UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

GABRIEL BARROS RODRIGUES

DESENVOLVIMENTO E AVALIAÇÃO DE SISTEMAS DE LIBERAÇÃO CONTROLADA DE DIETILCARBAMAZINA NANOENCAPSULADA EM DIFERENTES FASES DE INFLAMAÇÃO HEPÁTICA EXPERIMENTAL

Recife
2018

GABRIEL BARROS RODRIGUES

DESENVOLVIMENTO E AVALIAÇÃO DE SISTEMAS DE LIBERAÇÃO CONTROLADA DE DIETILCARBAMAZINA NANOENCAPSULADA EM DIFERENTES FASES DE INFLAMAÇÃO HEPÁTICA EXPERIMENTAL

Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas.

Orientadora: Profª. Dra. Christina Alves Peixoto

Recife

2018

Dados Internacionais de Catalogação na Publicação (CIP) de acordo com ISBD

Rodrigues, Gabriel Barros

Desenvolvimento e avaliação de sistemas de liberação controlada de dietilcarbamazina nanoencapsulada em diferentes fases de inflamação hepática experimental / Gabriel Barros Rodrigues - 2018.

56 folhas: il., fig.
Orientadora: Christina Alves Peixoto
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Ciências Biológicas. Recife, 2018.
Inclui referências e anexos

1. Dietilcarbamazina 2. Fígado - doenças 3. Nanobiotecnologia I. Peixoto, Christina Alves II. Título

616.362 CDD (22.ed.) UFPE/CB-2018-391

Elaborado por Claudina Karla Queiroz Ribeiro CRB4/1752

GABRIEL BARROS RODRIGUES

DESENVOLVIMENTO E AVALIAÇÃO DE SISTEMAS DE LIBERAÇÃO CONTROLADA DE DIETILCARBAMAZINA NANOENCAPSULADA EM DIFERENTES FASES DE INFLAMAÇÃO HEPÁTICA EXPERIMENTAL

Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas.

Aprovada em: 07/08/2018.

BANCA EXAMINADORA:

_____
Dra. Christina Alves Peixoto (Orientadora) - IAM/FIOCRUZ

_____
Dra. Ana Célia Oliveira dos Santos – UPE

_____
Dra. Sheilla Andrade de Oliveira - IAM/FIOCRUZ

_____
Dra. Márcia Vanusa da Silva - UFPE

_____
Dra. Marcia Regina Piuvezam – UFPB

Dedico este trabalho à **Deus** que está presente em todos os momentos da minha vida, realizando tudo da melhor maneira possível. Aos meus pais **Antonio** e **Anete**, por sempre confiarem em mim, nunca duvidarem da minha capacidade e não medirem esforços pelo meu sucesso. À minha irmã **Germana**, por sua paciência e eterna palavra de incentivo e carinho em todas as horas. À **Ingrid**, por se tornar o amor da minha vida, sendo a companhia de todas as horas e ter papel fundamental na conclusão deste trabalho.

AGRADECIMENTO

Agradeço primeiramente a DEUS, que tornou todo este esforço possível e direcionou todas as coisas da maneira mais perfeita possível, que só Ele sabe fazer.

Agradeço a minha família por todo amor e carinho a mim concedidos, pois só vocês sabem o quanto foram importantes para mim em todos os momentos da minha vida e em especial nestes últimos meses.

Agradeço a minha namorada Ingrid pela sua compreensão e inestimável apoio, não só durante este trabalho, como também pelas diversas coisas pelas quais passamos. Sem você, nada disso teria sido possível.

Agradeço a minha orientadora Christina não apenas pelo inestimável apoio intelectual e material, mas sobre tudo por sua amizade.

Agradeço as minhas colegas de trabalho pelas risadas, experiências compartilhadas, paciência e todos os bons momentos dessa caminhada.

Por fim, agradeço aos meus amigos, por entenderam e perdoarem minha ausência, pelo incentivo e companhia que sempre me foram gratos.

“Se eu vi mais longe (...) e se cheguei até aqui,

foi porque me apoiei no ombro de gigantes.”

(Isaac Newton)

RESUMO

Apesar do avanço econômico e sanitário, pouco progresso tem sido alcançado no tratamento de pacientes em estágios avançados de doenças hepáticas, sendo muitas vezes o transplante hepático a única alternativa. A dietilcarbamazina (DEC) é um medicamento amplamente utilizado na terapêutica da filariose e apresenta excelente tolerabilidade e poucos efeitos colaterais. Além disso, sua eficaz ação anti-inflamatória tem sido bastante explorada em nosso laboratório, especialmente no fígado. O uso de nanoestruturas como carreadores de fármacos, tais como nanocápsulas, que criam sistemas de liberação controlada, já estão na clínica ou em estudos pré-clínicos, de forma a melhorar significativamente a eficácia de muitos protocolos terapêuticos e diagnósticos. O objetivo do trabalho foi desenvolver e caracterizar o nanoencapsulamento da DEC (NANO-DEC) nas doses de 5 e 12.5 mg/kg e avaliar pela primeira vez sua ação em diferentes fases da inflamação hepática em murinos induzida por tetracloreto de carbono (CCl_4). Foram avaliados três fases de inflamação hepática: 1 - inflamação aguda, com tratamento prévio com a NANO-DEC por 6 dias e ao final, uma única injeção de CCl_4, 2 - inflamação crônica e 3 – fibrose, que consistiram em duas injeções semanais de CCl_4 por 6 e 8 semanas respectivamente, e nos 6 últimos dias de cada modelo foi realizado o tratamento com a NANO-DEC. Os resultados demonstraram a NANO-DEC 12.5 mg/kg apresentou ação anti-inflamatória e anti-fibrótica superior que sua formulação tradicional em todos os modelos avaliados, restaurando a função hepática e diminuindo a expressão de marcadores inflamatórios (IL-1β, TNF-α, COX-2, iNOS e NO) e fibróticos (TGF-β, TIMP-1 e MMP-2), com metade do regime de tratamento e apenas um quarto da dose da formulação tradicional. Estes resultados abrem novas possibilidades de investigação para o tratamento de doenças hepáticas crônicas.

Palavras-chave: Dietilcarbamazina. Nanopartículas. Inflamação. Fígado. Terapias. Via intraperitoneal.

ABSTRACT

Despite the economic and health advances, little progress has been achieved in the treatment of patients in advanced stages of liver disease, and liver transplantation is often the only alternative. Diethylcarbamazine (DEC) is a drug widely used in the treatment of filariasis and has excellent tolerability and few side effects. In addition, its effective anti-inflammatory action has been extensively explored in our laboratory, especially in the liver. The use of nanostructures as drug carriers, such as nanocapsules, which create controlled release systems, are already in clinical or preclinical studies, in order to significantly improve the efficacy of many therapeutic and diagnostic protocols. The aim of this study was to develop and characterize the nanoencapsulation of DEC (NANO-DEC) at doses of 5 and 12.5 mg/kg and to assess for the first time its action in different stages of the hepatic inflammation in murines induced by carbon tetrachloride (CCl_4). Three stages of hepatic inflammation were evaluated: 1 - acute inflammation, with a prior treatment with NANO-DEC for 6 days and at the end, a single injection of CCl_4, 2 - chronic inflammation and 3 - fibrosis, which consisted of two weekly injections of CCl_4 for 6 and 8 weeks respectively, and in the last 6 days of each model, the treatment with NANO-DEC was performed. The results demonstrated that NANO-DEC 12.5 mg/kg presented superior anti-inflammatory and anti-fibrotic action than its traditional formulation in all models, restoring hepatic function and decreasing the expression of inflammatory (IL-1β, TNF-α, COX-2, iNOS and NO) and fibrotic markers (TGF-β, TIMP-1 and MMP-2), with half the treatment regimen and only a quarter of the traditional formulation dose. These results open new possibilities of investigation for the treatment of chronic hepatic diseases.

Keywords: Diethylcarbamazine. Nanoparticles. Inflammation. Liver. Therapies. Intraperitoneal route.

