipo, compared with the animals treated only with NMD-Lipo, corroborates the hypothesis that the drug and the liposomal formulation act on benzodiazepine receptors (Table 3).

The performance of rodents receiving NMD-lipo 0.1 and NMD-lipo 1 in the elevated plus-maze test were similar to that of the animals treated with diazepam, while the behavior of the animals treated with NMD-Lipo 10 was much better than in those treated with diazepam. Moreover, the effects of nimodipine encapsulated into liposomes were significantly better

than the group treated with non-encapsulated nimodipine. These results suggest that the encapsulation of the nimodipine into liposomes increases its bioavailability, as well as the action of the drug on the animals.

5. Conclusion

This study suggests that the administration of a liposomal formulation containing nimodipine at the doses of 0.1, 1 and 10 mg/kg did not produce sedation and muscle relaxation in mice, showing anxiolytic-like activity in the open field, the light and dark and the elevated plus-maze test. The results of the treatment with NMD-Lipo were significantly better than the rodents treated with non encapsulated nimodipine, suggesting that the liposomes promoted a drug controlled release by increasing its bioavailability and consequently its effect. The decrease in the anxiolytic effect of NMP-Lipo in animals pretreated with flumazenil suggests that the formulation acts on benzodiazepine receptors.

Disclosure/conflict of interest

The authors have no conflicts of interest to disclose.

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References

Archer J. Tests for emotionality in rats and mice. A review. Anim Behav 1973; 21: 205–235.

Aslan A, Gurelik M, Cemek M, Goksel HM, Buyukokuroglu, ME. Nimodipine can improve cerebral metabolism and outcome in patients with severe head trauma. Pharmacol Res 2009; 59: 120–124.

Bartley CA, Hay M, Bloch MH. Meta-analysis: Aerobic exercise for the treatment of anxiety disorders. Prog Neuropsychopharmacol Biol Psychiatry 2013, 45, 34-39.

Casarrubea M, Roy V, Sorbera F, Magnusson MS, Santangelo A, Arabo A, Crescimanno G. Temporal structure of the rat's behavior in elevated plus maze test. Behav Brain Res 2013, 237, 290-299.

Chalikwar SS, Belgamwar VS, Talele VR, Surana SJ, Patil MU. Formulation and evaluation of Nimodipine-loaded solid lipid nanoparticles delivered via lymphatic transport system. Colloids Surf B 2012; 97; 109-116.

Crawley JN. Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev 1985; 9: 37–44.

Divljakovi´c J, Mili´c M, Namjoshi OA, Tiruveedhula VV, Timi´c, T, Cook JM, Savi´c MM. βCCT, an antagonist selective for α(1)GABA(A) receptors, reverses diazepam withdrawal-induced anxiety in rats. Brain Res Bull 2013; 91: 1–7.

Enkel T, Thomas M, Bartsch D. Differential effects of subchronic Phencyclidine on anxiety in the light-enhanced startle-, light/dark exploration- and open field tests. Behav Brain Res 2013; 263: 61–65.

Frye MA, Pazzaglia PJ, George MS, Luckenbaugh DA, Vanderham E, Davis CL, Rubinow DR, Post RM. Low CSF Somatostatin Associated with Response to Nimodipine in Patients with Affective Illness. Biol Psychiatry 2003; 53: 180–183.

Galdino PM, Nascimento MVM, Florentino IF, Lino FC, Fajemiroye JO, Chaibub BA, Paula JR, Lima TCM, Costa EA. The anxiolytic-like effect of an essential oil derived from Spiranthera odoratissima A. St. Hil. leaves and its major component, β-caryophyllene, in male mice. Progress in Neuro-Psychopharmacology & Biological Psychiatry 2012; 38; 276-284.

Kaplan HI, Sadock BJ. Comprehensive Textbook of Psychiatry. Lippincot Williams & Wilkins, Baltimore, MD. 2005.

Kumar N, Singh N, Jaggi AS. Anti-stress effects of cilnidipine and nimodipine in immobilization subjected mice. Physiol Behav 2012; 150: 1148–1155.

Kumar V, Bhat ZA, Kumar D. Animal models of anxiety: A comprehensive review. J. Pharmacol. Toxicol. Methods 2013; 68; 175–183.

Lira MCB, Siqueira-Moura MP, Rolim-Santos HML, Galetti, FCS, Simioni AR, Santos NP, Egito EST, Silva CL, Tedesco AC, Santos-Magalhães NS. In vitro uptake and antimycobacterial activity of liposomal usnic acid formulation. J Liposome Res 2009; 19: 49–58.

Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 1987; 92: 180–185.

Maack DJ, Tull MT, Gratz KL. Examining the incremental contribution of behavioral inhibition to generalized anxiety disorder relative to other Axis I disorders and cognitive-emotional vulnerabilities. J Anxiety Disord 2012; 26: 689–695.

Maigaard K, Hageman I, Jørgensen A, Jørgensen MB, Wörtwein G. Electroconvulsive stimulations prevent chronic stress-induced increases in L-type calcium channel mRNAs in the hippocampus and basolateral amygdala. Neurosci Lett 2012; 516; 24-28.

Marinho MMF, Bruin VMS, Souza FCF, Aguiar LMV, Pinho RSN, Viana GBS. Inhibitory action of a calcium channel blocker (nimodipine) on seizures and brain damage induced by pilocarpina and lithium-pilocarpine in rats. Neuroscience Letters 1997; 235; 13-16.

Mikati MA, Holmes GL, Werner S, Bakkar N, Carmant L, Liu Z, Stafstrom CE. Effects of nimodipine on the behavioral sequalae of experimental status epilepticus in prepubescent rats. Epilepsy & Behavior 2004, 5: 168–174.

Nascimento VS, D'alva MS, Oliveira AA, Freitas RM, Vasconcelos SMM, Sousa FCF, Fonteles MMF. Antioxidant effect of nimodipine in young rats after pilocarpine-induced seizures. Pharmacology, Biochemistry and Behavior 2005; 82: 11-16.

N'Gouemo P. Probing the role of the sodium/calcium exchanger in pentylenetetrazole-induced generalized seizures in rats. Brain Res Bull 2013; 90; 52-57.

Pazzaglia PJ, George MS, Post RM, Rubinow RD, Davis CL. Nimodipine increases CSF somatostatin in affectively ill patients. Neuropsychopharmacology 1995; 13: 75-83.

Quaglio G, Pattaro C, Gerra G, Mathewson S, Verbanck P, Jarlais DCD, Lugoboni F. High dose benzodiazepine dependence: Description of 29 patients treated with flumazenil infusion and stabilised with clonazepam. Psychiatry Res 2012; 198: 457–462.

Raupp IM, Sereniki A, Virtuoso S, Ghislandi C, Cavalcanti E, Silva EL, Trebien HA, Miguel O G, Andreatini R. Anxiolytic-like effect of chronic treatment with *Erythrina velutina* extract in the elevated plus-maze test. J Ethnopharmacol 2008; 118: 295-299

Richey JA, Schmidt NB, Hofmann SG, Timpano KR. Temporal and structural dynamics of anxiety sensitivity in predicting fearful responding to a 35% CO_2 challenge. J Anxiety Disord 2010; 24: 423–432.

Santos-Magalhães NS, Mosqueira VCF. Nanotechnology applied to the treatment of malaria. Adv Drug Deliv Rev 2010; 62: 560–575.

Salomons AR, Kortleve T, Reinders NR, Kirchhoff S, Arndt SS, Ohl F. Susceptibility of a potential animal model for pathological anxiety to chronic mild stress. Behav Brain Res 2010; 209: 241-248.

Sousa, FCF, Melo CTV, Citó COM, Félix FHC, Vasconcelos SMM, Fonteles MMF, Barbosa Filho JM, Viana GSB. Plantas medicinais e seus constituintes bioativos: Uma revisão da bioatividade e potenciais benefícios nos distúrbios da ansiedade em modelos animais. Rev Bras Farmacogn 2008; 18: 642–654.

Sun C, Wang J, Liu J, Qiu L, Zhang W, Zhang L. Liquid Proliposomes of Nimodipine Drug Delivery System: Preparation, Characterization, and Pharmacokinetics. AAPS PharmSciTech 2013; 14: 332–338.

Wong HL, Wu XY, Bendayan R. Nanotechnological advances for the delivery of CNS therapeutics. Adv Drug Delivery Ver 2012; 64; 686–700.

Yanpallewar SU, Hota D, Rai R, Kumar M, Acharya SB. Nimodipine attenuates biochemical, behavioral and histopathological alterations induced by acute transient and long-termbilateral common carotid occlusion in rats. Pharmacol Res 2004; 49; 143-150.

Zou JB, Dear BF, Titov N, Lorian CN, Johnston L, Spence J, Knight RG, Anderson T, Sachdev P. Brief internet-delivered cognitive behavioral therapy for anxiety in older adults: A feasibility trial. J Anxiety Disord 2012; 26; 650–655.

5. ACUTE TOXICITY AND ANTICONVULSANT ACTIVITY OF LIPOSOMES CONTAINING NIMODIPINE ON PILOCARPINE-INDUCED SEIZURES IN MICE

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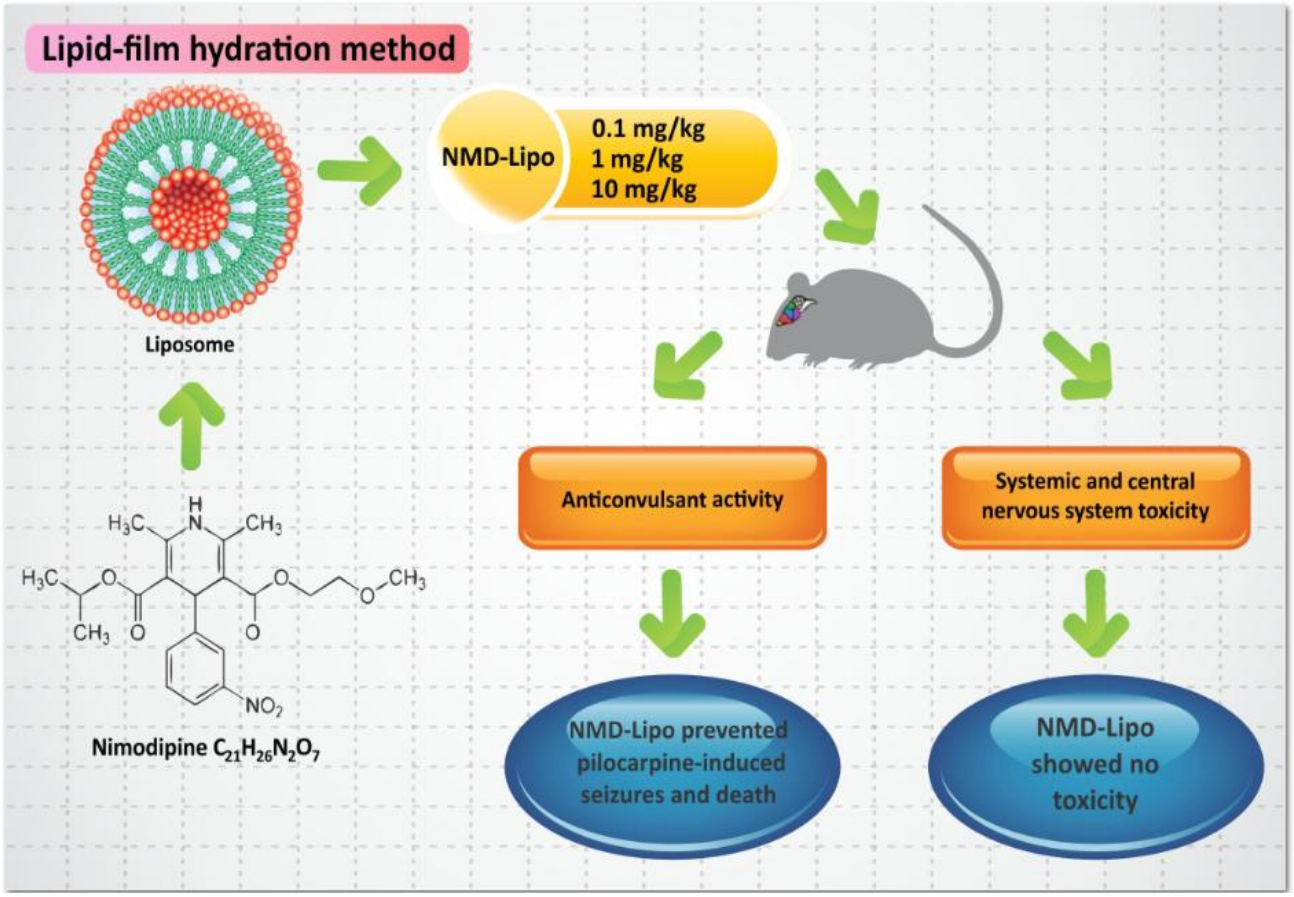
Abstract

Nimodipine has been shown to have an inhibitory action on seizures and brain damage in rodents. However, the pharmaceutical applicability of this drug is limited by its low solubility in gastrointestinal fluids and high first-pass effect in liver, which leads to low bioavailability. These difficulties can be overcome through the use of liposomes. The aim of the present study was the evaluation of liposomes containing nimodipine (NMD-Lipo) toxicity and anticonvulsant activity on pilocarpine-induced seizures. NMD-Lipo was prepared using lipid-film hydration method. Central nervous system toxicity of NMD-Lipo was assessed by the Hippocratic screening. Systemic toxicity was evaluated by analysis of biochemical and hematological parameters and by observing possible signs of acute toxicity. The possible anticonvulsant activity was tested by pilocarpine model. The administration of the NMD-Lipo at doses of 0.1, 1 and 10 mg/kg did not present toxicity. The administration of NMD-Lipo also prevented the installation of 100% of the pilocarpine-induced seizures and prevented the death of 100% of the mice treated with pilocarpine. The data suggest that NMD-Lipo showed anticonvulsant activity significantly superior to free NMD, suggesting that the liposomes promoted a drug controlled release by increasing its bioavailability and consequently its pharmacological activity.

Keywords: Anticonvulsant; Liposomes; Mice; Nimodipine; Toxicity.

Graphical Abstract



1. Introduction

Epilepsy is a chronic disease of the central nervous system characterized by recurrent seizures caused by excessive discharges of cerebral neurons. This condition is a health concern since the pathology is considered one of the most serious neurological disorders [1]. Clinically, patients with the disease experience a deterioration of one or more cognitive functions with or without motor behavior and / or psychomotor decrease [2].

Seizures can be completely controlled with medical therapy in two-thirds of patients, however one-third remains refractory to the medications [3]. Furthermore, the current antiepileptic drugs used in the treatment of epilepsy have a wide range of adverse reaction, toxicity and teratogenic effect. Based on these finds, new therapeutic agents, which allow more efficient control seizure in resistant patients and with fewer side effects, are greatly needed [4].

Research has shown that the intrinsic epileptiform activity is associated with calcium (Ca^{2+}) influx through NMDA receptor-operated Ca^{2+} channels and through voltage-operated Ca^{2+} channels. Therefore, the inhibition of the intracellular Ca^{2+} increase represents an important target in the development of antiepileptic and neuroprotective drugs [5]. From this perspective, calcium channel blockers may be considered as a possible therapeutic agent for the disease.

Nimodipine (NMD) is a dihydropyridine L-type Ca^{2+} channel antagonist that crosses the blood–brain-barrier easily than other calcium-channel-blockers and binds with high affinity and specificity to the calcium-channels receptors in the brain [6]. NMD has been shown to have an inhibitory action on seizures and brain damage in rodents [16-23]. However, the pharmaceutical applicability of nimodipine is limited by its low solubility in gastrointestinal fluids and high first-pass effect in liver, which leads to low bioavailability after oral administration [7, 8].

These difficulties can be overcome through the use of liposomes. These nanometer-scale pharmaceutical carriers are self-assembled colloidal vesicles consisting of one or more concentric phospholipid bilayers organized around an aqueous inner compartment, and are used to encapsulate drugs, biomolecules or diagnostic agent [9]. The aim of the present study was twofold: the evaluation of the nimodipine encapsulated into liposomes (NMD-Lipo) toxicity and the study of anticonvulsant activity of NMD-Lipo on pilocarpine-induced seizures.

2. Material and methods

2.1. Lipids, drugs and reagents

Cholesterol (CHOL), trehalose, nimodipine and pilocarpine hydrochloride were purchased from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (98% Epikuron 200) was obtained from Lipoid GMBH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Animals

Adult male Swiss mice (25-30 g; 2 months old) were obtained from Central Animal House of the Federal University of Piaui, Piaui, Brazil. They were maintained in a temperature controlled room (25 ± 1 °C), with a 12 h light/dark cycle (lights on 07:00–19:00 h) and food and water provided *ad libitum* (Nutrilabor, Campinas, Brazil). The experimental protocols and procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA/UFPI N° 014/11). All experiments were performed according to the Guide for the care and use of laboratory of the US Department of Health and Human Services, Washington, DC (1985).

2.3. Preparation and characterization of liposomes containing nimodipine (NMD-Lipo)

Liposomes containing nimodipine (NMD-Lipo) were prepared and characterized as previously described [10]. The liposomal formulation was produced using the method of hydrating the lipid film. The particle size and polydispersity of the liposomes were determined using photon correlation spectroscopy (Particle Analyzer™ Delsa Nano S, Beckman-Coulter, USA). The zeta potential of the liposomes was measured using a Zetatrac NC-148 apparatus (Microtrac, USA). The pH of the liposomes was measured using a digital pH meter (Bioblock Scientific 99, Prolabo, Paris, France). The encapsulation efficiency of nimodipine into liposomes was determined by the technique of ultrafiltration/ultracentrifugation using Ultrafree® units (Millipore, USA) and the content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm.

In order to improve stability, the liposomal formulations were lyophilized at $4x10^{-6}$ Barr at -80 °C (EZ-DRY, FTSS System, New York, USA) using trehalose (10%) as a cryoprotectant.

2.4. Studies of systemic and central nervous system toxicity of NMD-Lipo

Mice were divided into four groups of 16 animals each. The first group was treated with 0.9% saline. The second, third and fourth groups were treated with NMD-Lipo at doses of 0.1, 1 and 10 mg/kg. NMD is a widely used drug and its security is well-known, so the toxicity tests have not been conducted with free NMD, only with NMD-Lipo.

Central nervous system toxicity of NMD-Lipo was assessed by the Hippocratic screening. Systemic toxicity was evaluated by analysis of biochemical and hematological parameters and by observing possible signs of toxicity.

Half of the animals in each group (n = 8) was observed for 24 hours and subsequently were intended to implement the blood tests. During this period we proceeded to the observation of the mice at the time of 30 minutes, 1, 2, 4, 8, 12 and 24 hours for the purpose of quantifying the effect of NMD-Lipo on the following parameters: a) State of awareness and readiness; b) Motor coordination; c) Muscle tone; d) Reflection (atrial and cornea); e) Central nervous system activity; f) Autonomic nervous system activity. At the end of 24 hours, the animals were anesthetized with pentobarbital 40 mg/kg and blood was immediately collected from the retro-orbital plexus for the assessment of biochemical and hematologic parameters [11].

The other half (n = 8) was under observation for a period of 30 days for viewing and record of possible signs of toxicity of the formulation. During these 30 days, the consumption of water and feed were recorded daily, body weight of mice was measured every two days and the animals were evaluated for clinical signs of toxicity.

2.5. Studies of anticonvulsant activity of NMD-Lipo

Mice were divided into twenty two groups of 12 animals each. The negative control group was treated with 0.9% saline. The P400 group was treated with pilocarpine hydrochloride at a dose of 400 mg/kg to induce seizures. The third and fourth groups were treated with diazepam at a dose of 5 mg/kg and an association of diazepam with pilocarpine hydrochloride in a dose of 400 mg/kg. The fifth, sixth, and seventh groups were treated with empty liposomes at doses of 0.1, 1 and 10 mg/kg. The eighth, ninth and tenth groups were treated with empty liposomes at doses of 0.1, 1 and 10 mg/kg and 30 minutes after they received pilocarpine hydrochloride at the dose of 400 mg/kg. The eleventh, twelfth and thirteenth groups were treated with free nimodipine at doses of 0.1, 1 and 10 mg/kg. The fourteenth, fifteenth and sixteenth groups were treated with free nimodipine at doses of 0.1, 1

and 10 mg/kg and 30 minutes after they received pilocarpine hydrochloride at the dose of 400 mg/kg. The seventeenth, eighteenth and nineteenth groups were treated with NMD-Lipo at the doses of 0.1, 1 and 10 mg/kg. Finally, the animals of the twentieth, twenty first and twenty second groups received NMD-Lipo at the doses of 0.1, 1 and 10 mg/kg and 30 minutes after they received pilocarpine hydrochloride at the dose of 400 mg/kg.

After the treatments, the animals were recorded in 30 cm x 30 cm chambers with: appearance of peripheral cholinergic signs (miosis, piloerection, chromodacryorrhea, diarrhea and urination), stereotyped movements (continuous sniffing, paw licking and rearing), tremors, seizures, status epilepticus and mortality rate, during 24 h. We decided to observe possible changes in the behavior of mice for 24 h after pilocarpine administration because previous works showed that convulsions and deaths occurred within 1 and 24 h respectively post pilocarpine injection [12].

2.6. Statistical Analyses

The results were presented as percentage according with number of animals used in the experiments. Peripheral cholinergic signs, stereotypic movements, tremor, seizures, status epilepticus and mortality rate were presented as percentages and compared with a nonparametric test (Chi-Square test). In all situations statistical significance was reached at p less-than-or-equals, slant 0.05. The statistical analyses were performed with the software GraphPad Prism, Version 6.00 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Characterization of NMD-Lipo

NMD-Lipo presented mean vesicle sizes of 112.58 ± 1.21 nm (PDI = 0.28), zeta potencial of −5.32 ± 1.29 mV, pH of 7.4, and drug encapsulation efficiency of 99 ± 0.22%.

3.2. Systemic and central nervous system toxicity of NMD-Lipo

In the Hippocratic screening, NMD-Lipo did not cause any behavioral alterations in mice at the doses tested. Therefore, it was not observed any alteration in biochemical and hematologic parameters as any variation in weight of mice treated with the formulation. None of the animals treated with NMD-Lipo died.

3.3. Behavioral alterations after pretreatment with NMD-Lipo after 24 h of phase acute of pilocarpine-induced seizures

The results are summarized in Table 1. None of the mice that received injections of isotonic saline (negative control), diazepam, empty liposomes, free nimodipine and NMD-Lipo unassociated with pilocarpine showed peripheral cholinergic signs, stereotypic movements, tremor and seizures. None of the animals in these groups died.

All animals treated with P400 alone presented peripheral cholinergic signs and stereotyped movements followed by motor limbic seizures. The convulsive process persisted and built up to a status epilepticus in 75% of these mice, leading to death of 75% of the animals. Diazepam at the dose of 5 mg/kg did not significantly reduce the occurrence of peripheral cholinergic signs, stereotypic movements and tremors. The benzodiazepine was able to reduce by 50% the occurrence of seizures and 50% the mortality rate in mice. Empty liposomes at doses of 0.1, 1 and 10 mg/kg did not reduce the occurrence of peripheral cholinergic signs, stereotypic movements and tremor. Liposomes without nimodipine were unable to prevent the installation of the seizure and decrease the mortality rate in the rodent. Free NMD at the dose of 0.1 mg/kg did not reduce the occurrence of peripheral cholinergic signs, stereotypic movements and tremors. The unencapsulated drug at the dose of 0.1 mg/kg was unable to prevent the installation of the seizure, but reduced by 25% the mortality rate in mice. As with the mice pretreated with free NMD at the dose of 0.1 mg/kg, free NMD at the

dose of 1 mg/kg did not significantly reduce the occurrence of peripheral cholinergic signs, stereotypic movements and tremors. Moreover, free NMD at the dose of 1 mg/kg was unable to prevent the installation of the seizure but reduced by 25% the mortality rate in the mice. Free NMD at doses of 10 mg/kg did not significantly reduce the occurrence of peripheral cholinergic signs or tremors and was not able to prevent the installation of seizures. However, free NMD at the dose of 10 mg/kg was able to reduced 25% of stereotypic movements and reduced 25% the mortality rate compared to the mice of the P400 group.

On the other hand, NMD-Lipo at doses of 0.1, 1 and 10 mg/kg did not reduce the occurrence of peripheral cholinergic signs but decreased stereotypic movements and tremors in the mice. NMD encapsulated into liposomes at all doses tested was able to prevent the occurrence of 100% of the seizures. None of the mice pretreated with NMD-Lipo and subsequently given with pilocarpine died.

Table 1: Effect of pretreatment with NMD-Lipo, free nimodipine, liposomes and diazepam on pilocarpine-induced seizures and lethality in adult mice

Groups (n=12)	Peripheral Cholinergic Signs	Stereotypic Movements	Tremor	Seizures	Status Epilepticus	Mortality rate
	%	%	%	%	%	%
Negative control	00	00	00	00	00	00
P400	100[a]	100[a]	100[a]	100[a]	75[a]	75[a]
DZP 5 plus P400	100[a]	100[a]	100[a]	50[a,b]	50[a,b]	50[a,b]
DZP 5	00	00	00	00	00	00
Lipo 0.1	00	00	00	00	00	00
Lipo 1	00	00	00	00	00	00
Lipo 10	00	00	00	00	00	00
Lipo 0.1 plus P400	100[a,c]	100[a,c]	100[a,c]	100[a,c]	75[a,c]	75[a,c]
Lipo 1 plus P400	100[a,d]	100[a,d]	100[a,d]	100[a,d]	75[a,d]	75[a,d]
Lipo 10 plus P400	100[a,e]	100[a,e]	100[a,e]	100[a,e]	75[a,e]	75[a,e]
Free NMD 0.1	00	00	00	00	00	00
Free NMD 1	00	00	00	00	00	00
Free NMD 10	00	00	00	00	00	00
Free NMD 0.1 plus P400	100[a,f]	100[a,f]	100[a,f]	100[a,f]	50[a,b,f]	75[a,f]
Free NMD 1 plus P400	100[a,g]	100[a,g]	100[a,g]	100[a,g]	50[a,b,g]	75[a,g]
Free NMD 10 plus P400	100[a,h]	75[a,b,h,*,**]	100[a,h]	100[a,h]	75[a,h,*,**]	75[a,h]
NMD-Lipo 0.1	00	00	00	00	00	00
NMD-Lipo 1	00	00	00	00	00	00
NMD-Lipo 10	00	00	00	00	00	00
NMD-Lipo 0.1 plus P400	100[a,i]	65[a,b,i,&]	100[a,i]	00[b,&]	00[b,&]	00[b,&]
NMD-Lipo 1 plus P400	100[a,j]	40[a,b,j,#,&]	60[a,b,j,#,##,&]	00[b,&]	00[b,&]	00[b,&]
NMD-Lipo 10 plus P400	100[a,l]	30[a,b,l,#,##,&]	20[a,b,l,#,##,&]	00[b,&]	00[b,&]	00[b,&]

Mice (25-30 g; 2 months old) were treated acutely with vehicle (saline 0.25 ml, negative control), pilocarpine (400 mg/k.g, i.p, P400), diazepam (5 mg/kg, i.p., DZP 5, positive control), empty liposomes (Lipo), free nimodipine (Free NMD) and liposomal formulation containing nimodipine (NMD-Lipo) at doses 0.1, 1 e 10 mg/kg (i.p.). Others groups of mice were pretreated acutely with Lipo, Free NMD and NMD-Lipo at doses 0.1, 1 e 10 mg/kg (i.p.) or DZP and 30 minutes after treatment with pilocarpine 400 mg/kg, i.p. Results for peripheral cholinergic signs, stereotypic movements, tremor, seizures, status epilepticus and death are expressed as percentages of the number of animals from each group. [a]$p<0.05$, when compared with negative control; [b]$p<0.05$, when compared with P400 group; [c]$p<0.05$, when compared with Lipo 0.1; [d]$p<0.05$, when compared with Lipo 1; [e]$p<0.05$, when compared with Lipo 10; [f]$p<0.05$, when compared with Free NMD 0.1; [g]$p<0.05$, when compared with Free NMD 1; [h]$p<0.05$, when compared with Free NMD 10; [i]$p<0.05$, when compared with NMD-Lipo 0.1; [j]$p<0.05$, when compared with NMD-Lipo 1; [l]$p<0.05$, when compared with NMD-Lipo 10; [*]$p<0.05$, when compared with Free NMD 0.1 plus P400; [**]$p<0.05$, when compared with Free NMD 1 plus P400; [#]$p<0.05$, when compared with NMD-Lipo 0.1 plus P400; [##]$p<0.05$, when compared with NMD-Lipo 1 plus P400; [&]$p<0.05$, when compared with DZP 5 plus P400 (Chi-Square test).

4. Discussion

Animal models of seizure have been widely used in research to provide a better understanding of the pathophysiology of the disease, since they reproduce several components of human epilepsies. Pilocarpine-induced seizures is a model commonly used to investigate the anticonvulsant effect of antiepileptic drugs [13].

The administration of high doses of pilocarpine induces seizure activity, followed by a latent seizure-free period preceding the development of spontaneous recurrent focal seizures. The induction of status epilepticus by pilocarpine in rodents leads to neuropathological changes, such as hippocampal sclerosis and mossy fiber sprouting, resemble human temporal lobe epilepsy [14]. In the present study, we investigated the effects of a liposomal formulation containing nimodipine, a Ca^{2+} channel blocker, on susceptibility to seizures induced by pilocarpine in adult mice.

Antiepileptic drugs have different targets such as receptors, synaptic machinery and ion channels [15]. Previous studies have demonstrated that increased levels of intracellular Ca^{2+} in hippocampal neurons play an important role in the underlying mechanisms of neuronal hyperexcitability that leads to pilocarpine-induced seizures [16]. Research conducted with Ca^{2+} channel blocker NMD at the doses of 1 to 300 mg/kg have suggested that the drug present anticonvulsant activity on seizures induced by picrotoxin [17], kainic acid [18], aminophylline [19], pentylenetetrazole [20], phenytoin [21], pilocarpine and lithium-pilocarpine [22-24] in mice and rats. However, in all aforementioned studies, the drug was unable to prevent 100% of seizures.

One of the possible reasons explaining the lack of ability of nimodipine to prevent the installation of seizures in some rodents is that the drug has low bioavailability (4–13%) due to its high first-pass effect in liver [25]. This hypothesis is strengthened by our data, since NMD-Lipo showed anticonvulsant activity significantly superior to free NMD, suggesting that the

encapsulation of the nimodipine into liposomes increases its bioavailability, as well as the anticonvulsant activity of the drug on the animals.

Nimodipine is a very lipophilic drug, and because of this high lipophilicity, it can be incorporated into the lipid bilayer of the liposomes [26]. NMD-Lipo presented drug encapsulation efficiency of 99 ± 0.22%, showing that the formulation does not present significant amount of unload NMD. The treatment realized with NMD-Lipo at the doses of 0.1 to 10 mg/kg showed safe for mice, since do not cause changes in the hematological and biochemical parameters of the animals. Furthermore, NMD-Lipo did not cause any change in the weight of the animals and the reduction in body weight is a simple and sensitive index of toxicity after exposure to toxic substance [27].

The administration of the liposomal formulation at the doses of 0.1, 1 and 10 mg/kg was able to reduce stereotypic movements and tremors. Moreover, NMD-Lipo prevented the installation of 100% of the pilocarpine-induced seizures and prevented the death of 100% of the mice treated with pilocarpine. The results of the experiments suggest that NMD has a dose-dependent effect. Empty liposomes showed no anticonvulsant activity, suggesting that the activity of NMD-Lipo is due to a controlled release of the NMD afforded by the liposomes.

Animal studies indicate that seizures at an early stage of development can drastically affect the construction of networks of the hippocampus, which can cause the onset of other disorders such as schizophrenia [28]. The decrease in the occurrence of stereotypic movements and tremors and the ability to prevented seizures and death in rodents constitutes a major advance in drug development against epilepsy. Thus, a formulation that prevents the emergence of seizures appears very promising in the epilepsy therapy.

References

[1] O.O. Adeyemi, A.J. Akindele, O.K. Yemitan, F.R. Aigibe, F.I. Fagbo, Anticonvulsant, anxiolytic and sedative activities of the aqueous root extract of Securidaca longepedunculata Fresen, J. Ethnopharmacol. 130 (2010) 191–195.

[2] S. Fortini, L. Corredera, A.L. Pastrana, G. Reyes, L. Fasulo, R.H. Caraballo, Encephalopathy with hemi-status epilepticus during sleep or hemi-continuous spikes and waves during slow sleep syndrome: A study of 21 patients, Seizure 22 (2013) 565–571.

[3] P. Bhutada, Y. Mundhada, K. Bansod, P. Dixit, S. Umathe, D. Mundhada, Anticonvulsant activity of berberine, an isoquinoline alkaloid in mice, Epilepsy Behav. 18 (2010) 207–210.

[4] F. Hadizadeh, B. Rahimi, E. Taghiabadi, M. Razavi, G. Karimi, Evaluation of anticonvulsant effect of two novels 4-[1-(4-fluorobenzyl)-5-imidazolyl] dihydropyridine derivatives in mice, Res. Pharm. Sci. 8 (2013) 91-95.

[5] M. Ghasemi, H. Shafaroodi, S. Nazarbeiki, H. Meskar, P. Heydarpour, A. Ghasemi, S.S.Talab, P. Ziai, A. Bahremand, A.R. Dehpour, Voltage-dependent calcium channel and NMDA receptor antagonists augment anticonvulsant effects of lithium chloride on pentylenetetrazole-induced clonic seizures in mice, Epilepsy Behav. 18 (2010) 171–178.

[6] M.J. Bailey, B.A. Hutsell, M.C. Newlan, Dietary nimodipine delays the onset of methylmercury neurotoxicity in mice, NeuroToxicology 37 (2013) 108-117.

[7] N. Bege, T. Renette, T. Endres, M. Beck-Broichsitter, D. Hänggi, T.Kissel, In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm. Eur. J. Pharm. Biopharm. 84 (2013) 99–105.

[8] C. Sun, J. Wang, J. Liu, L. Qiu, W. Zhang, L. Zhang, Liquid Proliposomes of Nimodipine Drug Delivery System: Preparation, Characterization, and Pharmacokinetics, AAPS PharmSciTech 14 (2013) 332–338.

[9] P.G. Cadena, M.A. Pereira, R.B.S. Cordeiro, I.M.S. Cavalcanti, B. Barros-Neto, M.C.C.B. Pimentel, J.L. Lima-Filho, V.L. Silva, N.S. Santos-Magalhães, Nanoencapsulation of quercetin and resveratrol into elastic liposomes, Biochim. Biophys. Acta. 1828 (2013) 309–316.

[10] L.C.G.A.I Moreno, G.Z.S. Oliveira, I.M.F Cavalcanti, N.S. Santos-Magalhães, H.M.L. Rolim, R.M. Freitas, Development and evaluation of liposomal formulation containing nimodipine on anxiolytic activity in mice, Pharmacol. Biochem, Behav. 116 (2014) 64-68.

[11] B.H. Waynforth, Injection techniques. In: experimental and Surgical Techniques in the Rat, Academic Press, London, 1980.

[12] L.F.L. Santos, L.F.M. Freitas, S.M.L. Xavier, G.B. Saldanha, R.M. Freitas, Neuroprotective actions of vitamin C related to decreased lipid peroxidation and increased catalase activity in adult rats after pilocarpine-induced seizures, Pharmacol. Biochem. Behav. 89 (2008) 1–5.

[13] W. Zgrajka, D. Nieoczym, M. Czuczwar, J Ki´s, W. Brzana, P. Wla´z, W. A. Turski, Evidences for pharmacokinetic interaction of riluzole and topiramate with pilocarpine in pilocarpine-induced seizures in rats, Epilepsy Res. 88 (2010) 269—274.

[14] P. E. Schauwecker, Strain differences in seizure-induced cell death following pilocarpine-induced status epilepticus, Neurobiol. Dis.45 (2012) 297–304.

[15] A. J. Hill, N. A. Jones, I. Smith, C. L. Hill, C. M. Williams, G. J. Stephens, B. J. Whalley. Voltage-gated sodium (Na_V) channel blockade by plant cannabinoids does not confer anticonvulsant effects *per se*, Neurosci. Lett. 566 (2014) 269–274.

[16] Y. Martinez, P. N'Gouemo, Blockade of the sodium calcium exchanger exhibits anticonvulsant activity in a pilocarpine model of acute seizures in rats, Brain Res. 1366 (2010) 211-216.

[17] J. Thomas, The Effect of Nimodipine on Picrotoxin-Induced Seizures, Brain Res. Bull. 24 (1990) 11-15.

[18] R.P. Paczynski, F.B. Meyer, R.E. Anderson, Effects of the dihydropyridine Ca^{2+} channel antagonist nimodipine on kainic acid-induced lirnbic seizures, Epilepsy Res. 6 (1990) 33-38.

[19] A. Chakrabarti, H.S. Kaur, S.K. Garg, Dose-finding study with nimodipine: a selective central nervous system calcium channel blocker on aminophylline induced seizure model in rats, Brain Res. Bull. 45 (1998) 495–499.

[20] P. Zapater, J. Javaloy, J.F. Román, M.T. Vidal, J.F. Horga, Anticonvulsant effects of nimodipine and two novel dihydropyridines (PCA 50922 and PCA 50941) against seizures elicited by pentylenetetrazole and electroconvulsive shock in mice, Brain Res. 796 (1998) 311-314.

[21] C. Hocht, A. Lazarowski, N. N. Gonzalez, J. Auzmendi, J. A.W. Opezzo, G.F. Bramuglia, C.A. Taira, E. Girardi, Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model, Neurosci. Lett. 413 (2007) 168–172.

[22] M.M.F. Marinho, V.M.S. Bruin, F.C.F. Souza, L.M.V. Aguiar, R.S.N. Pinho, G.B.S. Viana, Inhibitory action of a calcium channel blocker (nimodipine) on seizures and brain damage induced by pilocarpina and lithium-pilocarpine in rats, Neurosci. Lett. 235 (1997) 13-16.

[23] M.A. Mikati, G.L. Holmes, S. Werner, N. Bakkar, L. Carmant, Z. Liu, C.E. Stafstrom, Effects of nimodipine on the behavioral sequalae of experimental status epilepticus in prepubescent rats, Epilepsy Behav. 5 (2004) 168–174.

[24] V.S. Nascimento, M.S. D'alva, A.A. Oliveira, R.M. Freitas, S.M.M. Vasconcelos, F.C,F. Sousa, M.M.F. Fonteles, Antioxidant effect of nimodipine in young rats after pilocarpine-induced seizures, Pharmacol. Biochem. Behav. 82 (2005) 11 – 16.

[25] S.S. Chalikwar, V.S. Belgamwar, V.R. Talele, S.J. Surana, M.U. Patil, Formulation and evaluation of Nimodipine-loaded solid lipid nanoparticles delivered via lymphatic transport system, Colloid Surface B. 97 (2012) 109– 116.

[26] T. Guan, Y. Miao, L. Xu, S. Yang, J. Wang, H. He, X. Tang, C.Cai, H. Xu. Injectable nimodipine-loaded nanoliposomes: Preparation, lyophilization and Characteristics, Int. J. Pharm. 410 (2011) 180–187.

[27] S. Thanabhorn, K. Jaijoy, S. Thamaree, K. Ingkaninan, A. Panthong, Acute and subacute toxicity study of the ethanol extract from *Lonicera japonica* Thunb, J. Ethnopharmacol. 107 (2006) 370–373.

[28] G. P. Labbate, A. V. Silva, R. C. Barbosa-Silva. Effect of severe neonatal seizures on prepulse inhibition and hippocampal volume of rats tested in early adulthood, Neurosci. Lett. 568 (2014) 62–66.

6. ANTIDEPRESSANT-LIKE ACTIVITY OF LIPOSOMAL FORMULATION CONTAINING NIMODIPINE TREATMENT IN THE TAIL SUSPENSION TEST, FORCED SWIM TEST AND MAO_B ACTIVITY IN MICE

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† Rivelilson Mendes Freitas *In memoriam*

Abstract

Previous studies have shown that intracellular calcium ion dysfunction may be an etiological factor in affective illness. Nimodipine (NMD) is a Ca^{2+} channel blocker that has been extensively investigated for therapy of central nervous system (CNS) disorders. In this work, we have evaluated the antidepressant-like activity of nimodipine encapsulated into liposomes (NMD-Lipo) in mice through tail suspension and forced swim assays, as well as MAO_B activity. During the tail suspension test, the administration of NMD-Lipo at 0.1, 1 and 10 mg/kg was able to promote a reduction in the immobility time of animals greater than the positive control (imipramine). In the forced swim test, the immobility time of mice treated with NMD-Lipo was reduced. This reduction was significantly greater than that found in the animals treated with imipramine and paroxetine. This may suggest that NMD-Lipo provides more antidepressant-like activity than in positive controls. The groups that received a combination of liposomal NMD and antidepressant drugs showed lower immobility time than the groups, which were treated only with imipramine or paroxetine. The mice treated with the combination of NMD-Lipo and reserpine presented an increase in the time of immobility compared with animals treated only with NMD-Lipo. There was a significant decrease in MAO_B activity in animals treated with NMD-Lipo compared with untreated animals. The results of the tail suspension test, forced swim test and MAO_B activity suggested that the antidepressant activity of NMD-Lipo may be related to an increase in the cerebral monoamine concentrations.

Keywords: Nimodipine, Liposomes, Tail suspension test, Forced swim test, Monoamine oxidase, Mice.

1. Introduction

Depression is a highly prevalent and recurrent mental disorder that impacts all aspects of human life (Socała et al., 2012). Research has shown that depression probably originated during the evolutionary process. Some studies have supported this thesis by arguing that being in a state of depression makes people focus on solving problems. Also, traces of depression may indicate that the affected person needs some help, besides discouraging individuals from risky behavior (Wittman, 2014). On the other hand, when depression progresses from a mild state to a severe condition, the disease becomes alarming. This pathology is one of the major causes of disability in the world, representing a heavy social burden, and is considered a lifetime risk (Wang et al., 2013).

Treatment-resistant depression is a frequent condition in patients. Statistics show that 50 to 60% of people treated with antidepressant drugs obtain clinically insignificant results (Fornaro et al., 2014). Therefore, strategies used to improve the treatment of this disease include the addition of one or more non-antidepressant drugs to an existing therapy or the use of agents to target specific symptoms of depression. Combined therapy involving the use of two or more antidepressant drugs has been increasingly implemented (Papakostas, 2009).

The pathogenesis of neuropsychiatric diseases and the mechanisms of action of drugs used in their treatments are still not well established. However, it is well known that alterations commonly occur in many diseases of the central nervous system (CNS). For example, rises in intracellular calcium concentration cause an increased contraction of the brain vasculature, which reduces blood flow in the organ (Xu et al., 2013). In addition, excessive penetration of calcium in neurons causes a process known as excitotoxicity that induces neuronal damage and cell death by necrosis or apoptosis (Justin et al., 2014 and Aslan et al., 2009).

Research has shown that the excessive flow of calcium through the membrane, which results in increased intracellular levels of this cation, may play a role in the pathophysiology of affective CNS disorders (Maigaard et al., 2012), epileptiform activity (N'Gouemo, 2013) and the induction of anxiety (Kumar et al., 2012).

In this way, an increase in calcium concentrations in platelets and lymphocytes of depressed and bipolar patients suggests that increased intracellular concentrations of this cation may play a role in the pathophysiology of depression and bipolar disorder (Dubovsky, Daurignac, Leonard, 2014; Dubovsky et al., 1994; Dubovsky et al., 1992 and Dubovsky et al., 1989). This finding was reinforced by the discovery that some drugs used for treating depression and bipolar disorder, such as tricycle antidepressants and lithium, cause a blockage in the calcium channels, indicating that this mechanism may contribute to their therapeutic effect (Roedding, Li, Warsh, 2006 and Grunze et al., 1996). Based on this data, calcium channel blockers may be considered a possible therapeutic agent for an effective treatment of affective illnesses of CNS.

Nimodipine (NMD) is a central Ca^{2+} channel blocker and has a high permeability through the blood brain barrier (BBB). Because of this, NMD has been extensively investigated in the therapy of CNS disorders (Sygnecka et al., 2015). It has been shown to have antidepressant properties in rats (Katagiri et al., 2001) and humans (Frye et al., 2003, Yingling et al., 2002 and Pazzaglia et al. 1995). However, the clinical use of NMD is limited by its low solubility in gastrointestinal fluids and high first-pass effect in the liver, which leads to a low bioavailability after oral administration (Bege et al., 2013 and Sun et al., 2013).