LISTA DE ILUSTRAÇÕES

Revisão da Literatura

Artigo I

Artigo II

LISTA DE TABELAS

Artigo I

LISTA DE ABREVIATURAS E SIGLAS

ALP	Fostatase Alcalina
α-SMA	Alpha Actina do Músculo Liso
ALT	Alanina Aminotransferase
AST	Aspartato Aminotransferase
CCL1	Ligante 1 de Quimiocina CC
CCL2	Ligante 2 de Quimiocina CC
CCl_3*	Radical Triclorometil
CCl3OO*	Radical Triclorometil Peróxido
CCl_4	Tetracloreto de Carbono
CYP2B	Citocromo P450 2B
CYP2E1	Citocromo P450 2E1
CYP3A	Citocromo P450 3A
COX-2	Ciclooxigenase 2
DEC	Dietilcarbamazina
DNA	Ácido Desoxirribonucleico
ECM	Matriz Extracelular
HCC	Carcinoma Hepatocelular
HDL	Lipoproteína de Alta Densidade
HSC	Célula Estrelada Hepática
IL-1β	Interleucina 1beta
iNOS	Óxido Nítrico-Sintase Induzível

LOX	Lipoxigenase
LPS	Lipopolissacarídeo
MMP	Metaloproteinase
NANO-DEC	Dietilcarbamazina nanoencapsulada
NF-κB	Fator Nuclear kappa B
NO	Óxido Nítrico
O_2	Oxigênio
PCL	Poli(ε-caprolactona)
PDGF	Fator de Crescimento Derivado de Plaquetas
PLA	Poliácido láctico
PLGA	Poli(ácido lático-co-ácido glicólico)
PNP	Nanopartícula polimérica
TIMP	Inibidor de Metaloproteinase
TGF-β	Fator de Transformação do Crescimento Beta
TNF-α	Fator de Necrose Tumoral Alpha

SUMÁRIO

1 INTRODUÇÃO

O fígado é o principal órgão responsável pelo metabolismo de drogas e outros metabólitos, consequentemente, é o principal órgão alvo para grande parte dos produtos químicos tóxicos. As doenças hepáticas representam um grave problema de saúde pública com alta morbidade e mortalidade. Por sua vez, a inflamação crônica do fígado é uma doença progressiva e lenta, causando disfunção do fígado e formação de fibrose (ROBINSON; HARMON; O 'FARRELLY, 2016).

Os tratamentos existentes para as hepatopatias são limitados e diferenciados, a depender da etiologia e/ ou persistência do estímulo. Quando as medidas terapêuticas não são eficientes, os pacientes podem evoluir para a cirrose (GE; RUNYON, 2016). O transplante de fígado passa, então, a ser o único tratamento disponível para os estes pacientes (MANNS, 2013).

O tetracloreto de carbono (CCl_4) é uma hepatotoxina bem conhecida que é amplamente utilizada para induzir lesões hepáticas tóxicas em animais experimentais. O desenvolvimento e estabilização da fibrose em modelo murino ocorre com a administração do CCl_4 durante 6 a 8 semanas (YANGUAS et al., 2016).

Dietilcarbamazina (DEC) é um derivado da piperazina (BUXTON; ROBERTSON; MARTIN, 2014). Devido à sua ação microfilaricida, a DEC tem sido utilizada no tratamento e controle da filariose desde 1947 (FISCHER et al., 2017; LANHAM; MWANRI, 2013). Apesar de mais de 70 anos de uso, o seu mecanismo de ação exato ainda não está claro (MCGARRY; PLANT; TAYLOR, 2005). No entanto, sabe-se que ela interfere na via de sinalização da ciclooxigenase (COX) e lipoxigenase (LOX) (PEIXOTO; SILVA, 2014).

Além disso, ao longo dos últimos 8 anos, nosso laboratório tem explorado a atividade anti-inflamatória de formualações tradicionais de DEC em modelos de eosinofilia pulmonar (QUETO et al., 2010), inflamação pulmonar aguda induzida por carragenina (RIBEIRO et al., 2014; SANTOS et al., 2014) e lipopolisacarídeo (FRAGOSO et al., 2017), inflamação hepática crônica induzida por álcool (DA SILVA et al., 2014; ROCHA, Sura Wanessa Santos; SILVA; et al., 2012; RODRIGUES et al., 2015), tetracloreto de carbono e desnutrição proteica (ROCHA, Sura Wanessa Santos; SANTOS; et al., 2012).

Nanoestruturas terapêuticas (carreadores) procuram direcionar fármacos ao sítio de ação desejado, podendo liberá-lo de forma controlada, mudando sua distribuição, para que se alcance maior eficácia terapêutica, com doses menores, e baixa toxicidade. Assim, tais sistemas de liberação controlada de fármacos, têm como objetivo tornar o composto ativo capaz de se acumular no órgão ou tecido-alvo de forma seletiva e quantitativa, independentemente do local e os métodos de sua administração. Estes sistemas podem retardar a liberação do princípio ativo, sustentar a sua liberação e/ou direcioná-lo a sítios específicos de ação (células, órgãos, micro- organismos) (KUMARI et al., 2014; MUDSHINGE et al., 2011).

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Analisar a ação de nanopartículas contendo dietilcarbarmazina (NANO-DEC) em diferentes fases do processo inflamatório hepático em camundongos C57BL/6.

1.1.2 Objetivos Específicos

1 – Desenvolver e caracterizar o efeito do nanoencapsulamento da DEC (12.5mg/kg e 5 mg/kg) na inflamação hepática aguda e crônica induzida por tetracloreto de carbono (CCl_4) em camundongos C57BL/6;

2 - Analisar o efeito do tratamento *in vivo* da DEC sobre a inflamação hepática induzida por CCl_4 através da microscopia óptica (histopatologia), bem como avaliar o conteúdo de colágeno;

3 - Examinar por meio de provas bioquímicas o funcionamento hepático (AST, ALT e ALP) e o perfil lipídico (colesterol, triglicerídeos e HDL);

4 - Analisar o efeito da NANO-DEC sobre o estresse oxidativo através das dosagns de óxido nítrico (NO) e glutationa redutase hepáticos.

4 - Caracterizar ação da NANO-DEC sobre a expressão dos marcadores inflamatórios e fibróticos: TNF-α, IL-1β, COX-2, iNOS, TGF-β; TIMP-1 e MMP-2.

2 REVISÃO DA LITERATURA

2.1 Função e Estrutura Hepática

O fígado, pesando cerca de 1,5 kg, é o segundo maior órgão do corpo (o maior é a pele). Situado no quadrante superior direito da cavidade abdominal, logo abaixo do diafragma, está subdividido em quatro lobos - direito, esquerdo, quadrado e caudado - dos quais os dois primeiros constituem a quase totalidade (Figura 1A). É um órgão multifuncional e dentre suas principais funções destacam-se a síntese, secreção e metabolismo proteico e lipídico, a secreção biliar, a regulação dos níveis glicêmicos e a metabolização de drogas e carboidratos (GARTNER; HIATT, 2007).