Nanotechnology offers an interesting approach for improving the therapeutic management of CNS diseases. In general, liposomal drug-loaded formulations are superior to free drugs in their pharmacokinetic and therapeutic effects (Wong et al., 2012). Our recent studies have demonstrated that NMD encapsulated into liposomes presents anxiolytic

(Moreno et al., 2014) and anticonvulsant activities in mice (Moreno et al., 2015). For these reasons, liposomes seem to be a suitable vehicle for the administration of NMD.

An animal model that perfectly mimics the symptoms of depression in human patients is hardly ever achieved. Animals not only lack consciousness of self, self-reflection and consideration of others, but also symptoms of depression such as suicidal tendencies and depressed mood are difficult to detect in non-human beings (Deussing, 2006). However, there are a number of symptoms of this disease that can be reproduced in animals such as anxiety, anhedonia, sleep disturbances, hormonal dysregulation, etc., which can be used for drug tests with the view to acquiring understanding of neurological diseases (Schmidt, 2011).

The tail suspension and the forced swim tests are widely used for evaluating antidepressant activity of compounds in preclinical trials (Rollema et al., 2009). The mechanism of the effects evaluated using these tests has yet to be well established, but it is believed that they may reflect conformational changes occurring in the neurotransmitter receptors and the signal transduction receptor mediated systems (Kim et al., 2010).

Monoamine oxidases (MAO) are a class of mitochondrial enzymes involved in the degradation of biogenic amines. Monoamine oxidase inhibitors (MAOIs) have been used in clinical therapy owing to their antidepressant effects. However, the use of MAOIs for the treatment of depression has diminished since the discovery that MAOIs ingested with tyramine can result in cardiovascular reaction ("cheese effect") (Ghazaleh et al., 2015). On the other hand, the finding that the selective inhibition of monoamine oxidase B (MAO_B) does not cause "cheese effect" suggests that drugs causing MAO_B inhibition are suitable for the therapy of affective disorders (Finberg, 2014).

Based on the abovementioned, we evaluated the antidepressant-like activity of nimodipine-loaded liposomes (NMD-Lipo) in treated mice using the tail suspension and

forced swim tests. Furthermore, we examined MAO_B activity in the hippocampus of treated mice after the forced swim test.

2. Results

2.1. Drug content and encapsulation rate of NMD-Lipo

NMD-Lipo presented a drug content of 0.96 ± 0.74 mg/ml and encapsulation efficiency of 99 ± 0.67%. These results are in agreement with our previous studies (Moreno at al., 2014 and Moreno et al., 2015).

2.2. Tail suspension test

The results are summarized in Table 1. In the tail suspension test, the animals treated with a dose of 50 mg/kg of imipramine showed a significant decrease in immobility time (68.1%) when compared with the saline-treated group. The mice treated with NMD-Lipo at the doses of 0.1,1 and 10 mg/kg also showed a reduction in immobility time (80.9%, 86.7% and 90.0%, respectively) in comparison with the saline-treated group.

Table 1: Effect of saline, imipramine and NMD-Lipo in tail suspension test in mice.

Groups	**Immobility time (seconds)**	**Values of Immobility (%)**
Saline	258.2 ± 0.44	-
Imipramine 50 mg/kg	82.3 ± 0.38[a]	↓ 68.1[a]
NMD-Lipo 0.1 mg/kg	49.2 ± 0.79[a,b]	↓ 80.9[a] ↓ 40.2[b]
NMD-Lipo 1 mg/kg	34.3 ± 0.28[a,b,c]	↓ 86.7[a] ↓ 58.3[b] ↓ 30.3[c]
NMD-Lipo 10 mg/kg	25.8 ± 1.94[a,b,c,d]	↓ 90.0[a] ↓ 68.6[b] ↓ 47.6[c] ↓ 24.8[d]

Values are mean ± E.P.M. of the immobility time in the tail suspension test of 8 mice (by group) used in the experiments. [a]$p<0.05$, when compared with saline group (negative control); [b]$p<0.05$, when compared with imipramine group (positive control); [c]$p<0.05$, when compared with NMD-Lipo 0.1 group; [d]$p<0.05$, when compared with NMD-Lipo 1 group (ANOVA followed by Student–Newman–Keuls *t*-test as *post hoc* test).

2.3. Forced swim test

The results are summarized in Table 2. In the forced swim test, there was a decrease in the immobility time of imipramine and paroxetine-treated mice (63.3% and 56.3%, respectively). In addition, an increase in total immobility duration in mice treated with reserpine (27.7%) when compared with saline-treated group was found. The animals receiving NMD-Lipo at doses of 0.1, 1 and 10 mg/kg showed a significant reduction in immobility time (88.6%, 95.2% and 97.4%, respectively) compared with the saline-treated group.

The mice treated with NMD-Lipo at a dose of 10 mg/kg plus imipramine, paroxetine and reserpine, showed an increase in swimming time (90.3%, 69.0% and 48.1%, respectively) when compared to the saline-treated group.

Table 2: Effect of saline, imipramine, paroxetine, reserpine, NMD-Lipo and their associations in forced swim test in mice.

Groups	**Immobility ime (seconds)**	**Values of Immobility (%)**
Saline	224.3 ± 0.86	-
Imipramine 50 mg/kg	75.00 ± 0.68[a]	↓66.56%[a]
Paroxetine 20 mg/kg	133.0 ± 3.28[a]	↓ 40.70%[a]
Reserpine 0.25 mg/kg	263.70 ± 2.15[a]	↑14.94%[a]
NMD-Lipo 0.1 mg/kg	49.25 ± 0.96[a,b,c,d]	↓78.04%[a] ↓34.33%[b] ↓62.97%[c] ↓81.32%[d]
NMD-Lipo 1 mg/kg	39.38 ± 0.88[a,b,c,d,e]	↓82.44%[a] ↓47.49%[b] ↓70.39%[c] ↓85.06%[d] ↓20.04%[e]
NMD-Lipo 10 mg/kg	26.88 ± 1.24[a,b,c,d,e,f]	↓88.01%[a] ↓64.16%[b] ↓79.78%[c] ↓89.80%[d] ↓45.42%[e] ↓31.74%[f]
NMD-Lipo 10 mg/kg plus Imipramine 50 mg/kg	18.40 ± 9.35[a,b,g]	↓91.8%[a] ↓75.47%[b] ↓31.54%[g]
NMD-Lipo 10 mg/kg plus Paroxetine 20 mg/kg	59.00 ± 10.05[a,c,g]	↓ 73.7%[a] ↓55.64%[c] ↑54,44%[g]
NMD-Lipo 10 mg/kg plus Reserpine 0.25mg/kg	98.90 ± 9.58[a,d,g]	↓ 55.91%[a] ↓ 62.49%[d] ↑ 72.82%[g]

Values are mean ± S.E.M. of the immobility time in forced swim test of 8 mice (by group) used in the experiments. [a]p<0.05, when compared with saline group (negative control); [b]p<0.05, when compared with imipramine group (positive control); [c]p<0.05, when compared with paroxetine group (positve control); [d]p<0.05, when compared with reserpine group (positive control). [e]p<0.05, when compared with NMD-Lipo 0.1 group; [f]p<0.05, when compared with NMD-Lipo 1 group; [g]p<0.05, when compared with NMD-Lipo 10 group (ANOVA followed by Student–Newman–Keuls *t*-test as *post hoc* test).

2.4. Measurement of MAO_B activity

The results are summarized in Figure 1. In the measurements of MAO_B activity, there were no significant differences in animals treated with imipramine, paroxetine and reserpine, when compared with the saline-treated group. There was a significant decrease in MAO_B activity of animals treated with NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (41.2%, 55.9% and 73.5% respectively) compared with the animals treated with saline ($p<0.05$). In addition, a significant decrease in enzymatic activity of mice treated with NMD-Lipo was observed at a dose of 10 mg/kg with the subsequent addition of imipramine, paroxetine and reserpine (44.1%, 56.2% and 61.8% respectively) when compared with the saline-treated group ($p<0.05$).

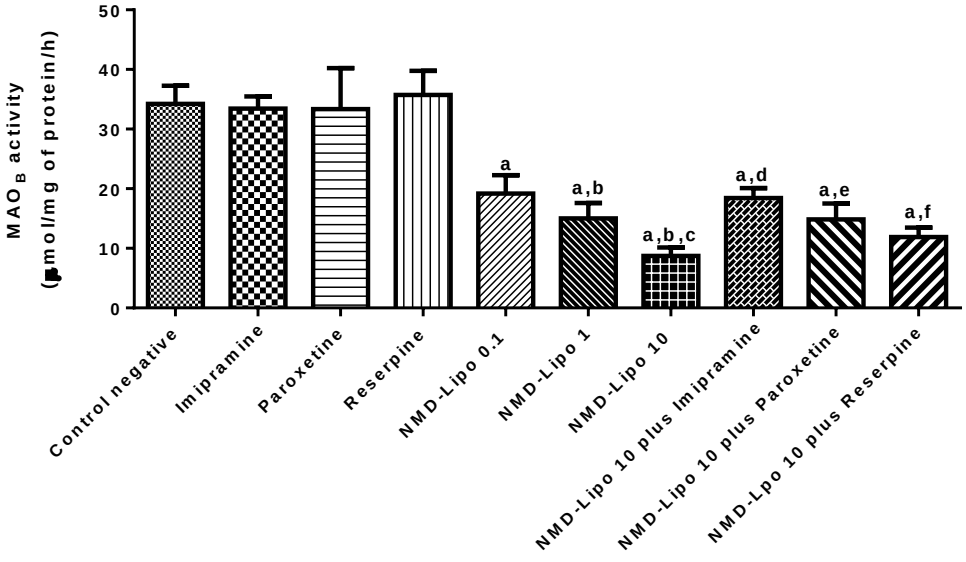

Figure 1: MAO_B activity in mice hippocampus after treatment with saline, imipramine, paroxetine, reserpine, NMD-Lipo and their combinations in the forced swim test.Values are mean ± E.P.M. of the MAO_B activity in mice hippocampus after forced swim test of 8 mice/group used in the experiments. [a]$p<0.05$, when compared with negative control (vehicle); [b]$p<0.05$, when compared with NMD-Lipo 0.1 group; [c]$p<0.05$, when compared with NMD-Lipo 1 group; [d]$p<0.05$, when compared with imipramine group (positive control); [e]$p<0.05$, when compared with paroxetine group (positive control); [f]$p<0.05$, when compared with reserpine group (positive control; ANOVA followed by Student–Newman–Keuls *t*-test as *post hoc* test).

3. Discussion

In the tail suspension test, the administration of NMD encapsulated into liposomes at doses of 0.1, 1 and 10 mg/kg was able to promote a greater reduction in immobility time of animals (40.2, 58,3 and 68.6%) than imipramine treatment. This result suggests that NMD-Lipo presents a significant dose-dependent antidepressant-like activity in mice, even better than that of the drug chosen as a positive control.

In the forced swim test, the immobility time of mice treated with NMD-Lipo at doses of 0.1, 1 and 10 mg/kg was reduced. Again, the effect was dose-dependent. This reduction was significantly greater than that found in the animals treated with imipramine (34.3%, 47.9% and 64.16% higher, respectively) and paroxetine (63.0%, 70.4% and 79.8% greater, respectively). These findings reinforced the hypothesis that states that liposomal NMD provides a better antidepressant-like activity in mice than that of the positive control.

Furthermore, the animal groups treated with the combination of NMD-Lipo and antidepressant drugs showed a lower immobility time and greater antidepressant-like effect than groups treated only with imipramine or paroxetine. The satisfactory results in the

administration of both NMD-Lipo and antidepressant drugs suggest an indication of combined therapy to treat depression.

Mice treated with the combination of NMD-Lipo at a dose of 10 mg/kg and reserpine showed an increase of 78.1% in immobility time compared with the animals treated only with NMD-Lipo. This finding can be explained by the fact that the administration of reserpine in rodents depletes brain monoamine oxidases (Sousa et al., 2013), which is the opposite effect of drugs used in the treatment of depression. These data may suggest a possible mechanism of action for the antidepressant activity of NMD-Lipo related to an increase in the concentration of brain monoamine oxidases.

In the measurement of MAO_B activity, it was observed that treatment with saline, imipramine, paroxetine, and reserpine did not cause the inhibition of MAO_B. On the other hand, treatment with NMD-Lipo at all doses tested (0.1, 1 and 10 mg/kg) resulted in a significant decrease in enzyme activity. This may suggest that NMD-Lipo causes a dose-dependent MAO_B inhibition, which might be responsible for the NMD antidepressant effect. Animals treated with combinations of NMD-Lipo and paroxetine, imipramine and reserpine showed lower MAO_B activity than untreated animals. However, those animals presented higher enzymatic activity than those treated only with the liposomal NMD at a dose of 10 mg/kg. This is due to the fact that paroxetine, imipramine and reserpine do not cause enzyme inhibition.

Depression has been recognized and treated by different therapeutic approaches over centuries (Rodrigues et al., 2014). In the present study, imipramine was chosen as a positive control because it is the oldest tricycle antidepressant, and is commonly used in the treatment of endogenous depression and illness-associated depression (Obuchowicz et al., 2014). The pharmacological action of this drug produces an increase in the concentration of monoamines through presynaptic monoamine reuptake inhibition (Yasuda et al., 2014).

Paroxetine has also been used as a positive control because it is a selective serotonin reuptake inhibitor widely used in therapy of depression (Murata el al., 2013). Reserpine is an alkaloid isolated from *Rauwolfia serpentine*, which was chosen as a negative control since it depletes monoamines by irreversibly blocking vesicular monoamine transporter. In mice, reserpine causes various symptoms presented in depression, such as hypoactivity, lethargy and anhedonia (Kyzar et al., 2013). The doses of imipramine, paroxetine and reserpine were chosen according to the literature (Campêlo et al., 2011 and Oliveira et al., 2013).

As reported, excessive calcium influx through the membrane and increased intracellular calcium concentrations play a role in the physiopathology of neuropsychiatric diseases (Dubovsk et al., 2014). In this scenario, nimodipine administered at a dose of 5 mg/kg showed antidepressant properties in rats, as previously reported (Katagiri et al., 2001). Even though this calcium channel antagonist has also proved to be useful in the treatment of affective illness in humans, it has never been administered as a single component of depression therapy; instead, it is always used in combination with other antidepressants (Frye et al., 2003, Grunze et al., 1996 and Pazzaglia et al., 1995). This is probably due to NMD high first-pass metabolism in the liver, which results in low bioavailability (Salgado-Figueroa, Gutiérrez, Sequella, 2015).

Previous research has shown that NMD encapsulated in liposomes presents effects on the CNS significantly greater than the free drug in several tests, suggesting that the liposomes promoted a controlled drug release by increasing its bioavailability and consequently its effect (Moreno el al., 2014 and Moreno et al., 2015). Hence, this liposomal formulation was chosen for the tests of antidepressant activity of the drug.

We have used the tail suspension test for determining a possible antidepressant-like activity of NMD-Lipo in treated mice. The forced swim test was carried out to confirm the NMD-Lipo antidepressant-like activity suggested in the tail suspension test. The doses were

chosen based on previous studies with free nimodipine (Frye et al., 2003, Grunze et al. 1996, Katagiri et al. 2001 and Pazzaglia et al. 1995) and liposomal NMD (Moreno el al., 2014 and Moreno et al., 2015).

The significant reduction in immobility time in mice treated with NMD-Lipo in the tail suspension and forced swim tests, as well as the increased immobility of rodents treated with reserpine and NMD-Lipo, are evidence that the liposomal NMD displays drug antidepressant-like activity by increasing the concentration of brain monoamines. The decrease in MAO_B activity in animals treated with the liposomal formulation containing NMD strengthens this hypothesis. More studies are needed to unravel the antidepressant activity of NMD-Lipo; however, NMD-loaded liposomes appear to be a promising approach for depression therapy.

4. Experimental procedure

4.1. Animals

Eight-week-old male Swiss mice (weighing 25-30 g) were obtained from Central Animal House of the Federal University of Piauí (Teresina, Brazil). Animals were housed in polypropylene cages with wire covers at 25 ± 2 °C under 12:12-h light–dark cycle (light on from 07:00 h to 19:00 h). They were supplied with commercial food pellets (Nutrilabor, Campinas, Brazil) and water ad libitum, and were acclimatized to laboratory conditions for 7 days before being subjected to experimental assays. The experimental protocol and procedures was approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA/UFPI N° 014/11). All experiments were performed according to the Guide of care and use of laboratory animals from the US Department of Health and Human Services (Washington, 1985).

4.2. Drugs and Chemicals

Cholesterol (Chol), trehalose, nimodipine, imipramine hydrochloride, paroxetine hydrochloride, reserpine and benzylamine were obtained from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Solvents and other chemicals supplied by from Merck (Darmstadt, Germany). All drugs were administered intraperitoneally. To study the association of the formulation with antidepressant drugs and reserpine, the drugs used as control (imipramine, paroxetine and reserpine) were administrated 30 minutes before NMD-Lipo.

4.3. Preparation and characterization of liposomes containing nimodipine (NMD-Lipo)

Liposomes containing nimodipine (NMD-Lipo) were prepared and characterized as previously reported (Moreno at al., 2014). The liposomal formulation was produced using the method of hydrating thin lipid film. The content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm. The encapsulation efficiency of nimodipine into liposomes was determined by the technique of ultrafiltration/ultracentrifugation using Ultrafree® units (Millipore, USA). A liposomal sample aliquot (400 µL) was transferred to filtering units and subjected to ultracentrifugation at 8776 g for 1 hour. The amount of encapsulated nimodipine was obtained from the difference between the total quantity measured in the formulation and the filtrate obtained after centrifugation.

4.4. Tail suspension test

The mice were divided into five groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), imipramine at a dose of 50 mg/kg

(positive control) and NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10 respectively). The animals were then subjected to the tail suspension test after 30 min of drug administration.

The total duration of immobility induced by tail suspension was measured according to a previously reported method (Steru et al., 1985). Mice were suspended 58 cm above the floor by adhesive tape placed approximately 1cm from the tip of the tail. The time during which mice remained immobile was quantified during a test period of 6 min. Mice were considered immobile only when they hung passively and completely motionless. Immobility was regarded as depression-like behavior (behavioral despair).

4.5. Forced swim test

The mice were divided into ten groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), imipramine at a dose of 50 mg/kg, paroxetine at a dose of 20 mg/kg, reserpine at a dose of 0.25 mg/kg (positive controls) and NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10, respectively). To study the effect of the formulation tested in combination with antidepressant drugs and reserpine, mice received NMD-Lipo at a dose of 10 mg/kg in combination with imipramine at a dose of 50 mg/kg (group NMD-Lipo 10 plus Imipramine), NMD-Lipo at a dose of 10 mg/kg in combination with paroxetine at a dose of 20 mg/kg (group NMD-Lipo 10 plus Paroxetine) and NMD-Lipo at a dose of 10 mg/kg in combination with reserpine at a dose of 0.25 mg/kg (group NMD-Lipo 10 plus Reserpine). Mice were subjected to the forced swim test 30 min after drug administration.

The test procedure was carried out according to the method describe by Porsolt et al. (1977). In brief, mice were individually forced to swim in glass beakers (height 25 cm, diameter 10 cm) containing 15 cm of water maintained at a temperature of 25 °C. The time

that the mice remained immobile was quantified during a test period of 5 min. The total immobility time was defined as the total amount of time during which the mice remained immobile or made only small limb movements necessary for floating. Immobility was regarded as depression-like behavior (behavioral despair).

4.6. Preparation of brain and liver mitochondria for MAO_B assay

After the forced swim test, the mice were sacrificed by cervical dislocation and their brains and livers were removed and dissected. Mitochondria were isolated from liver and brain of animals according to the method described by Gazzotti et al (1979). The organs were weighed and placed in a homogenization medium (pH: 7.4, temperature: 4 °C). Subsequently, the organs were sliced and grounded to obtain a uniform homogenate. Homogenates were centrifuged at 1100 g for 15 min at 4 °C and the supernatants were collected and centrifuged again at 1100 g rpm for 25 min at 4 °C. The pellets obtained were resuspended in 25 mL of homogenization medium and then centrifuged at 1100 g rpm for 25 minutes.

The mitochondrial pellets were resuspended in a homogenization medium to a final volume of 4 mL. The samples of isolated mitochondria were used to determine the protein concentration by applying the method described by Lowry et al. (1951).

4.7. Measurement of MAO_B activity

MAO_B activity was measured using benzylamine as substrate, according to the method of Tabour et al. (1954). A sample of 620 µg of mitochondrial proteins (90 µL) was dissolved in 2 mL of 0.1 M sodium phosphate buffer (pH 7.4). The reaction was started by the addition of 1 mM benzylamine and the progress of the reaction (benzaldehyde formation) was measured in a spectrophotometer UV/visible at 250 nm. The maximum rate of MAO_B activity

was expressed as micromoles per milligram of protein per minute. The activity of the MAO_B enzyme was assessed for 6 h after isolation of mitochondria.

Statistical analyses

Data values are expressed as mean ± standard error of mean (SEM) of the number of animals used in the experiments. Differences between groups were determined by analysis of variance (ANOVA) followed, when a difference was detected, by the Student–Newman–Keuls t-test with post hoc test. The significance level for rejecting the null hypothesis was always $p \leq 0.05$.

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Conflicts of interest

The authors declare no conflict of interest.

References

Aslan, A., Gurelik, M., Cemek, M., Goksel, H.M., Buyukokuroglu, M.E., 2009. Nimodipine can improve cerebral metabolism and outcome in patients with severe head trauma. Pharmacol. Res. 59, 120–124.

Campêlo, L.M.L., Sá, C.G., Almeida, A.A.C., Costa, J.P., Marques, T.H.C., Feitosa, C.M., Saldanha, C.B., Freitas, R.M., 2011. Sedative, anxiolytic and antidepressant activities of Citrus limon (Burn) essential oil in mice. Pharmazie. 66, 623-627.

Bege, N., Renette, T., Endres, T., Beck-Broichsitter, M., Hänggi, D., Kissel, T., 2013. In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm. Eur. J. Pharm. Biopharm. 84, 99–105.

Deussing, J. M., 2006. Animal models of depression. Drug. Discov. Today Dis. Models. 3, 375-383.

Dubovsky, S.L., Christlano, J., Daniell, L.C., Franks, R.D., Murphy, J., Adler, L., Baker, N., Harris, R.A., 1989. Increased intracellular calcium concentration in patients with bipolar affective disorders. Arch. Gen. Psychiatry. 46, 632-638.

Dubovsky, S.L., Daurignac, E., Leonard, K.E., 2014. Increased platelet intracellular calcium ion concentration is specific to bipolar disorder. J. Affect Disord. 164, 38-42.

Dubovsky, S.L., Murphy, J., Christiano, J., Lee, C., 1992. The calcium second messenger system in bipolar disorders: Data supporting new research directions. J. Neuropsychiatry Clin. Neurosci. 4, 3- 14.

Dubovsky, S.L., Thomas, M., Hijazi, A., Murphy, J., 1994. Intracellular calcium signalling in peripheral cells of patients with bipolar affective disorder. Eur. Arch. Psychiatry Clin. Neurosci. 241, 229- 234.

Finberg, J.P.M., 2014. Update on the pharmacology of selective inhibitors of MAO-A and MAO-B: Focus on modulation of CNS monoamine neurotransmitter release. Pharmacol. Ther. 143, 133–152.

Fornaro, M., Martino, M., Mattei, C., Prestia, D., Vinciguerra, V., Berardis, D., Pasquale, C., Iasevoli, F., Mungo, S., Fornaro, P., 2014. Duloxetine-bupropion combination for treatment-resistant atypical depression: A double-blind, randomized, placebo-controlled trial. Eur. Neuropsychopharmacol. 24, 1269–1278.

Frye, M.A., Pazzaglia, P.J., George, M.S., Luckenbaugh, D.A., Vanderham, E., Davis, C.L., Rubinow, D.R., Post, R.M., 2003. Low CSF somatostatin associated with response to nimodipine in patients with affective illness. Biol Psychiatry. 53, 180–183.

Gazzotti, P., Malmstrom, K., Crompton, M., 1979. Preparation and assay of animal mitochondria and submitochondrial vesicles. Membrane biochemistry. A laboratory manual on transport and bioenergetics. New York: Springer. 62–76.

Ghazaleh, H.A., Lalies, M.D., Nutt, D.J., Hudson, A.L., 2015. The modulatory action of harmane on serotonergic neurotransmission in rat brain. Brain. Res. 1597:56-64

Grunze, H., Walden, J., Wolf, R., Berger, M., 1996. Combined treatment with lithium and nimodipine in a bipolar i manic syndrome. Prog. Neuro-Psychopharmacal. &Biol. Psychiat. 20, 419-426.

Justin, A., Sathishkumar, M., Sudheer, A., Shanthakumari, S., Ramanathan, M., 2014. Non hypotensive dose of telmisartan and nimodipine produced synergistic neuroprotective effect in cerebral ischemic model by attenuating brain cytokine levels. Pharmacol. Biochem. Behav. 112, 61–73.

Katagiri, H., Kagaya, A., Nakae, S., Morinobu, S., Ymawaki, S., 2001. Modulation of serotonin2a receptor function in rats after repeated treatment with dexamethasone and L-type calcium channel antagonist nimodipine. Prog. Neuro-Psychopharmacal. & Biol. Psychiat. 25, 1269-1281.

Kim, S., Lee, Y., Kim, H., Lee, D., Woo, D., Choi, C., Chae, J., Choe, B., 2010. Desipramine attenuates forced swim test-induced behavioral and neurochemical alterations in mice: An in vivo 1 H-MRS study at 9.4 T. Brain.Res. 1348, 105-113.

Kumar, V., Bhat, Z.A., Kumar, D., 2013. Animal models of anxiety: a comprehensive review. J. Pharmacol. Toxicol. Methods. 68, 175–83.

Kyzar, E., Stewart, A.M., Landsman, S., Collins, C., Gebhardt, M., Robinson, K., Kalueff, A.V., 2013. Behavioral effects of bidirectional modulators of brain monoamines reserpine and d-amphetamine in zebra fish. Brain. Res. 1527, 108-11.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with follin phenol reagent. J. Biol. Chem. 193, 265-275.

Maigaard, K., Hageman, I., Jørgensen, A., Jørgensen, M.B., Wörtwein, G., 2012. Electroconvulsive stimulations prevent chronic stress-induced increases in L-type calcium channel mRNAs in the hippocampus and basolateral amygdala. Neurosci. Lett. 516, 24-28.

Moreno, L.C.G.A.I., Oliveira, G.Z.S., Cavalcanti, I.M.F., Santos-Magalhães, N.S., Rolim, H.M.L., Freitas, R.M., 2014. Development and evaluation of liposomal formulation containing nimodipine on anxiolytic activity in mice. Pharmacol. Biochem. Behav. 116, 64-68.

Moreno, L.C.G.A.I., Cavalcanti, I.M.F., Prabodh, S., Santos-Magalhães, N.S., Rolim, H.M.L., Freitas, R.M., 2015. Acute toxicity and anticonvulsant activity of liposomes containing nimodipine on pilocarpine-induced seizures in mice. Neurosci.Lett. 585, 38-42.

Murata, Y., Kamishioiri, Y., Tanaka, K., Sugimoto, H., Sakamoto, S., Kobayashi, D., Mine, K., 2013. Severe sleepiness and excess sleep duration induced by paroxetine treatment is a beneficial pharmacological effect, not an adverse reaction. J. Affect. Disord. 150, 1209-1212.

N'Gouemo, P., 2013. Probing the role of the sodium/calcium exchanger in pentylenetetrazole induced generalized seizures in rats. Brain Res. Bull.90, 52–7.

Obuchowicz, E., Bielecka, A.M., Paul-Samojedny, M., Pudełko, A., Kowalski, J., 2012. Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture. Pharmacol. Rep. 66, 34-43.

Oliveira, F.R.A.M., Cerqueira, G.S., Freitas, R.L.M., Costa-Júnior, J.S., Feitosa, C.M., Freitas, R.M., 2013. Anxiolytic- and antidepressant-like effects of the ethanolic

extract from Citrus limon plant widely used in Northeastern Brazil. Afr. J. Pharm. Pharmacol. 30, 2173-2179.

Pazzaglia, P.J., George, M.S., Post, R.M., Rubinow, D.R., Davis, C.L., 1995. Nimodipine increases CSF somatostatin in affectively ill patients. Neuropsychopharmacol. 13, 75-83.

Papakostas, G.I., 2009. Managing partial response or nonresponse: switching, augmentation, and combination strategies for major depressive disorder. J. Clin. Psychiatry 70 (6), 16–25.

Porsolt, R.D., Bertin, A., Jalfre, M., 1977. Behavioral despair in mice: a primary screening test for antidepressants. Arch. Into. Pharmacodynamic Thera. 229, 327-336.

Rodriguez, R., Petersen, R.B., Perry, G., 2014. Parallels between major depressive disorder and Alzheimer's disease: role of oxidative stress and genetic vulnerability. Cell. Mol. Neurobiol. 34, 925–949.

Roedding, A.S., Li, P.P., Warsh, J.J., 2006. Characterization of the transient receptor potential channels mediating lysophosphatidic acid-stimulated calcium mobilization in B lymphoblasts. Life Scien., 80, 89–97.

Rollema, H., Guanowsky, V., Mineur, Y.S., Shrikhande, A., Coe, J.W., Seymour, P.A., Picciotto, M. R., 2009. Varenicline has antidepressant-like activity in the forced swim test and augments sertraline's effect. Eu. J. Pharmacol. 605, 14-16.

Salgado-Figueroa, P., Gutiérrez, C., Squella, J.A., 2015. Carbon nanofiber screen printed electrode joined to a flow injectionsystem for nimodipine sensing. Sens. Actuators B: Chem. 220, 456–462.

Schmidt, M.V., 2011. Animal models for depression and the mismatch hypothesis of disease. Psychoneuroendocrinolo. 36, 330-338.

Socała, K., Nieoczym, D., Wyska, E., Poleszak, E., Wlaź, P., 2012. Influence of sildenafil on the antidepressant activity of bupropion and venlafaxine in the forced swim test in mice. Pharmacol. Biochem. Behav. 103, 273-278.

Souza, R.R., França, S.L., Bessa, M.M., Takahashi, R.N., 2013. The usefulness of olfactory fear conditioning for the study of early emotional and cognitive impairment in reserpine model. Behav. Process. 100, 67-73.

Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacol. (Berl). 85,367–370.

Sun, C., Wang, J., Liu, J., Qiu, L., Zhang, W., Zhang, L., 2013. Liquid Proliposomes of Nimodipine Drug Delivery System: Preparation, Characterization, and Pharmacokinetics. AAPS. PharmSciTech. 14, 332–338.

Sygnecka, K., Heinea, C., Scherf, N., Fasold, M., Binder, H., 2015. Nimodipine enhances neurite outgrowth in dopaminergic brain slice co-cultures. Int. J. Devl. Neuroscience. 40, 1–11.

Tabor, C.W., Tabor, H., Rosenthal, S.M., 1954. Purification of amine oxidase from beef plasma. J. Biol. Chem. 208, 645–662.

Xu, G.Z., Wang, M.D., Liu, K.G., Bai, Y.A., Wua, W., Li, W., 2013. A meta-analysis of treating acute traumatic brain injury with calcium channel blockers. Brain Res. Bull. 99, 41-47.

Wang, Z., Gu, J., Wang, X., Xie, K., Luan, Q., Wan, M., Zhang, Q., Jiang, H., Liu, D., 2013. Antidepressant-like activity of resveratrol treatment in the forced swim test and tail suspension test in mice: The HPA axis, BDNF expression and phosphorylation of ERK. Pharmacol. Biochem. Behav. 112, 104-110.

Wittman, D., 2014. Darwinian depression. J. Affect. Disorders. 168, 142-150.

Wong, H.L., Wu, X.Y., Bendayan, R., 2012. Nanotechnological advances for the delivery of CNS therapeutics. Adv. Drug. Deliv. Rev. 64, 686-700.

Yasuda, S., Yoshida, M., Yamagata, H., Iwanaga, Y., Suenaga, H., Ishikawa, K., Nakano, M., Okuyama, S., Furukawa, Y., Furukawa, S., Ishikawa, T., 2014. Imipramine ameliorates pain-related negative emotion via induction of brain-derived neurotrophic factor. Cell. Mol. Neurobiol. 34, 1199–1208.

Yingling, D.R., Utter, G., Vengalil, S., Mason, B., 2002. Calcium channel blocker, nimodipine, for the treatment of bipolar disorder during pregnancy. Am. J. Obstet. Gynecol. 187, 1711–1712.

7. GANTREZ AN NANOPARTICLES FOR THE ORAL DELIVERY OF NIMODIPINE: PHARMACOKINETICS STUDY AND EFFECT ON THE ANXIETY AND COGNITION OF MICE

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Abstract

It has been demonstrate that chronic stress increases the possibilities of developing neurodegenerative diseases, depression, skin disorders, rheumatoid arthritis, gastrointestinal diseases and cardiovascular problems. Nimodipine is a calcium channel blocker that restores some behavioral alterations and memory deficits produced by stress in mice. However nimodipine presents a low oral bioavailability. Thus, the aim of the present work was to develop and evaluate the capability of pegylated nanoparticles (based on Gantrez AN) as carrier to improve the oral bioavailability of nimodipine and test the effect of these formulations in the anxiolytic activity and cognitive ability of stressed mice. In the optimization of the preparative process of nimodipine-loaded nanoparticles we observed that the best results were obtained when we used an incubation time (between the drug and the polymer) of 15 min. Furthermore, it was observed that the higher payload and encapsulation efficiency were obtained when the nimodipine/poly(anhydride) ratio was fixed to 1:10. In these conditions, bare nanoparticles (NMD-NP) presented a mean size of 184 nm, zeta potential of -40 mV and a drug payload of 66 µg/mg nanoparticles with an encapsulation efficiency of 81%. In addition, pegylated nanoparticles (NMD-NP/PEG) presented a mean size of 191 nm, zeta potential of -23 mV and a drug payload of 68 µg/mg nanoparticles with an encapsulation efficiency of 84%. Concerning the in vitro release of NMD from the nanoparticles, it is noteworthy that under acidic conditions (SGF), no significant drug release was observed for any of the formulations tested. On the contrary, when nanoparticles were incubated under simulated intestinal fluid, nimodipine was released. This fact evidences the gastroresistent properties of these nanoparticles. In the pharmacokinetics studies, pegylated nanoparticles provided a high oral bioavailability of nimodipine (62%), which was calculated to be 7-times higher than a conventional solution of the drug (9%). This improvement was not observed for bare nanoparticles. These findings would be in line with previous observation demonstrating the mucus-penetrating properties of nanoparticles pegylated with PEG 2000 and their capability to reach the surface of the intestinal epithelium. On the other hand, bare nanoparticles have been reported to show mucoadhesive properties with a high interaction capability with the stomach mucus layer. So it is possible to imagine that the “success” with pegylated nanoparticles may be ascribed to their capacity to directly target the small intestine mucosa in which the drug would be released in a sustained way providing high levels of the calcium channel blocker in blood for prolonged periods of time. Encapsulated nimodipine showed a tendency to reverse the anxiety of treated mice, but this reversal was not statistically significant. Furthermore, NMD-NP/PEG was able to protect the cognitive deficits observed in stressed mice, improving not only the acquisition as well as the retention of memory.

Keywords: Nimodipine, nanoparticles, oral delivery, anxiety, cognitive deficit.

1. Introduction

Stress is a condition in which an individual is under a lot of personal or social pressure developing body reactions that disturb the normal physiological equilibrium, resulting in a state of threatened homeostasis and endanger the survival of the affected individual (Lkhagvasuren et al., 2014). An exposure to stress stimuli induces various changes including alteration in behavior, autonomic functions and hyper-activation of the hypothalamus-pituitary-adrenal (HPA) axis (Sparta et al., 2013). In addition, it has also been demonstrate that chronic stress increases the possibilities of developing neurodegenerative diseases (e.g. Alzheimer's and Parkinson's) (Sotiropoulos et al., 2011), depression (Munhoz et al., 2008), skin disorders (e.g. psoriasis, urticaria, alopecia and atopic dermatitis) (Zhang et al., 2009), rheumatoid arthritis (Steptoe, Hamer and Chida 2007), gastrointestinal diseases such as peptic ulcer and irritable bowel syndrome (Caso, Leza and Menchén, 2008) and cardiovascular problems including cardiomyopathy, ventricular arrhythmias and coronary artery disease (Geppert et al., 2010).

Calcium channels play an important role in brain activity, since it is demonstrated that neuronal degeneration during aging is thought to be mediated by changes in the level of intracellular calcium (Shen et al., 2016). Chronically elevated levels of intracellular calcium in neurons and reduced ability to buffer calcium levels during normal aging provoke subtle age-associated declines and mild impairment (Thibault, Gant, Landfield, 2007). Furthermore, increased intracellular calcium has been associated to the development of pathologies such as of depression (Maigaard et al., 2012), anxiety (Kumar, Bhat and Kumar, 2012) and epilepsy (N'Gouemo, 2013). In this sense, a drug that blocks calcium channels would be a good option for the treatment of brain damage triggered by stress.

Nimodipine, a dihydropyridine calcium channel blocker and classical compound ascribed to the class II of the Biopharmaceutical Classification System (BCS II), was

originally developed for the treatment of hypertension (Fu et al, 2013a). Nowadays, it is also used to prevent subarachnoid hemorrhagic complications and has been studied for the treatment of other pathologies of the central nervous system (Fu et al, 2013b). In this way, it has been proved that nimodipine restores some behavioral alterations and memory deficits produced by stress in mice (Kumar, Singh and Jaggi, 2012).

Despite the great versatility of nimodipine, the drug has some drawbacks. Therefore, due to its low solubility in gastrointestinal fluids and its extensive first-pass metabolism, nimodipine presents a low oral bioavailability (approximately 13%), which requires frequent dosage regimens (i.e., 60 mg every 6 h) (Sweetman, 2009). This high frequency in drug administration is inconvenient to patients, reducing their compliance and treatment adherence.

Recently, liposomal nimodipine, intraperitoneally administrated, has demonstrated a significantly higher ability to produce anxiolitic (Moreno et al, 2014), anticonvulsant (Moreno et al, 2015) and antidepressant (Moreno et al, 2016) effects than the free drug. These effects would be related to the capacity of these liposomes to sustain the nimodipine blood concentration for a long period of time and, then, provide high levels of the calcium blocker agent in the brain. However, liposomes cannot be administrated orally and this limitation is an inconvenience for the treatment of chronic diseases, as the pathologies mentioned above.

On the other hand, polymeric nanoparticles are widely investigated as carriers for oral drug delivery. Drug encapsulation in these devices may protect the compounds of interest from its premature degradation and, at the same time, offer a controlled release dosage form (Penalva et al, 2015). In addition, polymer nanoparticles may offer supplementary advantages such as the co-encapsulation of permeation enhancers (Huarte et al, 2016) and/or their "decoration" with ligands in other to modify their distribution in vivo (Salman, Irache and Gamazo, 2009). In this context, one possibility may be the use of poly(anhydride) nanoparticles coated with poly(ethylene glycol) (PEG). These nanocarriers possess mucus-

permeating properties and can reach the absorptive intestinal epithelium when administrated orally (Inchaurraga et al., 2015; Zabaleta et al, 2012). This effect may be of interest to promote the oral absorption of class II compounds of BCS (e.g., nimodipine).

Thus, the aim of the present work was to develop and evaluate the capability of pegylated nanoparticles (based on Gantrez AN) as carrier to improve the oral bioavailability of nimodipine. In addition, the effect of these formulations in the anxiolytic activity and cognitive ability of stressed mice was also studied.

2. Materials and methods

2.1. Chemicals, reagents and solutions

Poly(methyl vinyl ether-co-maleic anhydride) or poly (anhydride) (Gantrez® AN 119; MW 200,000) was kindly gifted by Ashland Inc. (Barcelona, Spain). Nimodipine, poly(ethylene glycol) 400 (PEG400), sodium chloride, monobasic potassium phosphate and Tween 80 were supplied by Sigma (Madrid, Spain). Corticosterone was supplied by Sigma-Aldrich (St Louis, USA). Poly(ethylene glycol) 2000 (PEG 2000) was provided by Fluka (Switzerland). Acetone and ethanol were obtained from VWR Prolabo (Fontenay sous Bois, France) and methanol and acetonitrile from Merck (Darmstadt, Germany). The slide-A-Lyzer® Dialysis cassette 10.000 MWCO and the filter nylon with pore of 0.45 μm were from Thermo scientific (Rockford, USA). The Microvette® 500K3E plasma tubes were from Sarstedt (Nümbrecht, Germany). The kit for measure serum corticosterone levels was from Cayman Chemical (Ann Arbor, USA). Sodium dodecyl sulphate-polyacrylamide gel nitrocellulose membrane was from Hybond-ECL; Amersham Bioscience (Barcelona, Spain). Anti-GR antibody was from Santa Cruz Biotechnology (Dallas, USA) and secondary antibodies conjugated to IRDye 800CW or IRDye 680CW were from LI-COR Biosciences (Lincoln, USA). All reagents and chemicals used were of analytical grade.

2.2. Preparation of nimodipine-loaded nanoparticles

In this study bare and pegylated poly(anhydride) nanoparticles were prepared. In all cases, these nanoparticles were prepared by a solvent displacement method previously described with some modifications (Calvo et al., 2011). In brief, for bare nanoparticles, 100 mg poly(anhydride) were firstly dissolved in 4 mL acetone. In parallel, a determined amount of nimodipine was dissolved in 1 mL acetone and, then, added to the solution of acetone containing the polymer. After incubation under magnetic stirring, nanoparticles were obtained by the addition of 20 mL of a mixture between ethanol and water (1:1 by vol.). The organic solvents were eliminated under reduced pressure (Büchi R210, Switzerland) and the nanosuspensions were purified by ultracentrifugation at 21000 rpm for 20 min (Sigma 3K3D, Germany). Supernatants were removed and pellets resuspended in water. Finally, the formulations were frozen and freeze-dried (Genesis 12EL, Virtis, USA) using sucrose (5% w/w) as cryoprotector. These nimodipine-loaded nanoparticles were named as NMD-NP.

On the other hand, pegylated nanoparticles were prepared in the same way as described before but after adding 12.5 mg PEG 2000 to the just formed nanoparticles. This mixture was maintained under agitation for 30 min. This formulation was identified as NMD-NP/PEG.

Control nanoparticles were also prepared by the same procedure but in the absence of nimodipine (NP/PEG) and PEG 2000 (NP).

2.3. Preparation of nimodipine solution

A nimodipine solution (NMD) was used as control. For this purpose, 60 mg nimodipine were dissolved in 6 mL PEG 400 under magnetic stirring. Then, 4 mL purified water were added and the final mixture was agitated in the dark for 10 min. This solution was hermetically closed and stored at 25ºC/RT in the dark until use.

2.4. Characterization of nanoparticles

2.4.1. Physicochemical characterization

The mean hydrodinamic diameter of the nanoparticles and the zeta potential were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a ZetaPlus analyzer system (Brookhaven Instruments Corporation, New York, USA). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1:10) and measured at 25°C by dynamic light scattering angle of 90°. The zeta potential was determined as follows: 200 µL of the same samples were diluted in 2 mL of a 0.1 mM KCl solution. Morphological analysis and the surface characteristics of nanoparticles were evaluated by transmission electron microscopy (TEM) using negative contrast to the surface view. The yield of the obtention process of nanoparticles was calculated by gravimetry as described previously (Arbos et al., 2002).

2.4.2. Nimodipine content in nanoparticles

The amount of nimodipine loaded into nanoparticles was quantified by HPLC. The chromatographic system was an Agilent 1100 series (Waldbornn, Germany), coupled with a UV diode array detection system. Data were analysed using the Chemstation G2171 program (B.01.03). The separation of nimodipine was carried out at 30°C on a reversed-phase 150 x 3 mm C18 Phenomenex Gemini column (particle size 5 µm). The mobile phase, pumped at 0.5 mL/min, was 70:30 acetonitrile-water and the effluent was monitored with UV detection at 236 nm. Under these conditions, the run time was 8 min and nimodipine eluted at 3.2 min. Calibration curves were designed over the range of 40-100000 ng/mL (R^2> 0.999). The limit of quantification was calculated to be 30 ng/mL with a relative standard deviation of 5%.