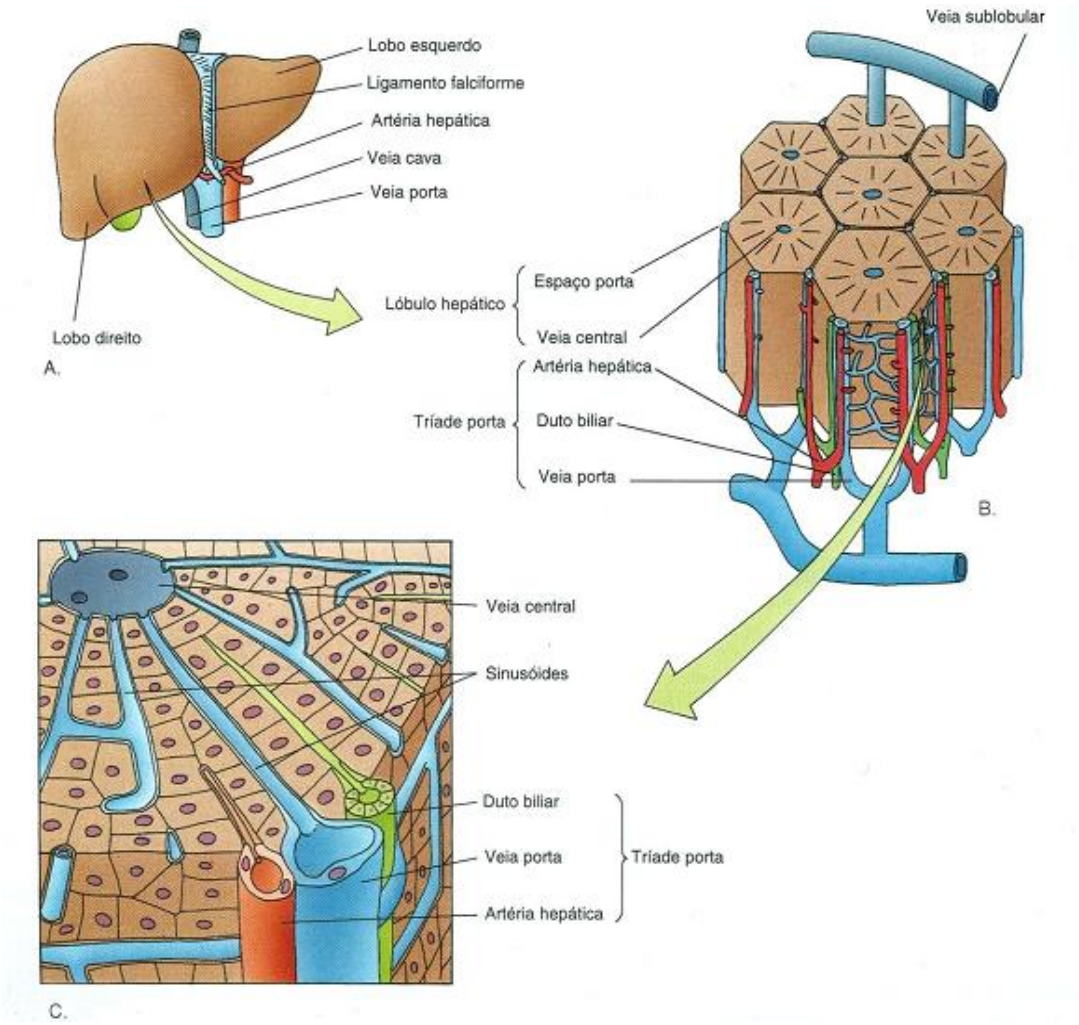
A célula hepática (hepatócito) é o componente estrutural básico do fígado, correspondendo a 80% da população celular. Estas células epiteliais estão agrupadas em placas interconectadas, os lóbulos hepáticos (Figura 1B,C e 2). Em certos animais (p. ex., porcos), os lóbulos são separados entre si por uma camada de tecido conjuntivo, o que não ocorre em humanos, nos quais os lóbulos estão em contato ao longo de grande parte de seu comprimento, tornando difícil o estabelecimento de limites exatos entre lóbulos diferentes. Em algumas regiões da periferia dos lóbulos existe tecido conjuntivo contendo ductos biliares, pequenos ramos da artéria hepática e ramos da veia porta (formando a tríade portal) (Figura 1C e 2), além de nervos e vasos linfáticos. Estas regiões, os espaços porta, estão presentes nos vértices dos lóbulos (JUNQUEIRA; CARNEIRO, 2013).

Os hepatócitos estão radialmente dispostos no lóbulo hepático formando fileiras, chamadas de cordões hepáticos, arranjados da periferia do lóbulo para o seu centro em torno de uma veia central, a veia centro-lobular (ou sublobular), anastomosando-se livremente. Entre os cordões de hepatócitos encontram-se capilares, os sinusóides hepáticos (Figura 1C). Capilares sinusóides são vasos irregularmente dilatados compostos por uma camada descontínua de células endoteliais fenestradas (JUNQUEIRA; CARNEIRO, 2013).

O fígado é envolvido pelo peritônio, que forma um epitélio pavimentoso simples cobrindo a cápsula de tecido conjuntivo denso não modelado (cápsula de Glisson) do órgão. A cápsula de Glisson se torna mais espessa no hilo, por onde a veia porta e a artéria hepática penetram no fígado e por onde saem os ductos hepáticos direito e esquerdo e os vasos linfáticos (GARTNER; HIATT, 2007).

O suprimento de sangue para o fígado deriva de duas fontes: da veia porta e da artéria hepática; a primeira fornece aproximadamente 75% do fluxo total de 1.500 mL/min, sangue pouco oxigenado e rico em nutrientes provenientes das vísceras abdominais. O restante, deriva da artéria hepática, que fornece sangue rico em oxigênio. Os pequenos ramos da vênula portal terminal e da arteríola hepática terminal, entram nos ácinos da tríade portal e então fluem através dos sinusóides entre placas de hepatócitos (BEERS; BERKOW, 2001).

Figura 1 - Desenho esquemático do fígado. A – anatomia macroscópica do fígado. B – lóbulos hepáticos. C – lóbulo hepático em maior aumento.

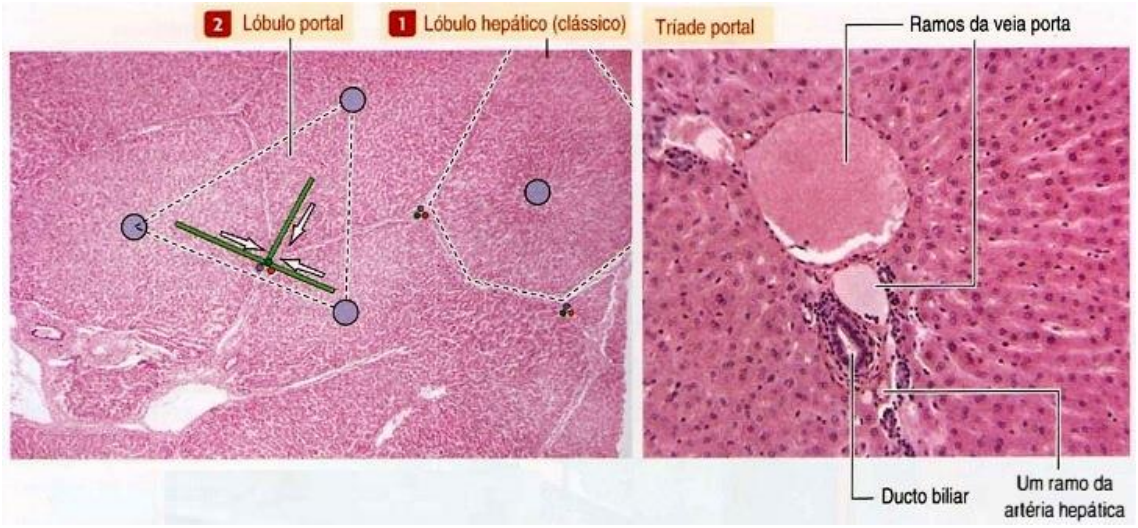


Fonte: GARTNER; HIATT, 2007.

A troca de nutrientes ocorre através dos espaços de Disse, que separam os hepatócitos do revestimento sinusoidal poroso. O fluxo sinusoidal se mistura nas vênulas hepáticas terminais que coalescem e finalmente formam a veia hepática, responsável pelo

transporte de todo o sangue eferente para a veia cava inferior. O fígado também é drenado por um suprimento rico de vasos linfáticos. É comum ocorrer interferência no suprimento sanguíneo hepático na cirrose e em outras doenças crônicas que se manifestam geralmente por hipertensão portal (BEERS & BERKOW, 2001).

Figura 2 - Fotomicrografia do fígado, evidenciando a estrutura do lóbulo hepático e a tríade portal.