For analysis, 10 mg of the lyophilized powder containing nanoparticles were solubilized in acetonitrile (10 mg/mL). The samples were transferred to auto-sampler vials,

capped and placed in the HPLC auto-sampler. Then, 10 µl aliquot was injected onto HPLC column. Each sample was assayed in triplicate, and results were expressed as the amount of nimodipine (µg) per mg nanoparticles. The payload was calculated as the quotient between the amount of nimodipine quantified in the samples and the weight of nanoparticles used in the analysis. The drug encapsulation efficiency (EE) was calculated as follows:

$$EE\ (\%) = [Q_{encapsulated}/Q_{initial}] \times 100 \qquad \text{[Equation 1]}$$

Where $Q_{initial}$ was the initial amount of nimodipine that it was supposed to be added per mg of polymer that forms the NP, and $Q_{encapsulated}$ was the amount of nimodipine in digested nanoparticles, which was calculated from the HPLC measurements.

2.4.3. *In vitro* release studies

Release experiments were conducted at 37°C using simulated gastric (SGF, pH 1.2) and intestinal (SIF, pH 6.8) fluids. In order to fulfill sink conditions, Tween® 80 (2% w/v) was added to both media. The studies were performed under agitation in a slide-A-Lyzer® Dialysis cassette 10.000 MWCO (Thermo Scientific, Rockford, IL, USA). For the study, 3 mg nimodipine loaded in nanoparticles were dispersed in 10 mL water and introduced in the cassette, which was then placed into a vessel containing 500 mL SGF for 2 hours. After this time, the same cassette was removed from the SGF and introduced in a second vessel with 500 mL SIF until the end of the experiment. At different time points, samples of 1 mL were collected and filtered to 0.45 µm (Filter nylon, Thermo Scientific, Rockford, USA) before quantification. The volume was recovered by the addition of an equal volume of gastric or intestinal simulated fluids.

The amount of nimodipine released from the formulations was quantified by HPLC (see above). Calibration curves of nimodipine were prepared in both release media over the range of 40-100000 ng/mL ($R^2 > 0.99$).

In order to determine the nimodipine release mechanism from nanoparticles, the obtained data were fitted to the Korsmeyer-Peppas and Peppas Shalin models. The Korsmeyer-Peppas equation is a simple semi-empirical model which relates drug released with the elapsed time (Korsmeyer et al, 1983; Siepman, Siepman, 2008):

$$M_t/M_\infty = K\, t^{\,n} \qquad \text{[Equation 2]}$$

In which M_t/M_∞ is the fraction of drug released at time t, K is the kinetic constant and n in the released exponent indicative of the release mechanism. Values of n close to 0.5 correspond to a Fickian diffusion mechanism, whereas values between 0.5 and 1.0 correspond with a non-Fickian transport. Only M_t/M_∞ values lower than 0.6 were fitted to this mathematical model.

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The Peppas-Shalin equation was employed to study the contribution of both diffusion and erosion mechanisms on the release of nimodipine from nanoparticles:

$$M_t/M_\infty = K_D\, t^{\,m} + K_E\, t^{\,2m} \qquad \text{[Equation 3]}$$

where K_D and K_E indicates the contribution of drug diffusion and erosion/relaxation phenomena of nanoparticles to drug release, respectively.

Finally, the released data were also fitted to a zero order kinetic model (Fredenberg et al, 2011; Mökel, Lippold, 1993):

$$M_t/M_\infty = K_0\, t \qquad \text{[Equation 4]}$$

2.5. In vivo pharmacokinetic studies in Wistar rats

2.5.1. Pharmacokinetic studies

Pharmacokinetic studies were performed in male Wistar rats (200-250 g) obtained from Harlan (Barcelona, Spain). Studies were approved by the Ethical Committee for Animal

Experimentation of the University of Navarra (protocol number 080-15) in accordance with the European legislation on animal experiments.

Previous to the oral administration of the formulations, animals were fasted overnight to avoid interference with the absorption, allowing free access to water. For the pharmacokinetic study, rats were randomly divided into 4 groups of 6 animals each. The experimental groups were as follows: (i) nimodipine solution in PEG 400:water (60:40 v/v), (ii) nimodipine-loaded poly(anhydride) nanoparticles (NMD-NP), and (iii) nimodipine loaded in pegylated nanoparticles (NMD-NP/PEG). As a control, the last group of animals received intravenously the solution of nimodipine in the mixture of PEG 400 and water. In all case, the dose of nimodipine (orally or intravenously) was 5 mg/kg body weight.

Blood samples (500 µL) were collected at different set-point times after administration in Microvette® 500K3E plasma tubes (Sarstedt, Nümbrecht, Germany). In all cases, the blood volume was recovered intraperitoneally with an equal volume of normal saline solution pre-heated at body temperature. Samples were immediately centrifuged at 10000 rpm for 10 min and plasma aliquots were frozen at -80 ºC until analysis.

2.5.2. Determination of nimodipine plasma concentration by HPLC

Prior the analysis, nimodipine was extracted from plasma samples. Both the extraction and the quantification of nimodipine in rat plasma followed the protocol stated by Huang and co-workers (2014) with minor modifications. For this purpose, 400 µL plasma was vortex-mixed with 200 µL 0.5 M NaOH for 1 min. Then 2 mL of a mixture between ether anhydrous and n-hexane (1:1 by vol.) was added and the solution was vortex-mixed at 25° C for 5 min. After centrifugation at 5000 rpm for 8 min, supernatants were recovered, transferred to a tube and evaporated at 50ºC. Lastly, 100 µL of methanol was added into the tube and was vortex-mixed for 5 min in order to dissolve the residues. Then, 20 µL of the solution was analyzed by HPLC.

Analyses were carried out in the same HPLC apparatus as described above with UV detection at 360 nm. The separation was also carried out at 30°C on a reversed-phase 150 x 3 mm C18 Phenomenex Gemini column (particle size 5 µm). However, in this case, the mobile phase, pumped at 1 mL/min, was 75:25 methanol-water. Under these conditions, the retention time for nimodipine was approximately 6.90 min. The same protocol was followed for the preparation of calibration curves and quality control standards, using blank plasma and different solutions of nimodipine in acetonitrile. Calibration curves were designed over the range of 40-100000 ng/mL (R2> 0.999). The limit of quantification was calculated to be 30 ng/mL. Linearity, accuracy and precision values during the same day (intraday assay) at low, medium and high concentrations of nimodipine were within the acceptable limits (relative error and coefficient of variation less than 15%).

2.5.3. Pharmacokinetic data analysis

Nimodipine plasma concentration was plotted against time, and pharmacokinetic analysis was performed using a non-compartmental model with the WinNonlin 5.2 software (Pharsight Corporation, USA). The following parameters were estimated: maximal serum concentration (C_{max}), time in which C_{max} is reached (T_{max}), area under the concentration-time curve from time 0 to the last sampling time (AUC), mean residence time (MRT), clearance (Cl), volume of distribution (V) and half-life in the terminal phase ($t_{1/2}$). Furthermore, the relative bioavailability (Fr %) of nimodipine was estimated by the following equation:

$$Fr\ (\%) = (AUC_{oral}/AUC_{iv}) \times 100 \qquad \text{[Equation 5]}$$

In which AUC_{iv} and AUC_{oral} are the areas under the curve for the iv and oral administrations, respectively.

2.6. Animals and experimental design

Male C57BL/J6 mice, 8 weeks old, were used. Animals were housed under a 12:12 h light/dark cycle at 21 ± 1ºC and 55 ± 5% relative humidity. Animal handling was conducted

in accordance with the principles of laboratory animal care as detailed in the European Communities Council Directive (2003/65/EC), Spanish legislation (Real Decreto 1201/2005) and approved by the Ethics Committee of the University of Navarra (protocol number 080-15).

Animals were randomly assigned to eight experimental groups with 10 mice each. Four groups of animals received drinking water containing a solution of 50 µg/mL corticosterone in 1% ethanol, whereas the other 4 groups of animals received drinking water containing 1% ethanol to counteract the effects of the chronic ethanol administration in the "corticosterone" (CORT) group. Then, the animals were treated with one of the following treatments: (i) saline orally administered every 24 hours, (ii) 10 mg/kg nimodipine orally every 24 hours, (iii) 10 mg/kg nimodipine formulated in NMD-NP/PEG orally administered every 24 hours, and (iv) empty nanoparticles (NP/PEG) in similar amount and posology to that administered with nimodipine-loaded nanoparticles. The treatments lasted seven weeks.

2.7. Behavioral experiments

After the 4 weeks of treatment and over 3 consecutive weeks, behavioural experiments were conducted between 9:00 am and 2:00 pm. Mice body weight measurements were taken once a week. During behavioural testing, for animals included in the "CORT" groups, corticosterone was maintained in the drinking water. Behavioural tests were always carried out in animals 20h after receiving the treatment (saline, NMD, NMD-NP/PEG or NP/PEG).

2.7.1. Open field test

Locomotor activity was measured for 30 min in an open field (65×65 cm^2, 45 cm height) made of black wood, using a video-tracking system (Ethovision 3.0, Noldus

Information Technology B.V., The Netherlands) in a softly illuminated room. The total distance walked was analyzed as general mobility parameter. The tendency of anxious animals to do not explore the periphery of the open field was observed measuring the distance traveled from the center of the box by mice.

2.7.2. Marble Burying

Compulsive-anxiety behaviour in mice was assessed with the marble burying test (Deacon, 2006). For this purpose, twelve marbles (1.5-cm diameter) were placed uniformly in a cage (45 × 28 × 20 cm) containing a constant amount of sawdust (3 cm deep). Mice were placed in the center of the cage and left for 30 min. The number of marbles buried was recorded.

2.7.3. Elevated-plus maze

The elevated plus-maze for mice consists of two opposing open arms (30 × 5 cm) and two closed arms (30 × 30× 5 cm), likewise opposing cross-shaped arms. The open and closed arms are connected by a central platform (5 × 5 cm) high and 38.5 cm from the floor. The animals were placed in the center of the apparatus with the head turned to towards one of the closed arms and their behavior was observed for 5 min. The following behavioral measure was recorded: the latency to enter in an open arm for the first time. To consider that the mice had entered into an arm, the four legs were inside the arm. Mice were video recorded and the total time spent exploring was also measured (Ethovision XT 5.0 plus multiple body point module).

2.7.4. Morris Water Maze (MWM)

The Morris water maze (MWM), a hippocampus-dependent learning task, was used to test spatial memory and to evaluate the working and reference memory functions. The maze consisted of a black circular tank (145 cm diameter) filled with water (20-22ºC) and made

opaque by the addition of non-toxic white paint. Mice underwent visible-platform training for 1 day (six trials) using a platform raised above the surface of the water (visible-platform). No distal visible cues were present during this phase. This was followed by hidden-platform training (with all visible cues present) during which mice were trained to locate a platform in the opposite quadrant and submerged 1 cm beneath the surface, conducted over 9 consecutive days (4 trials per day). In both visible- and hidden-platform versions, mice were placed pseudo-randomly in selected locations, facing towards the wall of the pool to eliminate the potentially confounding contribution of extramaze spacial cues. Each trial was terminated when the mouse reached the platform or after 60 s, whichever came first. Mice failing to reach the platform were guided onto it. After each hidden-platform trial, mice remained on the platform for 15 s. To test memory retention, probe trials were performed at the 4^{th}, 7^{th} and last day of the test (10^{th} day). In the probe trials the platform was removed from the pool and mice were allowed to swim for 60s. The percentage of time spent in the target quadrant was measured. All trials were monitored by a video camera set above the centre of the pool connected to a video tracking system (Ethovision 3.0; Noldus Information Technology B.V, Wageningen, Netherlands).

2.8. Analysis of corticosterone levels and glucocorticoid receptor

After last day of MWM test, mice were killed by decapitation between 09:00–12:00 am. Brains were removed and dissected on ice to obtain hippocampus and frontal cortex and stored at −80 °C. Blood samples were collected and were centrifuged for 10 min at 10000 rpm (4ºC). The serum was carefully collected in tubes and stored at – 80 ºC.

Corticosterone levels were measured in serum samples using the corticosterone enzyme immunoassay kit (Cayman Chemical), following the manufacturer's instructions. Western Blotting were performed in hippocampal and cortical tissue as described previously

(Solas et al. 2010). Brain protein was determined by Bradford protein assay. Samples (25 μg protein) were separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel (7.5%). Membranes were probed overnight at 4 °C with the anti-GR antibody (1:2000). Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW were diluted to 1/15,000 in TBS with 5% BSA. Bands were visualized using Odyssey Infrared Imaging System. β-actin was used as internal control. Results were calculated as the percentage of optical density values of the saline treated mice.

2.9. Statistical analysis

For the physicochemical and pharmaceutical characterization of formulations, data were expressed as the mean ± standard deviation (S.D.) of at least three experiments. The different behavioral paradigms and western blotting studies were analysed using One-way ANOVA analysis followed by Tukey post hoc. In the acquisition phase of the MWM, treatment effects were analyzed by 1-way analysis of variance (ANOVA) for repeated measures followed by Tukey post hoc test. For each day, a one-way ANOVA was carried out and significant differences among groups were analyzed with Tukey post hoc test. Treatment differences were considered statistically significant at $p < 0.05$. Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 15.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Optimization of the preparative process of nimodipine-loaded nanoparticles

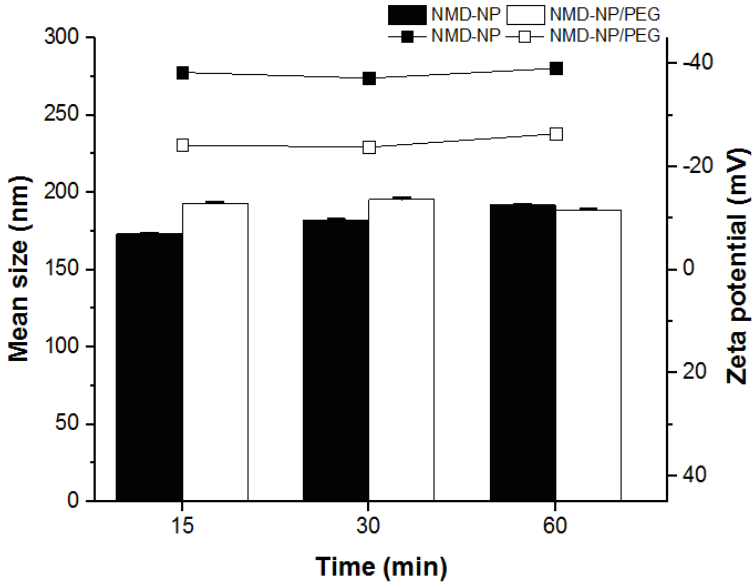
For the optimization of the preparative process of NMD-loaded nanoparticles, two parameters were studied: (i) incubation time between the drug and the polymer (Gantrez AN)

before the formation of nanoparticles by desolvation and (ii) the drug/poly(anhydride) ratio. All the studies were performed with an initial bulk amount of polymer of 100 mg.

Figure 1 shows the influence of the incubation time between NMD and the polymer, prior the formation of nanoparticles, on the physico-chemical properties of the resulting nanoparticles. For bare nanoparticles, the size of the nanoparticles significantly increased by increasing the time of incubation, whereas no influence of this parameter on the zeta potential or the PDI was observed (Figure 1A). Regarding the drug payload in these nanoparticles, a slightly (but no significant) decreased of the drug content was observed by increasing the time of incubation (Figure 1B). For pegylated nanoparticles, within the experimental conditions tested, the effect of the incubation time on the main physico-chemical properties of the resulting nanoparticles was negligible (Figure 1).

In a similar way, the effect of the drug/polymer ratio on the characteristics of nanoparticles was also studied (Figure 2). For these studies, the time of incubation between the drug and the polymer was, in all cases, 15 minutes. For both types of nanoparticles (bare and pegylated), the nimodipine loading and its encapsulation efficiency increased by increasing the drug/polymer ratio from 0.025 to 0.1. For drug/polymer ratios higher than 0.1, the yield of nanoparticle formation significantly decreased.

A



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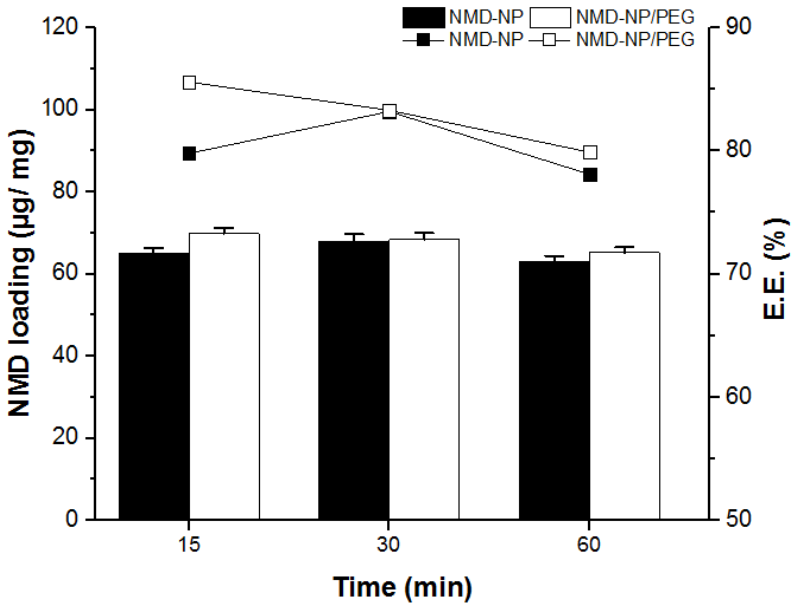


Figure 1: Influence of the incubation time between the drug and the polymer before the formation of nanoparticles on the physico-chemical properties of the resulting nanoparticles. Data expressed as mean ± SD, n = 3. Bars represent mean size (A) and drug loading (B), whereas lines refer to the zeta potential (A) and encapsulation efficiency (B). Black symbols represent data from bare nanoparticles (NMD-NP) and white cartoons are values obtained with pegylated nanoparticles (NMD-NP/PEG).

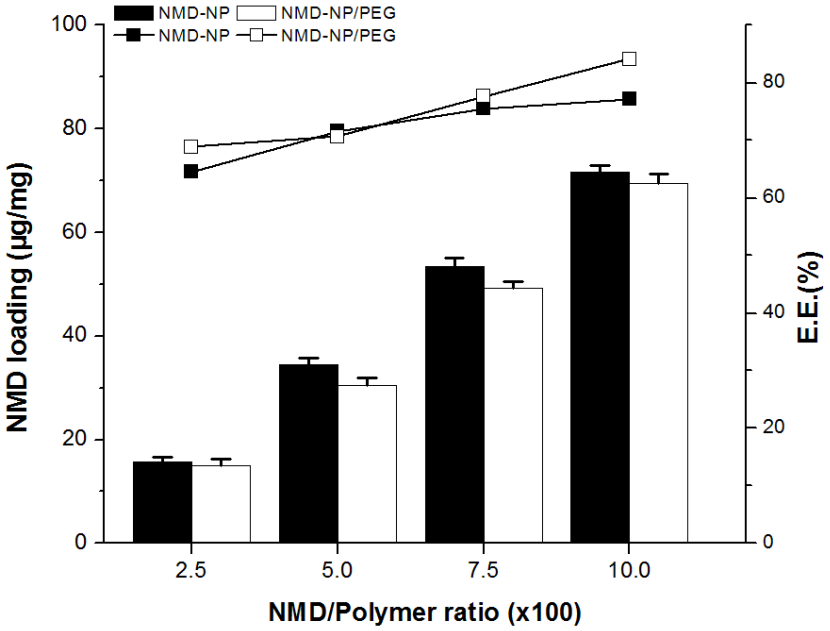


Figure 2: Influence of the nimodipine/polymer ratio on the payload and encapsulation efficience (EE) of poly(anhydride) nanoparticles. Data expressed as mean ± SD, n=6. Bars represent drug loading and lines the encapsulation efficiency. Black symbols are values from bare nanoparticles (NMD-NP), whereas white ones refers to pegylated nanoparticles (NMD-NP/PEG).

3.2. Characterization of the NMD-loaded nanoparticles

Based on the former optimization results, nanoparticles were prepared at a NMD/poly(anhydride) ratio of 0.1 with an incubation time (between the drug and the polymer) of 15 min. The physicochemical characteristics of the resulting poly(anhydride) nanoparticles containing NMD are summarized in Table 1. For bare nanoparticles, the encapsulation of nimodipine significantly increased the mean size (120 vs 184 nm), whereas the zeta potential was not affected (about -39 mV). The drug loading was calculated to be 66 µg/mg nanoparticles with an encapsulation efficiency of 81%. For pegylated nanoparticles, the mean size also increased by the encapsulation of the drug (124 vs 191 nm). Nanoparticles displayed a lower negative surface charge (about –23 mV) than uncoated nanoparticles and the payload was about 68 µg/mg (with encapsulation efficiency close to 90%). Interestingly,

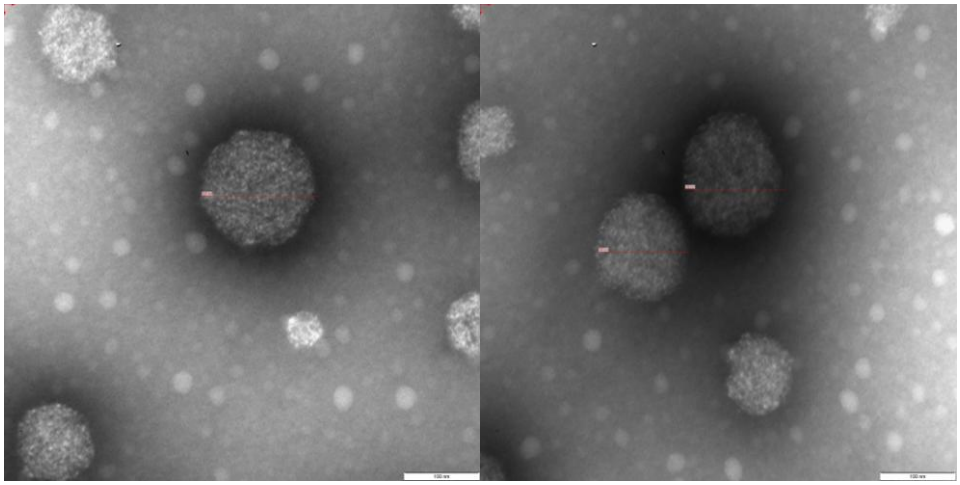
in all cases, the polydispersity index (PDI) was lower than 0.2, which implies homogeneous formulations (Table 1).

The morphological analysis by transmission electron microscopy (TEM; Figure 3) showed that nimodipine-loaded nanoparticles consisted of homogeneous population of spherical particles with a mean size similar to that obtained by spectroscopy photon correlation. No visible differences were observed for uncoated and pegylated nanoparticles.

Table 1: Physico-chemical characterization of poly(anhydride) nanoparticles. Data expressed as mean ± SD, n = 6.

	Size (nm)	**PDI**	**PZ (mV)**	**Payload (µg/mg)**	**EE (%)**	**Yield (%)**
NP	120 ± 0.5	0.133	-37 ± 1.1	-	-	87%
NP-NMD	184 ± 1.2	0.134	-40 ± 0.4	66	81%	83%
NP/PEG	124 ± 0.3	0.151	-28 ± 1,9	-	-	88%
NP-NMD/PEG	191 ± 1.7	0.081	-23 ± 0.5	68	84%	90%

A



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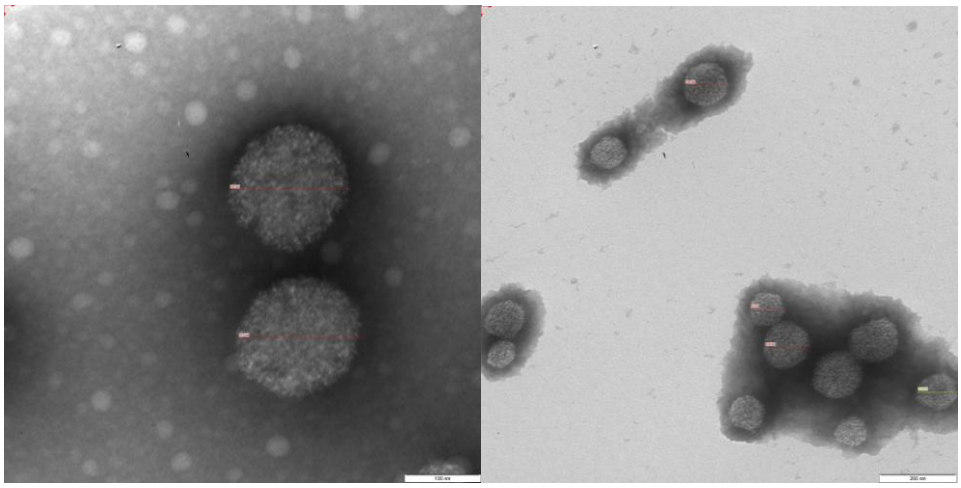

Figure 3: TEM-photographs of poly(anhydride) nanoparticles containing nimodipine. Figure 3A: NMD-NP Figure 3B: NMD-NP/PEG.

3.3. In vitro release study

Nimodipine release kinetics from nanoparticles was evaluated in simulated gastric and intestinal fluids (containing polysorbate 80 as solubilizing agent for NMD). Figure 4 represents the release profiles of nimodipine from the different assayed formulations as a function of time. Overall, all the nanoparticle formulations displayed a similar release behaviour. This pattern was characterised by a first non-release step, when nanoparticles were dispersed in SGF, and a release step (when nanoparticles were incubated in SIF). When nanoparticles (NMD-NP and NMD-NP/PEG) were dispersed in SIF, nimodipine was initially

slowly released. Thus, after 24 h of incubation in SIF, only about 15% of the loaded nimodipine was released from both bare and pegylated nanoparticles following an apparent linear profile that fitted well to a zero order equation (Equation 4; Table 2). This sustained release of nimodipine in this first step of incubation in SIF was associated to the presence of diffusion and erosion phenomena as presented in Table 2. Then, after 24 h, nimodipine was rapidly released from nanoparticles and, at the end of the experiment (98 h), the total content of nimodipine in bare and pegylated nanoparticles was completely released.

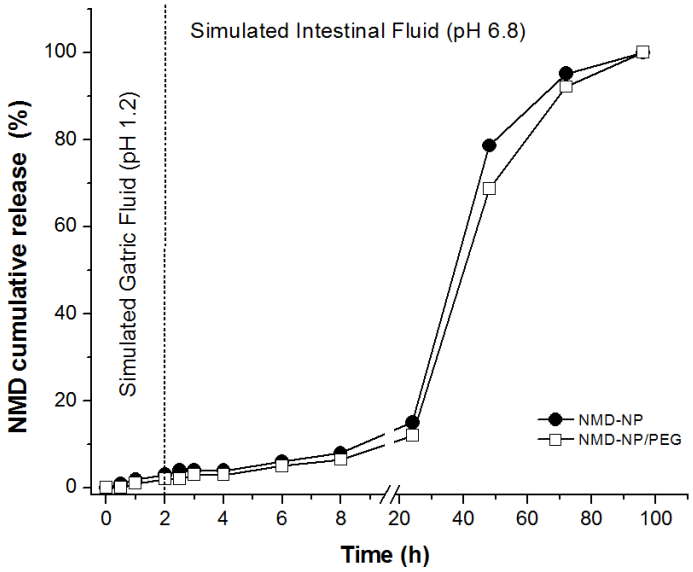

Figure 4: In vitro release profile of nimodipine from bare (NMD-NP) and pegylated nanoparticles (NMD-NP/PEG) in simulated gastric (pH: 1.2) and simulated intestinal media (pH: 6.8). Date are expressed as mean ± SD, n=3.

Table 2: Analysis of the nimodipine release from poly(anhydride) nanoparticles (when incubated in intestinal fluid).

pH 6.8	**K (min^{-n})**	**N**	**R^2**
NMD-NP	$1.7\ 10^{-4}$	0.90	0.987
NMD-NP/PEG	$7.\ 10^{-4}$	0.68	0.960

3.4. In vivo pharmacokinetic studies in Wistar rats

The plasma concentration-time profile of NMD after a single intravenous administration at a dose of 5 mg/kg is shown in Figure 5A. Data were analyzed by a non-compartmental model. NMD plasma concentration decreased rapidly, not being detectable 8 h after the administration. The drug plasma concentration reached 10250 ng/mL (C_{max}) and the values for AUC and $t_{1/2z}$, were 11743.9 ng h/mL and 1.35 h, respectively. The nimodipine clearance and the volume of distribution were calculated to be 145 mL/h and 284 mL, respectively (Table 2).

Figure 5B shows the plasma concentration profiles of NMD after the administration of a single oral dose (5 mg/kg) to rats as either a solution or loaded in nanoparticles. When the drug solution was administered orally, the bioavailability was about 9%. The drug plasma levels increased rapidly reaching the C_{max} (740.5 ng/mL) 30 min after administration (Table 2). Then the amounts of NMD in plasma decrease rapidly and 6 hours after the administration no detectable amounts of NMD were found.

For nimodipine-loaded in nanoparticles coated with PEG 2000 (NP-NMD/PEG), administered at the same dose (5 mg/kg), the plasma levels of NMD rapidly increased displaying a maximum of concentration 4 h post-administration. Then, the drug levels slowly decreased during the following 20 h, not being detected 30 h after the administration. For NMD-NP/PEG, the oral drug bioavailability was calculated to be 62%. Finally, in case of the oral administration of bare nanoparticles containing NMD, the typical plasma profile for conventional nanoparticles was observed. In this case, the oral bioavailability was 12%.

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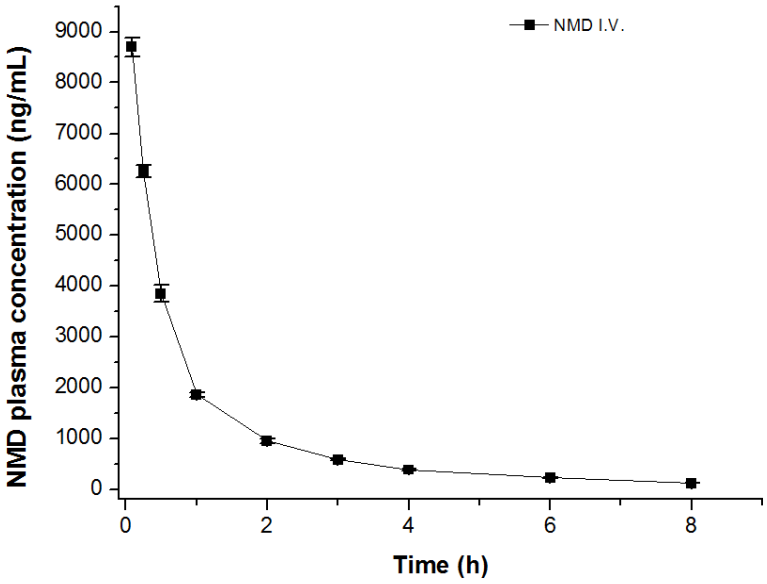

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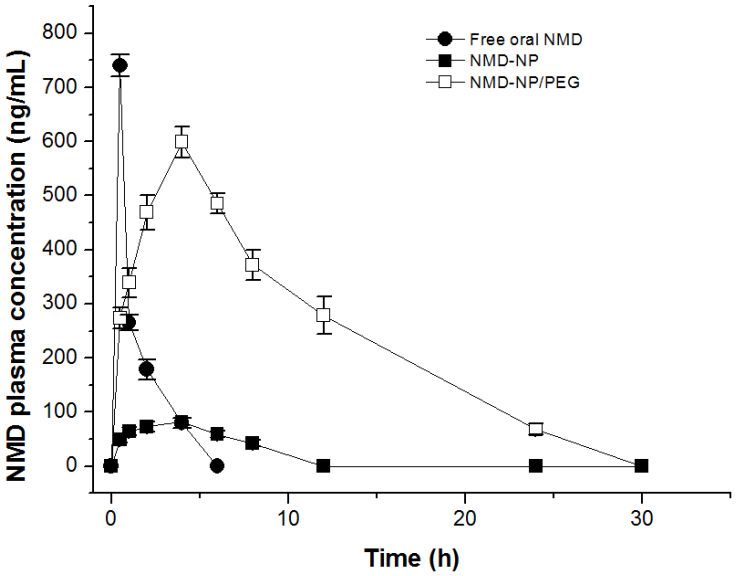

Figure 5: Pharmacokinetics of intravenous free nimodipine (NMD I.V.) (A), oral free nimodipine (NMD), poly(anhydride) nanoparticles containing nimodipine (NMD-NP), and poly(anhydride) nanoparticles containing nimodipine coated with PEG 2000 (NMD-NP/PEG)

(B) in rat. Animals received a single dose of 5 mg/kg. Data expressed as mean ± SD. n=6 per time point

Table 3: Pharmacokinetic parameters of nimodipine obtained after the administration of the different formulations tested at a dose of 5 mg/kg to Wistar male rats. i) free nimodipine intravenous (NMD-IV) ii) free nimodipine oral solution (NMD-O), iii) poly(anhydride) nanoparticles containing niodipine (NMD-NP) iv) poly(anhydride) nanoparticles containing nimodipine coated with PEG 2000 (NMP-NP/PEG). Data expressed as mean ± SD; (n=6).

	Tmax (h)	**Cmax (µg/mL)**	**T ½ (h)**	**AUC (µg h/mL)**	**Vz (mL)**	**Cl (mL/h)**	**MRT (h)**	**F %**
NMD IV	0	10250.2	1.35	11743.9	284.4	145.7	1.69	100
NMD O	0.5	740.5	1.24	1038.7	2115.22	1178.11	1.95	8.8
NMD-NP	4	81.8	2.32	1514.3	2857.40	607.00	4.06	12.8
NMD-NP/PEG	4	599.26	9.78	7276.0	2236.32	158.40	11.85	61.9

3.5. Effects of oral nimodipine on the behavior of mice

In order to study the effects of oral nimodipine (formulated in pegylated nanoparticles) on the anxiolytic and cognitive capabilities of mice, animals were chronically treated with corticosterone to induce anxiety and deficit cognition. Interestingly, this chronic administration of corticosterone did not affect the weight gain of animals during the experiment (data not shown). In a similar way, neither the administration of corticosterone nor the oral treatment with nimodipine affected the animal mobility (Figure 6).

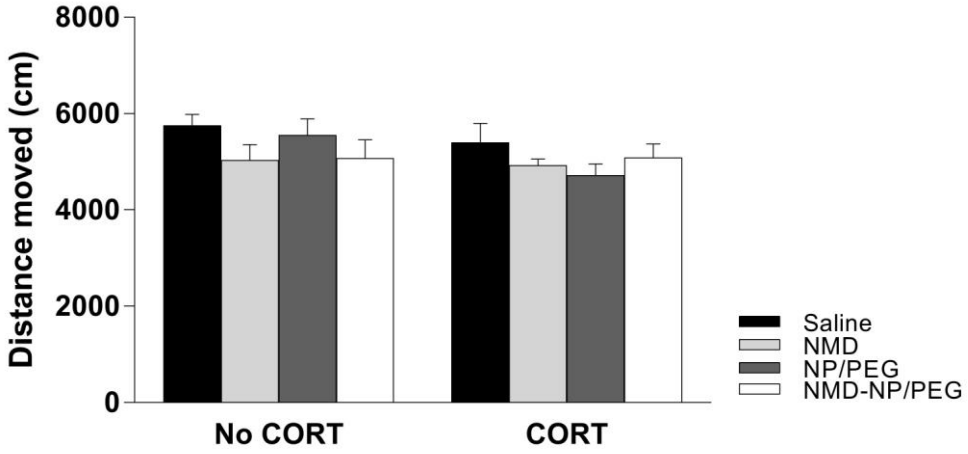

Figure 6: Effect of the chronic treatment with corticosterone (CORT), free nimodipine (NMD) and poly(anhydride) nanoparticles containing nimodipine coated with PEG 2000 (NMD-NP/PEG) in mice mobility.

3.6. Effects of chronic CORT and nimodipine treatment in anxiety like behaviour

Corticosterone treated mice showed anxiety-like behavior characterized by a strong tendency to increase the number of marbles buried (two way ANOVA, main effect of CORT, $F_{1,65}$=3.612; p=0.06) (Figure 7A), an increased latency to first entry in the open arms in the elevated plus maze test (two way ANOVA, main effect of CORT, $F_{1,65}$=17.86; p<0.001) (Figure 7B) and a decrease in the distance moved in the centre area in the open field test (two way ANOVA, main effect of CORT, $F_{1,66}$=27.81; p<0.001) (Figure 7C). The effects observed in these tests did not appear to be associated with differences in locomotor activity because of the total distance travelled in the open field was not affected by the administration of either corticosterone ($F_{1,64}$ = 2.066, p=0.1555) or nimodipine treatment ($F_{3,64}$ = 1.389, p=0.2542) (Figure 7d). Nimodipine treatment did not restore the anxiety-like behavior in any of the test (Figure 7).

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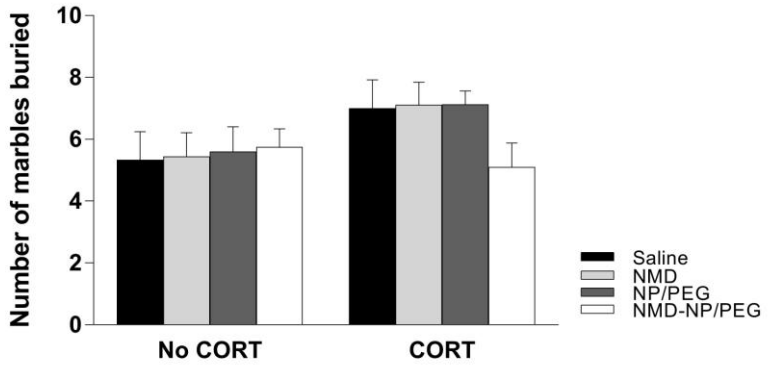

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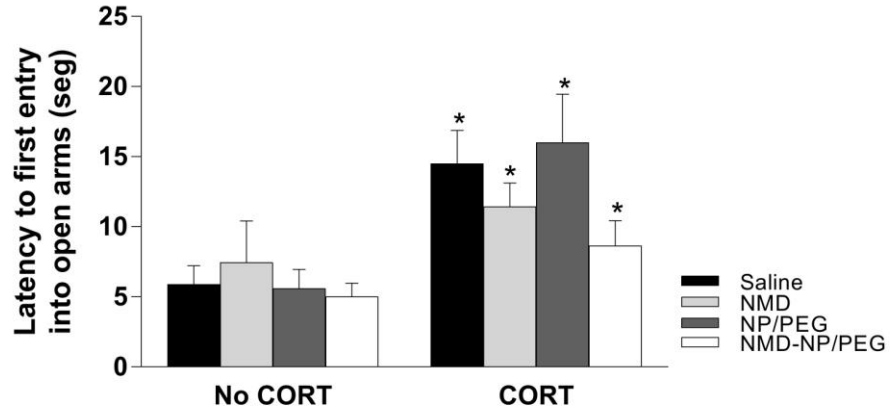

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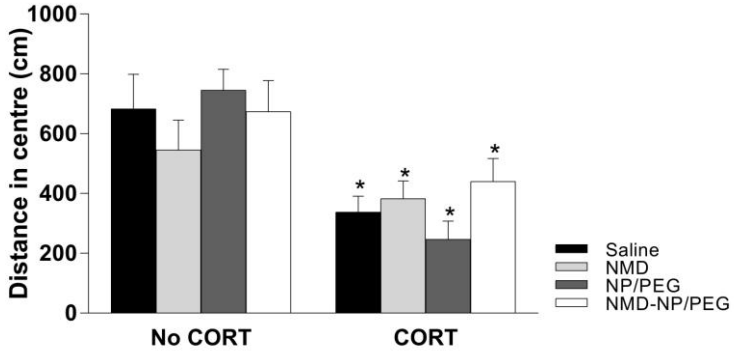

Figure 7: Effects of free nimodipine (NMD) and poly(anhydride) nanoparticles containing nimodipine coated with PEG 2000 (NMD-NP/PEG) in anxiety like behavior induced by the chronic CORT administration.

3.7. Effects of chronic CORT and nimodipine treatment in cognition

In the Morris water maze test (MWM), no significant differences among groups were found during the days of visible-platform training, indicating that all groups of animals were able to perform the test correctly (data not shown).

On the contrary, in the acquisition phase of the MWM, a significant effect of corticosterone was found (repeated measures ANOVA, main effect of genotype, $F_{7,66}$=2.387; p<0.05). In fact, mice receiving cortiscosterone showed a higher scape latency compared to non-CORT treated mice, indicating a cognitive impairment (Figure 8). Interestingly, this cognitive deficiency was completely reversed by the oral treatment with NMD-NP/PEG (Tukey's p<0.05, CORT NMD-NP/PEG group vs rest of CORT treated groups) (Figure 8B).

Finally, in the retention phase (Figure 8C), CORT-treated mice displayed a significantly lower capability to swim in the quadrant area in which the platform was located than control animals (two way ANOVA, main effect of CORT, $F_{1,66}$ = 4.634, p<0.05). Interestingly, in the last probe trial (Figure 8C), this memory deficit was completely reversed in animals treated with nimodipine-loaded nanoparticles (Tukey's p<0.05, CORT NMD-NP/PEG group vs rest of CORT treated groups).

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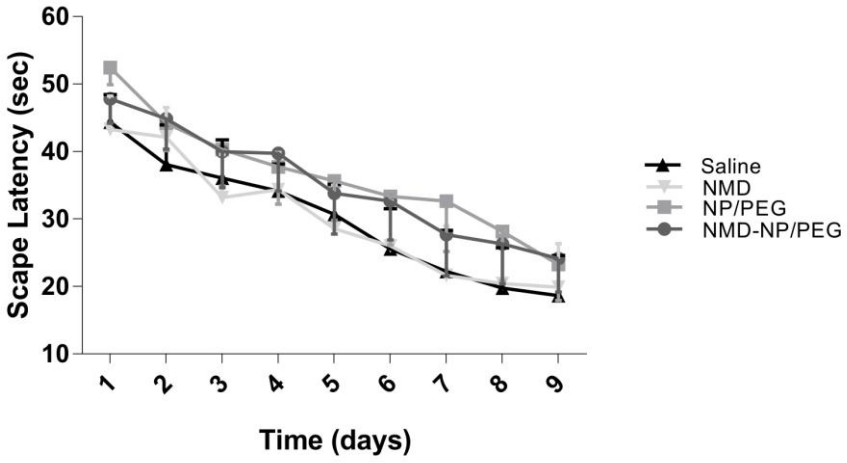

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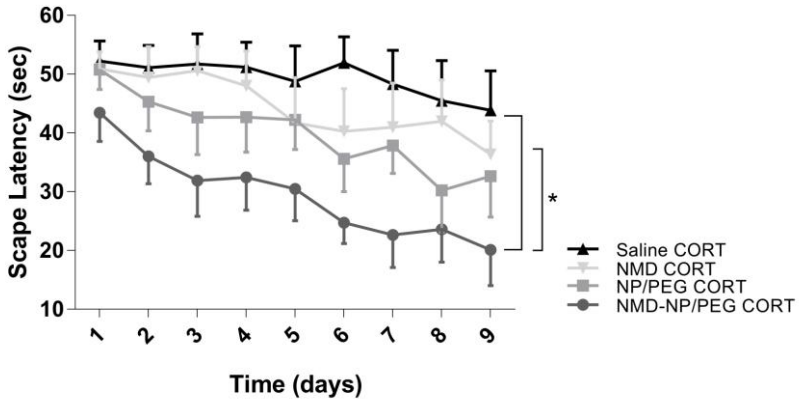

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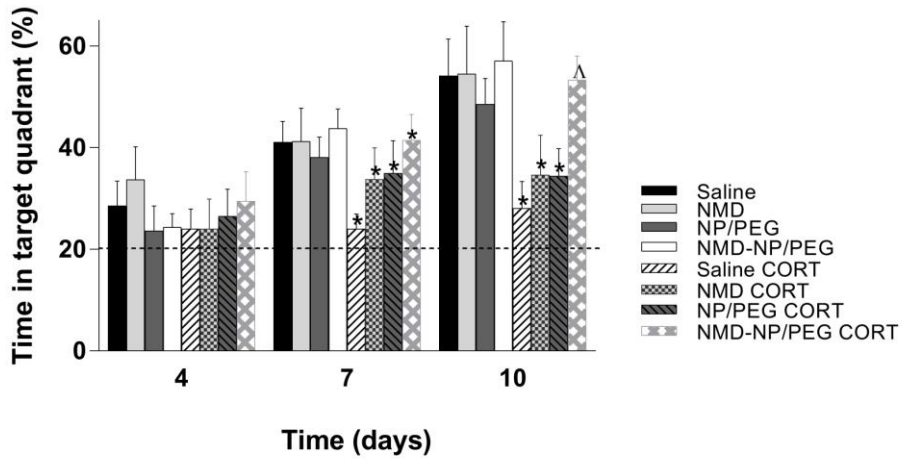

Figure 8: Effects of free nimodipine (NMD) and poly(anhydride) nanoparticles containing nimodipine coated with PEG 2000 (NMD-NP/PEG) in the cognition altered by the chronic CORT administration.

3.8. Effects of chronic CORT and nimodipine treatment in HPA axis

As depicted in Figure 9A, CORT treated mice showed increased CORT serum levels (two way ANOVA, main effect of CORT, $F_{1,56} = 5.797$, $p<0.05$) that were not reverted by nimodipine treatment. Furthermore, CORT treated mice showed decreased levels of glucocorticoid receptors at prefrontal cortex (two way ANOVA, main effect of CORT, $F_{1,40}=9,182$ $p<0,01$) (Figure 9B) and hippocampus (two way ANOVA, main effect of CORT, $F_{1,}$

$_{40}$=5,971, p<0,0150) (Figure 9C). Again, these elevated levels were not reverted by nimodipine treatment.

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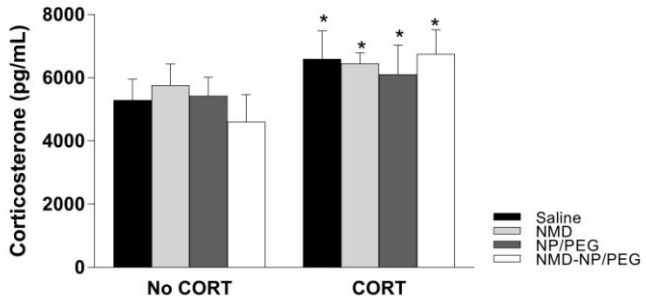

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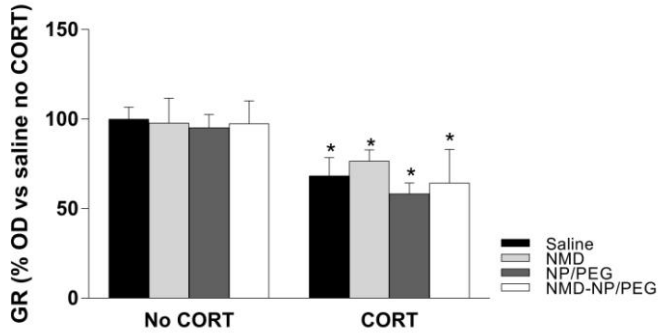

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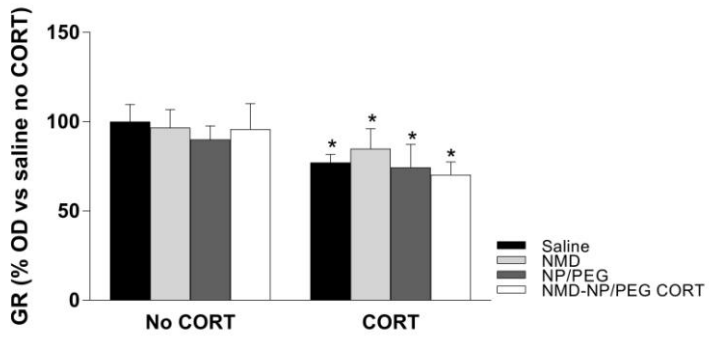

Figure 9: Effects of chronic CORT and nimodipine treatment in HPA axis.

4. Discussion

Nimodipine is an L-type Ca^{2+} blocker which can cross the blood brain barrier (Mohamed, Riva, Contin, 2016) and with an excellent selectivity for the central nervous system (Hoffman, Newland, 2016). The drug seems to be a suitable option to protect the brain

against CNS damage provoked by stress (Kumar, Singh and Jaggi, 2012), however nimodipine present low bioavailability when administrated orally (Scheller et al, 2014).

The oral route is the most widespread way for drug administration owing to being noninvasive, painless and presents high patient acceptance. This route is especially used when prolonged drug exposure or chronic treatments are needed (Pasquier, Kavallaris, Andre, 2010). Based on the above, the aim of this work was to evaluate the effects of the oral treatment with nimodipine encapsulated in poly(anhydride) nanoparticles in anxiety and memory of stressed mice.

In the optimization of the preparative process of nimodipine-loaded nanoparticles we observed that the best results were obtained when we used an incubation time (between the drug and the polymer) of 15 min (Figure 1). Furthermore, it was observed that the higher payload and encapsulation efficiency were obtained when the nimodipine/poly(anhydride) ratio was fixed to 1:10 (Figure 2). Nanoparticles coated with PEG 2000 displayed a less negative zeta potential ($p < 0.05$) (Table 1), which is suggestive of the presence of PEG in the structure of the nanocarriers (Chaudhari et al, 2012).

Concerning the in vitro release of NMD from the nanoparticles, it is noteworthy that under acidic conditions (SGF), no significant drug release was observed for any of the formulations tested (Figure 4). This observation would be related with the compact structure adopted for poly(anhydride) nanoparticles when incubated under acid conditions (Agüeros et al., 2009; Huarte et al, 2016). On the contrary, when nanoparticles were incubated under SIF, nimodipine was released. Under these SIF conditions, nimodipine was slightly released during the first 24 h, following zero order kinetics (Table 2). Then, the remaining content of nimodipine in the nanoparticles was rapidly expelled (Figure 4). As these nanoparticles may be considered as degradable matrix devices in which nimodipine would be uniformly distributed, the release of the drug should be affected by both diffusion and erosion/relaxation

phenomena. In accordance with the Korsmeyer-Peppas model, important differences were observed regarding the release of nimodipine from bare and pegylated nanoparticles. Thus, for bare nanoparticles, the release exponent (n = 0.9) was indicative that nimodipine release was more affected by the erosion/relaxation phenomena of the nanoparticles matrix than for the diffusion of the drug through the pores of the polymer structure constituting the nanoparticles. On the contrary, for pegylated nanoparticles the release of nimodipine would be mainly due to a diffusion phenomenon rather than the erosion of the nanoparticle matrix. In this context, it is plausible to think that the incorporation of PEG on the surface of nanoparticles prevents the erosion of the nanoparticles matrix but, at the same time, increases its porosity facilitating the diffusion of the drug.

Regarding the pharmacokinetic studies, is important to highlight that pegylated nanoparticles provided a high oral bioavailability of nimodipine, which was calculated to be 7-times higher than a conventional solution of the drug (Table 3). This improvement was not observed for bare nanoparticles. These findings would be in line with previous observation demonstrating the mucus-penetrating properties of nanoparticles pegylated with PEG 2000 (Calleja et al, 2014; He, Zhang, Shi, 2010) and their capability to reach the surface of the intestinal epithelium (Inchaurraga et al, 2015). On the contrary, bare nanoparticles have been reported to show mucoadhesive properties with a high interaction capability with the stomach mucus layer (Arbós et al., 2003; Gómez et al., 2007). So it is possible to imagine that the "success" with pegylated nanoparticles may be ascribed to their capacity to directly target the small intestine mucosa in which the drug would be released in a sustained way providing high levels of the calcium channel blocker in blood for prolonged periods of time. As consequence, for NMD-NP/PEG, a daily dose would be enough to maintain therapeutic levels of nimodipine.

Ca^{++} is a versatile cellular messenger that regulates a number of essential neuronal functions including the synaptic transmission, gene expression and synaptic plasticity processes involved in learning and memory (Hidalgo, Carrasco, 2011). The involvement of Ca^{++} in induction of anxiety (Biała, Kruk, 2009), dementia (López-Arrieta, Birks, 2002) and cerebral injury has been described (Kaur, Jaggi, Singh, 2010). Based on this, we studied the effect of calcium channel blocker nimodipine in behavioral changes caused by chronic stress induced by the addition of corticosterone to the mice drinking water. This approach was used as a non-invasive way of altering plasma corticosterone levels at the same time as retaining some integrity in the diurnal rhythm, present in normal animals. It has been shown that this approach has advantages over methods involving constant corticosterone pellets or corticosterone injections (Karatsoreos et al. 2010).

Corticosterone treatment, as well as nimodipine treatment, did not affect the weight gain and the mice mobility. The results obtained in the open field, Marble burying and elevated plus maze tests showed that corticosterone chronic ingestion caused anxiety in animals (Figure 7). In all tests, encapsulated nimodipine showed a tendency to reverse the anxiety of treated mice, but this reversal was not statistically significant. Previous studies had shown that nimodipine presented anxiolytic activity in mice (Kaygisiz, Ozatik, Erol, 2014; Moreno et al., 2014) and individuals (Wang, Dai, 2001); although, in our case, the obtained results may be related to the fact that the nimodipine dose was not sufficient to promote a clear anxiolytic effect in this animal model of anxiety.

On the other hand, in the Morris water maze test it was possible to observe that the treatment with cortiscosterone caused deficits in cognitive ability and memory of treated mice (Figure 8). These damages were fully reversed by oral nimodipine, when formulated in pegylated poly(anhydride) nanoparticles. In the MWM, mice presented not only an important amelioration in the acquisition of memory, but also showed an improvement in memory

retention (Figure 8C). The best of our knowledge this is the first time that oral nimodipine would be of interest to ameliorate senescence symptoms. In any case further investigations is needed to establish the real potential of pegylated nanoparticles as carriers for the oral delivery of nimodipine as well as the mechanisms by which this calcium channel blocker may improve the memory capabilities.

5. Conclusion

Poly(anhydride) nanoparticles containing nimodipine coated with PEG 2000 were able to load a significant amount of nimodipine and provide gastrorresistant properties to the resulting formulation (NMD-NP/PEG). After the oral administration of these nanoparticles to rats, the nimodipine plasma levels were high and sustained in time up to 24 hours offering a relative oral bioavailability which was calculated to be at least 7 fold higher than that observed for the free drug. Morover, nimodipine encapsulated in nanoparticles was able to protect the cognitive deficits observed in stressed mice, improving not only the acquisition as well as the retention of memory.

References:

A. N. Shen, C. Cummings, D. Pope, D. Hoffman, M.C. Newland. A bout analysis reveals age-related methylmercury neurotoxicity and nimodipine neuroprotection. Behav Brain Res 311 (2016) 147–159

A. Steptoe, M. Hamer, Y. Chida. The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. Brain Behav Immun 21 (2007) 901–912.

B. Kaygisiz, F. Y. Ozatik, K. Erol. Interaction of Sertraline and Nimodipine on Some Behavioural Tests in Rats. Adv Clin Exp Med 23 (2014) 169-175.

B. Lkhagvasuren, T. Oka, Y. Nakamura, H. Hayashi, N. Sudo, K. Nakamura. distribution of fos-immunoreactive cells in rat forebrain and midbrain following social defeat stress and diazepam treatment. Neuroscience 272 (2014) 34–57.

C.D. Munhoz, B. Garcia-Bueno, J.L. Madrigal, L.B. Lepsch, C. Scavone, J.C. Leza. Stress-induced neuroinflammation: mechanisms and new pharmacological targets. Braz J Med Biol Res 41 (2008) 1037–1046.

C. Hidalgo, M.A. Carrasco. Redox control of brain calcium in health and disease. Antioxid Redox Signal 14 (2011) 1203–1207.

C. Scheller, A. Wienke, F. Wurm, A.S. Vogel, S. Simmermacher, J. Prell, J. Rachinger, G. Koman, C. Strauss, K. Scheller. Enteral or parenteral nimodipine treatment: a comparative pharmacokinetic study. J Neurol Surg A Cent Eur Neurosurg 75 (2014), 84–90.

D.J. Hoffman, M.C. Newland. Chronic methylmercury exposure decreases the ability: but not the motivation to run: a microstructural analysis and protection by nimodipine. Neurotoxicology 54 (2016) 127–129.

D.R. Sparta, J.H. Jennings, R.L. Ung, G.D. Stuber. Optogenetic strategies to investigate neural circuitry engaged by stress. Behav Brain Res 255 (Sparta) 19–25.

E. Pasquier, M. Kavallaris, E. Andre. Metronomic chemotherapy: new rationale for new directions. Nat Rev Clin Oncol 7 (2010) 455-465.

G. Biała, M. Kruk. Influence of bupropion and calcium channel antagonists on the nicotine-induced memory-related response of mice in the elevated plus maze. Pharmacol Rep 61 (2009) 236–244.

G. Geppert, P.W. Radke, V. Kurowski, P. Hunold, H. Schunkert. Wasp sting, adrenaline injection and acute thoracic pain: an un usual case of stress-induced (tako-tsubo) cardiomyopathy. Med Klin (Munich) 105 (2010) 246–8.

G. Kaur, A.S. Jaggi, N. Singh. Exploring the potential effect of Ocimum sanctum in vincristine-induced neuropathic pain in rats. J Brachial Plex Peripher Nerve Inj 5 (2010) 1-9.

H. H. Salman, J. M. Irache, C. Gamazo. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccine 27 (2009) 4784–4790.

I. N. Karatsoreos, S. M. Bhagat, N. P. Bowles, Z. M. Weil, D. W. Pfaff, B. S. McEwen. Endocrine and Physiological Changes in Response to Chronic Corticosterone: A Potential Model of the Metabolic Syndrome in Mouse. Endocrinology 151 (2010) 2117–2127.

I. Sotiropoulos, C. Catania, L.G. Pinto, R. Silva, G.E. Pollerberg, A. Takashima, et al. Stress acts cumulatively to precipitate Alzheimer's disease-like tau pathology and cognitive deficits. J Neurosci 31 (2011) 7840–7847.

J. Calvo, J.L. Lavandera, M. Agüeros, J.M. Irache. Cyclodextrin/poly(anhydride) nanoparticles as drug carriers for the oral delivery of atovaquone. Biomed. Microdevices, 13 (2011), 1015–1025.

J. E. Mökel, B. C. Lippold. Zero-order drug release from hydrocolloid matrices. Pharmaceut Res 10 (1993) 1066 – 1070.

J. Huarte, S. Espuelas, Y. Lai, B. He, J. Tang, J. M. Irache. Oral delivery of camptothecin using cyclodextrin/poly(anhydride) nanoparticles. Int. J. Pharm 506 (2016) 116–128.

J. M. López-Arrieta, J. Birks. Nimodipine for primary degenerative, mixed and vascular dementia. Cochrane Database Syst Rev 3 (2002) 1-87.

J.R. Caso, J.C. Leza, L. Menchén. The effects of physical and psychological stress on the gastro-intestinal tract: lessons from animal models. Curr Mol Med 8 (2008) 299–312.

J. Siepman, F. Siepman. Mathematical modeling in drug delivery. Int J Pharm 364 (2008) 328-343.

K. Maigaard, I. Hageman, A. Jørgensen, M.B. Jørgensen, G. Wörtwein. Electroconvulsive stimulations prevent chronic stress-induced increases in L-type calcium channel mRNAs in the hippocampus and basolateral amygdala. Neurosci Lett 516 (2012) 24-28.

K. R. Chaudhari, M. Ukawala, A. S. Manjappa, A. Kumar, P. K. Mundada, A. K. Mishra, R. Mathur, J. Monkkonen, R.S. Murthy. Opsonization, biodistribution, cellular uptake and

apoptosis study of PEGylated PBCA nanoparticle as potential drug delivery carrier. Pharmaceut Res 29 (2012) 53-68.

L. C. G. A. I. Moreno, G. Z. S. Oliveira, I. M. F. Cavalcanti, N. S. Santos-Magalhães, H. M. L. Rolim, R. M. Freitas. Development and evaluation of liposomal formulation containing nimodipine on anxiolytic activity in mice. Pharmacol Biochem Behav 116 (2014) 64–68.

L. C. G. A. I. Moreno, H. M. L. Rolim, R. M. Freitas, N. S. Santos-Magalhães. Antidepressant-like activity of liposomal formulation containing nimodipine treatment in the tail suspension test, forced swim test and MAOB activity in mice. Brain Res 1646 (2016) 235–240.

L. C. G. A. I. Moreno, I. M. F. Cavalcanti, P. Satyal, N. S. Santos-Magalhães, H. M. L. Rolim, R. M. Freitas. Acute toxicity and anticonvulsant activity of liposomes containing nimodipine on pilocarpine-induced seizures in mice. Neurosci Lett 585 (2015) 38–42.

L. Inchaurraga, N. Martín-Arbella, V. Zabaleta, G. Quincoces, I. Peñuelas, J. M. Irache. In vivo study of the mucus-permeating properties of PEG-coated nanoparticles following oral administration. Eur J Pharma Biopharm 97 (2015) 280–289.

L. Wang, G. Dai. The effect of deanxit and nimodipine on anxiety and depression neurosis with headache. Chin J Clin Psychol 9 (2001) 121-123.

M. Agüeros, L. Ruiz-Gatón, C. Vauthier, K. Bouchemal, S. Espuelas, G. Ponchel, J.M. Irache. Combined hydroxypropyl-beta-cyclodextrin and poly (anhydride) nanoparticles improve the oral permeability of paclitaxel. Eur J Pharm Sci 38 (2009) 405–413.

M. Solas, B. Aisa, M.C. Mugueta, J. Del Río, R.M. Tordera, M.J. Ramírez. Interactions between age, stress and insulin on cognition: implications for Alzheimer's disease. Neuropsychopharmacology 38 (2010) 1664-73.

N. Elizalde, F.J. Gil-Bea, M. J. Ramírez, B. Aisa, B. Lasheras, J. Del Rio, R.M. Tordera. Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. Psychopharmacology (Berl). 1999 (2008) 1-14.

N. Kumar, N. Singh, A. S. Jaggi. Anti-stress effects of cilnidipine and nimodipine in immobilization subjected mice. Physiol Behav 105 (2012) 1148–1155.

O. Thibault, J.C. Gant, P.W. Landfield. Expansion of the calcium hypothesis of brain aging and Alzheimer's disease: minding the store. Aging Cell 6 (3) (2007), 307–317.

P. Arbos, M.A. Arangoa, M.A. Campanero, J.M. Irache. Quantification of the bioadhesive properties of protein-coated PVM/MA nanoparticles. Int J Pharm 242 (2002) 129–136.

P. Arbós, M. A. Campanero, M. A. Arangoa, M. J. Renedo, J. M. Irache, Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties. J Control Release 89 (2003) 19-30.

P. Calleja, S. Espuelas, C. Vauthier, G. Ponchel, J. M. Irache. Controlled Release, Intestinal Transport, and Oral Bioavailablity of Paclitaxel Can be Considerably Increased Using

Suitably Tailored Pegylated Poly(Anhydride) Nanoparticles. J Pharm Sci 104 (2015) 2877–2886.

P. N'Gouemo. Probing the role of the sodium/calcium exchanger in pentylenetetrazole-induced generalized seizures in rats. Brain Res Bull 90 (2013) 52-57.

Q Fu, J. Sun, D. Zhang, M. Li, Y. Wang, G. Ling, X. Liu, Y. Sun, X. Sui, C. Luo, L. Sun, X. Han, H. Lian, M. Zhu, S. Wang, Z. He. Nimodipine nanocrystals for oral bioavailability improvement: preparation, characterization and pharmacokinetic studies, Colloids Surface B, v. 109, p. 161–166, 2013a.

Q Fu, J. Sun, X. Ai, P. Zhang, M. Li, Y. Wang, X. Liu, Y. Sun, X. Sui, L. Sun, X. Han, M. Zhu, Y. Zhang, S. Wang, Z. He. Nimodipine nanocrystals for oral bioavailability improvement: role of mesenteric lymph transport in the oral absorption, International Journal of Pharmaceutics, v. 448, p. 290–297, 2013b.

Q. He, J. Zhang, J. Shi. The effect of PEGylation of mesoporous silica nanoparticles on nonspecific binding of serum proteins and cellular responses. Biomaterials 31 (2010) 1085-1092.

R.M. Deacon. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. Nat Protoc 1 (2006) 122-124.

R. Penalva, I. Esparza, M. Agüeros, C.J. Gonzalez-Navarro, C. Gonzalez-Ferrero, J.M. Irache, Casein nanoparticles as carriers for the oral delivery of folic acid. Food Hydrocoll 44 (2015) 399-406.

R. W. Korsmeyer, R. Gurny, E. M. Doelker, P. Buri, N. A. Peppas. Mechanisms of solute release from porous hydrophilic polymers. Int J Pharm 15 (1983) 25 – 35.

S.C. Sweetman. Nimodipine, in: S.C. Sweetman (Ed.), Martindale: the complete drug reference, Pharmaceutical Press, London 1 (2009) 1357–1358.

S. Fredenberg, M. Wahlgren, M. Reslow, A. Axelsson. The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery system- A review Int J Pharm 415 (2011) 34-52.

S. Gómez, C. Gamazo, B.-S. Roman, M. Ferrer, M.-L., Sanz, J.-M. Irache, S. Gómez, Gantrez® AN nanoparticles as an adjuvant for oral immunotherapy with allergens. Vaccine 25 (2007) 5263–5271.

S. Huang, X. Yu, L. Yang, F. Song, G. Chen, Z. Lv, T. Li, D. Chen, W. Zhu, A. Yu, Y. Zhang, F. Yang. The efficacy of nimodipine drug delivery using mPEG-PLA micelles and mPEG-PLA/TPGS mixed micelles. Eur . Pharm Sci 63 (2014) 187–198.

S. Mohamed, R. Riva, M. Contin. Simple and validated UHPLC–MS/MS analysis of nimodipine in plasma and cerebrospinal fluid of patients with subarachnoid haemorrhage. J Chromatogr B 1028 (2016) 94–99

V. Kumar, Z.A. Bhat, D. Kumar. Animal models of anxiety: A comprehensive review. J. Pharmacol. Toxicol. Methods 68 (2013) 175–183.

V. Zabaleta, G. Ponchel, H. Salman, M. Agüeros, C. Vauthier, J.M. Irache, Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: Permeability and pharmacokinetic study. European Journal of Pharmaceutics and Biopharmaceutics 81 (2012) 514–523.

X. Zhang, M. Yu, W. Yu, J. Weinberg, J. Shapiro, K.J. McElwee. Development of alopecia areata is associated with higher central and peripheral hypothalamic-pituitaryadrenal tone in the skin graft induced C3H/HeJ mouse model. J Invest Dermatol 129 (2009) 1527–38.

8. EFFECT OF THE ORAL ADMINISTRATION OF NANOENCAPSULATED QUERCETIN ON A MOUSE MODEL OF ALZHEIMER'S DISEASE

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Abstract

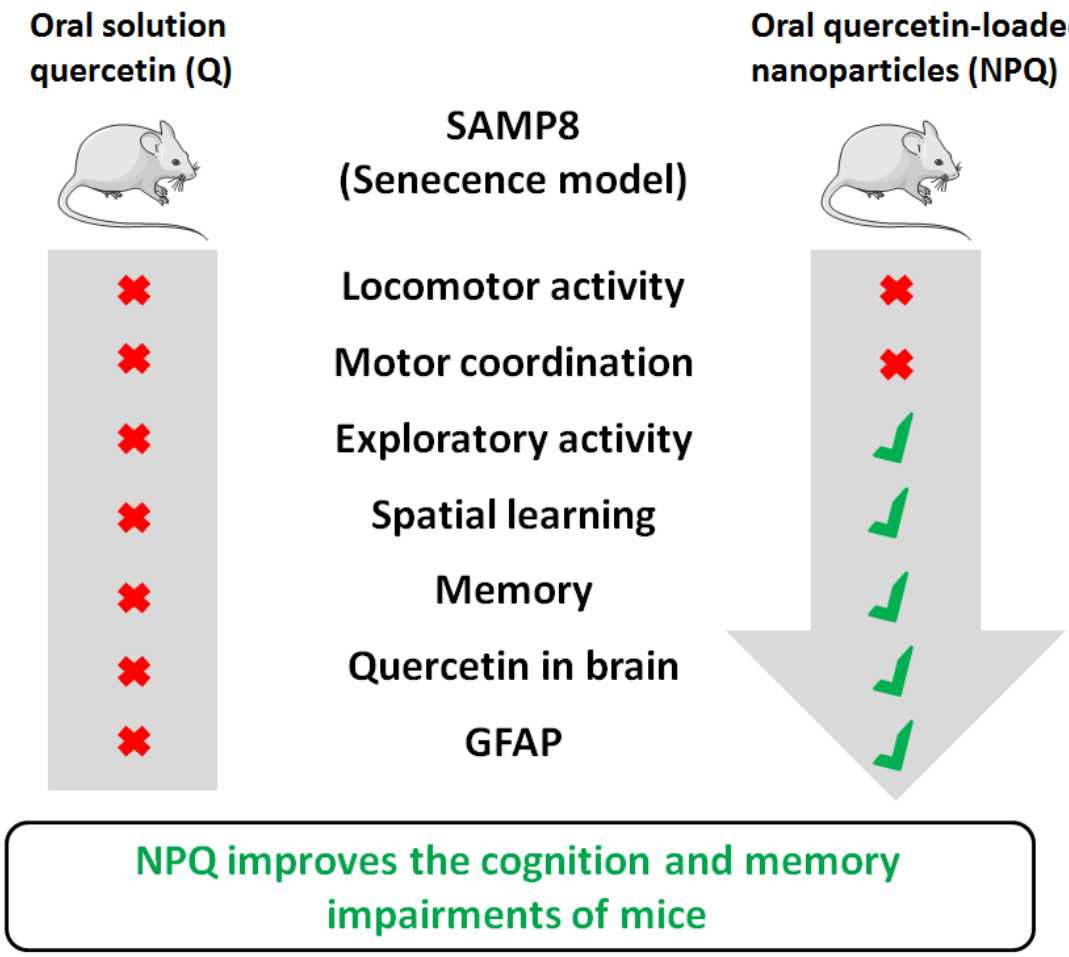
Quercetin has been identified as a promising compound with a neuroprotective potential against age-related neurodegenerative diseases such as Alzheimer's disease (AD). Nevertheless, the clinical application of quercetin is hampered by its low oral bioavailability. The aim of this work was to evaluate the capability of nanoencapsulated quercetin in zein nanoparticles (NPQ) as potential oral treatment for AD. For this purpose, 5-month old SAMP8 mice were orally treated for two months with either NPQ at a dose of 25 mg/kg every 48 hours or a solution of quercetin (Q) at the same dose but administered every 24 hours. NPQ displayed a size of 260 nm, negative zeta potential and a payload of about 70 µg/mg. Any significant effect was observed in the animals treated with Q. On the contrary, the oral administration of NPQ attenuated learning and memory impairments shown by SAMP8 mice in the Morris Water Maze test. These observations appeared to be related with a decreased expression of the hippocampal astrocyte marker GFAP. Furthermore, significant levels of quercetin were quantified in the brain of mice treated with nanoparticles. These findings highlight the potential of zein nanoparticles to promote the oral absorption of quercetin as well as the therapeutic potential of this flavonoid in AD pathogenesis.

Key words: quercetin, nanoparticles, SAMP8 mice, oral delivery, Alzheimer’s disease

Chemical compounds studied in this article

Quercetin (PubChem CID: 5280343); Zein (PubChem SID: 47208016); 2-hydroxypropyl-β-cyclodextrin (PubChem CID: 44134771).

Graphical abstract



1. Introduction

Alzheimer's disease (AD) is a deadly neurodegenerative brain disorder characterized by the progressive cognitive impairment and memory loss, which is considered the most common cause of dementia in elderly (Najafi et al., 2016). The onset is gradual, with continuing cognitive decline that significantly impairs social or occupational behaving and represents a significant decrease from a previous level of functioning (Boada et al., 2014).

Due to the aging of the human population, the number of patients affected by AD is increasing rapidly and, in the last decades, the prevalence is doubling every 20 years (Khunnawutmanotham et al., 2016). Thus, in 2010, 36 million people were living with this dementia in the world, whereas, for 2050, 115 million people are estimated to be affected by AD (Luo et al., 2016). Nowadays, AD would be responsible for reduction of life expectancy by 50% from the time of diagnosis in patients (Larson et al., 2004).

According to recent estimates, the costs of AD worldwide are approximately US$604 billion annually and only in Europe the direct costs of AD amounts to €105 billion for the community per year (Dodel el., 2015).

AD is characterized by destruction of the functional activity of neurons in different areas of the brain, including those accounted for memory, learning, emotional reactions and behavior (Nelson et al., 2012). The mechanisms responsible for AD onset and the secondary development thereafter include amyloid plaques and neurofibrillary tangles, which are caused by deposits of β-amyloid fragments(Aβ) and hyperphosphorylated tauproteins (Qi et al., 2016) . The small clusters of beta-amyloid plaque can block the signaling between cells at synapses and may also activate the immune cells that cause inflammation and trigger the destruction of neurons (López et al., 2012).

In the last years, some factors have been identified as responsible on the onset of this pathology such as the low levels of acetylcholine (García-Ayllón et al., 2011), oxidative stress

(Ishisaka et al., 2014) and neuroinflammation (Helmfors et al., 2015). Neuroinflammation is directly related to the decrease in synapses which causes cognitive decline (Hong et al., 2016). From this perspective, a compound that treats one or more of AD triggering factors can be a promising strategy for the treatment of this dementia.

Flavonoids have been identified as compounds with a neuroprotective potential by preventing the onset or by slowing the progression of age-related neurodegenerative diseases (Williams and Spencer, 2012). These capabilities of flavonoids seems to be related to their ability to interact with intracellular neuronal and glial signaling pathways, thus influencing the peripheral and cerebral vascular system, protecting vulnerable neurons, enhancing existing neuronal function, or stimulating neuronal regeneration (Spencer, 2009).

In this context, quercetin (3,5,7,30,40-penta hydroxyflavone) is a flavonoid with important antioxidant and anti-inflammatory properties (Oliveira et al., 2016). Furthermore, quercetin can cross the blood brain barrier (Youdim et al., 2004) and would exert a neuroprotective effect, increasing the resistance of neurons to oxidative stress and excitotoxicity by modulating the mechanisms of cell death (Choi et al., 2014; Liu et al., 2013). Very recently, Sabogal-Guaqueta and coworkers have been demonstrated that quercetin, intraperitoneally administered every two days during three months, reversed the histopathological hallmarks of AD and ameliorated cognitive and emotional impairments in a mice model of this disease (Sabogal-Guáqueta et al., 2015).

Nevertheless, the use of quercetin for clinical application is highly hampered by its low oral bioavailability (Patel et al., 2012). In fact, apart its low aqueous solubility, quercetin is a substrate of both the intestinal efflux pumps (e.g., P-glycoprotein and MRP2) (Chabane et al., 2009) and the cytochrome P450 enzymes (CYP3A) (Choi et al., 2011). As a consequence, the oral bioavailability of this flavonoid in humans would be approximately 2% (Suna et al., 2016).

In order to overcome these problems, one possibility may be the co-encapsulation of quercetin and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) in zein nanoparticles as oral carriers for this flavonoid. In a very recent work, this combination between zein nanoparticles and HP-β-CD has demonstrated to be adequate to significantly improve the oral bioavailability of quercetin (close to 60%) and, more important, provide high and sustained plasma levels of the flavonoid for at least 2 days (Penalva et al., 2016). On one side, zein nanoparticles would offer a prolonged residence in close contact with the gut mucosa due to their mucoadhesive properties (Penalva et al., 2015). On the other hand, the simultaneous release of HP-β-CD with quercetin would facilitate the inhibitory effect of the cyclodextrin in the activity of both the intestinal efflux pumps (Zhang et al., 2011) and the cytochrome P450 (Ishikawa et al., 2005).

In this context, the aim of this work was to evaluate quercetin-loaded in zein nanoparticles containing HP-β-CD as potential treatment for AD. For this purpose, free quercetin and quercetin nanoparticles were administrated orally during 2 months in SAMP8 mice and motor activity and memory tests were conducted.

2. Material and methods

2.1. Materials

Zein, quercetin, lysine, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), mannitol, poly(ethylene glycol) 400 (PEG400), Tween 20, Tris buffer saline, sodium chloride and rabbit polyclonal anti-β-actin were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol, methanol and acetic acid (HPLC grade) were obtained from Merck (Darmstadt, Germany). PBS (Phosphate-Buffered saline) was from Gibco by Life Technologies Corp. (New York, USA). Tris hydrocloride, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), Nonidet P-40, phosphatase and protease inhibitor cocktail set II were from

Calbiochem, (Darmstadt, Germany). Bradford protein assay was from Bio-Rad (Hercules, CA, USA). Sodium dodecyl sulphate-polyacrylamide gel nitrocellulose membrane was from Hybond-ECL; Amersham Bioscience (Barcelona, Spain). Mouse monoclonal anti-glial fibrillary acidic protein was from Cell Signaling Technology (Beverly, USA). Rabbit polyclonal (CD11b) was from Thermo Scientific (Rockford, USA). Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW were LI-COR Biosciences (Lincoln, USA). All reagents and chemicals used were of analytical grade.

2.2. Preparation of quercetin-loaded nanoparticles

Zein nanoparticles were prepared by a controlled desolvation procedure followed by a purification step by ultrafiltration and subsequent drying in a Spray-drying apparatus (Penalva et al, 2016). This formulation of nanoparticles was identified as NPQ.

For the preparation of nanoparticles, 600 mg zein and 60 mg lysine were dissolved in 88 mL ethanol 60%. In parallel, 60 mg quercetin and 302.5 mg HP-β-CD were dissolved in 10 mL absolute ethanol and added under magnetic stirring to the zein solution. After incubation, nanoparticles were formed by the addition of 88 mL purified water. The resulting suspension of nanoparticles was purified and concentrated by ultrafiltration through a polysulfone membrane cartridge of 50 kDa pore size (Medica SPA, Italy). Then, 12 mL of an aqueous solution of mannitol (100 mg/mL) were added. Finally, the suspension of zein nanoparticles was dried in a Buchi Mini Spray Drier B-290 apparatus (Buchi Labortechnik AG, Switzerland) under the following experimental conditions: (i) inlet temperature, 90°C; (ii) outlet temperature, 45-50°C; (iii) air pressure, 2-5 bar; (iv) pumping rate, 5 mL/min; (v) aspirator, 100%; and (vi) air flow, 900 L/h.

2.3. Preparation of quercetin solution

A quercetin solution (Q) was used as control. For this purpose, 50 mg of quercetin were dissolved in 6 mL of PEG 400 under magnetic stirring. Then 4 mL of purified water were added and the final mixture was agitated in the dark for 10 min.

2.4. Characterization of nanoparticles

2.4.1. Size, zeta potential and morphology

The particle size and the zeta potential of spray-dried nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetaplus apparatus (Brookhaven Instruments Corporation). In all cases, the size was measured after dispersion of nanoparticles in ultrapure water whereas the zeta potential was quantified in KCl 0.1 M. The yield of the process (amount of protein transformed into nanoparticles) was calculated as described previously (Penalva et al., 2015).

2.4.2. Quercetin quantification

The amount of quercetin loaded into the nanoparticles was quantified by HPLC-UV following a method previously described (Penalva et al., 2016). All analysis were carried out in an Agilent model 1100 series LC and a diode-array detector set at 370 nm. The chromatographic system was equipped with a reversed-phase C18 Alltima column (150 mm x 2.1 mm, particle size = 5 μm; Altech, USA) and a Gemini AJO-7596 C18 precolumn (Phenomenex, CA, USA). The mobile phase, pumped at 0.25 mL/min, consisted of a mixture of methanol, water and acetic acid under gradient condition. The column was placed at 40°C and the injection volume was 10 μL.

For analysis, 10 mg of nanoparticles were dispersed in 1 mL water and centrifuged 30,500 g for 20 min. The amount of encapsulated quercetin was calculated by dissolution of

the pellets with 1 mL of ethanol 75%. In addition, the total content of quercetin in the formulation was also quantified. Thus, 10 mg of the formulation were dissolved in 1 mL of ethanol 75% and an aliquot was quantified. Each sample was assayed in triplicate and the payload was expressed as the amount of quercetin quantified in the pellets (in μg) per mass (in mg) of nanoparticles. The encapsulation efficiency (EE), expressed in percentage, was calculated as the ratio between the amount of quercetin calculated in the pellets and the amount of the flavonoid calculated in the overall formulation.

2.5. Animals, treatments, and experimental design

Experiments were carried out in male SAMP8 and SAMR1 mice (28–30 g; 5-month old) obtained from Harlan (Harlan Iberica, Barcelona, Spain). Animals were housed (5 per cage) in constant conditions of humidity and temperature (22 ± 1 °C) with a 12-hour/12-hour light–dark cycle (lights on at 7:00 h). Food and water were available ad libitum. All the procedures followed in this work were in compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethical Committee of the University of Navarra (E36-15(170-14E1).

To study the effect of quercetin on the cognitive impairment of SAMP8 mice, four groups of 8 animals were employed. The first group received by the oral route a daily dose of quercetin as oral solution (Q, 25 mg/kg). Animals, of the second group of SAMP8 mice, were orally treated with the quercetin-loaded nanoparticles (NPQ) at a dose of 25 mg/kg administered every two days. In both cases, the animals were treated for 2 consecutive months. Quercetin administration started 4 weeks before the beginning of the behavioral tests and was extended throughout the duration of the assays. The last dose for animals treated with free quercetin (Q) and quercetin-loaded nanoparticles was administered 24 h and 48 h before their sacrifice, respectively.

As controls, two groups of animals were used. The first group included SAMP8 mice whereas the second one was of SAMR1 mice. In both cases, the animals received orally 1 mL of saline every day.

2.6. Behavioural test

In this work, four different behavioural tests were conducted: (i) open field, (ii) rotarod, (iii) marble burying and (iv) Morris water maze. In all cases, these behavioural experiments were conducted between 09:00 h and 15:00 h and the animals were always randomized.

2.6.1. Open field test

Locomotor activity was measured for 30 min in an open field (65×65 cm^2, 45 cm height) made of black wood, using a video-tracking system (Ethovision 3.0, Noldus Information Technology B.V., The Netherlands) in a softly illuminated room. The total distance walked for the animals as well as their velocity were analysed.

2.6.2. Rotarod test

Motor coordination and balance were measured in a rotarod apparatus (LE8200, Panlab). The animals were evaluated for 3 trials on 2 consecutive days. In each session, the animals were placed on the rod which was scheduled to accelerate gradually (4 to 40 rpm) for 5 minutes. The time that each mouse takes to fall was scored and then the mean of the three trails was obtained. A fall is counted every time the animal fell completely off the rotating rod.

2.6.3. Marble burying test

This test was employed to assess the exploratory motivation of animals. For this purpose, twelve marbles (1.5-cm diameter) were placed uniformly in a cage (45 × 28 × 20 cm) containing a constant amount of sawdust (3 cm deep). Mice were placed in the center of the cage and left for 30 min. The number of marbles buried was recorded.

2.6.4. Morris water maze

The Morris water maze (MWM), a hippocampus-dependent learning task, was used to analyze the spatial memory and to evaluate the working and reference memory functions in the animals. The water maze is a circular pool (diameter of 145 cm) filled with water (21–22° C) and virtually divided into four equal quadrants identified as northeast, northwest, southeast, and southwest. To test the learning capacity of animals, a hidden-platform (1 cm below the water surface) was placed in the northeast quadrant of the pool. In addition, several large visual cues were placed in the room to guide the mice to the platform. For the test, a mouse was introduced in the pool and the trial was finished when the animal reached the platform (escape latency). If after 60 seconds in the pool, the mouse was unable to reach the platform, it was guided onto it. Then, after each trial, mice remained on the platform for 15 seconds.

For each animal, the test was conducted over 8 consecutive days (4 trials per day).

On the other hand, and in order to test the memory of animals, probe trials were performed at the 4^{th} and last day of the test (8^{th} day). For this purpose, the platform was removed from the pool and animals were allowed to swim for 60 s. Then, the percentage of time spent in the northeast quadrant (in which the platform was localized during the learning phase of the experiment) was recorded. All trials were monitored by a video camera set above

the center of the pool and connected to a video tracking system (Ethovision 3.0; Noldus Information Technology B.V, Wageningen, Netherlands).

2.7. Sacrifice of SAMP8 mice and brain analysis

Mice treated with saline, free quercetin and quercetin-loaded in zein nanoparticles containing HP-β-CD were sacrificed by decapitation 1 h after the last probe trial of the MWM. The hippocampi of the animals were extracted for analysis of GFAP (glial fibrillary acidic protein) and CD11b by Western blot. In addition, the other portions of the brains were used for the quantification of quercetin. Biological samples were stored at -80 until the time of analysis.

2.7.1. Western Blotting Analysis

Western blot analysis was carried out in hippocampal tissues collected from mice killed. Hippocampal tissues were homogenized in 10 volumes of lysis buffer. This buffer contained (in mmol/l) 50 Tris–HCl (pH 8), 150 NaCl, 2 EGTA, Nonidet P-40, 1:100 of phosphatase and protease inhibitor cocktail set II. Lysates were left for 30 min on ice with shaking and were then centrifuged at 4° C for 20 min at 12,000 g. The supernatant was collected and protein was determined by Bradford protein assay. Equal amounts of protein (20 µg per lane) were separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel (8%) under reducing conditions and transferred onto a nitrocellulose membrane. The trans-blots were blocked for 1 h with 10% not-fat milk in buffer TBS containing 0.1% Tween 20 and then probed overnight at 4 °C with one of the following primary antibodies: mouse monoclonal anti-glial fibrillary acidic protein (GFAP; 1:1,000), CD11b (rabbit polyclonal, 1:500) and rabbit polyclonal anti-β-actin (1:10,000). Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW were diluted to 1/15,000 in TBS with 5% BSA. Bands were

visualized using Odyssey Infrared Imaging System. β-Actin was used as internal control. Results were calculated as the percentage of optical density values of SAMR1 mice and, when stated, normalized to total levels of no phosphorylated protein.

2.7.2. Quercetin quantification in brain

To study the amount of quercetin in SAMP8 mice brains, the extracted brains were weighed and homogenized in 1 ml of phosphate buffered saline, pH 7.4. Then, the homogenized brains were centrifuged at 10,000 g for 10 min and the supernatants were collected (Calleja et al., 2014). For the quantification of quercetin in the brain of animals, a liquid-liquid extraction method followed by reverse-phase HPLC analysis was performed. The extraction was adapted from Li et al. (2009) with minor modifications. Aliquots of 100 µl of homogenized brains were mixed with 125 µl methanol and 50 µl HCl (25% by vol.) under vigorous shaking at 2500 rpm for 10 min in order to induce protein precipitation. Then, samples were hydrolyzed in a water bath at 50° for 15 min and, finally centrifuge at 10,000 g for 10 min. The resulting supernatants were filtered (Filter nylon, 0.45 µm, Thermo scientific, Rockford, USA) and 50 µl was injected onto the HPLC column.

The same protocol was followed for the preparation of calibration curves, using blank brains and different solutions of quercetin in methanol. Calibration curves were designed over the range between 100 and 5000 ng/mL (R^2 = 0,996). The samples were analyzed using HPLC at 370 nm in the same conditions observed in the quantification of quercetin-loaded in zein nanoparticles. The limit of quantification was calculated as 200 ng/mg tissue.

2.8. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). The different behavioral paradigms and western blotting studies were analysed using One-way ANOVA

analysis followed by Tukey post hoc. In the acquisition phase of the MWM, treatment effects were analyzed by 1-way analysis of variance (ANOVA) for repeated measures followed by Tukey post hoc test. For each day, a one-way ANOVA was carried out and significant differences among groups were analyzed with Tukey post hoc test. Treatment differences were considered statistically significant at $p < 0.05$. Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 15.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Physicochemical characteristics of quercetin-loaded zein nanoparticles

Quercetin nanoparticles (NPQ) displayed a mean size of 260 ± 8 nm, a polydispersity index of 0.24 and a negative zeta potencial of -43.0 ± 1.2 mV. The quercetin payload in zein nanoparticles was 70 ± 1.3 µg/mg, representing an encapsulation efficiency of 81.2 ± 1.3%.