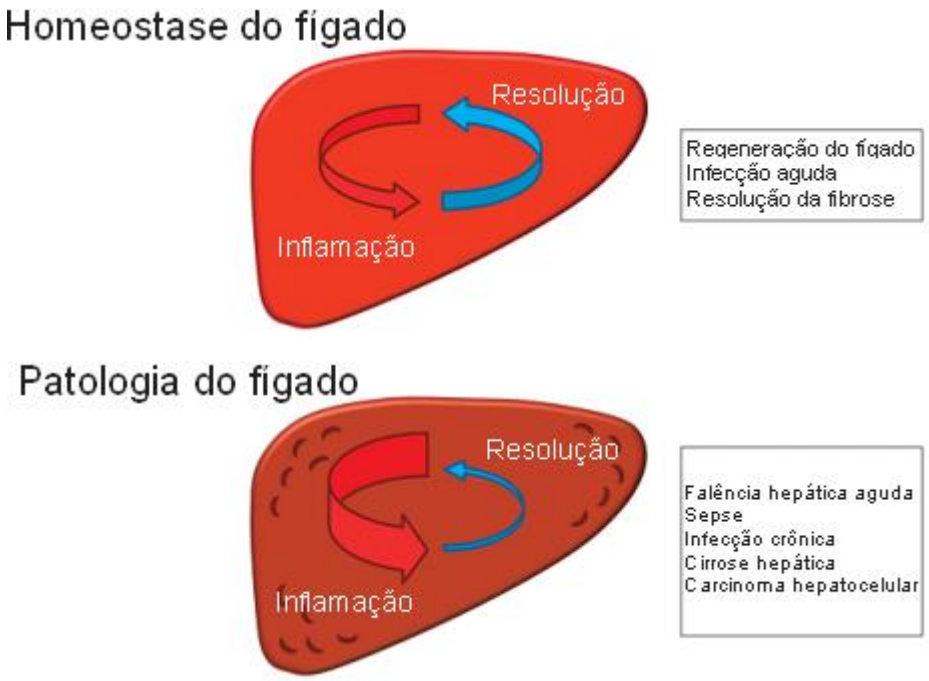


Fonte: KIERSZENBAUM; TRES, 2012.

2.2 Inflamação Hepática

A inflamação hepática é um processo complexo que se origina em resposta a uma variedade de estímulos e acompanha a maioria dos distúrbios hepáticos agudos e crônicos. Trata-se, portanto, de um mecanismo para proteger os hepatócitos de lesões, favorecer o reparo do dano tecidual e promover o restabelecimento da homeostase, exercendo efeitos hepatoprotetores consistentes (Figura 3). No entanto, as respostas inflamatórias que são muito intensas ou mantêm-se por longos períodos (inflamação crônica) tornam-se prejudiciais, podendo exacerbar a condição hepática e levar uma perda elevada de hepatócitos e causar um dano irreversível ao parênquima hepático (CZAJA, 2014; ROBINSON; HARMON; O ’FARRELLY, 2016).

Figura 3 – Papel do processo inflamatório na homeostase e na patologia do fígado.



Fonte: ROBINSON; HARMON; O 'FARRELLY, 2016.

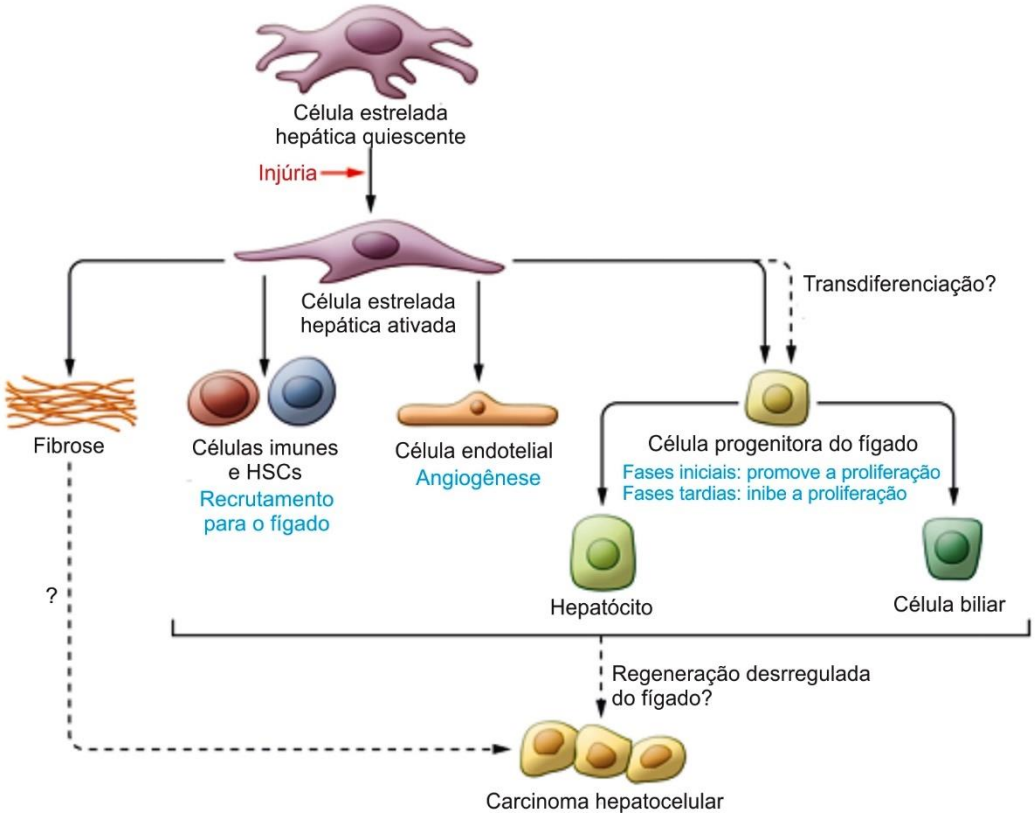
Dentre os principais estímulos que promovem respostas inflamatórias hepáticas desreguladas, encontra-se o consumo excessivo de álcool (OSNA et al., 2017), intoxicações por drogas (CHEN et al., 2015) ou metais pesados (tais como o cobre e o mercúrio) (JAISHANKAR et al., 2014), infecções virais (CHRISTENSON; MANALOOR, 2016) e condições metabólicas sistêmicas, como a doença hepática gordurosa não alcoólica (BENEDICT; ZHANG, 2017), obesidade (PARK et al., 2010) e diabetes (MOHAMED et al., 2016).

Mais de 90% dos pacientes que desenvolvem um episódio agudo de hepatite recuperam-se espontaneamente (SALIBA; SAMUEL, 2013). No entanto, a fibrose hepática é o resultado patológico de doenças inflamatórias crônicas persistentes, podendo levar ao desenvolvimento da cirrose e em último estágio, evoluir para o carcinoma hepatocelular (HCC). Este processo fibrótico, é caracterizado pela proliferação e diferenciação de células estreladas hepáticas (HSCs) em um fenótipo semelhante a miofibroblastos que depositam matriz extracelular (ECM) e colágeno (YIN et al., 2013).

A ativação das HSCs é mediada por espécies reativas de oxigênio e citocinas, como o fator de transformação do crescimento beta (TGF-β), o fator de necrose tumoral alpha (TNF-α) o fator de crescimento derivado de plaquetas (PDGF), bem como outros

fatores que são liberados pelos hepatócitos danificados e células de Kupffer ativadas (JIANG; TÖRÖK, 2013).

Figura 4 - Ativação das células estreladas e seus processos biológicos.