3.2. Effect of quercetin formulations in the spontaneous motor activity test and coordination

Figure 1 shows the effect of Q and NPQ in the motor activity of mice. Interestingly, neither Q nor NPQ had any effect on the distance traveled by (Figure 1A) and the speed of mice (Figure 1B). On the other hand, the performance on the Rotarod was analyzed by measuring the time that mice were able of supporting the accelerating rod. With this test it was evident that SAMP8 mice held a lower time in the rod than SAMR1 mice (Day 1: $F = 8.309$, $p < 0.05$; Day 2: $F = 5.178$, $p < 0.05$) (Figure 1C). On the other hand, the quercetin formulations did not show any effect on the coordination of SAMP8 mice.

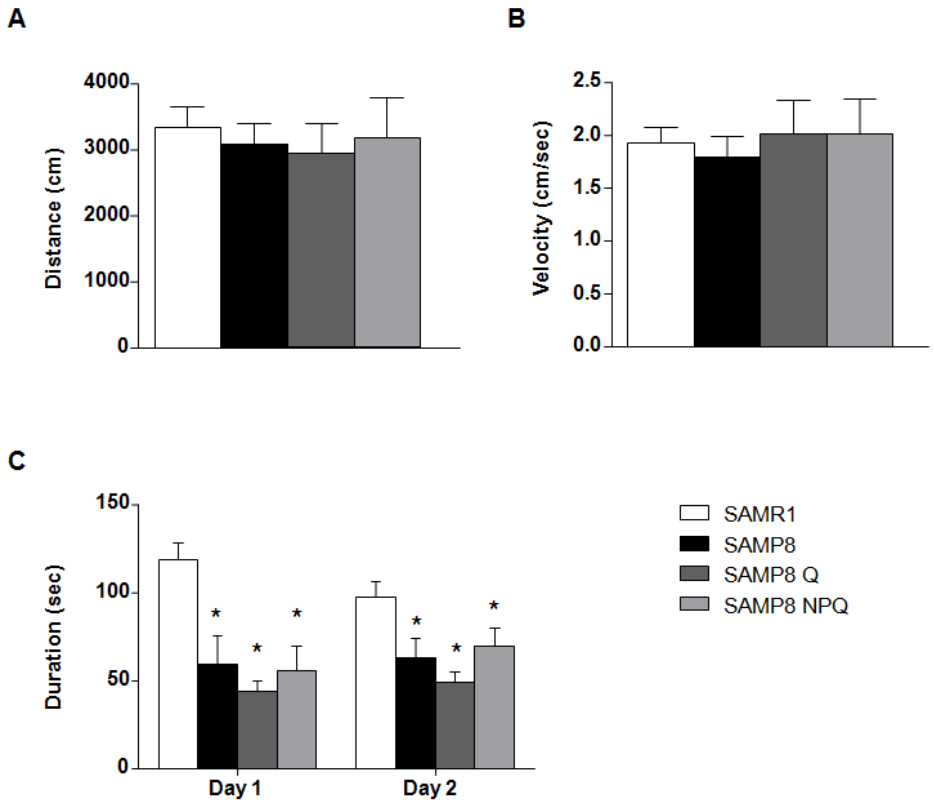


Figure 1. Effects of free quercetin and quercetin-loaded nanoparticles on motor performance. SAMP8 mice were treated with saline (SAMP8), free quercetin (25 mg/kg once per day, p.o, SAMP8 Q) or quercetin-loaded in nanoparticles (25 mg/kg every other day, p.o., SAMP8 NPQ). No significant differences were observed among all four groups when distance (A) or velocity (B) were analysed in the spontaneous motor activity test. (C) Effect on motor coordination in the rotarod assay. The duration remaining on rotarod in two consecutive days is shown. Data show the mean ± SEM. *p<0.05 *vs* SAMR1 group (One-Way ANOVA followed by Tukey test).

3.3. Effect of quercetin formulations on SAMP8 mice in the marble burying test

Results of the marble burying tests are summarized in Figure 2. The mean number of marbles buried by SAMR1 mice were significantly higher than the corresponding values of saline-treated SAMP8 mice (10.25 and 6.28 respectively; F= 7.221, p< 0.05). SAMP8 treated with the solution of quercetin showed a mean of buried marbles similar to that observed for saline-treated SAMP8. However, this altered normal exploratory behavior was fully reverted by quercetin-loaded nanoparticles treatment.

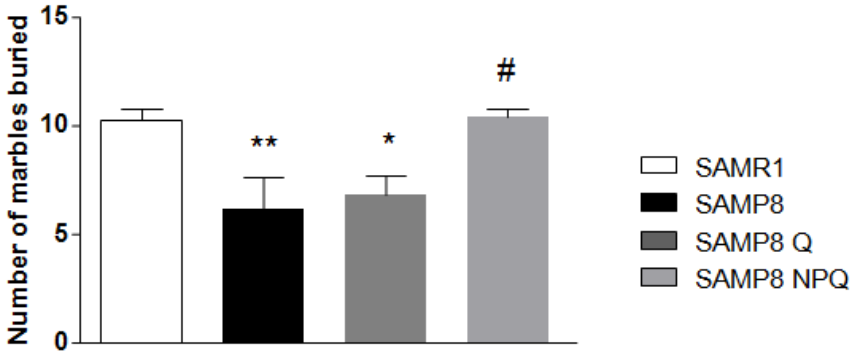


Figure 2. Effect of free quercetin and quercetin-loaded nanoparticles on the marble burying test. SAMP8 mice were treated with saline (SAMP8), free quercetin (25 mg/kg once per day, p.o, SAMP8 Q) or quercetin-loaded in nanoparticles (25 mg/kg every other day, p.o., SAMP8 NPQ). Data show the mean ± SEM. *p<0.05 , *p<0.01 *vs* SAMR1 group; #p<0.05 *vs* SAMP8 group (One-Way ANOVA followed by Tukey test).

3.4. Effects of quercetin formulations on learning and memory deficits of SAMP8 mice

Figure 3 summarizes the results obtained in the Morris water maze test. In the acquisition phase of this test (Figure 3A), as expected, the time spent to reach the platform was significantly lower for SAMR1 mice than for SAMP8 mice. On day 1, SAMR1 mice needed about 50% less time than SAMP8 to reach the platform. In addition, over trials, SAMR1 reduced the time required to reach the platform. Thus, on day 7, these mice were able of reaching the platform in a third of the initial time. On the contrary, the latencies exhibited by the saline-treated SAMP8 group did not significantly decrease over trials, indicating the existence of learning deficits in these mice. However, SAMP8 mice treated with NPQ showed a marked improvement in their behavioral performance as their escape latencies (on days 4, 6 and 7) were significantly shorter than for SAMP8 mice treated with saline. No significant differences were found between saline or free quercetin-treated SAMP8 mice (Figure 3A).

At the beginning of the fourth and eighth day, all mice were subjected to a probe trial in which they swam in the pool with the platform removed as a putative measurement of memory retention (Figure 3B). Upon removal of the platform, SAMP8 control mice spent significantly less time in the target quadrant than SAMR1 mice [$F = 3.390$, $p < 0.05$ for day 4 and $F = 6.695$, $p < 0.05$ for day 8]. Moreover, on day 4 no significant differences were found among saline, free quercetin or quercetin-loaded nanoparticles-treated SAMP8. However, on day 8 (24 h after the final trial on the learning test), post-hoc analysis revealed that SAMP8 mice treated with NPQ spent more time in the target quadrant than saline SAMP8 mice, whereas no differences were observed between saline or free quercetin -treated SAMR8 mice.

On the other hand, no differences were detected in the visual, motor or motivational skills of the animals during the visible test between the experimental groups (data not shown).

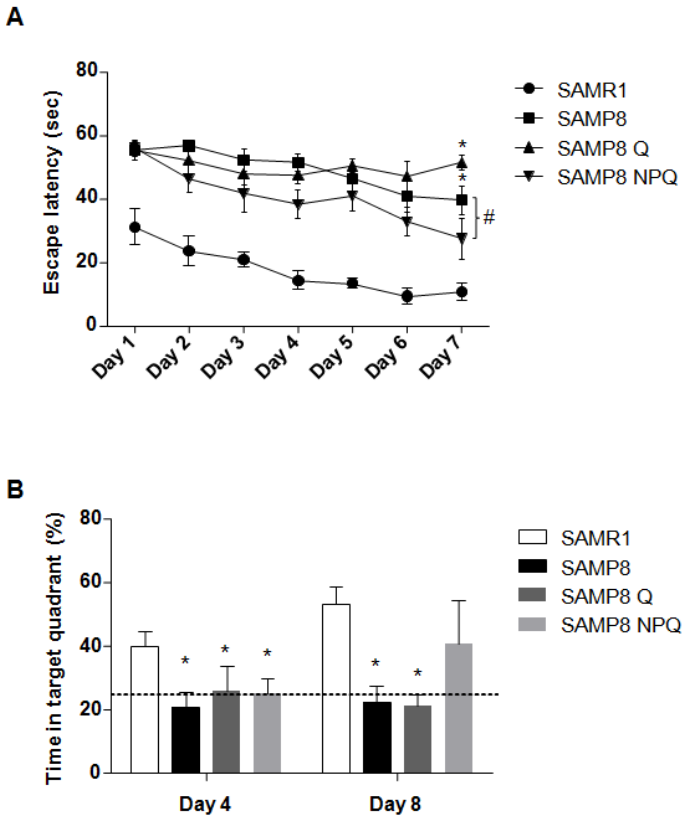


Figure 3. Quercetin-loaded nanoparticles treatment improves spatial learning and memory function in SAMP8 mice in the Morris Water Maze test. SAMP8 mice were treated with saline (SAMP8), free quercetin (25 mg/kg once per day, p.o, SAMP8 Q) or quercetin loaded

in nanoparticles (25 mg/kg every other day, p.o., SAMP8 NPQ). **(A)** Mean latency in reaching the hidden platform on the spatial learning task. Only statistic symbols for the last day are represented. **(B)** In the probe trial data are presented as percentage of time spent in the target quadrant. The data are expressed as the mean ± SEM. *p<0.05 *vs* SAMR1 group; #p<0.05 *vs* SAMP8 group (One-Way ANOVA followed by Tukey test).

3.5. Effect of quercetin formulations on astrogliosis in SAMP8 mice

In order to gain insight about the mechanisms underlying the differences found in the behavior of SAMP8 mice treated with quercetin formulations, two different markers of brain inflammation were analyzed. The first one was the astrocyte marker GFAP. GFAP displayed a significant increase in the hippocampus of SAMP8 mice in comparison to the SAMR1 control strain ($F= 4.724$, $p < 0.05$). Interestingly, quercetin-loaded nanoparticles significantly reduced hippocampal GFAP expression whereas this effect was not observed in free quercetin-treated SAMP8 mice (Figure 4A).

The second one was the hippocampal CD11b. When this microglia activation marker was analyzed, no differences were found among all four groups (Figure 4B).

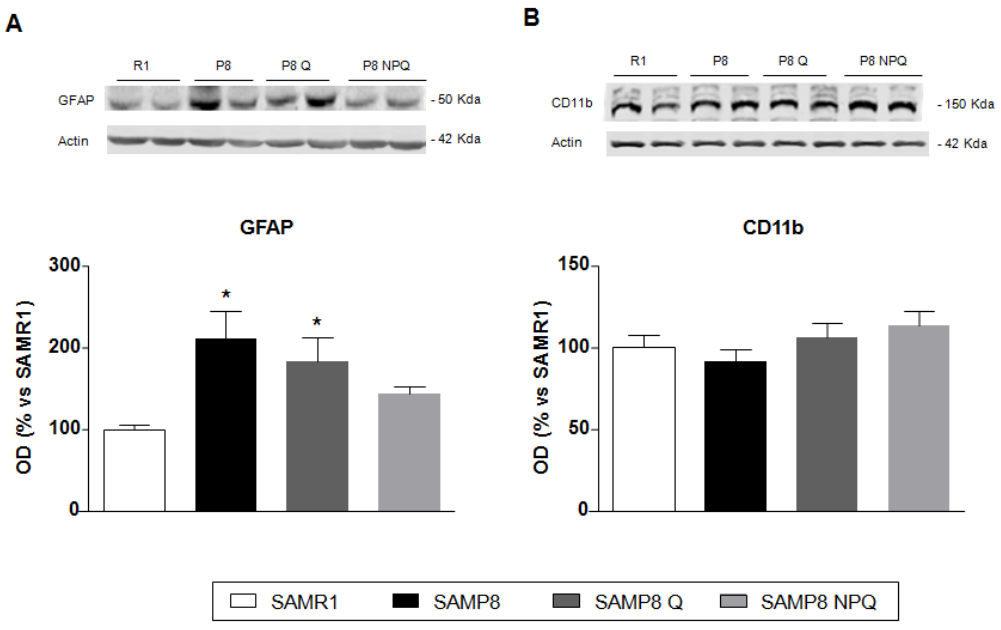


Figure 4. Effect of free quercetin and quercetin-loaded nanoparticles on astrogliosis. SAMP8 mice were treated with saline (SAMP8), free quercetin (25 mg/kg once per day, p.o, SAMP8

Q) or quercetin loaded in nanoparticles (25 mg/kg every other day, p.o., SAMP8 NPQ). Representative Western blot and quantitative measurement of GFAP **(A)** and CD11 **(B)** normalized to β-actin and in the hippocampus of SAMP8 treated with saline, free quercetin or quercetin-loaded nanoparticles. Note that quercetin-loaded nanoparticles decreased GFAP levels in SAMP8 mice. Results are expressed as mean ± SEM.*$p<0.05$ *vs* SAMR1 group (One-Way ANOVA followed by Tukey test).

3.6. Brain quantification of quercetin

At the end of the study, after two consecutive mounts of treatment, the amount of quercetin in brain was quantified. For animals treated with nanoencapsulated quercetin (NPQ), the concentration of this flavonoid in SAMP8 mice brains was 371 ± 28.4 (ng/g tissue). In case of animals treated with the solution of quercetin, the amount of this flavonoid in their brains was significantly lower and close to the quantitation limit of the HPLC technique (200 ng/g tissue).

4. Discussion

The senescence-accelerated mouse prone 8 (SAMP8) has been widely used as a non-transgenic murine model for late-onset AD, which constitutes over 90% of all AD cases (Butterfield and Poon, 2005; Cheng et al., 2014; Morley et al., 2012). These mice exhibited age-related learning and memory deficits, as well as AD main neuropathological hallmarks such as amyloid-like deposits, increased expression of hyperphosphorylated tau and inflammation (Farr et al., 2016; Orejana et al., 2012; Takeda, 2009).

Consistent with previous reports (Orejana et al., 2012; Orejana et al., 2013), 6-month-old SAMP8 mice presented learning and memory impairments in the MWM when compared with age matched SAMR1 mice. Interestingly, we demonstrate for the first time that this memory impairment was markedly ameliorated by an oral treatment with quercetin

nanoencapsulated in zein nanoparticles (25 mg/kg every 48 h for 2 months), while the administration of free quercetin was not able to reverse the faulty behavior, despite a higher administration frequency. Same results were observed in the marble burying test, where NPQ treatment was able to completely reverse the altered normal exploratory behavior in SAMP8 mice. Importantly, swimming velocity and the distance travelled in the open field test was similar in all groups indicating that the beneficial effects caused by NPQ were independent of any effect on motor activity.

Our data are in agreement with previous studies showing that the administration of quercetin to different AD transgenic mouse models improves their performance in the MWM test. However, given its low oral bioavailability (Patel et al., 2012), in those studies quercetin was always administered at higher doses (40 mg/kg per day) (Wang et al., 2014) or intraperitoneally (Sabogal-Guáqueta et al., 2015).

The importance of neuroinflammatory processes in the AD has been emphasized during the past decade, as intensive investigations have examined pro-inflammatory mediators, free radical-mediated oxidative stress and glial cell activation in the brain of AD patients (Bhamra and Ashton, 2012; Glass et al., 2010). Therefore, astrogliosis has been considered as a promising therapeutic target for therapeutic interventions (Baune, 2015). In this sense, due to its antioxidant and anti-inflammatory properties, quercetin has been proposed as promising compound that might successfully target ongoing brain inflammation during AD progression (Ansari et al., 2009; Bureau et al., 2008; Costa et al., 2016).

According to these data and to the findings described herein, we next analyzed the effect of NPQ on the inflammatory marker GFAP. As previously reported (Wu et al., 2005), we observed increased GFAP levels in the hippocampus of SAMP8 compared to age-matched SAMR1. Interestingly, NPQ treatment significantly reduced hippocampal GFAP expression whereas this effect was not observed in free quercetin-treated SAMP8 mice. Our result are in

line with those of Sabogal-Guaqueta and co-workers who described an important decrease in the GFAP levels of a transgenic mouse model of AD after 3 months of intraperitoneal quercetin treatment (Sabogal-Guáqueta et al., 2015). Although the cellular and molecular mechanisms for the anti-inflammatory effects of quercetin are not known, previous studies have demonstrated that quercetin inhibits iNOS, COX-2 and IL-1b (Martinez-Florez et al., 2005; Sharma et al., 2007) and increases the GSH levels in astrocytes and neurons, contributing to a decrease in oxidative stress (Lavoie et al., 2009). However, further investigations are needed in order to decipher the role of the different pathways involved in quercetin induced neuroprotection.

In an attempt to elucidate the mechanisms underlying the beneficial effects of NPQ, the amount of quercetin in the brain of NPQ and Q treated mice was quantified. Although the free quercetin was administered daily and quercetin-loaded in nanoparticles was administered every two days, no quantifiable levels of quercetin were observed in the brain of animals treated with the solution of the flavonoid. On the contrary, animals treated with the nanoparticles, displayed an amount of quercetin in the brain that was about 2-times higher than the limit of quantitation of our HPC technique.These results agree well with the increased oral bioavailability and the observed sustained levels of the flavonoid in plasma when administered as nanoencapsulated in the nanoparticles used in this work (Penalva et al., 2016). In fact, these previous studies have clearly demonstrated that the oral bioavailability of quercetin formulated in nanoparticles (NPQ) was about 15-times higher than when administered as an oral solution in a mixture of PEG400 and water (Q). In the same way, nanoparticles provided sustained levels of the flavonoid in plasma for about 48 h, whereas the levels of quercetin in plasma were only quantifiable during the first eight hours after administration (Penalva et al., 2016).

Although further studies are required to elucidate the precise mechanisms and action of quercetin-loaded nanoparticles, the present study supports the hypothesis that this drug delivery system might be a potential therapeutic tool that could increase quercetin's beneficial effects by improving its oral bioavailability and, at the same time, offering prolonged and sustained therapeutic levels of the flavonoid.

5. Conclusion

In summary, the oral administration of quercetin-loaded in zein nanoparticles at the dose of 25 mg/kg every two days for 2 consecutive months improved the cognition and memory impairments and reduced the astrogliosis shown by SAMP8 mice. On the contrary, the same dose of free quercetin treatment administered orally everyday did not have any significant effect on the senescence of the animals.

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References

Ansari, M.A., Abdul, H.M., Joshi, G., Opii, W.O., Butterfield, D.A., 2009. Protective effect of quercetin in primary neurons against Abeta (1–42): relevance to Alzheimer's disease. J. Nutr. Biochem. 20, 269–275.

Baune, B.T., 2015. Inflammation and neurodegenerative disorders: is there still hope for therapeutic intervention? Curr. Opin. Psychiatry. 28, 148-154

Bhamra, M.S., Ashton, N.J., 2012. Finding a pathological diagnosis for Alzheimer's disease: are inflammatory molecules the answer? Electrophoresis. 33, 3598–3607.

Boada, M., Tárraga, L., Hernández, I., Valero, S., Alegret, M., Ruiz, A., Lopez, O.L., Becker, J.T., 2014. Design of a comprehensive Alzheimer's disease clinic and research center in Spain to meet critical patient and family needs. Alzheimers Dement. 10, 409-415.

Bureau, G., Longpre, F., Martinoli, M.G., 2008. Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. J. Neurosci. Res. 86, 403–410.

Butterfield, D.A., Poon, H.F., 2005. The senescence-accelerated prone mouse (SAMP8): a model of age-related cognitive decline with relevance to alterations of the gene expression and protein abnormalities in Alzheimer's disease. Exp. Gerontol. 40, 774–783.

Calleja, P., Espuelas, S., Corrales, L., Pio, R., Irache, J.M., 2014. Pharmacokinetics and antitumor efficacy of paclitaxel-cyclodextrin complexes loaded in mucus-penetrating nanoparticles for oral administration. Nanomedicine (Lond). 9, 2109-2121.

Chabane, M.N., Ahmad, A.A., Peluso, J., Muller, C.D., Ubeaud- Séquier, G., 2009. Quercetin and naringenin transport across human intestinal Caco- 2 cells, J. Pharm. Pharmacol. 61, 1473-1483.

Cheng, X.R., Zhou, W.X., Zhang, Y.X., 2014. The behavioral, pathological and therapeutic features of the senescence-accelerated mouse prone 8 strain as an Alzheimer's disease animal model. Ageing Res. Rev. 13, 13–37.

Choi, J., Piao, Y., Kang, K. W., 2011. Effects of quercetin on the bioavailability of doxorubicin in rats: role of CYP3A4 and P-gp inhibition by quercetin. Arch. Pharm. Res. 34, 607-613.

Choi, S.M., Kim, B.C., Cho, Y.H., Choi, K.H., Chang, J., Park, M.S., Kim, M.K., Cho, K.H., Kim, J.K., 2014. Effects of flavonoid compounds on beta-amyloid-peptide-induced neuronal death in cultured mouse cortical neurons. Chonnam Med. J. 50, 45–51.

Costa, L.G., Garrick, J.M., Roquè, P.J., Pellacani, C., 2016. Mechanisms of neuroprotection by quercetin: counteracting oxidative stress and more. Oxid. Med. Cell. Longev. 2016, 1-10.

Dodel, R., Belger, M., Reed, C., Wimo, A., Jones, R.W., Happich, M., Argimon, J.M., Bruno, G., Vellas, B., Haro, J.M., 2015. Determinants of societal costs in Alzheimer's disease: GERAS study baseline results. Alzheimers Dement. 11, 933-945.

Farr, S.A., Niehoff, M.L., Ceddia, M.A., Herrlinger, K.A., Lewis, B.J., Feng, S., Welleford, A., Butterfield, D.A., Morley, J.E., 2016. Effect of botanical extracts containing carnosic acid or rosmarinic acid on learning and memory in SAMP8 mice. Physiol. Behav. 165, 328–338.

García-Ayllón, M., Small, D., Avila, J., Sáez-Valero, J., 2011. Revisiting the role of acetylcholinesterase in Alzheimer's disease: cross-talk with P-tau and β-amyloid. Front. Mol. Neurosci. 4, 1-9.

Glass, C.K., Saijo, K., Winner, B., Marchetto, M.C., Gage, F.H., 2010. Mechanisms underlying inflammation in neurodegeneration. Cell. 140, 918–934.

Helmfors, L., Boman, A., Civitelli, L., Nath, S., Sandin, L., Janefjord, C., McCann, H., Zetterberg, H., Blennow, K., Halliday, G., Brorsson, A., Kågedal, K., 2015. Protective properties of lysozyme on β-amyloid pathology: implications for Alzheimer disease. Neurobiol. Dis. 83, 122–133.

Hong, S., Beja-Glasser, V.F., Nfonoyim, B.M., Frouin, A., Li, S., Ramakrishnan, S., Merry, K. M., Shi, Q., Rosenthal, A., Barres, B. A., Lemere, C. A., Selkoe, D.J., Stevens, B., 2016. Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science. 352, 712-716.

Ishikawa, M., Yoshii, H., Furuta, T., 2005. Interaction of modified cyclodextrins with cytochrome P-450. Biosci Biotechnol Biochem. 69, 246–248.

Ishisaka, A., Mukai, R., Terao, J., Shibata, N., Kawai, Y., 2014. Specific localization of quercetin-3-O-glucuronide in human brain. Arch. of Biochem. Biophys. 557, 11–17.

Khunnawutmanotham, N., Chimnoi, N., Saparpakorn, P., Techasakul, S., 2016. Synthesis and anti-acetylcholinesterase activity of scopoletin derivatives. Bioorg. Chem. 65, 137-145.

Larson, E.B., Shadlen, M.F., Wang, L., McCormick, W.C., Bowen, J.D., Teri, L., Kukull, W.A., 2004. Survival after initial diagnosis of Alzheimer disease. Ann. Intern Med. 140, 501-509.

Lavoie, S., Chen, Y., Dalton, T.P., Gysin, R., Cuenod, M., Steullet, P., Do, K.Q., 2009. Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit. J. Neurochem. 108, 1410-1422.

Li, H., Zhao, X., Ma, Y., Zhai, G., Li, L., Lou, H., 2009. Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. J. Control. Release. 133, 238-244.

Liu, C.M., Zheng, G.H., Cheng, C., Sun, J.M., 2013. Quercetin protects mouse brain against lead-induced neurotoxicity. J. Agric. Food Chem. 61, 7630–7635.

López, L.C., Reis, S.R., Espargaro, A., Carrodeguas, J.A., Maddelein, M.L., Ventura, S., Sancho, J., 2012. Discovery of novel inhibitors of amyloid β-peptide 1–42 aggregation. J. Med. Chem. 55, 9521–9530.

Luo, W., Wang, T., Hong, C., Yang, Y., Chen, Y., Cen, J., Xie, S., Wang, C., 2016. Design, synthesis and evaluation of 4-dimethylamine flavonoid derivatives as potential multifunctional anti-Alzheimer agents. Eur. J. Med. Chem. 122, 17-26.

Martinez-Florez, S., Gutierrez-Fernandez, B., Sanchez-Campos, S., Gonzalez-Gallego, J., Tunon, M.J., 2005. Quercetin attenuates nuclear factor-kappaB activation and nitric oxide production in interleukin-1beta-activated rat hepatocytes. J. Nutr. 135, 1359-1365.

Morley, J.E., Armbrecht, H.J., Farr, S.A., Kumar, V.B., 2012. The senescence accelerated mouse (SAMP8) as a model for oxidative stress and Alzheimer's disease. Biochim. Biophys. Acta 1822, 650–656.

Najafi, Z., Saeedi, M., Mahdavi, M., Sabourian, R., Khanavi, M., Tehrani, M.B., Moghadam, F.M., Edraki,N., Karimpor-Razkenari,E., Sharifzadeh, M., Foroumadi, A., Shafiee, A., Akbarzadeh, T., 2016. Design and synthesis of novel anti-Alzheimer’s agents: Acridine-chromenone and quinoline-chromenone hybrids. Bioorg. Chemis. 67, 84–94.

Nelson, P.T., Alafuzoff, I., Bigio, E.H., Bouras, C., Braak, H., Cairns, N.J., Castellani, R.J., Crain, B.J., Davies, P., Tredici, K.D., Duyckaerts, C., et al., 2012. Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. J. Neuropathol. Exp. Neurol. 71, 362-381.

Oliveira, M. R., Nabavi, S. M., Braidy, N., Setzer, W. N., Ahmed, T., Nabavi, S.F., 2016. Quercetin and the mitochondria: a mechanistic view. Biotechnol. Adv. 34, 532–549.

Orejana, L., Barros-Miñones, L., Aguirre, N., Puerta, E., 2013. Implication of JNK pathway on tau pathology and cognitive decline in a senescence-accelerated mouse model. Exp. Gerontol. 48, 565–571.

Orejana, L., Barros-Minones, L., Jordán, J., Puerta, E., Aguirre, N., 2012. Sildenafil ameliorates cognitive deficits and tau pathology in a senescence-accelerated mouse model. Neurobiol. Aging. 33, 11-20.

Paliwal, R., Palakurthi, S., 2014. Zein in controlled drug delivery and tissue engineering, J. Control. Release. 189, 108-122.

Patel, A.R., Heussen, P.C.M., Hazekamp, J., Drost, E., Velikov, K.P., 2012. Quercetin loaded biopolymeric colloidal particles prepared by simultaneous precipitation of quercetin with hydrophobic protein in aqueous medium. Food Chem. 133, 423–429.

Peñalva, R., Esparza, I., González-Navarro, C.J., Quincoces, G., Peñuelas, I., Irache, J.M., 2015. Zein nanoparticles for oral folic acid delivery. J. Drug Deliv. Sci. Technol. 30, 450-457.

Penalva, R., González-Navarro, C.J., Gamazo, C., Esparza, I., Irache, J.M., (2016) Zein nanoparticles for oral delivery of quercetin: pharmacokinetic studies and preventive anti-inflammatory effects in a mouse model of endotoxemia. Nanomedicine. doi: 10.1016/j.nano.2016.08.033.

Qi, L., Ke, L., Liu, X., Liao, L., Ke, S., Liu, X., Wang, Y., Lin, X., Zhou, Y., Wu, L., Chen, Z., Liu, L., 2016. Subcutaneous administration of liraglutide ameliorates memory impairment by modulating tau hyperphosphorylation via the glycogen synthase kinase-3β pathway in an amyloid β protein induced Alzheimer disease mouse model. Eur. J. Pharmacol. 783, 23–32.

Sabogal-Guáqueta, A.M.., Munõz-Manco, J.I., Ramírez-Pineda, J.R., Lamprea-Rodriguez, M., Osorio, E., Cardona-Gómez, G.P., 2015. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacol. 133, 423–429.

Sharma, V., Mishra, M., Ghosh, S., Tewari, R., Basu, A., Seth, P., Sen, E., 2007. Modulation of interleukin-1beta mediated inflammatory response in human astrocytes by flavonoids: implications in neuroprotection. Brain Res. Bull. 73, 55-63.

Spencer, J.P., 2009. The impact of flavonoids on memory: physiological and molecular considerations. Chem. Soc. Rev. 38, 1152–1161.

Sun, M., Gao, Y., Pei, Y., Guo, C., Li, H., Cao, F., Yu, A., Zhai, G., 2010. Development of nanosuspension formulation for oral delivery of quercetin. J. Biomed. Nanotechnol. 6, 325-332.

Suna, D., Li, N., Zhanga, W., Zhaoa, Z., Moua, Z., Huanga, D., Liub, J., Wang, W., 2016. Design of PLGA-functionalized quercetin nanoparticles for potential use in Alzheimer’s disease. Colloids Surf. B Biointerfaces. 148:116-129.

Takeda, T., 2009. Senescence-accelerated mouse (SAM) with special references to neurodegeneration models, SAMP8 and SAMP10 mice. Neurochem Res. 34, 639-59.

Youdim, K.A., Shukitt-Hale, B., Joseph, J.A., 2004. Flavonoids and the brain: interactions at the blood-brain barrier and their physiological effects on the central nervous system. Free Radic. Biol. Med. 37, 1683–1693.

Wang, D.M., Li, S.Q., Wu, W.L., Zhu, X.Y., Wang, Y., Yuan, H.Y., 2014. Effects of long-term treatment with quercetin on cognition and mitochondrial function in a mouse model of Alzheimer's disease. Neurochem. Res. 39, 1533-1543.

Williams, R.J., Spencer, J.P.E., 2012. Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. Free Radic. Biol. Med. 52, 35-45.

Wu, Y., Zhang, A.Q,, Yew, D.T., 2005. Age related changes of various markers of astrocytes in senescence-accelerated mice hippocampus. Neurochem. Int. 46, 565–574.

Zhang, Y., Meng, F., Cui, Y., Song, Y., 2011. Enhancing effect of hydroxypropyl-β-cyclodextrin on the intestinal absorption process of genipin. J. Agric. Food Chem. 59, 10919-10926.

9. CONCLUSÕES E PERSPECTIVAS

9.1. Conclusões

- A formulação lipossomal contendo nimodipina (NMD-Lipo) apresenta lipossomas unilamerares pequenos, com tamanhos médios que variam entre 105 e 120 nm de diâmetro, potencial zeta de −5,32 e taxas de encapsulação que variam entre 99 ± 0.22% e 99 ± 0,67% (0,96 a 0,98 mg de nimodipina por mL de formulação lipossomal);

- A administração intraperitoneal de NMD-Lipo não causou sedação ou relaxamento muscular em camundongos e apresenta atividade ansiolítica igual ou superior ao fármaco usado como controle positivo (diazepam) e significativamente superior à nimodipina livre. A diminuição do efeito ansiolítico de NMD-Lipo nos animais pré-tratados com flumazenil indica que a nimodipina encapsulada atua nos receptores benzodiazepínicos;

- A administração intraperitoneal de NMD-Lipo não apresentou toxicidade em camundongos. NMD-Lipo apresentou atividade anticonvulsivante significativamente superior ao fármaco usado como controle positivo (diazepam) e significativamente superior à nimodipina livre no modelo de convulsão induzida por pilocarpina. A administração de NMD-Lipo impediu a instalação do processo convulsivo e a morte de 100% dos animais tratados;

- A administração intraperitoneal de NMD-Lipo apresentou atividade antidepressiva significativamente superior aos fármacos usados como controle positivo (imipramina e paroxetina). A diminuição do efeito antidepressivo de NMD-Lipo nos animais pré-tratados com reserpina, bem como a inibição da enzima MAO_B, sugere que a atividade antidepressiva da nimodipina encapsulada está relacionada com um aumento nas concentrações das monoaminas cerebrais.

- As nanopartículas de polianidrido contendo nimodipina (NMD-NP) apresentaram diâmetros médios que variam entre 120 e 180 nm, com conteúdos de fármaco próximos a 7%, potenciais zeta entre -37 e -50, eficiência de encapsulação de aproximadamente 81% (66 µg de nimodipina/mg nanopartículas) e rendimentos do processo de fabricação superiores a 83%.

Por outro lado, as nanopartículas de polianidrido contendo nimodipina recobertas com PEG 2000 (NMD-NP/PEG) apresentaram diâmetros médios que variam entre 124 e 191 nm, potenciais zeta entre -28 e -23, eficiência de encapsulação de aproximadamente 84% (68 µg de nimodipina/mg nanopartículas) e rendimentos do processo de fabricação superiores a 90%. O recubrimento das ditas nanopartículas com PEG 2000 deu lugar a sistemas muito parecidos, com um tamanho ligeiramente superior e uma carga negativa inferior as nanopartículas sem recobrimento. A peguilação não apresentou nenhuma influência na taxa de encapsulação da nimodipina.

- A administração oral de uma solução de nimodipina apresentou uma biodisponilibilidade de 8,8%, enquanto a administração de NMD/NP apresentou uma biodisponibilidade de 12,8 e a administração de NMD-NP/PEG mostrou uma biodisponibilidade de 61,9.

- A administração oral de NMD-NP/PEG foi capaz de proteger os déficits cognitivos observados em camundongos estressados, melhorando não apenas a aquisição, bem como a retenção de memória.

- A administração oral de quercetina encapsulada em nanopartículas de zeína melhorou a cognição e as limitações de memória, bem como reduziu a astrogliose mostrada em um modelo murino de Alzheimer (SAMP8).

9.2. Perspectivas

- Exploração das capacidades mucopenetrantes, assim como a distribuição no trato gastrointestinal, das nanopartículas recobertas com PEG 2000. Estudar a influência do grau de peguilação na liberação do fármaco encapsulado, assim como seus efeitos no SNC.

- Determinação das características físico-químicas (espectroscopia de infravermelho, difração de raios-X, calorimetria diferencial exploratória e termogravimetría) de NMD-NP e NMD-NP/PEG.

- Estudo dos mecanismos de ação pelos quais a nimodipina encapsulada em nanopartículas evitou os déficits cognitivos dos camundongos estressados.

- Delineamento de nanopartículas de zeína peguiladas como veículos da administração oral de quercetina. Avaliação das suas capacidades mucopenetrantes e como sistemas de liberação controlada para a administração oral desse flavonoide no tratamento de transtornos cognitivos.

REFERÊNCIAS

ADEYEMI, O.O.; AKINDELE, A.J.; YEMITAN, O.K.; AIGIBE, F.R.; FAGBO, F.I. Anticonvulsant, anxiolytic and sedative activities of the aqueous root extract of securidaca longepedunculata fresen. **Journal of Ethnopharmacology**, v. 130, p. 191–195, 2010.

AGÜEROS, M.; ARESES, P.; CAMPANEROA, M.A.; SALMANA, H.; QUINCOCES, G.; PENUELAS, I.; IRACHE, J.M. Bioadhesive properties and biodistribution of cyclodextrin–poly(anhydride) nanoparticles. **European Journal of Pharmaceutical Sciences**, v. 37, p. 231–240, 2009.

AIT-OUDHIA, S.; MAGER, D.E.; STRAUBINGER, R.M. Application of Pharmacokinetic and Pharmacodynamic Analysis to the Development of Liposomal Formulations for Oncology. **Pharmaceutics**, v. 6, p. 137-174, 2014.

ALYAUDTIN, R.N.; REICHEL, A.; LÖBENBERG, R.; RAMGE, P.; KREUTER, J.; BEGLEY, D.J. Interaction of poly(butylcyanoacrylate) nanoparticles with the blood–brain barrier in vivo and in vitro. **Journal of Drug Targeting**, v. 9, p. 209–221, 2001.

ARBOS, P.; CAMPANERO, M.A.; ARANGOA, M.A.; IRACHE, J.M. Nanoparticles with specific bioadhesive properties to circumvent the pre-systemic degradation of fluorinated pyrimidines. **Journal of Controled Release**, v. 96, p. 55–65, 2004.

ASLAN, A.; GURELIK, M.; CEMEK, M.; GOKSEL, H. M.; BUYUKOKUROGLU, M. E. Nimodipine can improve cerebral metabolism and outcome in patients with severe head trauma. **Pharmacological Research,** v. 59, p. 120–124, 2009.

BABU, V.R.; PATEL, P.; MUNDARGI, R.C.; RANGASWAMY, V.; AMINABHAVI, T.M. Developments in polymeric devices for oral insulin delivery. **Expert Opinion on Drug Delivery**, v.5, p. 403–415, 2008.

BAILEY, M. L.; HUTSELL, B. A.; NEWLAN, M. C. Dietary nimodipine delays the onset of methylmercury neurotoxicity in mice. **NeuroToxicology**, v. 37, p.108-117, 2013.

BALASUBRAMANIAM, M.; TELLES, S.; DORAISWAMY, M. Yoga on Our Minds: A Systematic Review of Yoga for Neuropsychiatric Disorders. **Frontiers in Psychiatry**, v.3, p. 117, 2012.

BANERJEE, T.; VAN DER VLIET, A.; ZIBOH, V.A. Downregulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. **Prostaglandins, Leukotrienes and Essential Fatty Acids** v. 66, p. 485-492 2002.

BANGHAM, A.D.; STANDISH, M.M.; WATKINS, J.C. Diffusion of univalent ions across the lamellae of swollen phospholipids. **Journal of Molecular Biology**, v. 13, p. 238-252, 1965.

BATISTA, C. M.; CARVALHO, C. M. B.; SANTOS-MAGALHÃES, N. S. Lipossomas e suas aplicações terapêuticas: Estado da arte. **Revista Brasileira de Ciências Farmacêuticas,** v. 43, p 167-179, 2007.

BARTLEY, C. A.; HAY, M.; BLOCH, M.H. Meta-analysis: Aerobic exercise for the treatment of anxiety disorders. **Progress in Neuro-Psychopharmacology & Biological Psychiatry**, v. 45, p.34–39, 2013.

BASSIL, N.; GROSSBERG, G. T. Novel regimens and delivery systems in the pharmacological treatment of Alzheimer's disease. **CNS Drugs**, v. 23, p. 293-307, 2009.

BEGE, N.; RENETTE, T.; ENDRES,T.; BECK-BROICHSITTER, M.; HÄNGGI, D.; KISSEL, T. In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm. **European Journal of Pharmaceutics and Biopharmaceutics**, v.84, p. 99–105, 2013.

BHUTADA, P.; MUNDHADA, Y.; BANSOD , K.; DIXIT, P.; UMATHE, S.; MUNDHADA, D. Anticonvulsant activity of berberine, an isoquinoline alkaloid in mice. **Epilepsy & Behavior**, v. 18, p. 207–210, 2010.

BRIGHTMAN, M. W.; REESE, T. S. Junctions between intimately apposed cell membranes in the vertebrate brain. **The Journal of Cell Biology**, v.40, p. 648–677, 1969**.**

BUCHWEITZ, M.; KROON, P.A.; RICH, G.T.; WILDE, P.J. Quercetin solubilisation in bile salts: A comparison with sodium dodecyl sulphate. **Food Chemistry**, v. 211, p. 356-364, 2016.

CADENA, P.G.; PEREIRA, M.A.; CORDEIRO, R.B.S.; CAVALCANTI, I.M.F.; BARROS-NETO, PIMENTEL, M.C.C.B.; LIMA-FILHO, J.L.; SILVA, V.L.; SANTOS-MAGALHÃES, N.S. Nanoencapsulation of quercetin and resveratrol into elastic liposomes. **Biochimica et Biophysica Acta**, v. 1828, p. 309–316, 2013.

CARDOSO, F. L.; BRITES, D.; BRITO, M. A.; Looking at the blood–brain barrier: Molecular anatomy and possible investigation approaches. **Brain Research Reviews**, v. 64, p. 328-363, 2010.

CAVALCANTI, I.M.F.; MENDONÇA, E.A.M.; LIRA, M;C.B.; HONORATO, S.B.; CAMARA, C.A.; AMORIM, R.V.S.; MENDES FILHO, J.; RABELLO, M.M; HERNANDES, M.Z.; AYALA, A.P.; SANTOS-MAGALHÃES, N.S. The encapsulation of β-lapachone in 2-hydroxypropyl-β-cyclodextrin inclusion complex into liposomes: A physicochemical evaluation and molecular modeling approach. **European Journal of Pharmaceutical Sciences**, v. 4, p. 332-340, 2011.

CHAKRABARTI, A.; KAUR, H. S.; GARG, S. K. Dose-finding study with nimodipine: a selective central nervous system calcium channel blocker on aminophylline induced seizure model in rats, **Brain Research Bulletin, v.** 45, p. 495–499, 1998.

CHALIKWAR, S. S.; BELGAMWAR, V.S.; TALELE, V.R.; SURANA, S.J.; PATIL, M. U. Formulation and evaluation of nimodipine-loaded solid lipid nanoparticles delivered via lymphatic transport system. **Colloids and Surfaces B: Biointerfaces**, v. 9, p.109–16, 2012.

CHOI, S.M.; KIM, B.C.; CHO, Y.H.; CHOI, K.H.; CHANG, J.; PARK, M.S.; KIM, M.K.; CHO, K.H.; KIM, J.K. Effects of flavonoid compounds on beta-amyloid-peptideinduced neuronal death in cultured mouse cortical neurons. **Chonnam Medical Journal**, v. 50, p. 45-51, 2014.

CHRISTOFORIDIS, J.B.; CHANG, S.; JIANG, A.; WANG, J.; CEBULLA, C.M. INTRAVITREAL Devices for the Treatment of Vitreous Inflammation. **Mediators of Inflammation**, v. 2012, p. 1-8, 2012.

COSTA, J.P. **Bioprospecção do diterpenoide fitol com ênfase nas propriedades antioxidante, ansiolítica e antidepressiva**. 2014. 275f. Tese do Doutorado do Programa de Pós-graduação em Biotecnologia (RENORBIO), Teresina, 2014.

COUVREUR, P.; BARRATT, G.; FATTAL, E.; LEGRAND, P.; VAUTHIER, C. Nanocapsule technology: A review. **Critical Reviews in Therapeutic Drug Carrier Systems**, v. 19, p. 99–134, 2002.

COUVREUR, P.; VAUTHIER, C. Nanotechnology: Intelligent design to treat complex disease. **Pharmaceutical Research**, v. 23, p. 1417–1450, 2006.

DAMGE, C.; MICHEL, C.; APRAHAMIAN, M.; COUVREUR, P. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. **Diabetes**, v. 37, p. 246–251, 1988

DE PASCUAL-TERESA, S.; JOHNSTON, K. L.; DUPONT, M. S.; O'LEARY, K.A.; NEEDS, P.W.; MORGAN, L.M.; CLIFFORD, M.N.; BAO, Y.; WILLIAMSON, G. Quercetin metabolites downregulate cyclooxygenase-2 transcription. in human lymphocytes ex vivo but not in vivo. **Journal of Nutrition**, v. 134, p. 552-557, 2004.

DEL PORTO, José Alberto. Conceito e diagnóstico. **Revista Brasileira de Psiquiatria**, v.21, p. 6-11, 1999.

DORIA, S.; VUONO, A.; SANLORENZO, R.; IRTELLI, F.; MENCACCI, C. Anti-anxiety efficacy of Sudarshan Kriya Yoga in general anxiety disorder: A multicomponent, yoga based, breath intervention program for patients suffering from generalized anxiety disorder with or without comorbidities. **Journal of Affective Disorders**, v. 184, p. 310–317, 2015.