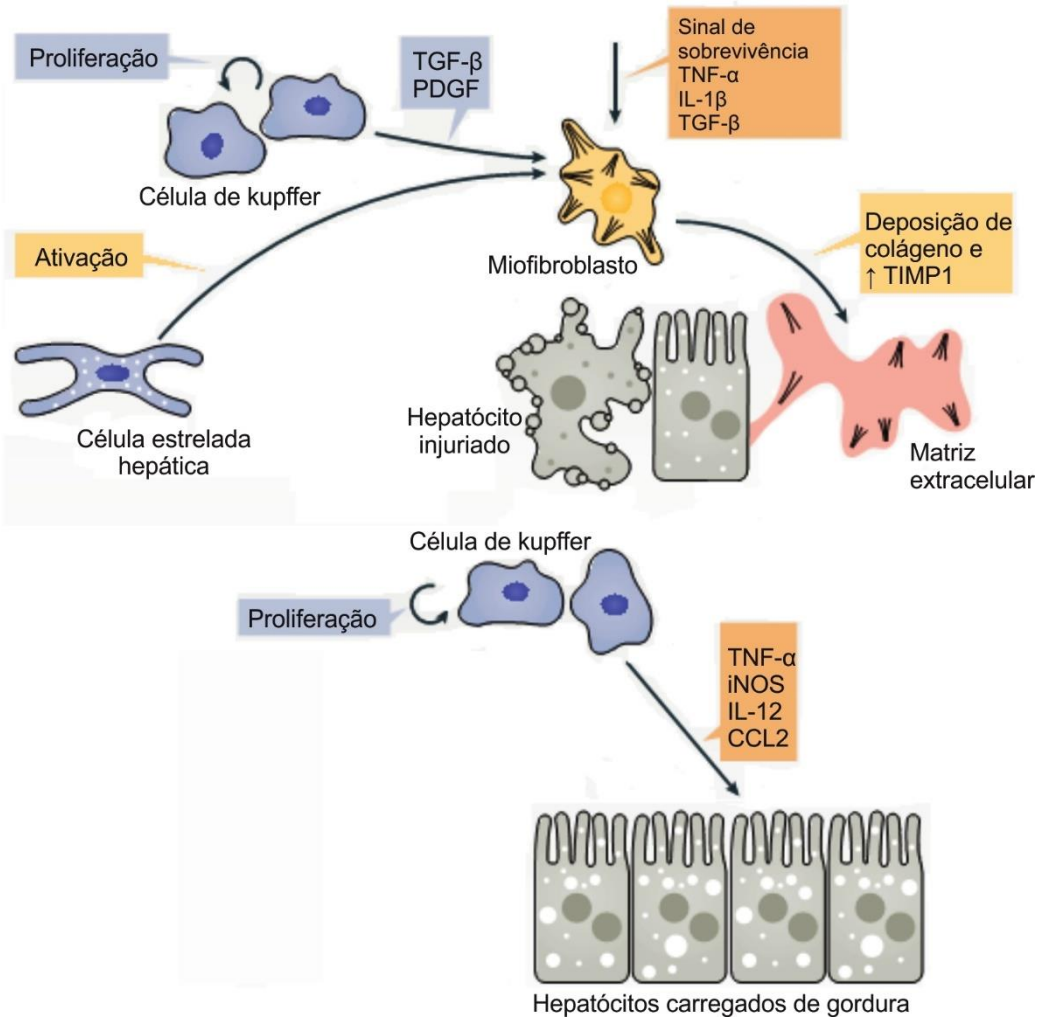
Fonte: YIN *et al.*, 2013.

Quando ativadas, as HSCs apresentam maior capacidade de proliferação e motilidade, passando a produzir grandes quantidades de componentes da ECM, como também de expressar marcadores fibróticos como a alpha actina do músculo liso, TGF-β, fator de crescimento derivado de plaquetas (PDGF) e outras citocinas, além de contribuir para angiogênese e a regulação do estresse oxidativo. Dependendo do tipo e duração do estímulo, tem-se um processo de *feedback positivo*, onde a liberação de tais fatores estimula que a própria célula continue a produzi-los, contribuindo para perpetuação do processo (TRAUTWEIN et al., 2015; WELLS; SCHWABE, 2015).

Além das células estreladas, os macrófagos hepáticos conhecidos como células de Kupffer, desempenham papel crucial na patogênese da lesão hepática mediada por substâncias químicas, toxinas e agentes farmacológicos, como o tetracloreto de carbono (CCl_4), lipopolissacarídeo (LPS), galactosamina e acetaminofeno. Durante o processo

inflamatório, as células de Kupffer ativadas são uma importante fonte de mediadores inflamatórios, incluindo citocinas (IL-1β e TNF-α), superóxido, óxido nítrico, eicosanóides, quimiocinas (CCL1 e CCL2), enzimas (COX-2 e iNOS) e demonstram aumento da citotoxicidade e quimiotaxia (DIXON et al., 2013; TACKE, 2017).

Figura 5 - Papel das células de Kupffer no processo inflamatório.



Fonte: adaptado de KRENKEL; TACKE, 2017.

As células de Kupffer também contribuem para ativação das HSCs através da produção de TGF-β e de PDGF como também regulam a produção de metaloproteinases (MMPs) e seus inibidores (TIMPs), responsáveis pela regulação da matriz extracelular (JU; TACKE, 2016).

2.3 Tetracloreto de Carbono

O tetracloreto de carbono (CCl_4) também conhecido como perclorometano ou tetraclorometano, é um líquido claro de odor doce que evapora muito facilmente. Não inflamável e imiscível com água, o CCl_4 não ocorre naturalmente no ambiente sendo formado pela cloração de metano, etano, propano ou propeno (BASU, 2003).

O CCl_4 é amplamente utilizado em modelos agudos e crônicos de injúria hepática, desempenhando papel preponderante na elucidação de mecanismos hepatotóxicos, como a esteatose, fibrose, morte de hepatócitos e carcinogênese (YANGUAS et al., 2016).

Após a absorção, o tetracloreto de carbono é rapidamente metabolizado pela citocromo P-450 (CYP2E1 em seres humanos), e em menor extensão por outras CYPs (CYP2B e CYP3A) com a produção do radical triclorometil (CCl_3*). O fígado é o alvo mais sensível e o órgão principal do metabolismo do CCl_4, independente da via de administração, devido à abundância de CYP2E1 e outros citocromos (WEBER; BOLL; STAMPFL, 2003).

O radical triclorometil é capaz de ligar-se a moléculas celulares (ácidos nucleicos, proteínas e lipídios), interferindo em processos celulares cruciais, como o metabolismo lipídico, levando ao acúmulo de gordura (esteatose). Estudos sugerem que a ligação irreversível entre o CCl3* e o DNA (formação de adutos) seria uma das vias que levam a formação do carcinoma hepatocelular (WEBER; BOLL; STAMPFL, 2003). O CCl_3* também pode reagir com oxigênio (O_2) para formar o radical triclorometil peróxido (CCl_3OO*), altamente reativo. O CCl_3OO* é capaz de promover peroxidação lipídica, que ataca e destrói os ácidos graxos poli-insaturados, afetando a permeabilidade das membranas plasmáticas com consequente dano tecidual (BASU, 2003).

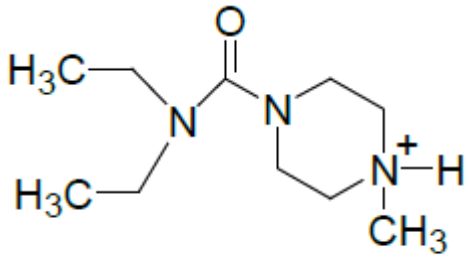
Além do estresse oxidativo, dentro da célula o CCl_4 estimula a expressão de TNF-α, óxido nítrico (NO) e TGF-β, processos que levam a apoptose das células, inflamação e promoção da fibrose.

2.4 Dietilcarbamazina

A dietilcarbamazina (DEC) é um derivado da piperazina sintetizada como 1-dietilcarbamil-4-metilpiperazina e preparada na forma de cloridrato, citrato (Figura 6) ou fosfato. A partir de 1950, foi distribuída como sal citratado por inúmeras companhias farmacêuticas sob diferentes nomes, como Hetrazan, Banocide, Caricide, Carbilazine, entre outros. É um pó branco, muito solúvel em água, estável, mesmo em condições de

umidade e temperatura muito elevadas, e resiste, inclusive, à autoclavagem. A denominação dietilcarbamazina genericamente se refere à sua forma citratada, uma vez que é mais comumente utilizada (DREYER; NORÕES, 1997).

Figura 6. Citrato de dietilcarbamazina.