DUYCKAERTS, C.; DELATOUR, B.; POTIER, M.C. Classification and basic pathology of Alzheimer disease. **Acta Neuropathologica**. v. 118, p. 5-36, 2009.

ELSABAHY, M.; WOOLEY, K. Design of polymeric nanoparticles for biomedical delivery applications, **Chemical Society Reviews**, v. 41, p. 2545-2561, 2012.

EL-SHABOURI, M.H. Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. **International Journal of Pharmaceutics**, v. 249, p. 101–108, 2002.

ERLUND, I. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. **Nutrition Research**, v. 24, p. 851–874, 2004.

FIORANI, M.; GUIDARELLI, A.; BLASA, M.; AZZOLINI, C.; CANDIRACCI, M.; PIATTI, E.; CANTONI, O. Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid. **Journal of Nutrition and Biochemistry**, v. 21, p. 397–404, 2010.

FONTINELLE, L. Neurobiologia na adolescencia. **Jornal de Pediatria**, v.77, p. 205-216, 2001.

FORNARO, M.; MARTINO, M.; MATTEI, C.; PRESTIA, D.; VINCIGUERRA, V.; BERARDIS, D.; PASQUALE, C.; IASEVOLI, F.; MUNGO, S.; FORNARO, P. Duloxetine-bupropion combination for treatment-resistant atypical depression: A double-blind, randomized, placebo-controlled trial. **European Neuropsychopharmacology**, v. 24, p. 1269-1278, 2014.

FORTINI, S.; CORREDERA, L.; PASTRANA, A. L.; REYES, G.; FASULO, L.; CARABALLO, R. H. Encephalopathy with hemi-status epilepticus during sleep or hemi-continuous spikes and waves during slow sleep syndrome: A study of 21 patients. **Seizure**, v. 22, p. 565–571, 2013.

FRYE, M.A.; CALABRESE, J.R.; REED, M.L.; HIRSCHFELD, R.M. Healthcare resource utilization in bipolar Depression compared with unipolar Depression: Results of a United States population based study. **CNS Spectrums**, v.11, p. 704–710, 2006.

FRYE, M. A.; PAZZAGLIA, P .J.; GEORGE, M. S.; LUCKENBAUGH, D. A.; VANDERHAM, E.; DAVIS, C. L.; RUBINOW, D. R.; POST, R. M. Low CSF somatostatin associated with response to nimodipine in patents with affective illness. **Biological Psychiatry,** v. 53, p. 180-183, 2003.

GARCIA-MEDIAVILLA, V.; CRESPO, I.; COLLADO, P.S.; ESTELLER, A.; SANCHEZ-CAMPOS, S.; TUNON, M.J.; GONZALEZ-GALLEGO, J. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappa B pathway in Chang liver cells. **European Journal of Pharmacology**, v.557, p. 221- 229, 2007.

GHANBARZADEH, B.; MUSAVI, M.; OROMIEHIE, A.R.; REZAYI, K.; RAZMI RAD, E.; MILANI, J. Effect of plasticizing sugars on water vapor permeability, surface energy and microstructure properties of zein films. **LWT - Food Science and Technology**, v. 40, p. 1191-1197, 2007.

GHARIBA, R.; GREIGE-GERGES, H.; FOURMENTINB, S.; CHARCOSSET, C.; AUEZOVA, L. Liposomes incorporating cyclodextrin–drug inclusion complexes: Current state of knowledge. **Carbohydrate Polymers**, v. 129, p. 175–186, 2015.

HADIZADEH, F.; RAHIMI, B.; TAGHIABADI, E.; RAZAVI, M.; KARIMI, G. Evaluation of anticonvulsant effect of two novels 4-[1-(4-fluorobenzyl)-5-imidazolyl] dihydropyridine derivatives in mice. **Research in Pharmaceutical Sciences**, v. 8, p. 91-95, 2013.

HEFFREN, J.; MCINTOSH, A. M.; REITER, P. D. Nimodipine for the Prevention of cerebral vasospasm after subarachnoid hemorrhage in 12 children. pediatric neurology, v. 52, p. 356–360, 2015.

HOCHT, C.; LAZAROWSKI, A.; GONZALEZ, N. N.; AUZMENDI, J.; OPEZZO, J. A.W.; BRAMUGLIA, G. F.; TAIRA, C. A.; GIRARDI, E. Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model, **Neurosciense Letters**, v. 413, p. 168–172, 2007.

HUANG, S.; YU, X.; YANG, L.; SONG, F.; CHEN, G.; LV, Z.; LI, T.; CHEN, D.; ZHU, W.; YU, A.; ZHANG, Y.; YANG, F. The efficacy of nimodipine drug delivery using mPEG-PLA micelles and mPEG-PLA/TPGS mixed micelles, **European Journal of Pharmaceutical Sciences**, v. 63, p. 187-198, 2014.

IQBAL, K.; GRUNDKE-IQBAL, I. Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention. **Journal of Cellular and Molecular Medicine.** v.12, p.38-55, 2008.

INCHAURRAGA, L.; MARTÍN-ARBELLA, N.; ZABALETA, V.; QUINCOCES, G.; PEÑUELAS, I.; IRACHE, J.M. In vivo study of the mucus-permeating properties of PEGcoated nanoparticles following oral administration. **European Journal of Pharmaceutics and Biopharmaceutics**, In Press, Corrected Proof, 2014.

INVERNICI, G.; CRISTINI, S.; ALESSANDRI, G.; NAVONE, S.E.; CANZI, L.; TAVIAN, D.; REDAELLI, C.; ACERBI, F.; PARATI, E.A. Nanotechnology advances in brain tumors: The state of the art. **Recent Patents on Anticancer Drug Discovery**, v.6, p. 58–69, 2011.

IRACHE, J.M.; ESPARZA, I.; GAMAZO, C.; AGÜEROS, M.; ESPUELAS, S. Nanomedicine: Novel approaches in human and veterinary therapeutics. **Veterinary Parasitology**, v. 180, p. 47– 71, 2011.

IRACHE, J.M.; HUICI, M.; KONECNY, M.; ESPUELAS, S.; CAMPANERO, M.A.; ARBOS, P. Bioadhesive properties of gantrez nanoparticles. **Molecules**, v. 10, p. 126-145, 2005.

ISHISAKA, A.; ICHIKAWA, S.; SAKAKIBARA, H.; PISKULA, M.K.; NAKAMURA, T.; KATO, Y.; ITO, M.; MIYAMOTO, K.I.; TSUJI, A.; KAWAI, Y.; TERAO, J. Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats. **Free Radical Biology & Medicine**, v. 51, p. 1329-1336, 2011.

ITTNER, L.M.; GOTZ, J. Amyloid-beta and tauea toxic pas de deux in Alzheimer's disease. **Nature Reviews Neuroscience**. v.12, p. 65-72, 2011.

ITALIA, J.L.; BHATT, D.K.; BHARDWAJ, V.; TIKOO, K.; KUMAR, M.N. PLGA nanoparticles for oral delivery of cyclosporine: Nephrotoxicity and pharmacokinetic studies in comparison to Sandimmune Neoral. **Journal of Controlled Release**, v. 119, p. 197–206, 2007.

KANTER, M.; UNSAL, C.. AKTAS, C.; ERBOGA, M. 2013. Neuroprotective effect of quercetin against oxidative damage and neuronal apoptosis caused by cadmium in hippocampus. **Toxicology and Industrial Health**, v. 32, p. 541-550, 2016.

KHRESTCHATISKY, M.; TOKAY, A. Nervous system diseases: an ever increasing cost for society. **Pharmaphorum**, 2014.

<www.pharmaphorum.com/articles/nervoussystem-diseases-an-ever-increasing-cost-for-society> (accesado em 03.03.15).

KIM, D.H.; MARTIN, D.C. Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery. **Biomaterials**, v. 27, p. 3031–3037, 2006.

KOSKINEN, L.D.; RUNNERSTAM, M.; KOCH, M.; KARLSSON, B. M. Cerebral microvascular effects of nimodipine in combination with soman. **Environmental Toxicology and Pharmacology**, v. 34, p. 905–T910, 2012.

KULKARNI, P.R.; YADAV, J.D.; VAIDYA, K.A. Liposomes: A novel drug delivery system. **International Journal of Current Pharmaceutical Research**, v. 3, p. 10-8, 2011.

KUMAR, N.; SINGH, N.; JAGGI, A. S. Anti-stress effects of cilnidipine and nimodipine in immobilization subjected mice. **Physiology & Behavior**, v. 150, p. 1148-1144, 2012.

KUO, Y.; LIU, Y. Cardiolipin-incorporated liposomes with surface CRM197 for enhancing neuronal survival against neurotoxicity. **International Journal of Pharmaceutics**, v. 473, p. 334–344, 2014.

LEI, W.; SI, W.; HAO, Q.; HAN, Z.; ZHANG, Y.; XIA, M. Nitrogen-doped graphene modified electrode for nimodipine sensing. **Sensors and Actuators B: Chemical**, v. 212, p. 207-213, 2015.

LI, L.; AN, X.; YAN, X. Folate-polydiacetylene-liposome for tumor targeted drug delivery and fluorescent tracing. **Colloids and Surfaces B: Biointerfaces**, v. 134, p. 235–239, 2015.

LIU, L.; ZHU, C.; TIAN, H.; ZHENG, T.; YE, M.; LI, H. Correlations of PTEN genetic polymorphisms with the risk of depression and depressive symptoms in a Chinese population. **Gene**, v. 595, p. 77–82, 2016.

LIU, Z.; NIU, W.; YANG, X.; WANG, Y. Effects of combined acupuncture and eugenol on learning-memory ability and antioxidation system of hippocampus in Alzheimer disease rats via olfactory system stimulation. **Journal of Tradicional Chinese Medicine**, v. 33, p. 399-402, 2013a.

LIU, C.M.; ZHENG, G.H.; CHENG, C.; SUN, J.M. Quercetin protects mouse brain against lead-induced neurotoxicity. **Journal of Agriculture Food and Chemistry** v. 61 p.7630-7635, 2013b.

LOSCHER, W. New visions in the pharmacology of anticonvulsion. **European Journal of Pharmacoly**, v. 342, p. 1-13, 1998.

MAACK, D.J.; TULL, M.T.; GRATZ, K.L. Examining the incremental contribution of behavioral inhibition to generalized anxiety disorder relative to other Axis I disorders and cognitiveemotional vulnerabilities. **Journal of Anxiety Disorders**, v.26, p. 689–695, 2012.

MAIGAARD, K.; HAGEMAN, I.; JØRGENSEN, A.; JØRGENSEN,M. B.; WÖRTWEIN, G. Electroconvulsive stimulations prevent chronic stress-induced increases in L-type calcium channel mRNAs in the hippocampus and basolateral amygdala. **Neurosciense Letters**, v. 516, p. 24–28, 2012.

MARINHO, M. M. F.; BRUIN, V. M. S.; SOUZA, F. C. F.; AGUIAR, L. M. V.; PINHO, R. S. N.; VIANA, G. B. S. Inhibitory action of a calcium channel blocker (nimodipine) on seizures and brain damage induced by pilocarpina and lithium pilocarpine in rats. **Neurosciense Letters**, v. 235, p. 13-16, 1997.

MARTINEZ-FLOREZ, S.; GUTIERREZ-FERNANDEZ, B.; SANCHEZ-CAMPOS, S.; GONZALEZ-GALLEGO, J.; TUNON, M.J. Quercetin attenuates nuclear factor-kappaB activation and nitric oxide production in interleukin-1beta-activated rat hepatocytes. **Journal of Nutrition**, v.135, p. 1359-1365, 2005.

MENON, P.; YIN, T.Y.; MISRAN, M. Preparation and characterization of liposomes coated withDEAE-Dextran. **Colloids and Surfaces A: Physicochemical and Engineering Aspects**, v. 481, p.345–350, 2015.

MIKATI, M. A.; HOLMES, G. L.; WERNER, S.; BAKKAR, N.; CARMANT, L.; LIU, Z.; STAFSTROM, C. E. Effects of nimodipine on the behavioral sequalae of experimental status epilepticus in prepubescent rats, **Epilepsy Behavior**, v. 5, p. 168–174, 2005.

MOVAHEDI, F.; HU, R.G.; BECKER, D.L.; XU, C. Stimuli-responsive liposomes for the delivery of nucleic acid therapeutics. **Nanomedicine: Nanotechnology, Biology, and Medicine**, v. 11, p. 1575–1584, 2015.

MULLEN, W.; ROUANET, J. M.; AUGER, C.; TEISSÈDRE, P. L.; CALDWELL, S. T.; HARTLEY, R. C.; et al. Bioavailability of [2-14C] quercetin-40-glucoside in rats. **Journal of Agricultural and Food chemistry**, v. 56, p. 12127-12137, 2008.

NASCIMENTO, V. S.; D'ALVA, M. S.; OLIVEIRA, A. A.; FREITAS, R. M.; VASCONCELOS, S. M. M.; SOUSA, F. C. F.; FONTELES, M. M. F. Antioxidant effect of nimodipine in young rats after pilocarpine-induced seizures, **Pharmacology Biochemistry Behavior**, v. 82, p. 11 – 16, 2005.

NEUTEL, C.I.; SKURTVEIT, S.; BERG, C. What is the point of guidelines? Benzodiazepine and z-hypnotic use by an elderly population. **Sleep Medicine**, v. 13, p. 893–897, 2012.

NINOMIYA, K.; YAMASHITA, T.; TANABE, Y.; IMAI, M.; TAKAHASHI,K.; SHIMIZU, N. Targeted and ultrasound-triggered cancer cell injury using perfluorocarbon emulsion-loaded liposomes endowed with cancer cell-targeting and fusogenic capabilities. **Ultrasonics Sonochemistry**, v. 28, p. 54–61, 2016.

N'GOUEMO, P. Probing the role of the sodium/calcium exchanger in pentylenetetrazole induced generalized seizures in rats. **Brain Research Bulletin**, v. 90, p.52–57, 2013.

NÓBREGA, F.F.F. **Monoterpeno 4-terpineol – uma molécula com atividade anticonvulsivante: estudos comportamentais e eletrofisiológicos**. 2012. 159f. Tese do Doutorado do Programa de Pós – graduação em Biotecnologia (RENORBIO), João Pessoa, 2012.

OYEBODE, F.; RASTOGI, A.; BERRISFORD, G.; COCCIA, F. Psychotropics in pregnancy: Safety and other considerations. **Pharmacology & Therapeutics**, v. 135, p. 71–77, 2012.

ONOCKO-CAMPOS, R.S.; PASSOS, E.; PALOMBINI, A.L.; SANTOS, D.V.D.; STEFANELLO, S.; GONÇALVES, L.L.M.; ANDRADE, P.M.; BORGES, L.R. A Gestão Autônoma da Medicação: uma intervenção analisadora de serviços em saúde mental. **Ciência & Saúde Coletiva**, v. 18, n. 10, p. 2889-2898, 2013.

OREJANA, L.; BARROS-MINONES, L.; JORDÁN, J.; PUERTA, E.; AGUIRRE, N. Sildenafil ameliorates cognitive deficits and tau pathology in a senescenceaccelerated mouse model. **Neurobiology Aging**, v. 33, p. 11-20, 2013.

PACZYNSKI, R. P.; MEYER, F. B.; ANDERSON, R. E. Effects of the dihydropyridine Ca^{2+} channel antagonist nimodipine on kainic acid-induced lirnbic seizures. **Epilepsy Research,** v. 6, p. 33-38, 1990.

PADUA, G. W.; WANG, Q. Controlled self-organization of zein nanostructures for encapsulation of food ingredients. **Micro/Nanoencapsulation of Active Food Ingredients**, v. 1007, p. 143-155, 2009.

PAOLINO, D.; COSCO, D.; MOLINARO, R.; CELIA,C .; FRESTA, M. Supramolecular devices to improve the treatment of brain diseases. **Drug Discovery Today**, v. 16, p. 311-324, 2011.

PARK, C.H.; CHANG, J.Y.; HAHM, E.R.; PARK, S.; KIM, H.K.; YANG, C.H. Quercetin, a potent inhibitor against beta-catenin/Tcf signaling in SW480 colon cancer

cells**. Biochemical and Biophysical Research Communications**, v. 328, p. 227-234, 2005.

PATHAK, R.; DASH, R.P.; MISRA, M.; NIVSARKAR, M. Role of mucoadhesive polymers in enhancing delivery of nimodipine microemulsion to brain via intranasal route. **Acta Pharmaceutica Sinica B**, v. 4 (2), p. 151-160, 2014.

PAZZAGLIA, P. J.; GEORGE, M. S.; POST, R. M.; RUBINOW, R. D.; DAVIS, C. L. Nimodipine increases CSF somatostatin in affectively ill patients. **Neuropsychopharmacology**, v. 13, p. 75-83, 1995.

PEÑALVA, R.; ESPARZA, I.; GONZÁLEZ-NAVARRO, C.J.; QUINCOCES, G.; PEÑUELAS, I.; IRACHE, J.M. Zein nanoparticles for oral folic acid delivery. **Journal of Drug Delivery Science and Technology**, v.30, p. 450-457, 2015.

PEÑALVA, R.; GONZÁLES-NAVARRO, C. J.; GAMAZO, C.; ESPARZA, I.; IRACHE, J.M. Zein nanoparticles for oral delivery of quercetin: Pharmacokinetic studies and preventive anti-inflammatory effects in a mouse model of endotoxemia. **Nanomedicine: Nanotechnology, Biology and Medicine**, v.13, p. 103-110, 2017.

PERIYASAMY, P.C.; LEIJTEN,J.C.H.; DIJKSTRA, P.J.; KARPERIEN, M.; POST, J.N. Nanomaterials for the Local and Targeted Delivery of Osteoarthritis Drugs. **Journal of Nanomaterials**, v. 2012, p.1-13, 2012.

PETERSEN, B., EGERT, S.; BOSY-WESTPHAL, A.; MÜLLER, M.J.; WOLFFRAM, S.; HUBBERMANN, E.M.; RIMBACH, G.; SCHWARZ, K. Bioavailability of quercetin in humans and the influence of food matrix comparing quercetin capsules and different apple sources. **Food Research International**, v. 88, p. 159–165, 2016.

PETROVIC, Z. K.; ERJAVEC, G. N.; PERKOVIC, M. N.; PERAICA, T.; PIVAC, N. No association between the serotonin transporter linked polymorphic region polymorphism and severity of posttraumatic stress disorder symptoms in combat

veterans with or without comorbid depression. **Psychiatry Research**, v. 244, p. 376–381, 2016.

PINHEIRO, M.; LUCIO, M.; LIMA, J.L.; REIS, S. Liposomes as drug delivery systems for the treatment of TB. **Nanomedicine (Lond)**, v. 6, p. 1413-28, 2011.

PONCHEL, G., IRACHE, J.M. Specific and non-specific bioadhesive particulates for oral delivery. **Advanced Drug Delivery Reviews**, v. 34, p. 191–219, 1998.

PORFIRE, A.S.; ZABALETA, V.; GAMAZO, C.; LEUCUTA, S.E.; IRACHE, J.M. Influence of dextran on the bioadhesive properties of poly(anhydride) nanoparticles. **International Journal of Pharmaceutics**, v. 390, p. 37–44, 2010.

PRINCE, M.; BRYCE, R.; FERRI, C. A.; JACKSON, J. Alzheimer's Disease International. World Alzheimer Report 2011. The Benefits of Early Diagnosis and Intervention. Institute of Psychiatry, King's College London, London, p 2-32, 2011.

PSAHOULIA, F.H.; MOUMTZI, S.; ROBERTS, M.L.; SASAZUKI, T.; SHIRASAWA, S.; PINTZAS, A. Quercetin mediates preferential degradation of oncogenic Ras and causes autophagy in Ha-RAS-transformed human colon cells. **Carcinogenesis**, v. 28, p. 1021-1031, 2007.

QIN, Y.; CHEN, H.; YUAN, W.; KUAI, R.; ZHANG, Q.; XIE, F.; ZHANG, L.; ZHANG, Z.; LIUB, J.; HE Q. Liposome formulated with TAT-modified cholesterol for enhancing the brain delivery. **International Journal of Pharmaceutics**, v. 419, p. 85-95, 2011.

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

RAHMAN, I.; BISWAS, S. K.; KIRKHAM, P. A. Regulation of inflammation and redox signaling by dietary polyphenols. **Biochemical Pharmacology**, v. 72, p. 1439-1452, 2006.

RAUPP, I.M.; SERENIKI, A.; VIRTUOSO, S.; GHISLANDI, C.; CAVALCANTI, E.; SILVA, E.L.; TREBIEN, H. A.; MIGUEL, O. G.; ANDREATINI, R. Anxiolytic-like effect of chronic treatment with *Erythrina velutina* extract in the elevated plus-maze test. **Journal of Ethnopharmacology**, v. 118, p. 295-299, 2008.

Real farmacopea española. 5ª edição. 2015.

REDDY, L.H.; COUVREUR, P. Novel approaches to deliver gemcitabine to cancers. **Current Pharmaceutical Design**, v. 14, p. 1124–1137, 2008.

RICHEY, J.A.; SCHMIDT, N.B.; HOFMANN, S.G.; TIMPANO, K.R. Temporal and structural dynamics of anxiety sensitivity in predicting fearful responding to a 35% CO_2 challenge. **Journal of Anxiety Disorders**, v. 24, p. 423–32, 2010.

RIEKES, M.K.; CAON, T.; DA SILVA, J.; SORDI, R.; KUMINEK, G.; BERNARDI, L.S.; RAMBO, C.R.; DE CAMPOS, C.E.M.; FERNANDES, D.; STULZER, H.K. Enhanced hypotensive effect of nimodipine solid dispersions produced by supercritical CO_2 drying. **Powder Technology**, v. 278, p. 204-210, 2015.

ROLLER, L.; GAUAU, J. Epilepsy. **Australian Journal of Pharmacy**, v. 97, p. 62-72, 2016.

RUSSO, G.L.; RUSSO, M.; SPAGNUOLO, C.; TEDESCO, I.; BILOTTO, S.; IANNITTI, R.; PALUMBO, R. Quercetin: a pleiotropic kinase inhibitor against Cancer. **Cancer Treatement Research**, v.159, p. 185-205, 2014.

RUSSO, M.; SPAGNUOLO, C.; TEDESCO, I.; BILOTTO, S.; RUSSO, G.L. The flavonoid quercetin in disease prevention and therapy: facts and fancies. **Biochemistry Pharmacology**, v. 83, p. 6-15, 2012.

SABOGAL-GUÁQUETA, A. M..; MUNÕZ-MANCO, J. I.; RAMÍREZ-PINEDA, J.R.; LAMPREA-RODRIGUEZ, M.; OSORIO, E.; CARDONA-GÓMEZ, G.P. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive

and emotional function in aged triple transgenic Alzheimer's disease model mice. **Neuropharmacology**, v. 133, p. 423–429, 2015.

SALGADO-FIGUEROA, P.; GUTIÉRREZ, C.; SQUELLA, J.A. Carbon nanofiber screen printed electrode joined to a flow injectionsystem for nimodipine sensing. **Sensors and Actuators B: Chemical**, v. 220, p. 456–462, 2015.

SALOMONS, A.R.; KORTLEVE, T.; REINDERS, N.R.; KIRCHHOFF, S.; ARNDT, S.S.; OHL, F. Susceptibility of a potential animal model for pathological anxiety to chronic mild stress. **Behavariour Brain Research**, v. 209, p.241–248, 2010.

SANTOS-MAGALHÃES, N.S.; MOSQUEIRA, V.C.F. Nanotechnology applied to the treatment of malaria. **Advanced Drug Delivery Reviews**, v. 62, p. 560–575, 2010.

SEBAALY, C.; JRAIJ, A.; FESSI, H.; CHARCOSSET, C.; GREIGE-GERGES, H. Preparation and characterization of clove essential oil-loaded liposomes. **Food Chemistry**, v. 178, p. 52–62, 2015.

ŠEGOTA, S.; TEŽAK, D. Spontaneous formation of vesicles. **Advances in Colloid and Interface Science.** v. 121, p. 51-75, 2006.

SHARMA, D. R.; WANI, W. Y.; SUNKARIA, A.;. KANDIMALLA, R. J; SHARMA, R. K.; VERMA, D.; BAL, A.; GILL, K. D. Quercetin attenuates neuronal death against aluminum-induced neurodegeneration in the rat hippocampus. **Neuroscience**, v. 324, 163–176, 2016.

SHEN, A. N.; CUMMINGS, C.; POPE, D.; HOFFMAN, D.; NEWLAND, M.C. A bout analysis reveals age-related methylmercury neurotoxicity and nimodipine neuroprotection. **Behavioural Brain Research**, v. 311, p. 147–159, 2016.

SOSNIK, A,; CARCABOSO, A.M. Nanomedicines in the future of pediatric therapy. **Advanced Drug Delivery Reviews**, v. 73, p. 140-161, 2014.

SODDU, E.; RASSU, G.; GIUNCHEDI, P.; SARMENTO, B.; GAVINI, E. From naturally-occurring neurotoxic agents to CNS shuttles for drug delivery. **European Journal of Pharmaceutical Sciences**, v. 74, p. 63-76, 2015.

STELLA, F. Depressão no idoso: Diagnóstico, tratamento e benefícios da atividade física. **Revista Motriz**, v. 18, p. 91-98, 2002.

SUN, C.; WANG, J.; LIU, J.; QIU, L.; ZHANG, W.; ZHANG, L. Liquid proliposomes of nimodipine drug delivery system: Preparation, characterization, and pharmacokinetics. **AAPS PharmSciTech**, v. 14, p. 332–8, 2013.

SUN, D.; LI, N., ZHANG, W.; ZHAO, Z.; MOU, Z.; HUANG, D.; LIU, J.; WANG, W. Design of PLGA-functionalized quercetin nanoparticles for potentialuse in Alzheimer's disease. **Colloids and Surfaces B: Biointerfaces**, v. 148, p. 116–129, 2016.

REBOUÇAS, J.S.; IRACHE, J.M., CAMACHO, A.I.; ESPARZA, I.; POZO, V.; SANZ, M.L.; FERRER, M.; GAMAZO, C. Development of poly(anhydride) nanoparticles loaded with peanut proteins: The influence of preparation method on the immunogenic properties. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 82, p. 241–249, 2012. .

THIBAULT, O.; GANT, J.C.; LANDFIELD, P.W. Expansion of the calcium hypothesis of brain aging and Alzheimer's disease: minding the store. **Aging Cell,** v. 6 (3), p. 307–317, 2007.

THOMAS, J. The effect of nimodipine on picrotoxin-induced seizures, **Brain Research Bulletin**, v. 24, p.11-15, 1990.

TORCHILIN, V. Multifunctional and stimuli-sensitive pharmaceutical nanocarriers. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 71, p. 431-444, 2009.

VANGILDER, R. L.; ROSEN, C.L.; BARR, T.L.; HUBER, J.D. Targeting the neurovascular unit for treatment of neurological disorders. **Pharmacology & Therapeutics**, v.130, p. 239–247, 2011.

VEMURI, S.; RHODES, C. T. Preparation and characterization of liposomes as therapeutic delivery systems: a review. **Pharmaceutica Acta Helvetiae**, v. 70, p.95-111, 1995.

VERSTER, J.C.; RAMAEKERS, J.G. Antidepressants and traffic safety. In: VERSTER, J.C.; PANDI-PERUMAL, S.R.; RAMAEKERS, J.G.; DE GIER, J.J. eds. **Drugs, Driving and Traffic Safety**. Basel-Boston- Berlin, Switzerland: Birkaeuser Verlag AG, p. 307-313, 2009.

WANG, Y.; FANG, Y.; CHEN, X.; CHEN, J.; WU, Z.; YUAN, C.; YI, Z.; HONG, W.; ZHANG, C.; CAO, L. A follow-up study on features of sensory gating P50 in treatmentresistant depression patients. **Chinese Medical Journal**, v. 122, p. 2956–2960, 2009.

WILLNER, P.; SCHEEL-KRÜGER, J.; BELZUNG, C. The neurobiology of depression and antidepressant action. **Neuroscience and Biobehavioral Reviews**, v. 37, p. 2331–237, 2013

WITTMAN, D. Darwinian depression. **Journal of Affective Disorders**, v. 168, p. 142-150, 2014.

WITTCHEN, H.U.; JACOBI, F. Size and burden of mental disorders in Europe: a critical review and appraisal of 27 studies. **European Neuropsychopharmacology**, v. 15, p. 357–367, 2005.

WITTCHEN, H.U.; JACOBI, F.; REHM, J.; GUSTAVSSON, A.; SVENSSON, M.; JÖNSSON, B.; OLESEN, J.; ALLGULANDER, C.; ALONSO, J.; FARAVELLI, C.; FRATIGLIONI, L.; JENNUM, P.; LIEB, R.; MAERCKER, A.; VAN OS, J.; PREISIG, M.; SALVADOR-CARULLA, L.; SIMON, R.; STEINHAUSEN, H.C. The size and burden of mental disorders and other disorders of the brain in Europe 2010. **European Neuropsychopharmacology**, v. 21, p. 655–679, 2011.

WORLD HEALTH ORGANIZATION (WHO). **The Global Burden of Disease**: 2004 Update. Geneva: WHO, 2008.

WONG, C. H.; SHAH, Z. U. B. S.; TENG, C. L.; LIN, T. Q.; MAJEED, Z.A.; CHAN, C. W. A systematic review of anxiety prevalence in adults within primary care and community settings in Malaysia. **Asian Journal of Psychiatry**, v. 24, p. 110–117, 2016.

WONG, H.L.; BENDAYAN, R.; RAUTH, A.M.; LI, Y.; WU, X.Y. Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles. **Advanced Drug Delivery Reviews**, v. 59, p. 491–504, 2007.

WONG, H. L.; WU, X. Y.; BENDAYAN, R. Nanotechnological advances for the delivery of CNS therapeutics. **Advanced Drug Delivery Reviews**, v. 64, p. 686–700, 2012.

YAO, R. Q.; QI, D. S.; YU, H. L.; LIU, J.; YANG, L.H.; WU, X.X. Quercetin attenuates cell apoptosis in focal cerebral ischemia rat brain via activation of BDNF-TrkB -PI3K/Akt signaling pathway. **Neurochemical Research**, v. 37, p. 2777-2786, 2012.

YOUDIM, K.A.; SHUKITT-HALE, B.; JOSEPH, J.A. Flavonoids and the brain: Interactions at the blood-brain barrier and their physiological effects on the central nervous system. **Free Radical Biology & Medicine**, v. 37, p. 1683-1693, 2004.

YONCHEVA, K.; LIZARRAGA, E.; IRACHE, J.M. Pegylated nanoparticles based on poly(methyl vinyl ether-co-maleicanhydride): Preparation and evaluation of their bioadhesive properties. **European Journal of Pharmaceutical Sciences**, v.24, p. 411–419, 2005.

ZABALETA, V.; PONCHEL, G.; SALMAN, H.; AGÜEROS, M.; VAUTHIER, C.; IRACHE, J.M. Oral administration of paclitaxel with pegylated poly(anhydride)

nanoparticles: Permeability and pharmacokinetic study. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 81, p. 514–523, 2012.

ZAPATER, P.; JAVALOY, J.; ROMÁN, J. F.; VIDAL, M. T.; HORGA, J. F. Anticonvulsant effects of nimodipine and two novel dihydropyridines (PCA 50922 and PCA 50941) against seizures elicited by pentylenetetrazole and electroconvulsive shock in mice. **Brain Research**, v. 796, p. 311-314, 1998.

ZHANG, X.; CHEN, G.; WEN, L.; YANG, F.; SHAO, A.; LI, X.; LONG, W.; MU, L. Novel multiple agents loaded PLGA nanoparticles for brain delivery via inner ear administration: In vitro and in vivo evaluation. **European Journal of Pharmaceutical Sciences**, v. 48, p. 595–603, 2013.

ZHAO, Y.; JIN, R.; YANG, W.; XIANG, Q.; YU, W.; LIN, Q.; TIAN, F.; MAO, K.; LV, C.; WÁNG, Y. J.; LU. C. Using gelatin nanoparticle mediated intranasal delivery of neuropeptide substance P to enhance neurorecovery in hemiparkinsonian rats. **PLoS one**, v.11, p. 1-18, 2016.

ZHAO, Y.; XIN, T.; YE, T.; YANG, X.; PAN, W. Solid dispersion in the development of a nimodipine delayed-release tablet formulation. **Asian journal of pharmaceutical sciences**, v. 9, p. 35-41, 2014.

ZIELLO, J.E.; HUANG, Y.; JOVIN, I.S. Cellular endocytosis and gene delivery. **Molecular Medicine**, v. 16, p. 222-229, 2010

ZOU, J.B.; DEAR, B.F.; TITOV, N.; LORIAN, C.N.; JOHNSTON, L.; SPENCE, J. Brief internet-delivered cognitive behavioral therapy for anxiety in older adults: a feasibility trial. **Journal of Anxiety Disorders**, v. 26, p. 650–655, 2012.

ZOUHAR, A.; MARES, P.; LISKOVA-BERNASDOVA, K.; MUDROCHOVA, M.; Motor and electrocorticographic activity induced by bicuculline in developing rats. **Epilepsia,** v. 20, p. 501-510, 1989.

ZU, Y.; LI, N.; ZHAO,X.; LI, Y.; GE,Y.; WANG, W.; WANG, K.; LIU Y. In vitro dissolution enhancement of micronized l-nimodipine by antisolvent re-crystallization from its crystal form H. **International Journal of Pharmaceutics**, v. 464, p. 1-9, 2014.

ANEXO A: Carta de aprovação do comitê de ética em experimentação com animas da Universidade Federal do Piauí.

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PIAUÍ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO COM ANIMAIS
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Teresina, 20 de setembro de 2011.

Ao (A)

Prof (a): Dr. Rivelilson Mendes de Freitas
Departamento de Bioquímica e Farmacologia/ CCS / UFPI
Sr. (a) Pesquisador (a)

Declaro para os devidos fins que o projeto de pesquisa intitulado: **"Estudo da atividade farmacológica e toxicológica de uma preparação lipossomal derivada de diidropiridinas (LCN1)",** foi avaliado pelo Comitê de ética em Experimentação com Animais – CEEA/UFPI teve parecer **APROVADO sob o nº. 014/11**. Esclarecemos que o mesmo se encontra de acordo com os requisitos exigidos para apreciação de projetos de pesquisa.

Atenciosamente,

Profª. Ivete L. de Mendonça
Comitê de Ética em Experimentação Animal-UFPI
Coordenadora

ANEXO B: Carta de aprovação do comitê de ética em experimentação com animas da Universidad de Navarra.

GOBIERNO DE NAVARRA
R.S.(Unidad: 10002014)
DEPARTAMENTO DE SALUD
SECCION DE SEGURIDAD ALIMENTARIA
ISPLN
Instituto de Salud Pública y Laboral de Navarra

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LA DIRECTORA GERENTE DEL INSTITUTO DE SALUD PÚBLICA Y LABORAL DE NAVARRA ha dictado la siguiente Resolución:

"RESOLUCION 188/2015 de 14 de octubre de la Directora Gerente del Instituto de Salud Pública y Laboral de Navarra, por la que se autoriza al centro usuario Universidad de Navarra-CIMA, el proyecto "Nanopartículas para la administración oral de nimodipina".

La Resolución 647/2006, de 7 de abril, del Director General de Salud, autorizó el funcionamiento como centro usuario para la utilización de animales de experimentación y otros fines científicos del establecimiento, titularidad Universidad de Navarra, NIF R3168001J, con domicilio en Pío XII, 55, 31008 Pamplona y código de identificación ES312010000132.

El día 24 de septiembre de 2015, Don Juan Manuel Irache de la Universidad de Navarra, presentó la documentación Técnica del proyecto de experimentación animal "Nanopartículas para la administración oral de nimodipina", procedimiento dirigido por Don Juan Manuel Irache responsable de su ejecución. La documentación incluye los elementos del Anexo X del Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. En la documentación se incluye la descripción y el diseño experimental del proyecto de investigación aplicada, las actividades a realizar, un análisis del balance de daño y beneficio, la justificación de que la finalidad del procedimiento no puede conseguirse utilizando animales diferentes de los propuestos, la aplicación de los principios de reemplazo, reducción y refinamiento incluyendo métodos de eutanasia, anestesia y analgesia, las referencias bibliográficas de referencia en la aplicación de dichos principios y que avalan el fundamento científico del proyecto y el "Resumen no técnico del proyecto".

El proyecto ha sido revisado, corregido y finalmente evaluado y aprobado, por el Comité de Ética para la Experimentación Animal del centro Universidad de Navarra, en su reunión de 18 de septiembre de 2015. Se acompaña certificado de la Secretaria del Comité de los acuerdos adoptados en relación con el proyecto. Este Comité dispone de habilitación para la evaluación de proyectos a partir del 15 de octubre de 2013, de acuerdo con lo dispuesto en Real Decreto 53/2013, de 1 de febrero.

La Sección de Seguridad Alimentaria del Instituto de Salud Pública y Laboral de Navarra informa favorablemente la realización del procedimiento solicitado por cumplirse los requisitos mínimos establecidos en el Real Decreto 53/2013, de 1 de febrero.

En el ejercicio de las competencias que el Decreto Foral 196/1996, 29 de abril, por el que se asignan, a los Departamentos de Medio Ambiente, Ordenación del Territorio y Vivienda y al Departamento de Salud, las derivadas del desarrollo de la Ley Foral 7/1994, de 31 de mayo, de protección de los animales, atribuye al Departamento de Salud en cuanto al control de la utilización de animales con fines de investigación.

De conformidad con lo expuesto, en ejercicio de las atribuciones conferidas en el artículo 22.1., de la Ley Foral 15/2004, de 3 de diciembre, de la Administración de la Comunidad Foral de Navarra y de las competencias delegadas por la Resolución 1496/2012, de 29 de agosto, de la Directora General de Salud,

RESUELVO:

1. Autorizar por un periodo máximo de cinco años el proyecto "Nanopartículas para la administración oral de nimodipina" a los efectos de lo dispuesto en el artículo 33 Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, con las siguientes condiciones:

- Usuario que llevará a cabo el proyecto, Don Juan Manuel Irache.
- Responsable del Proyecto: Doña María Javier Ramírez y Don Juan M. Irache.
- El órgano habilitado deberá hacer una evaluación retrospectiva del proyecto en base a la preceptiva documentación presentada por el usuario y se evaluará los resultados del proyecto que se relacionan en el apartado 3 del artículo 35 del Real Decreto 53/2013.

-El procedimiento se realizará en las instalaciones del centro registrado con número ES312010000132, de titularidad Universidad de Navarra y autorizado mediante Resolución 647/2006, de 7 de abril, del Director General de Salud.

Cualquier cambio del proyecto que pueda tener un impacto negativo en el bienestar de los animales implicará una nueva evaluación y, cuando proceda, autorización del proyecto.

Anexo C: Artigo publicado na revista: "**Pharmacology, Biochemistry and Behavior**".

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Development and evaluation of liposomal formulation containing nimodipine on anxiolytic activity in mice

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ABSTRACT

Nimodipine has been investigated in the treatment of anxiety. Its administration, however, presents a number of limitations, particularly by low bioavailability, low aqueous solubility and photosensitivity. These difficulties can be resolved by the use of nanometer-scale pharmaceutical carriers. The goal of the present study was thus to develop a liposomal formulation containing nimodipine (NMD-Lipo) and evaluate anxiolytic activity using models of anxiety (open-field, light and dark and elevated plus-maze test). The results suggest that administration of NMD-Lipo has no sedative or muscle relaxant effect in animals, since there was no reduction in the number of crossings, grooming and rearings. The increased residence time of the animals treated with NMD-Lipo in the bright field is a reflection of the anxiolytic-like activity of the formulation. Furthermore, the reduction in residence time of rodents treated with the combination of flumazenil and NMD-Lipo in the illuminated box suggests that NMD-Lipo acts on benzodiazepine receptors. The increase in the number of entries and length of stay in the open arms of mice treated with NMD-Lipo suggests that anxiolytic activity of formulation and reduction in number of entries and length of stay in open arms of rodents treated with a combination of flumazenil and NMD-Lipo suggest that NMD-Lipo act on benzodiazepine receptors.

1. Introduction

Anxiety is an emotional state that is part of human existence, since normal circumstances in people's lives, such as the development of some kind of physical and mental suffering, as well as changes in everyday life, may be associated with its onset. It is a type of emotion that has been shaped by natural selection, since it makes people alert to impending dangers (Richey et al., 2010). However, anxiety may cease to be a natural occurrence and progress to a pathological condition when it occurs disproportionately to the triggering event that causes it or when there is no apparent reason for its onset (Salomons et al., 2010).

Pathological anxiety is characterized by excessive and uncontrollable worry about a considerable number of factors, in which the individuals involved have experienced at least three of the following symptoms: feeling keyed up or on edge, sleep disturbance, muscle tension, being easily fatigued, difficulty concentrating or having one's mind go blank, and irritability (Maack et al., 2012). The pathological form is debilitating, reduces the quality of life of patients and is associated with an increased risk of death and suicide (Zou et al., 2012).

Treatments currently applied for anxiety disorders include pharmacotherapy and cognitive behavioral therapy (Bartley et al., 2013). The pharmacological treatment of pathological anxiety consists of the use of benzodiazepines, buspirone and antidepressants. Although this drugs shows great efficacy in the therapy of pathology, its administration has many drawbacks. For example, benzodiazepines can cause some side effects such as amnesia, induction of dependence and sedation which cause inconveniences for the patients (Raupp et al., 2008). The search for new therapeutic agents with anxiolytic properties is therefore of paramount importance.

Research has shown that the excessive flow of calcium through the membrane, which results in increased levels of intracellular ion, may play a role in the pathophysiology of affective disorders (Maigaard et al., 2012), epileptiform activity (N'Gouemo, 2013) and in the induction of anxiety (Kumar et al., 2012). From this perspective, the application of nimodipine, a selective antagonist of L-type calcium channels, has been investigated in the treatment of numerous neurological disorders (Yanpallewar et al., 2004).

Nimodipine has high lipophilicity and hence easily crosses the blood brain barrier. Studies have concluded that this drug has the ability to increase cerebral blood flow and its use in the treatment of ischemia present in numerous pathologies affecting the brain (Aslan et al.,

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2009), besides being useful in the therapy of mood disorders (Frye et al., 2003; Pazzaglia et al., 1995) and in the treatment of senile dementia (Chalikwar et al., 2012), and in displaying anticonvulsant properties (Marinho et al., 1997; Mikati et al., 2004; Nascimento et al., 2005).

However, the administration of nimodipine has a number of limitations, owing chiefly to its high first-pass effect in the liver, which results in decreased bioavailability, low aqueous solubility and photosensitivity (Sun et al., 2013). These difficulties can be overcome through the use of nanometer-scale pharmaceutical carriers. These nanosystems are useful tools to improve the pharmacokinetic profile of drugs that have limited pharmaceutical applicability (Santos-Magalhães and Mosqueira, 2010). Furthermore, nanotechnology is great in improving the therapy of diseases that affect the central nervous system because the drugs applied in those treatments normally cannot cross the blood–brain barrier and could substantially benefit from the use of nanocarriers (Wong et al., 2012).

Based on these findings, the goal of the present study was twofold: the design of a liposomal formulation containing nimodipine and the evaluation of the drug's anxiolytic effects tested in three animal models of anxiety (open field, the light and dark and the elevated plus-maze test).

2. Material and methods

2.1. Material

Cholesterol (Chol), trehalose, nimodipine, diazepam and flumazenil were purchased from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Production of liposomal formulation derived from nimodipine (NMD-Lipo)

Liposomes containing nimodipine (NMD-Lipo) were prepared using the method of hydrating the lipid film (Lira et al., 2009) at the Immunopathology Keizo-Asami Laboratory, Federal University of Pernambuco. NMD-Lipo was produced using the lipids soybean phosphatidylcholine and cholesterol (117.6 mM) at 8:2 ratio and drug concentration of 1.0 mg/mL. These constituents were dissolved in a mixture of chloroform: methanol (3:1 v/v) under magnetic stirring. The solvents were removed by vacuum evaporation at 80 rpm for 60 min at 37 ± 1 °C, resulting in a thin lipid film. This film was then hydrated with 10 mL of pH 7.4 phosphate buffer solution resulting in the production of large multilamellar vesicles (MLV). This liposomal suspension was then subjected to sonication (Vibra Cell, Branson, USA) at 200 W and 300 Hz for 40 s to obtain small unilamellar liposomes (SUV).