Fonte: adaptado de QARAH; K; ABDULRAHMAN, 2017.

A DEC é rapidamente absorvida pelo trato gastrointestinal e atinge o pico da sua concentração plasmática entre uma e três horas após a ingestão e está quase ausente na urina, plasma e saliva de humanos após 24h da ingestão (ILONDU et al., 2000). Horii e Aoki (1997) descreveram o nível plasmático de DEC em ratos após a administração de 200 mg/kg, registrando valores de 30 µg/ml após 30-60 min da injeção, decrescendo rapidamente para 1,5 µg/ml após 4h e atingindo 0,1 µg/ml após 8h do tratamento.

Devido a sua ação micro e macrofilaricida (PEIXOTO; SILVA, 2014), a DEC é utilizada como tratamento padrão para filariose linfática desde meados de 1940. Sendo o regime de tratamento recomendado consistindo de um curso de 12 dias de tratamento com DEC (BRASIL, 2009; LANHAM; MWANRI, 2013).

Apesar de mais de 70 anos do seu uso, o seu mecanismo de ação exato ainda não está claro (MCGARRY; PLANT; TAYLOR, 2005).

Ciclooxigenases são enzimas envolvidas na conversão do ácido araquidônico em prostaglandina H2, precursora de vários compostos que são importantes mediadores inflamatórios (RICCIOTTI; FITZGERALD, 2011). Estudos prévios do nosso laboratório demonstraram a efetiva inibição da COX-2 pela DEC (ROCHA, S.W.S. et al., 2014; RODRIGUES et al., 2015).

Além disso, estudos do nosso laboratório apontam para uma possível inibição da via de sinalização do fator nuclear kappa beta (NF-κB), um importante fator de

transcrição de agentes pró-inflamatórios, pela DEC. Em um modelo de inflamação pulmonar aguda induzida por carragenina, o tratamento com DEC reduziu a fosforilação do NF-κB (SANTOS et al., 2014). Em um estudo de inflamação hepática crônica induzida por álcool, a redução expressão do NF-κB fosforilado pela DEC foi similar a produzida pela pirrolidina ditiocarbamato (RODRIGUES et al., 2015), um inibidor específico do NF-κB, sugerindo uma ação específica e possível mecanismo de ação da DEC sobre este fator de transcrição.

2.5 Nanopartículas

O adequado fornecimento dos compostos terapêuticos ao local alvo é um grande problema no tratamento de muitas doenças. As formulações convencionais de drogas em geral apresentam eficácia limitada, baixa biodistribuição e falta de seletividade. Nos sistemas de liberação controlada de fármacos, a droga é transportada para o local de ação, e portanto, sua influência em tecidos vitais e efeitos colaterais indesejáveis podem ser minimizados (ALVAREZ-LORENZO; CONCHEIRO, 2014).

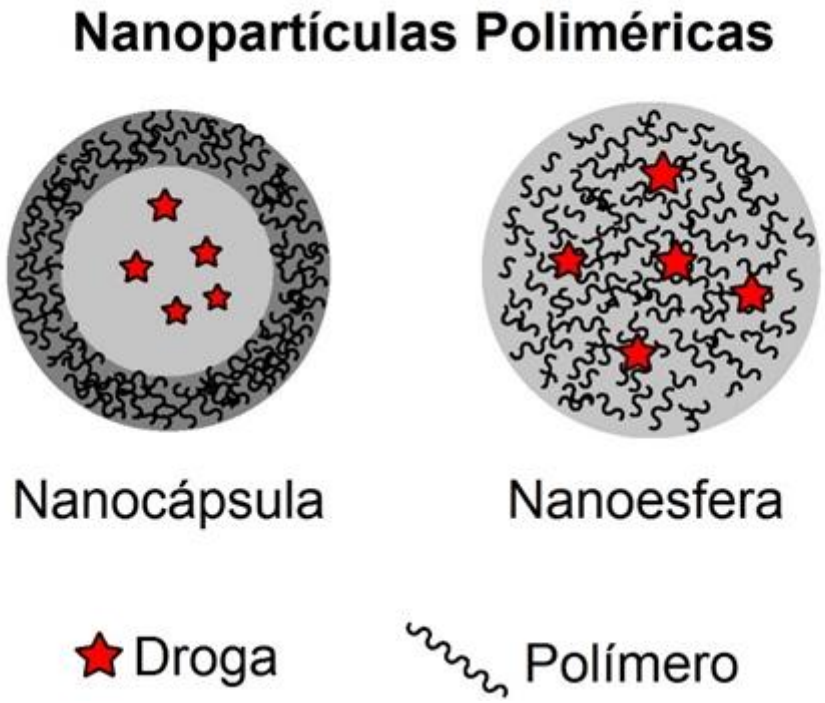
Além disso, tais sistemas de entrega e liberação de drogas, protegem o fármaco de uma degradação ou depuração rápida e aumentam sua a concentração nos tecidos alvo, sendo dessa forma utilizadas doses mais baixas. Esta moderna forma de terapia é especialmente importante em tratamentos de longo prazo, ou quando há uma discrepância entre uma dose ou concentração de um medicamento e seus resultados terapêuticos ou efeitos tóxicos (KUMARI et al., 2014; MUDSHINGE et al., 2011).

As nanopartículas são dispersões particuladas ou partículas sólidas com uma faixa de tamanho que varia entre 10 e 1000 nm. O fármaco ou principio ativo é dissolvido, aprisionado, encapsulado ou ligado a uma matriz de nanopartículas (NIKAM; RATNAPARKHIAND; CHAUDHARI, 2014).

As nanopartículas poliméricas (PNPs) quando aplicadas como sistemas de liberação controlada de fármacos, a depender do método de preparação, pode-se obter dois tipos de estruturas diferentes: as nanoesferas e as nanocápsulas. Nanoesferas são sistemas em que o fármaco se encontra disperso de forma homogênea no interior de uma matriz polimérica, criando-se um sistema monolítico, onde não é possível identificar um núcleo diferenciado. Nanocápsulas, são sistemas em que a droga é confinada em uma

cavidade cercada por uma membrana polimérica, sendo possível identificar um núcleo (CRUCHO; BARROS, 2017; MOHANRAJ; CHEN, 2006).

Figura 7 - Diferenças estruturais entre as nanopartículas poliméricas.



Fonte: CRUCHO; BARROS, 2017.

As PNPs podem ser obtidas a partir de uma variedade de polímeros. Os não-biodegradáveis, como a poliacrilamida, poliestireno e o polimetil-metacrilato, foram os primeiros a serem utilizados como carreadores. Toxicidade crônica e reações inflamatórias eram observadas com frequência, devido a estes materiais apresentarem baixo *clearence* no organismo, além de problemas na distribuição e acumulação em níveis tóxicos nos tecidos (BANIK; FATTAHI; BROWN, 2016; MAITZ, 2015).

Dentre os principais polímeros biodegradáveis existe o poliácido láctico (PLA), o poli(ácido lático-co-ácido glicólico) (PLGA) e o poli(ε-caprolactona) (PCL), além dos polímeros naturais como a quitosana, alginato, gelatina e albumina. Estes polímeros apresentam toxicidade reduzida, melhor compatibilidade e capacidade de influenciar o padrão cinético de liberação (BANIK; FATTAHI; BROWN, 2016; MAITZ, 2015).

Espera-se, portanto, que tais preparações permitam a modificação dos parâmetros farmacocinéticos da DEC, tais como o tempo de meia-vida, volume de distribuição aparente e o *clearence*, permitindo doses menores e toxicidade reduzida.