2.3. Characterization of NMD-Lipo

After 24 h of production, NMD-Lipo was characterized by evaluating its features: macroscopic aspects, pH, particle size, polydispersity index, zeta potential, drug content and encapsulation efficiency. The pH of the liposomes was measured using a digital pH meter (Bioblock Scientific 99, Prolabo, Paris, France) at room temperature. The particle size and polydispersity of the liposomes were determined using photon correlation spectroscopy (Particle Analyzer™ Delsa Nano S, Beckman-Coulter, USA). For this analysis 300 μL of the liposomal suspension was diluted in 1 mL of deionized water (Milli Q Plus, Millipore, USA). The zeta potential of the liposomes, corresponding to the surface charge of the vesicles, was measured using a Zetatrac NC-148 apparatus (Microtrac, USA). A sample of the liposomes (50 μL) was diluted in 5 mL of deionized water before analysis.

The content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm. A standard curve of nimodipine was prepared at concentrations of 0.5, 1, 2, 3, 4, 5 and 6 μg/mL of nimodipine using methanol as solvent. Subsequently, an aliquot of liposomes (30 μL) was diluted in methanol to a final concentration of theoretical 3 μg/mL of nimodipine.

The encapsulation efficiency of nimodipine into liposomes was determined by the technique of ultrafiltration/ultracentrifugation using Ultrafree® units (Millipore, USA). A liposomal sample aliquot (400 μL) was transferred to filtering units and subjected to ultracentrifugation at 8776 *g* for 1 h. The amount of encapsulated nimodipine was obtained from the difference between the total quantity measured in the formulation and that of the filtrate obtained after centrifugation. The readings were performed at 237 nm.

2.4. Studies of anxiolytic activity of NMD-Lipo

2.4.1. The experimental units

Animal models of anxiety are applied for the evaluation of anxiolytic or anxiogenic compounds, as well as the identification of their mechanisms of action and study of the neurobiology of disease. We used male Swiss mice 2 months of age and weighing 25–30 g, from the Central Animal Facility of the Center for Agricultural Sciences, Federal University of Piauí. The animals used in the experiment remained on the premises of the Experimental Neurochemistry Laboratory Research, for 7 days, for proper acclimatization. The experimental units received water and diet (Labina ®) *ad libitum* and were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) and temperature (25 ± 1 °C). The experimental protocols and procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA/UFPI No. 014/11).

2.4.2. Treatments

The mice were divided into thirteen groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), diazepam at a dose of 2 mg/kg (positive control), nimodipine at doses of 0.1, 1 and 10 mg/kg (groups Free NMD 0.1, 1 and 10 respectively), NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10 respectively), flumazenil at a dose 2.5 mg/kg (group Flu), flumazenil in combination with diazepam (group Flu + DZP) and flumazenil in combination with NMD-Lipo (groups Flu + NMD-Lipo 0.1, Flu + NMD-Lipo 1, and Flu + NMD-Lipo 10). The behavioral assessments were carried out 30 min after drug administration.

2.4.3. Open field test

The motor activity of the animals was observed by means of an acrylic open field (transparent walls and black floor, 30 × 30 × 15 cm) and divided into nine equal quadrants based on the model described by Archer (1973). After 30 min of treatment, the animals were placed, one at a time, in the center of the field for quantification of the number of crossings with four legs (spontaneous locomotor activity), number of self-cleaning behavior (grooming) and the number of lifting (rearing) without abutting the wall during the period of 5 min.

2.4.4. The light and dark test

The apparatus used is made of acrylic divided into two compartments (bright field and dark field) that communicate through a small door 5 by 5 cm (Crawley, 1985). The dark field (black acrylic, 27 × 18 × 29 cm) is poorly lit. The bright field (acrylic, 27 × 18 × 29 cm) is illuminated by ambient light. Each animal was observed for 5 min. The parameter used is the dwell time in the bright field in seconds.

2.4.5. Elevated plus-maze test

The elevated plus-maze for mice (Lister, 1987) consists of two opposing open arms (30 × 5 cm) and two closed (30 × 25 × 5 cm), likewise opposing cross-shaped arms. The open and closed arms are connected by a central platform (5 × 5 cm) high and 45 cm from the floor. The animals were placed in the center of the apparatus with the

head turned to towards one of the closed arms and their behavior was observed for 5 min. The behavioral measures were recorded: number of entries in the open arms (NEOA) and length of stay of the animal in the open arms (LSOA).

2.5. Statistical analyses

Values are expressed as mean ± standard error of mean (SEM) of the number of animals used in the experiments. Differences between groups were determined by analysis of variance (ANOVA) followed, when a difference was detected, by the Student–Newman–Keuls *t*-test with *post hoc* test. The significance level for rejecting the null hypothesis was always $p \leq 5\%$.

3. Results

3.1. Characterization of NMD-Lipo

The data of NMD-Lipo properties indicates that the liposomes are small unilamellar vesicles (SUV) with size 107.17 ± 1.53 nm, homogeneity (PDI = 0.303), pH of 7.4, surface charge of −5.32 ± 1.29 mV and drug encapsulation efficiency of 99 ± 0.45%.

3.2. Open field test

The results are summarized in Table 1. In the open field test, there was a decrease in the number of crossings, rearings and groomings of diazepam-treated mice compared to the control group. The animals receiving both free NMD and NMD-Lipo at doses of 0.1, 1 and 10 mg/kg showed no reduction in the number of crossings, rearings and groomings. The groups treated with flumazenil and flumazenil combinations with diazepam and NMD-Lipo also showed no significant differences when compared to the control group.

3.3. Light and dark test

The results are summarized in Table 2. In the light and dark test, the animals treated with a 2 mg/kg dose of diazepam showed a significant increase in the time spent in the light field when compared to the control group. The mice treated with free NMD at the doses of 0.1, 1 and 10 mg/kg showed no change in time spent in the light field. For their part, rodents treated with NMD-Lipo at the doses of 0.1, 1 and 10 mg/kg were shown to spend a significantly longer time in the bright field than the control group. The mice pretreated with flumazenil and subsequently given diazepam and NMD-Lipo showed a reduction in the time spent in the light field, compared to animals that did not receive the antagonist.

Table 1
Effects of free NMD, NMD-Lipo, flumazenil, diazepam and their associations in mice using the open-field test.

Groups	Number of crossings	Number of rearings	Number of groomings
Control	63.14 ± 2.60	3.80 ± 0.75	25.60 ± 1.63
DZP	30.43 ± 1.21[a]	1.80 ± 0.24[a]	12.80 ± 0.97[a]
Free NMD 0,1	63.14 ± 2.36[b]	3.80 ± 0.37[b]	25.60 ± 1.69[b]
Free NMD 1	63.86 ± 3.04[b]	4.00 ± 0.44[b]	25.20 ± 1.77[b]
Free NMD 10	63.71 ± 3.38[b]	4.00 ± 0.32[b]	24.80 ± 1.35[b]
NMD-Lipo 0,1	66.71 ± 1.60[b]	4.00 ± 0.32[b]	24.20 ± 1.88[b]
NMD-Lipo 1	67.86 ± 2.44[b]	4.00 ± 0.32[b]	24.20 ± 2.31[b]
NMD-Lipo 10	66.57 ± 3.95[b]	4.00 ± 0.32[b]	25.20 ± 1.68[b]
Flu	67.29 ± 4.79[b]	4.20 ± 0.37[b]	26.60 ± 1.29[b]
Flu + DZP	74.29 ± 3.97[b]	4.20 ± 0.37[b]	27.00 ± 1.76[b]
Flu + NMD-Lipo 0,1	71.57 ± 3.43[b]	4.20 ± 0.37[b]	27.60 ± 1.07[b]
Flu + NMD-Lipo 1	72.00 ± 2.86[b]	4.20 ± 0.49[b]	27.40 ± 0.98[b]
Flu + NMD-Lipo 10	71.71 ± 4.97[b]	4.20 ± 0.37[b]	25.60 ± 1.88[b]

Values are mean ± S.E.M. of number of crossings, rearings and groomings of mice (n = 8).
[a] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from control group.
[b] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from diazepam group.

Table 2
Effects of free NMD, NMD-Lipo, flumazenil, diazepam and their associations in mice in the light and dark test.

Groups	Time spent in the bright field (s)
Conrol	92.50 ± 2.59
DZP	124.5 ± 6.75[a]
Free NMD 0.1	94.83 ± 3.26
Free NMD 1	98.17 ± 3.20
Free NMD 10	109.2 ± 5.60
NMD-Lipo 0.1	124.3 ± 4.85[a,c]
NMD-Lipo 1	148.7 ± 8.36[a,b,d]
NMD-Lipo 10	149.0 ± 7.20[a,b,e]
Flu	92.50 ± 0.76
Flu + DZP	91.83 ± 2.69[b]
Flu + NMD-Lipo 0.1	95.00 ± 1.21[f]
Flu + NMD-Lipo 1	104.5 ± 7.20[g]
Flu + NMD-Lipo 10	102.8 ± 2.81[h]

Values are mean ± S.E.M. of the time spent in the bright field of 8 mice (by group) used in the experiments.
[a] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from control.
[b] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from diazepam group.
[c] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test), significantly different from free NMD 0.1 group.
[d] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from free NMD 1 group.
[e] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from free NMD 10 group.
[f] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from NMD-Lipo 0,1 group.
[g] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from NMD-Lipo 1 group.
[h] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from NMD-Lipo 10 group.

3.4. Elevated plus-maze test

The results are summarized in Table 3. In the elevated plus-maze test, the animals treated with a 2 mg/kg dose of diazepam showed a significant increase in the number of entries and length of stay in the open arms when compared to the control group. The rodents that received free NMD at the doses of 0.1, 1 and 10 mg/kg showed no change in the number of entries and a modest increase in length of stay in the open arms when compared to the control group. The mice treated with NMD-Lipo at the doses of 0.1, 1 and 10 mg/kg exhibited a significant increase in the number of entries and length of stay when compared to the control group. The mice pretreated with flumazenil and subsequently given diazepam and NMD-Lipo showed a reduction in the number of entries and length of stay in the open arms, compared to animals that did not receive the antagonist.

3.5. Statistical analysis

Results were expressed as the mean ± the standard error of the mean (SEM) of number of animals that were used in the experiments. The values that followed a parametric distribution were analyzed by the analysis of variance (ANOVA) and *t*–Student–Newman–Keuls test as *post hoc* test, using the program GraphPad Prism version 5.00 for Windows. Differences were considered statistically significant at $p < 0.05$.

4. Discussion

Anxiety is usually diagnosed based on reports by patients, which demonstrates the subjective nature of the disorder and represents a

Table 3
Effects of free NMD, NMD-Lipo, flumazenil, diazepam and their associations in mice in the elevated plus maze test.

Groups	Number of entries in the open arms	Length of stay in the open arms
Control	5.86 ± 0.40	40.43 ± 3.75
DZP	7.57 ± 0.65[a]	93.71 ± 1.51[a]
Free NMD 0.1	3.29 ± 0.36	68.71 ± 5.41[a]
Free NMD 1	3.71 ± 0.68	87.43 ± 3.68[a]
Free NMD 10	4.57 ± 0.43	83.29 ± 10.02[a]
NMD-Lipo 0.1	7.14 ± 0.88[a,c]	111.7 ± 2.05[a,b,c]
NMD-Lipo 1	7.86 ± 0.86 [a,d]	118.7 ± 5.74[a,b,d]
NMD-Lipo 10	8.29 ± 0.92[a,b,e]	128.0 ± 8.98[a,b,e]
Flu	5.86 ± 0.55	39.14 ± 3.54
Flu + DZP	5.86 ± 0.74[b]	41.00 ± 1.76[b]
Flu + NMD-Lipo 0.1	5.71 ± 0.68[f]	43.86 ± 1.10[f]
Flu + NMD-Lipo 1	6.29 ± 0.60[g]	42.43 ± 1.34[g]
Flu + NMD-Lipo 10	5.57 ± 0.37[h]	43.14 ± 1.30[h]

Values are mean ± S.E.M. of the number of entries and length of stay in the open arms of 8 mice (by group) used in the experiments.
[a] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from control.
[b] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from diazepam group.
[c] $p < 0.05$ (ANOVA followed by Student-Newman-Keuls *t*-test with *post hoc* test), significantly different from NMD 0.1 group.
[d] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from NMD 1 group.
[e] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from nimo 10 group.
[f] $p < 0.05$ (ANOVA followed by *t*-test Student–Newman–Keuls with *post hoc* test) significantly different from NMD-Lipo 0,1 group.
[g] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from NMD-Lipo 1 group.
[h] $p < 0.05$ (ANOVA followed by Student–Newman–Keuls *t*-test with *post hoc* test) significantly different from NMD-Lipo 10 group.

challenge for the development of animal models for this disease. However, since the introduction of anxiolytics, which enabled pharmacological validation tests and a better understanding of the pathophysiology of the disease, the conducting of experiments that mimic anxiety has received a considerable boost (Kumar et al., 2013).

Over the past 50 years, anxiolytic benzodiazepines have been the drugs of choice for treatment of anxiety. However, despite showing benefits well described and characterized, benzodiazepines can also cause numerous side effects, such as sedation, muscle relaxation, anterograde amnesia and physical dependence, which lead patients to seek alternative therapies (Kaplan and Sadock, 2005; Sousa et al., 2008). In this context, the production of liposomes containing nimodipine has emerged as a possible alternative for the treatment of anxiety.

In the present study, diazepam was chosen as a positive control because it is the benzodiazepine reference drug. Benzodiazepines are widely used in the treatment of anxiety, sleep disorders, seizures and other diseases, and are among the most commonly prescribed psychotropic medications worldwide, with a prevalence of use of around 2–7%. Despite the well-described benefits, the misuse of benzodiazepines can cause a number of problems, such as deficits in learning, attention, and memory and depression, as well as falls, car accidents and other hazards to the user (Quaglio et al., 2012). Furthermore, the risk of dependence after long-term use has been described, as reflected in the appearance of a series of symptoms when the drug is abruptly withdrawn (Galdino et al., 2012).

Flumazenil was used in the experiment because it is a nonselective antagonist of the benzodiazepine binding site of GABAA receptors (Divljakovi'c et al., 2013). Its administration assists in elucidating the mechanism of action of the formulation, since the possible decrease in anxiolytic effects in animals treated with flumazenil before receiving NMD-Lipo, and the resulting channel antagonism of benzodiazepines, is indicative of the action of nanoencapsulated nimodipine in such receptors.

As sedation and muscle relaxation are major annoyances reported by patients undergoing treatments fort anxiety, it is vital that new anxiolytic formulations do not present these adverse reactions. Thus, the open field test is sorely needed in the search for new compounds to combat the aforementioned disorder, since it enables the assessment of the animal's motor activity, detecting whether the administration of the test compound caused changes in the consciousness or muscle tone of mice.

The decrease in the number of crossings, rearings and groomings of diazepam-treated mice compared to the control group suggests that diazepam reduced the animals' mobility. The absence of reduction in the number of crossings, rearings and groomings of animals that received both free NMD and NMD-Lipo suggests that the drug and the liposomal formulation which it contains do not alter the mobility of the animals, as occurs with diazepam.

The absence of changes in the mobility of rodents in the groups treated with NMD-Lipo constitutes a major advance in drug development against anxiety, as sedation and muscle relaxation are major complaints of patients using the anxiolytic drugs currently available on the market.

The light and dark test anxiety is generated by conflict resulting from the tendency to explore both compartments and their innate aversion to bright environments. Thus, the increase in light–dark transitions and the increase in stay of the animals in the bright field are indicative of the anxiolytic activity of the formulation (Enkel et al., 2013).

The mice treated with free NMD showed no change in time spent in the light field, which suggests that the drug has no anxiolytic activity at the doses tested. On the other hand, the increased residence time of the animals treated with NMD-Lipo in the bright field is evidence of the nimodipine anxiolytic-like activity when encapsulated into liposomes. The results of mice treated with the liposomal formulation were similar (NMD-Lipo 0.1) and better (NMD-Lipo 1 e 10) to the group treated with diazepam and much better than in the animals treated with non-encapsulated nimodipine, which suggests that the encapsulation of the drug into liposomes promoted its controlled release, increasing its bioavailability and improving its effect. Furthermore, the reduction in residence time in the illuminated box of rodents treated with the combination of flumazenil and NMD-Lipo, compared with the animals treated only with NMD-Lipo, suggests that NMD-Lipo act on benzodiazepine receptors (Table 2).

The elevated plus-maze test use as a measure of anxiety is based on the natural aversion of rodents for open spaces. The larger the exploratory capacity of the animals and the longer it remains in the open arms, the lower is their level of anxiety. The validity of the elevated plus-maze animal model of anxiety is based on the animal's sensitivity to a number of anxiolytic and anxiogenic drugs and treatment for stressors (Casarrubea et al., 2013).

The increase in the number of entries and length of stay in the open arms of the animals treated with NMD-Lipo demonstrated the anxiolytic-like activity of the formulation. In addition, the reduction in the number of entries and length of stay in the open arms of rodents treated with the combination of flumazenil and NMD-Lipo, compared with the animals treated only with NMD-Lipo, corroborates the hypothesis that the drug and the liposomal formulation act on benzodiazepine receptors (Table 3).

The performance of the rodents receiving NMD-Lipo 0.1 and NMD-Lipo 1 in the elevated plus-maze test were similar to that of the animals treated with diazepam, while the behavior of the animals treated with NMD-Lipo 10 was much better than in those treated with diazepam. Moreover, the effects of nimodipine encapsulated into liposomes were significantly better than the group treated with non-encapsulated nimodipine. These results suggest that the encapsulation of the nimodipine into liposomes increases its bioavailability, as well as the action of the drug on the animals.

5. Conclusion

This study suggests that the administration of a liposomal formulation containing nimodipine at the doses of 0.1, 1 and 10 mg/kg did not produce sedation and muscle relaxation in mice, showing anxiolytic-like activity in the open field, the light and dark and the elevated plus-maze test. The results of the treatment with NMD-Lipo were significantly better than the rodents treated with non encapsulated nimodipine, suggesting that the liposomes promoted a drug controlled release by increasing its bioavailability and consequently its effect. The decrease in the anxiolytic effect of NMP-Lipo in animals pretreated with flumazenil suggests that the formulation acts on benzodiazepine receptors.

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References

Archer J. Tests for emotionality in rats and mice. A review. Anim Behav 1973;21:205–35.
Aslan A, Gurelik M, Cemek M, Goksel HM, Buyukokuroglu ME. Nimodipine can improve cerebral metabolism and outcome in patients with severe head trauma. Pharmacol Res 2009;59:120–4.
Bartley CA, Hay M, Bloch MH. Meta-analysis: aerobic exercise for the treatment of anxiety disorders. Prog Neuropsychopharmacol Biol Psychiatry 2013;45:34–9.
Casarrubea M, Roy V, Sorbera F, Magnusson MS, Santangelo A, Arabo A, et al. Temporal structure of the rat's behavior in elevated plus maze test. Behav Brain Res 2013;237:290–9.
Chalikwar SS, Belgamwar VS, Talele VR, Surana SJ, Patil MU. Formulation and evaluation of nimodipine-loaded solid lipid nanoparticles delivered via lymphatic transport system. Colloids Surf B 2012;97:109–16.
Crawley JN. Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev 1985;9:37–44.
Divljakovi'c J, Mili'c M, Namjoshi OA, Tiruveedhula VV, Timi'c T, Cook JM, et al. βCCT, an antagonist selective for α(1)GABA(A) receptors, reverses diazepam withdrawal-induced anxiety in rats. Brain Res Bull 2013;91:1–7.
Enkel T, Thomas M, Bartsch D. Differential effects of subchronic phencyclidine on anxiety in the light-enhanced startle-, light/dark exploration- and open field tests. Behav Brain Res 2013;263:61–5.
Frye MA, Pazzaglia PJ, George MS, Luckenbaugh DA, Vanderham E, Davis CL, et al. Low CSF somatostatin associated with response to nimodipine in patients with affective illness. Biol Psychiatry 2003;53:180–3.
Galdino PM, Nascimento MVM, Florentino IF, Lino FC, Fajemiroye JO, Chaibub BA, et al. The anxiolytic-like effect of an essential oil derived from *Spiranthera odoratissima* A. St. Hil. leaves and its major component, β-caryophyllene, in male mice. Prog Neuropsychopharmacol Biol Psychiatry 2012;38:276–84.
Kaplan HI, Sadock BJ. Comprehensive textbook of psychiatry. Baltimore, MD: Lippincot Williams & Wilkins; 2005.
Kumar N, Singh N, Jaggi AS. Anti-stress effects of cilnidipine and nimodipine in immobilization subjected mice. Physiol Behav 2012;150:1148–55.
Kumar V, Bhat ZA, Kumar D. Animal models of anxiety: a comprehensive review. J Pharmacol Toxicol Methods 2013;68:175–83.
Lira MCB, Siqueira-Moura MP, Rolim-Santos HML, Galetti FCS, Simioni AR, Santos NP, et al. In vitro uptake and antimycobacterial activity of liposomal usnic acid formulation. J Liposome Res 2009;19:49–58.
Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987;92:180–5.
Maack DJ, Tull MT, Gratz KL. Examining the incremental contribution of behavioral inhibition to generalized anxiety disorder relative to other Axis I disorders and cognitive-emotional vulnerabilities. J Anxiety Disord 2012;26:689–95.
Maigaard K, Hageman I, Jørgensen A, Jørgensen MB, Wörtwein G. Electroconvulsive stimulations prevent chronic stress-induced increases in L-type calcium channel mRNAs in the hippocampus and basolateral amygdala. Neurosci Lett 2012;516:24–8.
Marinho MMF, Bruin VMS, Souza FCF, Aguiar LMV, Pinho RSN, Viana GBS. Inhibitory action of a calcium channel blocker (nimodipine) on seizures and brain damage induced by pilocarpina and lithium-pilocarpine in rats. Neurosci Lett 1997;235:13–6.
Mikati MA, Holmes GL, Werner S, Bakkar N, Carmant L, Liu Z, et al. Effects of nimodipine on the behavioral sequelae of experimental status epilepticus in prepubescent rats. Epilepsy Behav 2004;5:168–74.
N'Gouemo P. Probing the role of the sodium/calcium exchanger in pentylenetetrazole-induced generalized seizures in rats. Brain Res Bull 2013;90:52–7.
Nascimento VS, D'alva MS, Oliveira AA, Freitas RM, Vasconcelos SMM, Sousa FCF, et al. Antioxidant effect of nimodipine in young rats after pilocarpine-induced seizures. Pharmacol Biochem Behav 2005;82:11–6.
Pazzaglia PJ, George MS, Post RM, Rubinow RD, Davis CL. Nimodipine increases CSF somatostatin in affectively ill patients. Neuropsychopharmacology 1995;13:75–83.
Quaglio G, Pattaro C, Gerra G, Mathewson S, Verbanck P, Jarlais DCD, et al. High dose benzodiazepine dependence: description of 29 patients treated with flumazenil infusion and stabilised with clonazepam. Psychiatry Res 2012;198:457–62.
Raupp IM, Sereniki A, Virtuoso S, Ghislandi C, Cavalcanti E, Silva EL, et al. Anxiolytic-like effect of chronic treatment with *Erythrina velutina* extract in the elevated plus-maze test. J Ethnopharmacol 2008;118:295–9.
Richey JA, Schmidt NB, Hofmann SG, Timpano KR. Temporal and structural dynamics of anxiety sensitivity in predicting fearful responding to a 35% CO_2 challenge. J Anxiety Disord 2010;24:423–32.
Salomons AR, Kortleve T, Reinders NR, Kirchhoff S, Arndt SS, Ohl F. Susceptibility of a potential animal model for pathological anxiety to chronic mild stress. Behav Brain Res 2010;209:241–8.
Santos-Magalhães NS, Mosqueira VCF. Nanotechnology applied to the treatment of malaria. Adv Drug Deliv Rev 2010;62:560–75.
Sousa FCF, Melo CTV, Citó COM, Félix FHC, Vasconcelos SMM, Fonteles MMF, et al. Plantas medicinais e seus constituintes bioativos: Uma revisão da bioatividade e potenciais benefícios nos distúrbios da ansiedade em modelos animais. Rev Bras Farmacogn 2008;18:642–54.
Sun C, Wang J, Liu J, Qiu L, Zhang W, Zhang L. Liquid proliposomes of nimodipine drug delivery system: preparation, characterization, and pharmacokinetics. AAPS PharmSciTech 2013;14:332–8.
Wong HL, Wu XY, Bendayan R. Nanotechnological advances for the delivery of CNS therapeutics. Adv Drug Deliv Rev 2012;64:686–700.
Yanpallewar SU, Hota D, Rai R, Kumar M, Acharya SB. Nimodipine attenuates biochemical, behavioral and histopathological alterations induced by acute transient and long-term bilateral common carotid occlusion in rats. Pharmacol Res 2004;49:143–50.
Zou JB, Dear BF, Titov N, Lorian CN, Johnston L, Spence J, et al. Brief internet-delivered cognitive behavioral therapy for anxiety in older adults: a feasibility trial. J Anxiety Disord 2012;26:650–5.

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Short communication

Acute toxicity and anticonvulsant activity of liposomes containing nimodipine on pilocarpine-induced seizures in mice

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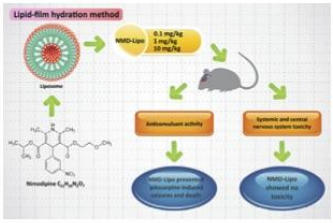
HIGHLIGHTS

- NMD-Lipo did not produce acute toxicity in mice.
- NMD-Lipo has anticonvulsant activity on pilocarpine-induced seizures in mice.
- NMD-Lipo showed anticonvulsant activity significantly major that free NMD.

GRAPHICAL ABSTRACT



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Toxicity

ABSTRACT

Nimodipine has been shown to have an inhibitory action on seizures and brain damage in rodents. However, the pharmaceutical applicability of this drug is limited by its low solubility in gastrointestinal fluids and high first-pass effect in the liver, which leads to low bioavailability. These difficulties can be overcome through the use of liposomes. The aim of the present study is to evaluate the toxicity and anticonvulsant activity of liposomes containing nimodipine (NMD-Lipo) on pilocarpine-induced seizures. NMD-Lipo was prepared using the lipid-film hydration method. Central nervous system toxicity of NMD-Lipo was assessed by Hippocratic screening. Systemic toxicity was evaluated by analyses of biochemical and hematological parameters and by observing possible signs of toxicity. The possible anticonvulsant activity was tested by the pilocarpine model. The administration of the NMD-Lipo at doses of 0.1, 1, and 10 mg/kg caused no toxicity in animals. Furthermore, NMD-Lipo prevented the installation of 100% of the pilocarpine-induced seizures and prevented the death of 100% of the mice treated with pilocarpine. These data shown that NMD-Lipo has an anticonvulsant activity significantly superior to free NMD, suggesting that the liposomes promoted a drug controlled release by improving its bioavailability and consequently increasing its pharmacological activity.

1. Introduction

Epilepsy is a chronic disease of the central nervous system characterized by recurrent seizures caused by excessive discharges of cerebral neurons. This condition is a health concern, as it is considered one of the most serious neurological disorders [1]. Clinically, patients with the disease experience a deterioration of one or more

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cognitive functions, with or without motor behavior and/or psychomotor decrease [2].

Seizures can be completely controlled with medical therapy in two-thirds of patients; however, one-third remains refractory to the medications [3]. Furthermore, the current antiepileptic drugs used in the treatment of epilepsy have a wide range of adverse reactions, toxicity, and teratogenic effects. Based on these findings, new therapeutic agents, which allow more efficient seizure control in resistant patients and with fewer side effects, are greatly needed [4].

Research has shown that the intrinsic epileptiform activity is associated with calcium (Ca^{2+}) influx through NMDA receptor-operated Ca^{2+} channels and through voltage-operated Ca^{2+} channels. Therefore, the inhibition of the intracellular Ca^{2+} increase represents an important target in the development of antiepileptic and neuroprotective drugs [5]. From this perspective, calcium channel blockers may be considered as a possible therapeutic agent for the disease.

Nimodipine (NMD) is a dihydropyridine L-type Ca^{2+} channel antagonist that crosses the blood–brain-barrier more easily than other calcium-channel-blockers and binds with high affinity and specificity to the calcium-channel receptors in the brain [6]. NMD has been shown to have an inhibitory action on seizures and brain damage in rodents [16–23]. However, the pharmaceutical applicability of nimodipine is limited by its low solubility in gastrointestinal fluids and high first-pass effect in the liver, which leads to low bioavailability after oral administration [7,8].

These difficulties can be overcome through the use of liposomes. These nanometer-scale pharmaceutical carriers are self-assembled colloidal vesicles consisting of one or more concentric phospholipid bilayers organized around an aqueous inner compartment, and are used to encapsulate drugs, biomolecules or diagnostic agents [9]. The aim of the present study is two fold: the evaluation of the nimodipine encapsulated into liposomes (NMD-Lipo) toxicity and the study of anticonvulsant activity of NMD-Lipo on pilocarpine-induced seizures.

2. Materials and methods

2.1. Reagents

Cholesterol (CHOL), trehalose, nimodipine, and pilocarpine hydrochloride were purchased from Sigma–Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (98% Epikuron 200) was obtained from Lipoid GMBH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Animals

Adult male Swiss mice (25–30 g; 2 months old) were obtained from Central Animal House of the Federal University of Piaui, Piaui, Brazil. They were maintained in a temperature controlled room ($25 \pm 1\,^{\circ}C$), with a 12 h light/dark cycle (lights on 07:00–19:00 h), and food and water provided *ad libitum* (Nutrilabor, Campinas, Brazil). The experimental protocols and procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA/UFPI N° 014/11). All experiments were performed according to the guide for the care and use of laboratory of the US, Department of Health and Human Services, Washington, DC (1985).

2.3. Preparation and characterization of liposomes containing nimodipine

Liposomes containing nimodipine (NMD-Lipo) were prepared and characterized as previously described [10]. The content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm and the encapsulation efficiency of nimodipine into liposomes was determined after the submission of samples to ultrafiltration/ultracentrifugation using Ultrafree® units (Millipore, USA), for the separation of the drug encapsulated and non encapsulated into liposomes [10]. The content of nimodipine in the supernatant was then determined and the drug encapsulation ratio was calculated as:

$$\%EE = \frac{[NMD]_{content} - [NMD]_{free}}{NMD_{content}} \times 100.$$

2.4. Systemic and central nervous system toxicity of NMD-Lipo

Mice were divided into four groups, with 16 animals in each group. The first group was treated with 0.9% saline. The second, third, and fourth groups were treated with NMD-Lipo at doses of 0.1, 1, and 10 mg/kg. NMD is a widely used drug and its security is well-known, so the toxicity tests have not been conducted with free NMD, only with NMD-Lipo.

Central nervous system toxicity of NMD-Lipo was assessed by Hippocratic screening. Systemic toxicity was evaluated by analysis of biochemical and hematological parameters and by observing possible signs of toxicity.

Half of the animals in each group ($n = 8$) were observed for 24 h and subsequently were intended to implement the blood tests. During this period we proceeded to the observation of the mice at the time of 30 min, 1, 2, 4, 8, 12, and 24 h for the purpose of quantifying the effect of NMD-Lipo on the following parameters: (a) state of awareness and readiness; (b) motor coordination; (c) muscle tone; (d) reflection (atrial and cornea); (e) central nervous system activity; (f) autonomic nervous system activity. At the end of 24 h, the animals were anesthetized with pentobarbital 40 mg/kg and blood was immediately collected from the retro-orbital plexus for the assessment of biochemical and hematologic parameters [11].

The other half ($n = 8$) was under observation for a period of 30 days for viewing and the recording of possible signs of toxicity of the formulation. During these 30 days, the consumption of water and feed was recorded daily, body weight of mice was measured every two days and the animals were evaluated for clinical signs of toxicity.

2.5. Anticonvulsant activity of NMD-Lipo

Mice were divided into twenty-two groups, with each group containing 12 animals. The negative control group was treated with 0.9% saline. The P400 group was treated with pilocarpine hydrochloride at a dose of 400 mg/kg to induce seizures. The third and fourth groups were treated with diazepam at a dose of 5 mg/kg and an association of diazepam with pilocarpine hydrochloride in a dose of 400 mg/kg. The fifth, sixth, and seventh groups were treated with empty liposomes at doses of 0.1, 1, and 10 mg/kg. The eighth, ninth, and tenth groups were treated with empty liposomes at doses of 0.1, 1, and 10 mg/kg and after 30 min they received pilocarpine hydrochloride at the dose of 400 mg/kg. The eleventh, twelfth, and thirteenth groups were treated with free nimodipine at doses of 0.1, 1, and 10 mg/kg. The fourteenth, fifteenth, and sixteenth groups were treated with free nimodipine at doses of 0.1, 1, and 10 mg/kg and after 30 min they received pilocarpine hydrochloride at the dose of 400 mg/kg. The seventeenth, eighteenth, and nineteenth groups were treated with NMD-Lipo at the doses of 0.1, 1, and 10 mg/kg. Finally, the animals of the twentieth, twenty-first, and twenty-second groups received NMD-Lipo at the doses of 0.1, 1, and 10 mg/kg and after 30 min they received pilocarpine hydrochloride at the dose of 400 mg/kg.

After the treatments, the animals were recorded in 30 cm × 30 cm chambers with: appearance of peripheral cholinergic signs (miosis, piloerection, chromodacryorrhea, diarrhea, and urination), stereotyped movements (continuous sniffing, paw licking, and rearing), tremors, seizures, status epilepticus, and mortality rate, during 24 h. We decided to observe possible changes in the behavior of mice for 24 h after pilocarpine administration because previous works showed that convulsions and deaths occurred within 1 and 24 h, respectively, post pilocarpine injection [12].

2.6. Statistical analyses

The results were presented as a percentage according to the number of animals used in the experiments. Peripheral cholinergic signs, stereotypic movements, tremor, seizures, status epilepticus, and mortality rate were presented as percentages and compared with a nonparametric test (Chi-Square test). In all situations statistical significance was reached at p less-than-or-equals, slant 0.05. The statistical analyses were performed with the software GraphPad Prism, version 6.00 for windows, GraphPad software (San Diego, CA, USA).

3. Results

3.1. Nimodipine-loaded liposomes

NMD-Lipo presented a drug content of 0.98 ± 0.58 mg/ml and encapsulation efficiency of 99 ± 0.22%.

3.2. Systemic and central nervous system toxicity of NMD-Lipo

In the Hippocratic screening, NMD-Lipo did not cause any behavioral alterations in mice at the doses tested. Therefore, no alteration was observed in biochemical and hematologic parameters as any variation in weight of mice treated with the formulation. None of the animals treated with NMD-Lipo died.

3.3. Behavioral alterations after pretreatment with NMD-Lipo

The results of the behavioral alterations of animals after pretreatment with NMD-Lipo after 24 h of phase acute of pilocarpine-induced seizures are summarized in Table 1. None of the mice that received injections of isotonic saline (negative control), diazepam, empty liposomes, free nimodipine, and NMD-Lipo unassociated with pilocarpine showed peripheral cholinergic signs,

Table 1
Effect of pretreatment with NMD-Lipo, free nimodipine, liposomes, and diazepam on pilocarpine-induced seizures and lethality in adult mice.

Groups (n = 12)	Peripheral cholinergic signs %	Stereotypic movements %	Tremor %	Seizures %	Status epilepticus %	Mortality rate %
Negative control		00	00	00	00	00
P400	100[a]	100[a]	100[a]	100[a]	75[a]	75[a]
DZP 5 plus P400	100[a]	100[a]	100[a]	50[a,b]	50[a,b]	50[a,b]
DZP 5	00	00	00	00	00	00
Lipo 0.1	00	00	00	00	00	00
Lipo 1	00	00	00	00	00	00
Lipo 10	00	00	00	00	00	00
NMD-Lipo 0.1 plus P400	100[a]	65[a,b,c,d,e]	100[a]	00[b,c,d,e]	00[b,c,d,e]	00[b,c,d,e]
Lipo 0.1 plus P400	100[a,c]	100[a,c]	100[a,c]	100[a,c]	75[a,c]	75[a,c]
Lipo 1 plus P400	100[a,d]	100[a,d]	100[a,d]	100[a,d]	75[a,d]	75[a,d]
Lipo 10 plus P400	100[a,e]	100[a,e]	100[a,e]	100[a,e]	75[a,e]	75[a,e]
Free NMD 0.1	00	00	00	00	00	00
Free NMD 1	00	00	00	00	00	00
Free NMD 10	00	00	00	00	00	00
Free NMD 0.1 plus P400	100[a,f]	100[a,f]	100[a,f]	100[a,f]	50[a,b,f]	75[a,f]
Free NMD 1 plus P400	100[a,g]	100[a,g]	100[a,g]	100[a,g]	50[a,b,g]	75[a,g]
Free NMD 10 plus P400	100[a,h]	75[a,b,h,*,**]	100[a,h]	100[a,h]	75[a,h,*,**]	75[a,h]
NMD-Lipo 0.1	00	00	00	00	00	00
NMD-Lipo 1	00	00	00	00	00	00
NMD-Lipo 10	00	00	00	00	00	00
NMD-Lipo 0.1 plus P400	100[a,i]	65[a,b,i,&]	100[a,i]	00[b,&]	00[b,&]	00[b,&]
NMD-Lipo 1 plus P400	100[a,j]	40[a,b,j,#,&]	60[a,b,j,#,##,&]	00[b,&]	00[b,&]	00[b,&]
NMD-Lipo 10 plus P400	100[a,l]	30[a,b,l,#,##,&]	20[a,b,l,#,##,&]	00[b,&]	00[b,&]	00[b,&]

Mice (25–30 g; 2 months old) were treated acutely with vehicle (saline 0.25 ml, negative control), pilocarpine (400 mg/k.g, i.p, P400), diazepam (5 mg/kg, i.p., DZP 5, positive control), empty liposomes (Lipo), free nimodipine (Free NMD), and liposomal formulation containing nimodipine (NMD-Lipo) at doses 0.1, 1 e 10 mg/kg (i.p.). Others groups of mice were pretreated acutely with Lipo, free NMD and NMD-Lipo at doses 0.1, 1 e 10 mg/kg (i.p.) or DZP and 30 min after treatment with pilocarpine 400 mg/kg, i.p. Results for peripheral cholinergic signs, stereotypic movements, tremor, seizures, status epilepticus, and death are expressed as percentages of the number of animals from each group.

[a] $p < 0.05$, when compared with negative control.
[b] $p < 0.05$, when compared with P400 group.
[c] $p < 0.05$, when compared with Lipo 0.1.
[d] $p < 0.05$, when compared with Lipo 1.
[e] $p < 0.05$, when compared with Lipo 10.
[f] $p < 0.05$, when compared with Free NMD 0.1.
[g] $p < 0.05$, when compared with Free NMD 1.
[h] $p < 0.05$, when compared with Free NMD 10.
[i] $p < 0.05$, when compared with NMD-Lipo 0.1.
[j] $p < 0.05$, when compared with NMD-Lipo 1.
[l] $p < 0.05$, when compared with NMD-Lipo 10.
[*] $p < 0.05$, when compared with Free NMD 0.1 plus P400.
[**] $p < 0.05$, when compared with Free NMD 1 plus P400.
[#] $p < 0.05$, when compared with NMD-Lipo 0.1 plus P400.
[##] $p < 0.05$, when compared with NMD-Lipo 1 plus P400.
[&] $p < 0.05$, when compared with DZP 5 plus P400 (Chi-Square test).

stereotypic movements, tremor, and seizures. None of the animals in these groups died.

All animals treated with P400 alone presented peripheral cholinergic signs and stereotyped movements followed by motor limbic seizures. The convulsive process persisted and built up to a status epilepticus in 75% of these mice, leading to death of 75% of the animals. Diazepam at the dose of 5 mg/kg did not significantly reduce the occurrence of peripheral cholinergic signs, stereotypic movements, and tremors. The benzodiazepine was able to reduce by 50% the occurrence of seizures and by 33.33% the mortality rate in mice. Empty liposomes at doses of 0.1, 1, and 10 mg/kg did not reduce the occurrence of peripheral cholinergic signs, stereotypic movements, and tremor. Liposomes without nimodipine were unable to prevent the installation of the seizure and decrease the mortality rate in rodent. Free NMD at the dose of 0.1 mg/kg did not reduce the occurrence of peripheral cholinergic signs, stereotypic movements, and tremors. The unencapsulated drug at the dose of 0.1 mg/kg was unable to prevent the installation of the seizure and reduce the mortality rate in mice. As with the mice pretreated with free NMD at the dose of 0.1 mg/kg, free NMD at the dose of 1 mg/kg did not significantly reduce the occurrence of peripheral cholinergic signs, stereotypic movements, and tremors. Moreover, free NMD at the dose of 1 mg/kg was unable to prevent the installation of the seizure and reduce the mortality rate in the mice. Free NMD at doses of 10 mg/kg did not significantly reduce the occurrence of peripheral cholinergic signs or tremors and was not able to prevent the installation of seizures. Free NMD at the dose of 10 mg/kg was able to reduce by 25% of stereotypic movements but was unable to prevent the installation of the seizure and reduce the mortality rate in the mice.

NMD-Lipo at doses of 0.1, 1, and 10 mg/kg did not reduce the occurrence of peripheral cholinergic signs, but decreased stereotypic movements and tremors in the mice. NMD encapsulated into liposomes at all doses tested was able to prevent the occurrence of 100% of the seizures. None of the mice pretreated with NMD-Lipo and subsequently given with pilocarpine died.

4. Discussion

Animal models of seizure have been widely used in research to provide a better understanding of the pathophysiology of the disease, since they reproduce several components of human epilepsies. Pilocarpine-induced seizures is a model commonly used to investigate the anticonvulsant effect of antiepileptic drugs [13].

The administration of high doses of pilocarpine induces seizure activity, followed by a latent seizure-free period preceding the development of spontaneous recurrent focal seizures. The induction of status epilepticus by pilocarpine in rodents leads to neuropathological changes, such as hippocampal sclerosis and mossy fiber sprouting, resembling human temporal lobe epilepsy [14]. In the present study, we investigated the effects of a liposomal formulation containing nimodipine, a Ca^{2+} channel blocker, on susceptibility to seizures induced by pilocarpine in adult mice.

Antiepileptic drugs have different targets such as receptors, synaptic machinery, and ion channels [15]. Previous studies have demonstrated that increased levels of intracellular Ca^{2+} in hippocampal neurons play an important role in the underlying mechanisms of neuronal hyperexcitability that leads to pilocarpine-induced seizures [16]. Research conducted with Ca^{2+} channel blocker NMD at the doses of 1 to 300 mg/kg have suggested that the drug presents anticonvulsant activity on seizures induced by picrotoxin [17], kainic acid [18], aminophylline [19], pentylenetetrazole [20], phenytoin [21], pilocarpine, and lithium-pilocarpine [22–24] in mice and rats. However, in all aforementioned studies, the drug was unable to prevent 100% of seizures.

One of the possible reasons explaining the lack of ability of nimodipine to prevent the installation of seizures in some rodents is that the drug has low bioavailability (4–13%) due to its high first-pass effect in the liver [25]. This hypothesis is strengthened by our data, since NMD-Lipo showed anticonvulsant activity significantly superior to free NMD, suggesting that the encapsulation of the nimodipine into liposomes increases its bioavailability, as well as the anticonvulsant activity of the drug on the animals.

Nimodipine has a high lipophilicity and it can be easily incorporated into the lipid bilayer of the liposomes [26]. NMD-Lipo presented drug encapsulation efficiency of $99 \pm 0.22\%$, showing that the formulation does not present a significant amount of unloaded NMD. The treatment with NMD-Lipo at the doses of 0.1–10 mg/kg was demonstratedto be safe for mice, since these treatments do not cause changes in the hematological and biochemical parameters of the animals are found. Furthermore, NMD-Lipo did not cause any change in the weight of the animals, a significant factor as the reduction in body weight is a simple and sensitive index of toxicity after exposure to a toxic substance [27].

The administration of the liposomal formulation at the doses of 0.1, 1, and 10 mg/kg was able to reduce stereotypic movements and tremors. Moreover, NMD-Lipo prevented the installation of 100% of the pilocarpine-induced seizures and prevented the death of 100% of the mice treated with pilocarpine, showing even better results than the rodents treated with diazepam. The results of the anticonvulsant activity suggest that NMD has a dose-dependent effect. As expected, empty liposomes showed no anticonvulsant activity, suggesting that the liposomes potentiate the anticonvulsant effect of nimodipine.