Resultados

ARTIGO I - Characterization and evaluation of nanoencapsulated diethylcarbamazine in model of acute hepatic inflammation

International Immunopharmacology 50 (2017) 330–337

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Characterization and evaluation of nanoencapsulated diethylcarbamazine in model of acute hepatic inflammation

Gabriel Barros Rodrigues[a,b], Elquio Eleamen Oliveira[c], Francisco Jaime Bezerra Mendonça Junior[c], Laise Aline Martins dos Santos[a], Wilma Helena de Oliveira[a,b], Maria Eduarda Rocha de França[a,b], Deniele Bezerra Lós[a,d], Brennda Martins Gabínio[c], Fábia Cristiane Melo Leite de Lira[e], Christina Alves Peixoto[a,*]

[a] Laboratório de Ultraestrutura, Instituto Aggeu Magalhães – FIOCRUZ, Recife, Brazil
[b] Programa de Pós-graduação em Ciências Biológicas, Centro de Biociências, Universidade Federal de Pernambuco - UFPE, Recife, Brazil
[c] Laboratório de Síntese e Vetorização de Moléculas (LSVM), Universidade Estadual da Paraíba, João Pessoa, Brazil
[d] Programa de Pós-graduação em Biotecnologia/RENORBIO, Universidade Federal de Pernambuco - UFPE, Recife, Brazil
[e] Laboratório de Biologia Celular e Ultraestrutura, Centro de Tecnologias Estratégicas do Nordeste – CETENE, Recife, Brazil

ARTICLE INFO

Keywords:
Diethylcarbamazine
Nanoencapsulation
Liver
Inflammation
Carbon tetrachloride

ABSTRACT

Previous studies from our laboratory have demonstrated that Diethylcarbamazine (DEC) is a potent anti-inflammatory drug. The aim of the present study was to characterize the nanoencapsulation of DEC and to evaluate its effectiveness in a model of inflammation for the first time. C57BL/6 mice were divided into six groups: 1) Control; 2) Carbon tetrachloride (CCl4); 3) DEC 25 mg/kg + CCl4; 4) DEC 50 mg/kg + CCl4; 5) DEC-NANO 05 mg/kg + CCl4 and 6) DEC-NANO 12.5 mg/kg + CCl4. Liver fragments were stained with hematoxylin-eosin, and processed for Western blot, ELISA and immunohistochemistry. Serum was also collected for biochemical measurements. Carbon tetrachloride induced hepatic injury, observed through increased inflammatory markers (TNF-α, IL-1β, PGE2, COX-2 and iNOS), changes in liver morphology, and increased serum levels of total cholesterol, triglycerides, TGO and TGP, LDL, as well as reduced HDL levels. Nanoparticles containing DEC were characterized by diameter, polydispersity index and zeta potential. Treatment with 12.5 nanoencapsulated DEC exhibited a superior anti-inflammatory action to the DEC traditional dose (50 mg/kg) used in murine assays, restoring liver morphology, improving serological parameters and reducing the expression of inflammatory markers. The present formulation of nanoencapsulated DEC is therefore a potential therapeutic tool for the treatment of inflammatory hepatic disorders, permitting the use of smaller doses and reducing treatment time, while maintaining high efficacy.

1. Introduction

Acute hepatitis can occur without symptoms and may lead to high blood bilirubin levels (jaundice), a decreased sensation of appetite, and fatigue. About 90% of patients with acute hepatitis recover spontaneously. However, depending on the type and duration of the stimulus, the disease may progress to a serious condition, such as acute liver failure (ALF). ALF is characterized by acute inflammation with severe liver cell injury, where a previously normal liver fails within days or weeks. With a high mortality rate (40–50%), drug-induced injury is responsible for half the cases of ALF [1,2].

The hepatotoxin carbon tetrachloride (CCl_4) is widely used in models of liver inflammation. CCl_4 causes fatty liver, acute necrosis and oxidative stress. Studies have demonstrated that single doses of CCl_4 cause areas of necrosis and the generation of reactive radicals in 2 h following administration [3,4].

Diethylcarbamazine (DEC) is a piperazine derivative which, as a result of its microfilaricide action, has been used in the treatment and control of lymphatic filariasis since 1947. DEC also has anti-inflammatory properties due to its interference with arachidonic acid metabolism. Studies demonstrate the inhibition of enzymes of the 5-lipoxygenase pathway and cyclooxygenase, resulting in a reduction in prostaglandin E2 (PGE_2) production [5].

In a previous study, we characterized the anti-inflammatory action of DEC in a liver model of chronic inflammation induced by alcohol. The results demonstrated that treatment with 50 mg/kg of DEC for

* Corresponding author at: Instituto Aggeu Magalhães, Av Moraes Rego s/n Campus da UFPE FIOCRUZ, Recife CEP 52171-011, Brazil.
E-mail address: cpeixoto@cpqam.fiocruz.br (C.A. Peixoto).

http://dx.doi.org/10.1016/j.intimp.2017.07.014
Received 12 May 2017; Received in revised form 11 July 2017; Accepted 13 July 2017
1567-5769/ Published by Elsevier B.V.

12 days inhibited the transcription factor kappa B (NF-κB) reducing the inflammatory cytokines and the production of hepatic collagen [6].

Nanoparticles can be defined as particulate dispersions or solid particles with a size ranging from 10 to 1000 nm. With the advancement of nanotechnology in the field of health, nanomedicine offers many potential benefits for medical applications, including microfluidics, drug delivery systems, biosensors, microarrays and tissue micro-engineering [7,8].

Conventional pharmacological preparations have limitations such as limited biodistribution, the lack of a sustained effect and the absence of selectivity. Drug delivery systems (DDS) have been proposed as a solution to overcome such problems. These nano-carrier based systems transport the drug to its specific site of action, protecting it from rapid degradation or clearance. Lower doses are therefore required [9].

The objective of the present study was therefore to characterize DEC nanoencapsulation and evaluate for the first time its efficacy as an anti-inflammatory drug utilizing a quarter (12.5 mg/kg) and one tenth (5 mg/kg) of the usual dose for mice hepatic inflammation models and only half the treatment time (6 days).

2. Materials and methods

2.1. Animals

Forty-two male C57BL/6 mice (age: 5 weeks; weight: 15–17 g) were obtained from the Aggeu Magalhães Research Center–FIOCRUZ (Pernambuco, Brazil). The health status of the mice was examined and they were acclimated to a laboratory environment of 23 °C and a 12-h light and 12-h dark photoperiod. The animals were housed in metal cages and fed with a standard diet and water ad libitum. The Animal Studies Ethics Committee of the Oswaldo Cruz Institute approved all the experiments reported (n° 72/2014).

2.2. Diethylcarbamazine solutions

The solutions were composed of distilled water and DEC (Farmanguinhos, FIOCRUZ, Brazil) and were adjusted in accordance with the body weight of each animal. They were administered through intraperitoneal injection (100 μL/day). The control group (Cont) received only distilled water via the same administration route.

2.3. Preparation of DEC-loaded nanoparticles

The nanoparticles were prepared by the double emulsion method. One milliliter of an aqueous solution containing 5 or 12.5 mg/mL of DEC was emulsified in 10 mL of dichloromethane containing 50 μg of poly(ε-caprolactone) (PCL) (Mw = 14,000 g/mol) and 30 μg of Pluronic F68. The first emulsion (W1/O) was obtained using the Ultra-Turrax (Ultra-Turrax T18 basic, IKA) homogenizer at 25,000 rotations per minute (rpm) for 10 min. The first emulsion was then added to 40 mL of a poly(vinyl alcohol) (PVA) aqueous solution (5 mg/mL) and dispersed by using Ultra-Turrax (Ultra-Turrax T18 basic, IKA) at 25,000 rotations per minute (rpm) for 10 min until the formation of a double emulsion (W1/O/W2). Finally, solvent evaporation was carried out under reduced pressure using a rotary evaporator (RV 10 D, IKA). NANO-DEC formulation is under patent submission (BR 10 2017 002700 7).