Animal studies indicate that seizures at an early stage of development can drastically affect the construction of networks of the hippocampus, which can cause the onset of other disorders such as schizophrenia [28]. The decrease in the occurrence of stereotypic movements and tremors and the ability to prevented seizures, and death in rodents constitutes a major advance in drug development against epilepsy. Thus, a formulation that prevents the emergence of seizures appears promising in the epilepsy therapy.

References

[1] O.O. Adeyemi, A.J. Akindele, O.K. Yemitan, F.R. Aigibe, F.I. Fagbo, Anticonvulsant, anxiolytic and sedative activities of the aqueous root extract of *Securidaca longepedunculata* Fresen, J. Ethnopharmacol. 130 (2010) 191–195.
[2] S. Fortini, L. Corredera, A.L. Pastrana, G. Reyes, L. Fasulo, R.H. Caraballo, Encephalopathy with hemi-status epilepticus during sleep or hemi-continuous spikes and waves during slow sleep syndrome: a study of 21 patients, Seizure 22 (2013) 565–571.
[3] P. Bhutada, Y. Mundhada, K. Bansod, P. Dixit, S. Umathe, D. Mundhada, Anticonvulsant activity of berberine, an isoquinoline alkaloid in mice, Epilepsy Behav. 18 (2010) 207–210.
[4] F. Hadizadeh, B. Rahimi, E. Taghiabadi, M. Razavi, G. Karimi, Evaluation of anticonvulsant effect of two novels 4-[1-(4-fluorobenzyl)-5-imidazolyl] dihydropyridine derivatives in mice, Res. Pharm. Sci. 8 (2013) 91–95.
[5] M. Ghasemi, H. Shafaroodi, S. Nazarbeiki, H. Meskar, P. Heydarpour, A. Ghasemi, S.S. Talab, P. Ziai, A. Bahremand, A.R. Dehpour, Voltage-dependent calcium channel and NMDA receptor antagonists augment anticonvulsant effects of lithium chloride on pentylenetetrazole-induced clonic seizures in mice, Epilepsy Behav. 18 (2010) 171–178.
[6] M.J. Bailey, B.A. Hutsell, M.C. Newlan, Dietary nimodipine delays the onset of methylmercury neurotoxicity in mice, Neurotoxicology 37 (2013) 108–117.
[7] N. Bege, T. Renette, T. Endres, M. Beck-Broichsitter, D. Hänggi, T. Kissel, In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm, Eur. J. Pharm. Biopharm. 84 (2013) 99–105.
[8] C. Sun, J. Wang, J. Liu, L. Qiu, W. Zhang, L. Zhang, Liquid proliposomes of nimodipine drug delivery System: preparation, characterization, and pharmacokinetics, AAPS Pharm. Sci. Tech. 14 (2013) 332–338.
[9] P.G. Cadena, M.A. Pereira, R.B.S. Cordeiro, I.M.S. Cavalcanti, B. Barros-Neto, M.C.C.B. Pimentel, J.L. Lima-Filho, V.L. Silva, N.S. Santos-Magalhães, Nanoencapsulation of quercetin and resveratrol into elastic liposomes, Biochim. Biophys. Acta 1828 (2013) 309–316.
[10] L.C.G.A.I. Moreno, G.Z.S. Oliveira, I.M.F. Cavalcanti, N.S. Santos-Magalhães, H.M.L. Rolim, R.M. Freitas, Development and evaluation of liposomal

formulation containing nimodipine on anxiolytic activity in mice, Pharmacol. Biochem. Behav. 116 (2014) 64–68.
[11] B.H. Waynforth, Injection techniques, in: Experimental and Surgical Techniques in the Rat, Academic Press, London, 1980.
[12] L.F.L. Santos, L.F.M. Freitas, S.M.L. Xavier, G.B. Saldanha, R.M. Freitas, Neuroprotective actions of vitamin C related to decreased lipid peroxidation and increased catalase activity in adult rats after pilocarpine-induced seizures, Pharmacol. Biochem. Behav. 89 (2008) 1–5.
[13] W. Zgrajka, D. Nieoczym, M. Czuczwar, J. Kíís, W. Brzana, P. Wlaíz, W.A. Turski, Evidences for pharmacokinetic interaction of riluzole and topiramate with pilocarpine in pilocarpine-induced seizures in rats, Epilepsy Res. 88 (2010) 269–274.
[14] P.E. Schauwecker, Strain differences in seizure-induced cell death following pilocarpine-induced status epilepticus, Neurobiol. Dis. 45 (2012) 297–304.
[15] A.J. Hill, N.A. Jones, I. Smith, C.L. Hill, C.M. Williams, G.J. Stephens, B.J. Whalley, Voltage-gated sodium (Na_V) channel blockade by plant cannabinoids does not confer anticonvulsant effects *per se*, Neurosci. Lett. 566 (2014) 269–274.
[16] Y. Martinez, P. N'Gouemo, Blockade of the sodium calcium exchanger exhibits anticonvulsant activity in a pilocarpine model of acute seizures in rats, Brain Res. 1366 (2010) 211–216.
[17] J. Thomas, The effect of nimodipine on picrotoxin-induced seizures, Brain Res. Bull. 24 (1990) 11–15.
[18] R.P. Paczynski, F.B. Meyer, R.E. Anderson, Effects of the dihydropyridine Ca^{2+} channel antagonist nimodipine on kainic acid-induced lirnbic seizures, Epilepsy Res. 6 (1990) 33–38.
[19] A. Chakrabarti, H.S. Kaur, S.K. Garg, Dose-finding study with nimodipine: a selective central nervous system calcium channel blocker on aminophylline induced seizure model in rats, Brain Res. Bull. 45 (1998) 495–499.
[20] P. Zapater, J. Javaloy, J.F. Román, M.T. Vidal, J.F. Horga, Anticonvulsant effects of nimodipine and two novel dihydropyridines (PCA 50,922 and PCA 50,941) against seizures elicited by pentylenetetrazole and electroconvulsive shock in mice, Brain Res. 796 (1998) 311–314.
[21] C. Hocht, A. Lazarowski, N.N. Gonzalez, J. Auzmendi, J.A.W. Opezzo, G.F. Bramuglia, C.A. Taira, E. Girardi, Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model, Neurosci. Lett. 413 (2007) 168–172.
[22] M.M.F. Marinho, V.M.S. Bruin, F.C.F. Souza, L.M.V. Aguiar, R.S.N. Pinho, G.B.S. Viana, Inhibitory action of a calcium channel blocker (nimodipine) on seizures and brain damage induced by pilocarpina and lithium-pilocarpine in rats, Neurosci. Lett. 235 (1997) 13–16.
[23] M.A. Mikati, G.L. Holmes, S. Werner, N. Bakkar, L. Carmant, Z. Liu, C.E. Stafstrom, Effects of nimodipine on the behavioral sequalae of experimental status epilepticus in prepubescent rats, Epilepsy Behav. 5 (2004) 168–174.
[24] V.S. Nascimento, M.S. D'alva, A.A. Oliveira, R.M. Freitas, S.M.M. Vasconcelos, F.C.F. Sousa, M.M.F. Fonteles, Antioxidant effect of nimodipine in young rats after pilocarpine-induced seizures, Pharmacol. Biochem. Behav. 82 (2005) 11–16.
[25] S.S. Chalikwar, V.S. Belgamwar, V.R. Talele, S.J. Surana, M.U. Patil, Formulation and evaluation of nimodipine-loaded solid lipid nanoparticles delivered via lymphatic transport system, Colloids Surf. B 97 (2012) 109–116.
[26] T. Guan, Y. Miao, L. Xu, S. Yang, J. Wang, H. He, X. Tang, C. Cai, H. Xu, Injectable nimodipine-loaded nanoliposomes: preparation, lyophilization and characteristics, Int. J. Pharm. 410 (2011) 180–187.
[27] S. Thanabhorn, K. Jaijoy, S. Thamaree, K. Ingkaninan, A. Panthong, Acute and subacute toxicity study of the ethanol extract from *Lonicera japonica* Thunb, J. Ethnopharmacol. 107 (2006) 370–373.
[28] G.P. Labbate, A.V. Silva, R.C. Barbosa-Silva, Effect of severe neonatal seizures on prepulse inhibition and hippocampal volume of rats tested in early adulthood, Neurosci. Lett. 568 (2014) 62–66.

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Research report

Antidepressant-like activity of liposomal formulation containing nimodipine treatment in the tail suspension test, forced swim test and MAO_B activity in mice

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Mice

ABSTRACT

Previous studies have shown that intracellular calcium ion dysfunction may be an etiological factor in affective illness. Nimodipine (NMD) is a Ca^{2+} channel blocker that has been extensively investigated for therapy of central nervous system (CNS) disorders. In this work, we have evaluated the antidepressant-like activity of nimodipine encapsulated into liposomes (NMD-Lipo) in mice through tail suspension and forced swim assays, as well as MAO_B activity. During the tail suspension test, the administration of NMD-Lipo at 0.1, 1 and 10 mg/kg was able to promote a reduction in the immobility time of animals greater than the positive control (imipramine). In the forced swim test, the immobility time of mice treated with NMD-Lipo was reduced. This reduction was significantly greater than that found in the animals treated with imipramine and paroxetine. This may suggest that NMD-Lipo provides more antidepressant-like activity than in positive controls. The groups that received a combination of liposomal NMD and antidepressant drugs showed lower immobility time than the groups, which were treated only with imipramine or paroxetine. The mice treated with the combination of NMD-Lipo and reserpine presented an increase in the time of immobility compared with animals treated only with NMD-Lipo. There was a significant decrease in MAO_B activity in animals treated with NMD-Lipo compared with untreated animals. The results of the tail suspension test, forced swim test and MAOB activity suggested that the antidepressant activity of NMD-Lipo may be related to an increase in the cerebral monoamine concentrations.

1. Introduction

Depression is a highly prevalent and recurrent mental disorder that impacts all aspects of human life (Socała et al., 2012). Research has shown that depression probably originated during the evolutionary process. Some studies have supported this thesis by arguing that being in a state of depression makes people focus on solving problems. Also, traces of depression may indicate that the affected person needs some help, besides discouraging individuals from risky behavior (Wittman, 2014). On the other hand, when depression progresses from a mild state to a severe condition, the disease becomes alarming. This pathology is one of the major causes of disability in the world, representing a heavy social burden, and is considered a lifetime risk (Wang et al., 2013).

Treatment-resistant depression is a frequent condition in patients. Statistics show that 50–60% of people treated with antidepressant drugs obtain clinically insignificant results (Fornaro et al., 2014). Therefore, strategies used to improve the treatment of this disease include the addition of one or more non-antidepressant drugs to an existing therapy or the use of agents to target specific symptoms of depression. Combined therapy involving the use of two or more antidepressant drugs has been increasingly implemented (Papakostas, 2009).

The pathogenesis of neuropsychiatric diseases and the mechanisms of action of drugs used in their treatments are still not well established. However, it is well known that alterations commonly occur in many diseases of the central nervous system (CNS). For example, rises in intracellular calcium concentration cause an increased contraction of the brain vasculature, which reduces blood flow in the organ (Xu et al., 2013). In addition, excessive penetration of calcium in neurons causes a process known as excitotoxicity that induces neuronal damage and cell death by necrosis or apoptosis (Justin et al., 2014 and Aslan et al., 2009).

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Research has shown that the excessive flow of calcium through the membrane, which results in increased intracellular levels of this cation, may play a role in the pathophysiology of affective CNS disorders (Maigaard et al., 2012), epileptiform activity (N'Gouemo, 2013) and the induction of anxiety (Kumar et al., 2013).

In this way, an increase in calcium concentrations in platelets and lymphocytes of depressed and bipolar patients suggests that increased intracellular concentrations of this cation may play a role in the pathophysiology of depression and bipolar disorder (Dubovsky et al., 2014; Dubovsky et al., 1994; Dubovsky et al., 1992 and Dubovsky et al., 1989). This finding was reinforced by the discovery that some drugs used for treating depression and bipolar disorder, such as tricycle antidepressants and lithium, cause a blockage in the calcium channels, indicating that this mechanism may contribute to their therapeutic effect (Roedding et al., 2006 and Grunze et al., 1996). Based on this data, calcium channel blockers may be considered a possible therapeutic agent for an effective treatment of affective illnesses of CNS.

Nimodipine (NMD) is a central Ca^{2+} channel blocker and has a high permeability through the blood brain barrier (BBB). Because of this, NMD has been extensively investigated in the therapy of CNS disorders (Sygnecka et al., 2015). It has been shown to have antidepressant properties in rats (Katagiri et al., 2001) and humans (Frye et al., 2003, Yingling et al., 2002 and Pazzaglia et al. 1995). However, the clinical use of NMD is limited by its low solubility in gastrointestinal fluids and high first-pass effect in the liver, which leads to a low bioavailability after oral administration (Bege et al., 2013 and Sun et al., 2013).

Nanotechnology offers an interesting approach for improving the therapeutic management of CNS diseases. In general, liposomal drug-loaded formulations are superior to free drugs in their pharmacokinetic and therapeutic effects (Wong et al., 2012). Our recent studies have demonstrated that NMD encapsulated into liposomes presents anxiolytic (Moreno et al., 2014) and anticonvulsant activities in mice (Moreno et al., 2015). For these reasons, liposomes seem to be a suitable vehicle for the administration of NMD.

An animal model that perfectly mimics the symptoms of depression in human patients is hardly ever achieved. Animals not only lack consciousness of self, self-reflection and consideration of others, but also symptoms of depression such as suicidal tendencies and depressed mood are difficult to detect in non-human beings (Deussing, 2006). However, there are a number of symptoms of this disease that can be reproduced in animals such as anxiety, anhedonia, sleep disturbances, hormonal dysregulation, etc., which can be used for drug tests with the view to acquiring understanding of neurological diseases (Schmidt, 2011).

The tail suspension and the forced swim tests are widely used for evaluating antidepressant activity of compounds in preclinical trials (Rollema et al., 2009). The mechanism of the effects evaluated using these tests has yet to be well established, but it is believed that they may reflect conformational changes occurring in the neurotransmitter receptors and the signal transduction receptor mediated systems (Kim et al., 2010).

Monoamine oxidases (MAO) are a class of mitochondrial enzymes involved in the degradation of biogenic amines. Monoamine oxidase inhibitors (MAOIs) have been used in clinical therapy owing to their antidepressant effects. However, the use of MAOIs for the treatment of depression has diminished since the discovery that MAOIs ingested with tyramine can result in cardiovascular reaction ("cheese effect") (Ghazaleh et al., 2015). On the other hand, the finding that the selective inhibition of monoamine oxidase B (MAO_B) does not cause "cheese effect" suggests that drugs causing MAO_B inhibition are suitable for the therapy of affective disorders (Finberg, 2014).

Based on the abovementioned, we evaluated the antidepressant-like activity of nimodipine-loaded liposomes (NMD-Lipo) in treated mice using the tail suspension and forced swim tests. Furthermore, we examined MAO_B activity in the hippocampus of treated mice after the forced swim test.

Table 1
Effect of saline, imipramine and NMD-Lipo in tail suspension test in mice.

Groups	Immobility time (s)	Values of immobility (%)
Saline	224.3 ± 0.86	–
Imipramine 50 mg/kg	75.00 ± 0.68^{a}	↓ 66.56[a]
NMD-Lipo 0.1 mg/kg	$49.25 \pm 0.96^{a,b}$	↓ 78.04%[a] ↓ 34.33%[b]
NMD-Lipo 1 mg/kg	$39.38 \pm 0.88^{a,b,c}$	↓ 82.44%[a] ↓ 47.49%[b] ↓ 20.04%[c]
NMD-Lipo 10 mg/kg	$26.88 \pm 1.24^{a,b,c,d}$	↓ 88.01%[a] ↓ 64.16%[b] ↓ 45.42%[c] ↓ 31.74%[d]

Values are mean ± E.P.M. of the immobility time in the tail suspension test of 8 mice (by group) used in the experiments.

[a] $p < 0.05$, when compared with saline group (negative control).

[b] $p < 0.05$, when compared with imipramine group (positive control).

[c] $p < 0.05$, when compared with NMD-Lipo 0.1 group.

[d] $p < 0.05$, when compared with NMD-Lipo 1 group (ANOVA followed by Student–Newman–Keuls *t*-test as *post hoc* test).

2. Results

2.1. Drug content and encapsulation rate of NMD-Lipo

NMD-Lipo presented a drug content of 0.96 ± 0.74 mg/mL and encapsulation efficiency of $99 \pm 0.67\%$. These results are in agreement with our previous studies (Moreno et al., 2014, 2015).

2.2. Tail suspension test

The results are summarized in Table 1. In the tail suspension test, the animals treated with a dose of 50 mg/kg of imipramine showed a significant decrease in immobility time (66.6%) when compared with the saline-treated group. The mice treated with NMD-Lipo at the doses of 0.1, 1 and 10 mg/kg also showed a reduction in immobility time (78.0%, 82.4% and 88.0%, respectively) in comparison with the saline-treated group.

2.3. Forced swim test

The results are summarized in Table 2. In the forced swim test, there was a decrease in the immobility time of imipramine and paroxetine-treated mice (63.3% and 56.3%, respectively). In addition, an increase in total immobility duration in mice treated with reserpine (27.7%) when compared with saline-treated group was found. The animals receiving NMD-Lipo at doses of 0.1, 1 and 10 mg/kg showed a significant reduction in immobility time (88.6%, 95.2% and 97.4%, respectively) compared with the saline-treated group.

The mice treated with NMD-Lipo at a dose of 10 mg/kg plus imipramine, paroxetine and reserpine, showed an increase in swimming time (90.3%, 69.0% and 48.1%, respectively) when compared to the saline-treated group.

2.4. Measurement of MAO_B activity

The results are summarized in Fig. 1. In the measurements of MAO_B activity, there were no significant differences in animals treated with imipramine, paroxetine and reserpine, when compared with the saline-treated group. There was a significant decrease in MAO_B activity of animals treated with NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (41.2%, 55.9% and 73.5% respectively)

Table 2
Effect of saline, imipramine, paroxetine, reserpine, NMD-Lipo and their associations in forced swim test in mice.

Groups	Immobility time (s)	Values of Immobility (%)
Saline	224.3 ± 0.86	–
Imipramine 50 mg/kg	75.00 ± 0.68[a]	↓66.56%[a]
Paroxetine 20 mg/kg	133.0 ± 3.28[a]	↓ 40.70%[a]
Reserpine 0.25 mg/kg	263.70 ± 2.15[a]	↑14.94%[a]
NMD-Lipo 0.1 mg/kg	49.25 ± 0.96[a,b,c,d]	↓78.04%[a] ↓34.33%[b] ↓62.97%[c] ↓81.32%[d]
NMD-Lipo 1 mg/kg	39.38 ± 0.88[a,b,c,d,e]	↓82.44%[a] ↓47.49%[b] ↓70.39%[c] ↓85.06%[d] ↓20.04%[e]
NMD-Lipo 10 mg/kg	26.88 ± 1.24[a,b,c,d,e,f]	↓88.01%[a] ↓64.16%[b] ↓79.78%[c] ↓89.80%[d] ↓45.42%[e] ↓31.74%[f]
NMD-Lipo 10 mg/kg plus Imipramine 50 mg/kg	18.40 ± 9.35[a,b,g]	↓91.8%[a] ↓75.47% [b] ↓31.54%[g]
NMD-Lipo 10 mg/kg plus Paroxetine 20 mg/kg	59.00 ± 10.05[a,c,g]	↓ 73.7%[a] ↓55.64%[c] ↑54.44%[g]
NMD-Lipo 10 mg/kg plus Reserpine 0.25 mg/kg	98.90 ± 9.58[a,d,g]	↓ 55.91%[a] ↓ 62.49%[d] ↑ 72.82%[g]

Values are mean ± S.E.M. of the immobility time in forced swim test of 8 mice (by group) used in the experiments.

[a] $p < 0.05$, when compared with saline group (negative control).
[b] $p < 0.05$, when compared with imipramine group (positive control).
[c] $p < 0.05$, when compared with paroxetine group (positve control).
[d] $p < 0.05$, when compared with reserpine group (positive control).
[e] $p < 0.05$, when compared with NMD-Lipo 0.1 group.
[f] $p < 0.05$, when compared with NMD-Lipo 1 group.
[g] $p < 0.05$, when compared with NMD-Lipo 10 group (ANOVA followed by Student–Newman–Keuls *t*-test as *post hoc* test).

compared with the animals treated with saline ($p < 0.05$). In addition, a significant decrease in enzymatic activity of mice treated with NMD-Lipo was observed at a dose of 10 mg/kg with the subsequent addition of imipramine, paroxetine and reserpine (44.1%, 56.2% and 61.8% respectively) when compared with the saline-treated group ($p < 0.05$).

3. Discussion

In the tail suspension test, the administration of NMD encapsulated into liposomes at doses of 0.1, 1 and 10 mg/kg was able to promote a greater reduction in immobility time of animals (34.3%, 47.5% and 64.2%) than imipramine treatment. This result suggests that NMD-Lipo presents a significant dose-dependent antidepressant-like activity in mice, even better than that of the drug chosen as a positive control.

In the forced swim test, the immobility time of mice treated with NMD-Lipo at doses of 0.1, 1 and 10 mg/kg was reduced. Again, the effect was dose-dependent. This reduction was significantly greater than that found in the animals treated with imipramine (34.3%, 47.9% and 64.16% higher, respectively) and paroxetine (63.0%, 70.4% and 79.8% greater, respectively). These findings reinforced the hypothesis that states that liposomal NMD provides a better antidepressant-like activity in mice than that of the positive control.

Furthermore, the animal groups treated with the combination of NMD-Lipo and antidepressant drugs showed a lower immobility time and greater antidepressant-like effect than groups treated only with imipramine or paroxetine. The satisfactory results in the administration of both NMD-Lipo and antidepressant drugs suggest an indication of combined therapy to treat depression.

Mice treated with the combination of NMD-Lipo at a dose of 10 mg/kg and reserpine showed an increase of 78.1% in immobility time compared with the animals treated only with NMD-Lipo. This finding can be explained by the fact that the administration of reserpine in rodents depletes brain monoamine oxidases (Souza et al., 2013), which is the opposite effect of drugs used in the treatment of depression. These data may suggest a possible mechanism of action for the antidepressant activity of NMD-Lipo related to an increase in the concentration of brain monoamine oxidases.

In the measurement of MAO_B activity, it was observed that treatment with saline, imipramine, paroxetine, and reserpine did not cause the inhibition of MAO_B. On the other hand, treatment with NMD-Lipo at all doses tested (0.1, 1 and 10 mg/kg) resulted in a significant decrease in enzyme activity. This may suggest that NMD-Lipo causes a dose-dependent MAO_B inhibition, which might be responsible for the NMD antidepressant effect. Animals treated with combinations of NMD-Lipo and paroxetine, imipramine and reserpine showed lower MAO_B activity than untreated animals. However, those animals presented higher enzymatic activity than those treated only with the liposomal NMD at a dose of 10 mg/kg. This is due to the fact that paroxetine, imipramine and reserpine do not cause enzyme inhibition.

Depression has been recognized and treated by different therapeutic approaches over centuries (Rodrigues et al., 2014). In the

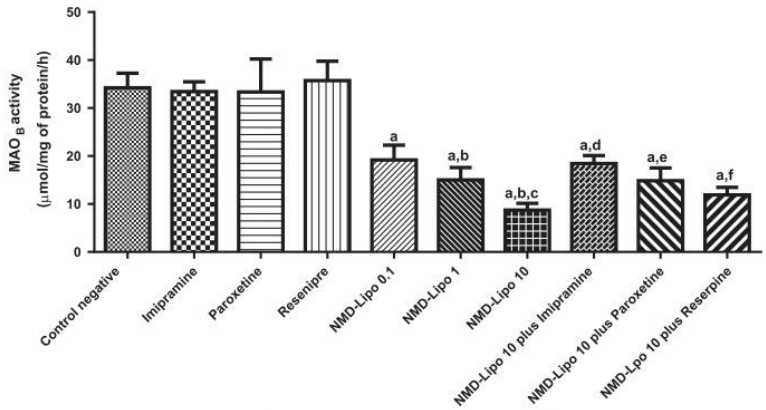

Fig. 1. MAOB activity in mice hippocampus after treatment with saline, imipramine, paroxetine, reserpine, NMD-Lipo and their combinations in the forced swim test. Values are mean ± E.P.M. of the MAO_B activity in mice hippocampus after forced swim test of 8 mice/group used in the experiments. [a]$p < 0.05$, when compared with negative control (vehicle); [b]$p < 0.05$, when compared with NMD-Lipo 0.1 group; [c]$p < 0.05$, when compared with NMD-Lipo 1 group; [d]$p < 0.05$, when compared with imipramine group (positive control); [e]$p < 0.$)05, (when compared with paroxetine group (positive control); [f]$p < 0.05$,) when compared with reserpine group (positive control; ANOVA followed by Student–Newman–Keuls *t*-test as *post hoc* test).

present study, imipramine was chosen as a positive control because it is the oldest tricycle antidepressant, and is commonly used in the treatment of endogenous depression and illness-associated depression (Obuchowicz et al., 2012). The pharmacological action of this drug produces an increase in the concentration of monoamines through presynaptic monoamine reuptake inhibition (Yasuda et al., 2014).

Paroxetine has also been used as a positive control because it is a selective serotonin reuptake inhibitor widely used in therapy of depression (Murata et al., 2013). Reserpine is an alkaloid isolated from *Rauwolfia serpentina*, which was chosen as a negative control since it depletes monoamines by irreversibly blocking vesicular monoamine transporter. In mice, reserpine causes various symptoms presented in depression, such as hypoactivity, lethargy and anhedonia (Kyzar et al., 2013). The doses of imipramine, paroxetine and reserpine were chosen according to the literature (Campêlo et al., 2011 and Oliveira et al., 2013).

As reported, excessive calcium influx through the membrane and increased intracellular calcium concentrations play a role in the physiopathology of neuropsychiatric diseases (Dubovsky et al., 2014). In this scenario, nimodipine administered at a dose of 5 mg/kg showed antidepressant properties in rats, as previously reported (Katagiri et al., 2001). Even though this calcium channel antagonist has also proved to be useful in the treatment of affective illness in humans, it has never been administered as a single component of depression therapy; instead, it is always used in combination with other antidepressants (Frye et al., 2003, Grunze et al., 1996 and Pazzaglia et al., 1995). This is probably due to NMD high first-pass metabolism in the liver, which results in low bioavailability (Salgado-Figueroa et al., 2015).

Previous research has shown that NMD encapsulated in liposomes presents effects on the CNS significantly greater than the free drug in several tests, suggesting that the liposomes promoted a controlled drug release by increasing its bioavailability and consequently its effect (Moreno et al., 2014, 2015). Hence, this liposomal formulation was chosen for the tests of antidepressant activity of the drug.

We have used the tail suspension test for determining a possible antidepressant-like activity of NMD-Lipo in treated mice. The forced swim test was carried out to confirm the NMD-Lipo antidepressant-like activity suggested in the tail suspension test. The doses were chosen based on previous studies with free nimodipine (Frye et al., 2003, Grunze et al. 1996, Katagiri et al. 2001 and Pazzaglia et al. 1995) and liposomal NMD (Moreno et al., 2014', 2015).

The significant reduction in immobility time in mice treated with NMD-Lipo in the tail suspension and forced swim tests, as well as the increased immobility of rodents treated with reserpine and NMD-Lipo, are evidence that the liposomal NMD displays drug antidepressant-like activity by increasing the concentration of brain monoamines. The decrease in MAOB activity in animals treated with the liposomal formulation containing NMD strengthens this hypothesis. More studies are needed to unravel the antidepressant activity of NMD-Lipo; however, NMD-loaded liposomes appear to be a promising approach for depression therapy.

4. Experimental procedure

4.1. Animals

Eight-week-old male Swiss mice (weighing 25–30 g) were obtained from Central Animal House of the Federal University of Piauí (Teresina, Brazil). Animals were housed in polypropylene cages with wire covers at 25 ± 2 °C under 12:12-h light–dark cycle (light on from 07:00 h to 19:00 h). They were supplied with commercial food pellets (Nutrilabor, Campinas, Brazil) and water ad libitum, and were acclimatized to laboratory conditions for 7 days before being subjected to experimental assays. The experimental protocol and procedures was approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA/UFPI N° 014/11). All experiments were performed according to the Guide of care and use of laboratory animals from the US Department of Health and Human Services (Washington, 1985).

4.2. Drugs and chemicals

Cholesterol (Chol), trehalose, nimodipine, imipramine hydrochloride, paroxetine hydrochloride, reserpine and benzylamine were obtained from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Solvents and other chemicals supplied by from Merck (Darmstadt, Germany). All drugs were administered intraperitoneally. To study the association of the formulation with antidepressant drugs and reserpine, the drugs used as control (imipramine, paroxetine and reserpine) were administrated 30 min before NMD-Lipo.

4.3. Preparation and characterization of liposomes containing nimodipine (NMD-Lipo)

Liposomes containing nimodipine (NMD-Lipo) were prepared and characterized as previously reported (Moreno et al., 2014). The liposomal formulation was produced using the method of hydrating thin lipid film. The content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm. The encapsulation efficiency of nimodipine into liposomes was determined by the technique of ultrafiltration/ultracentrifugation using Ultrafree® units (Millipore, USA). A liposomal sample aliquot (400 μL) was transferred to filtering units and subjected to ultracentrifugation at 8776 g for 1 h. The amount of encapsulated nimodipine was obtained from the difference between the total quantity measured in the formulation and the filtrate obtained after centrifugation.

4.4. Tail suspension test

The mice were divided into five groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), imipramine at a dose of 50 mg/kg (positive control) and NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10 respectively). The animals were then subjected to the tail suspension test after 30 min of drug administration.

The total duration of immobility induced by tail suspension was measured according to a previously reported method (Steru et al., 1985). Mice were suspended 58 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The time during which mice remained immobile was quantified during a test period of 6 min. Mice were considered immobile only when they hung passively and completely motionless. Immobility was regarded as depression-like behavior (behavioral despair).

4.5. Forced swim test

The mice were divided into ten groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), imipramine at a dose of 50 mg/kg, paroxetine at a dose of 20 mg/kg, reserpine at a dose of 0.25 mg/kg (positive controls) and NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10, respectively). To study the effect of the formulation tested in combination with antidepressant drugs and reserpine,

mice received NMD-Lipo at a dose of 10 mg/kg in combination with imipramine at a dose of 50 mg/kg (group NMD-Lipo 10 plus Imipramine), NMD-Lipo at a dose of 10 mg/kg in combination with paroxetine at a dose of 20 mg/kg (group NMD-Lipo 10 plus Paroxetine) and NMD-Lipo at a dose of 10 mg/kg in combination with reserpine at a dose of 0.25 mg/kg (group NMD-Lipo 10 plus Reserpine). Mice were subjected to the forced swim test 30 min after drug administration.

The test procedure was carried out according to the method describe by Porsolt et al. (1977). In brief, mice were individually forced to swim in glass beakers (height 25 cm, diameter 10 cm) containing 15 cm of water maintained at a temperature of 25 °C. The time that the mice remained immobile was quantified during a test period of 5 min. The total immobility time was defined as the total amount of time during which the mice remained immobile or made only small limb movements necessary for floating. Immobility was regarded as depression-like behavior (behavioral despair).

4.6. Preparation of brain and liver mitochondria for MAO_B assay

After the forced swim test, the mice were sacrificed by cervical dislocation and their brains and livers were removed and dissected. Mitochondria were isolated from liver and brain of animals according to the method described by Gazzotti et al. (1979). The organs were weighed and placed in a homogenization medium (pH: 7.4, temperature: 4 °C). Subsequently, the organs were sliced and grounded to obtain a uniform homogenate. Homogenates were centrifuged at 1100 g for 15 min at 4 °C and the supernatants were collected and centrifuged again at 1100 g rpm for 25 min at 4 °C. The pellets obtained were resuspended in 25 mL of homogenization medium and then centrifuged at 1100 g rpm for 25 min.

The mitochondrial pellets were resuspended in a homogenization medium to a final volume of 4 mL. The samples of isolated mitochondria were used to determine the protein concentration by applying the method described by Lowry et al. (1951).

4.7. Measurement of MAO_B activity

MAO_B activity was measured using benzylamine as substrate, according to the method of Tabor et al. (1954). A sample of 620 μg of mitochondrial proteins (90 μL) was dissolved in 2 mL of 0.1 M sodium phosphate buffer (pH 7.4). The reaction was started by the addition of 1 mM benzylamine and the progress of the reaction (benzaldehyde formation) was measured in a spectrophotometer UV/visible at 250 nm. The maximum rate of MAO_B activity was expressed as micromoles per milligram of protein per minute. The activity of the MAO_B enzyme was assessed for 6 h after isolation of mitochondria.

4.7.1. Statistical analyses

Data values are expressed as mean ± standard error of mean (SEM) of the number of animals used in the experiments. Differences between groups were determined by analysis of variance (ANOVA) followed, when a difference was detected, by the Student–Newman–Keuls *t*-test with post hoc test. The significance level for rejecting the null hypothesis was always $p \leq 0.05$.

Conflicts of interest

The authors declare no conflict of interest.

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References

Aslan, A., Gurelik, M., Cemek, M., Goksel, H.M., Buyukokuroglu, M.E., 2009. Nimodipine can improve cerebral metabolism and outcome in patients with severe head trauma. Pharmacol. Res. 59, 120–124.

Bege, N., Renette, T., Endres, T., Beck-Broichsitter, M., Hänggi, D., Kissel, T., 2013. In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm. Eur. J. Pharm. Biopharm. 84, 99–105.

Campêlo, L.M.L., Sá, C.G., Almeida, A.A.C., Costa, J.P., Marques, T.H.C., Feitosa, C.M., Saldanha, C.B., Freitas, R.M., 2011. Sedative, anxiolytic and antidepressant activities of Citrus limon (Burn) essential oil in mice. Pharmazie 66, 623–627.

Deussing, J.M., 2006. Animal models of depression. Drug. Discov. Today Dis. Model. 3, 375–383.

Dubovsky, S.L., Daurignac, E., Leonard, K.E., 2014. Increased platelet intracellular calcium ion concentration is specific to bipolar disorder. J. Affect Disord. 164, 38–42.

Dubovsky, S.L., Murphy, J., Christiano, J., Lee, C., 1992. The calcium second messenger system in bipolar disorders: Data supporting new research directions. J. Neuropsychiatry Clin. Neurosci. 4, 3–14.

Dubovsky, S.L., Thomas, M., Hijazi, A., Murphy, J., 1994. Intracellular calcium signalling in peripheral cells of patients with bipolar affective disorder. Eur. Arch. Psychiatry Clin. Neurosci. 241, 229–234.

Dubovsky, S.L., Christlano, J., Daniell, L.C., Franks, R.D., Murphy, J., Adler, L., Baker, N., Harris, R.A., 1989. Increased intracellular calcium concentration in patients with bipolar affective disorders. Arch. Gen. Psychiatry 46, 632–638.

Finberg, J.P.M., 2014. Update on the pharmacology of selective inhibitors of MAO-A and MAO-B: Focus on modulation of CNS monoamine neurotransmitter release. Pharmacol. Ther. 143, 133–152.

Fornaro, M., Martino, M., Mattei, C., Prestia, D., Vinciguerra, V., Berardis, D., Pasquale, C., Iasevoli, F., Mungo, S., Fornaro, P., 2014. Duloxetine-bupropion combination for treatment-resistant atypical depression: a double-blind, randomized, placebo-controlled trial. Eur. Neuropsychopharmacol. 24, 1269–1278.

Frye, M.A., Pazzaglia, P.J., George, M.S., Luckenbaugh, D.A., Vanderham, E., Davis, C. L., Rubinow, D.R., Post, R.M., 2003. Low CSF somatostatin associated with response to nimodipine in patients with affective illness. Biol. Psychiatry 53, 180–183.

Gazzotti, P., Malmstrom, K., Crompton, M., 1979. Preparation and Assay of Animal Mitochondria and Submitochondrial Vesicles. Membrane biochemistry. A Laboratory Manual on Transport and Bioenergetics. Springer., New York, pp. 62–76.

Ghazaleh, H.A., Lalies, M.D., Nutt, D.J., Hudson, A.L., 2015. The modulatory action of harmane on serotonergic neurotransmission in rat brain. Brain Res. 1597, 56–64.

Grunze, H., Walden, J., Wolf, R., Berger, M., 1996. Combined treatment with lithium and nimodipine in a bipolar i manic syndrome. Prog. Neuro-Psychopharmacal. Biol. Psychiatry 20, 419–426.

Justin, A., Sathishkumar, M., Sudheer, A., Shanthakumari, S., Ramanathan, M., 2014. Non hypotensive dose of telmisartan and nimodipine produced synergistic neuroprotective effect in cerebral ischemic model by attenuating brain cytokine levels. Pharmacol. Biochem. Behav. 112, 61–73.

Katagiri, H., Kagaya, A., Nakae, S., Morinobu, S., Ymawaki, S., 2001. Modulation of serotonin2a receptor function in rats after repeated treatment with dexamethasone and L-type calcium channel antagonist nimodipine. Prog. Neuro-Psychopharmacal. Biol. Psychiatry 25, 1269–1281.

Kim, S., Lee, Y., Kim, H., Lee, D., Woo, D., Choi, C., Chae, J., Choe, B., 2010. Desipramine attenuates forced swim test-induced behavioral and neurochemical alterations in mice: an in vivo 1H-MRS study at 9.4 T. Brain Res. 1348, 105–113.

Kumar, V., Bhat, Z.A., Kumar, D., 2013. Animal models of anxiety: a comprehensive review. J. Pharmacol. Toxicol. Methods 68, 175–183.

Kyzar, E., Stewart, A.M., Landsman, S., Collins, C., Gebhardt, M., Robinson, K., Kalueff, A.V., 2013. Behavioral effects of bidirectional modulators of brain monoamines reserpine and D-amphetamine in zebra fish. Brain Res. 1527, 108–116.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with follin phenol reagent. J. Biol. Chem. 193, 265–275.

Maigaard, K., Hageman, I., Jørgensen, A., Jørgensen, M.B., Wörtwein, G., 2012. Electroconvulsive stimulations prevent chronic stress-induced increases in L-type calcium channel mRNAs in the hippocampus and basolateral amygdala. Neurosci. Lett. 516, 24–28.

Moreno, L.C.G.A.I., Oliveira, G.Z.S., Cavalcanti, I.M.F., Santos-Magalhães, N.S., Rolim, H.M.L., Freitas, R.M., 2014. Development and evaluation of liposomal formulation containing nimodipine on anxiolytic activity in mice. Pharmacol. Biochem.

Behav. 116, 64–68.
Moreno, L.C.G.A.I., Cavalcanti, I.M.F., Prabodh, S., Santos-Magalhães, N.S., Rolim, H. M.L., Freitas, R.M., 2015. Acute toxicity and anticonvulsant activity of liposomes containing nimodipine on pilocarpine-induced seizures in mice. Neurosci. Lett. 585, 38–42.
Murata, Y., Kamishioiri, Y., Tanaka, K., Sugimoto, H., Sakamoto, S., Kobayashi, D., Mine, K., 2013. Severe sleepiness and excess sleep duration induced by paroxetine treatment is a beneficial pharmacological effect, not an adverse reaction. J. Affect. Disord. 150, 1209–1212.
N'Gouemo, P., 2013. Probing the role of the sodium/calcium exchanger in pentylenetetrazole induced generalized seizures in rats. Brain Res. Bull. 90, 52–57.
Obuchowicz, E., Bielecka, A.M., Paul-Samojedny, M., Pudełko, A., Kowalski, J., 2012. Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture. Pharmacol. Rep. 66, 34–43.
Oliveira, F.R.A.M., Cerqueira, G.S., Freitas, R.L.M., Costa-Júnior, J.S., Feitosa, C.M., Freitas, R.M., 2013. Anxiolytic- and antidepressant-like effects of the ethanolic extract from Citrus limon plant widely used in Northeastern Brazil. Afr. J. Pharm. Pharmacol. 30, 2173–2179.
Papakostas, G.I., 2009. Managing partial response or nonresponse: switching, augmentation, and combination strategies for major depressive disorder. J. Clin. Psychiatry 70 (6), 16–25.
Pazzaglia, P.J., George, M.S., Post, R.M., Rubinow, D.R., Davis, C.L., 1995. Nimodipine increases CSF somatostatin in affectively ill patients. Neuropsychopharmacology 13, 75–83.
Porsolt, R.D., Bertin, A., Jalfre, M., 1977. Behavioral despair in mice: a primary screening test for antidepressants. Arch. Int. Pharmacodyn. Ther. 229, 327–336.
Rodrigues, R., Petersen, R.B., Perry, G., 2014. Parallels between major depressive disorder and Alzheimer's disease: role of oxidative stress and genetic vulnerability. Cell. Mol. Neurobiol. 34, 925–949.
Roedding, A.S., Li, P.P., Warsh, J.J., 2006. Characterization of the transient receptor potential channels mediating lysophosphatidic acid-stimulated calcium mobilization in B lymphoblasts. Life Sci. 80, 89–97.
Rollema, H., Guanowsky, V., Mineur, Y.S., Shrikhande, A., Coe, J.W., Seymour, P.A., Picciotto, M.R., 2009. Varenicline has antidepressant-like activity in the forced swim test and augments sertraline's effect. Eur. J. Pharmacol. 605, 14–16.
Salgado-Figueroa, P., Gutiérrez, C., Squella, J.A., 2015. Carbon nanofiber screen printed electrode joined to a flow injection system for nimodipine sensing. Sens. Actuators B: Chem. 220, 456–462.
Schmidt, M.V., 2011. Animal models for depression and the mismatch hypothesis of disease. Psychoneuroendocrinology 36, 330–338.
Socała, K., Nieoczym, D., Wyska, E., Poleszak, E., Wlaź, P., 2012. Influence of sildenafil on the antidepressant activity of bupropion and venlafaxine in the forced swim test in mice. Pharmacol. Biochem. Behav. 103, 273–278.
Souza, R.R., França, S.L., Bessa, M.M., Takahashi, R.N., 2013. The usefulness of olfactory fear conditioning for the study of early emotional and cognitive impairment in reserpine model. Behav. Process. 100, 67–73.
Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85, 367–370.
Sun, C., Wang, J., Liu, J., Qiu, L., Zhang, W., Zhang, L., 2013. Liquid proliposomes of nimodipine drug delivery system: preparation, characterization, and pharmacokinetics. AAPS PharmSciTech 14, 332–338.
Sygnecka, K., Heinea, C., Scherf, N., Fasold, M., Binder, H., 2015. Nimodipine enhances neurite outgrowth in dopaminergic brain slice co-cultures. Int. J. Dev. Neurosci. 40, 1–11.
Tabor, C.W., Tabor, H., Rosenthal, S.M., 1954. Purification of amine oxidase from beef plasma. J. Biol. Chem. 208, 645–662.
Wang, Z., Gu, J., Wang, X., Xie, K., Luan, Q., Wan, M., Zhang, Q., Jiang, H., Liu, D., 2013. Antidepressant-like activity of resveratrol treatment in the forced swim test and tail suspension test in mice: the HPA axis, BDNF expression and phosphorylation of ERK. Pharmacol. Biochem. Behav. 112, 104–110.
Wittman, D., 2014. Darwinian depression. J. Affect. Disord. 168, 142–150.
Wong, H.L., Wu, X.Y., Bendayan, R., 2012. Nanotechnological advances for the delivery of CNS therapeutics. Adv. Drug Deliv. Rev. 64, 686–700.
Xu, G.Z., Wang, M.D., Liu, K.G., Bai, Y.A., Wua, W., Li, W., 2013. A meta-analysis of treating acute traumatic brain injury with calcium channel blockers. Brain Res. Bull. 99, 41–47.
Yasuda, S., Yoshida, M., Yamagata, H., Iwanaga, Y., Suenaga, H., Ishikawa, K., Nakano, M., Okuyama, S., Furukawa, Y., Furukawa, S., Ishikawa, T., 2014. Imipramine ameliorates pain-related negative emotion via induction of brain-derived neurotrophic factor. Cell. Mol. Neurobiol. 34, 1199–1208.
Yingling, D.R., Utter, G., Vengalil, S., Mason, B., 2002. Calcium channel blocker, nimodipine, for the treatment of bipolar disorder during pregnancy. Am. J. Obstet. Gynecol. 187, 1711–1712.