2.4. Experimental groups

Acute inflammation was induced by intraperitoneal administration of CCl_4 0.1 μL/g of body weight (Sigma-Aldrich, St. Louis, MO, USA) dissolved in olive oil (final volume, 100 μL per mouse) [10]. A single injection was given on the last (sixth) day of treatment with DEC and DEC-NANO. DEC (25 mg/kg and 50 mg/kg) and DEC-NANO (12.5 mg/kg and 5 mg/kg) were administered through intraperitoneal injection for 6 days (1 injection/day) before CCl_4 administration. The control groups received distilled water via the same route. The C57BL/6 mice were separated into six groups (n = 7/group): (1) the control group (Cont), which received just distilled water; (2) the CCl_4 group (CCl_4); (3) the DEC 25 mg/kg plus CCl_4 group (CCl_4 + DEC 25); (4) the DEC 50 mg/kg plus CCl_4 group (CCl_4 + DEC 50); (5) the DEC-NANO 05 mg/kg plus CCl_4 group (CCl_4 + DEC-NANO 05) and (6) the DEC-NANO 12.5 mg/kg plus CCl_4 group (CCl_4 + DEC-NANO 12.5). 24 h after intraperitoneal administration of CCl_4, the animals were anesthetized (xylazine 10 mg/kg and ketamine 100 mg/kg) and sacrificed by cervical dislocation.

2.5. Characterization of the prepared nanoparticles

2.5.1. Particle size, size distribution and zeta potential

The mean hydrodynamic diameter, polydispersity index (PDI) and zeta potential of the prepared nanoparticles were determined using the Malvern particle size analyzer (Nano ZS, Malvern).

2.5.2. Morphology and surface characteristics

Nanoparticle morphology was examined with transmission electron microscopy (TEM) equipment. A drop of the nanoparticle suspension was withdrawn with a micropipette then placed on a carbon–coated copper grid and negatively stained with 2% phosphotungstic acid. The grid was dried at room temperature and observed by TEM (Tecnai G20, FEI).

2.6. Biochemical determinations

The lipid profile of the serum was assessed (total cholesterol, high-density lipoprotein-HDL, low-density lipoprotein-LDL and triglycerides), and hepatic damage was evaluated based on aspartate aminotransferase (AST) and alanine transaminase (ALT). Serum concentrations were measured by the spectrophotometric method with an Integra 400 (Roche, Basel, Switzerland). Data were compared by one-way ANOVA and Dunnett's or Tukey's tests, with GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA).

2.7. Histopathology

2.7.1. Hematoxylin–eosin staining

Liver fragments were fixed in 10% formalin for 24 h, processed, and embedded in paraffin. Sections of 4–5 μm were cut and mounted on glass slides. Slices were stained with hematoxylin–eosin and assessed by inverted microscopy (Observer Z1; Zeiss MicroImaging, Jena, Germany), a camera, and 4.7.4 image analysis software (AxionCam MRm; Zeiss, Jena, Germany) at a magnification of × 400.

2.7.2. Immunohistochemical assays

Five sections (5 μm thickness) from each group were cut and adhered to slides treated with 3-amino-propyl-triethoxy-silane (Sigma-Aldrich, St. Louis, MO, USA). The sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). The sections were then heated for 30 min in a sodium citrate buffer (0.01 M, pH 6.0) and treated with 0.3% (v/v) H_2O_2 in water for 5 min. The sections were washed with 0.01 M PBS (pH 7.2) and then blocked with 1% BSA and 0.2% Tween 20 in PBS for 1 h at room temperature. The sections were incubated for 12 h at 4 °C with the following antibodies: *anti*-PGE_2 antibody (1:100 dilution; ab2318; Abcam, Cambridge, UK), *anti*-TNF-α (1:100 dilution; 500-P64; PrepoTech, London, UK), *anti*-iNOS (1:250 dilution; ab3523; Abcam) and *anti*-COX-2 (1:400 dilution; ab15191; Abcam). The antigen-antibody reaction was visualized with avidin-biotin peroxidase (DakoUniversal LSAB + Kit, Peroxidase), using 3.3-diaminobenzidine as the chromogen. The slides were counterstained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, with the exception of the first

antibody, which was omitted. Five images at the same magnification were quantitatively analyzed using GIMP 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.8. Measurement of nitric oxide

The Griess colorimetric reaction was used for the measurement of nitric oxide (NO), which involved the detection of nitrite (NO_2^-) and the oxidation of NO in the liver. In duplicate, 50 μL liver homogenate was added to a 96-well ELISA plate, followed by the same volume of Griess reagent, composed of 1% sulfanilamide diluted in 2.5% H_3PO_4 (solution A) and *N*-1-naphtyl-ethylenediamine, also diluted in 2.5% H_3PO_4 (solution B). To prepare the standard curve, a solution of sodium nitrite in an initial concentration of 100 μM was serially diluted in PBS. After incubation for 10 min in the dark, a reading was performed in the spectrophotometer at 490 nm. The absorbance of different samples was compared with the standard curve and the results were expressed as mean ± standard error of the duplicate, using the GraphPad Prism program (v. 6.01).

2.9. Hepatic glutathione reductase activity

Liver tissues were obtained and weighed. Each piece was homogenized using the manufacturer's buffer (Abnova Corporation, Taipei, Taiwan, catalog number: KA0881) and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was used to estimate the activity of hepatic glutathione reductase using the Assay Kit, following the manufacturer's instructions.

2.10. Western blot

Livers were quickly dissected and then homogenized in a Wheaton Overhead Stirrer (model 903475; Wheaton Science Products, Millville, NJ, USA) using an extraction cocktail of 10-mmol/L ethylenediamine tetraacetic acid, 2-mmol/L phenylmethylsulfonyl fluoride, 100-mmol/L sodium fluoride, 10-mmol/L sodium pyrophosphate, 10-mmol/L sodium orthovanadate, 10-mg aprotinin, and 100-mmol/L Tris(hydroxymethyl) aminomethane (pH 7.4). Western blot assays were then performed according to the previously described methodology [6]. The antibodies used were *anti*-IL-1β antibody (1:1000 dilution; ab9722; Abcam, Cambridge, UK), TNF-α (1:1000 dilution; 500-P64; PrepoTech, London, UK), *anti*-iNOS (1:1000 dilution; ab3523; Abcam) and *anti*-COX-2 (1:1000 dilution; ab15191; Abcam).

2.11. Statistical analysis

GraphPad Prism version 6.01 was used for statistical analysis. Data were expressed as mean ± SD. Differences between the control and treated groups were analyzed by ANOVA and either Dunnett's test, Tukey's test, or the *t*-test as a post hoc test. Probability values lower than 0.05 were considered significant.

3. Results

3.1. Characterization of nanoparticles containing diethylcarbamazine

Three formulations were prepared in this study: drug free nanoparticles (DRUG-FREE-NPs), nanoparticles with an initial addition of 5 mg of DEC (DEC-NANO 05), and nanoparticles with an initial addition of 12.5 mg of DEC (DEC-NANO 12.5). The particle sizes, polydispersity index (PDI) and zeta potential of the nanoparticles were measured (Table 1). The mean particle size for the DRUG-FREE-NPs was around 283 nm, with a slight increase in the mean diameter size for the DEC-NANO 05 nanoparticles and a more prominent increase in the mean diameter size for the DEC-NANO 12.5 nanoparticles. The polydispersity indexes followed the same tendency as the mean hydrodynamic diameter, with a low value for DRUG-FREE-NPs and an increase in the PDI according to the amount of the drug used. Nevertheless, all samples exhibited a PDI below 0.3, indicating narrow size distribution [11]. The zeta potential for the samples varied from approximately −3 to −5 mV. The negative values could be attributed to the polymer PCL [12]. These low zeta potential values may suggest a propensity to form aggregate if the nanoparticles are stored in an aqueous suspension. The addition of DEC did not induce any drastic modification in the size and zeta potential of the nanoparticles.

Table 1
Characterization of the nanoencapsulated DEC. Data were presented as mean ± standard deviation (n = 3). PDI, polydispersity index.

Sample	Diameter (nm)	PDI	Zeta potential (mV)
DRUG-FREE-NPs	283.6 ± 1.68	0.138 ± 0.4	−3.24 ± 0.39
DEC-NANO 05	298.7 ± 3.86	0.168 ± 0.2	−2.40 ± 0.21
DEC-NANO 12.5	329.8 ± 1.08	0.260 ± 0.1	−5.39 ± 0.81

The morphological characteristics of the nanoparticles were observed by TEM and are shown in Fig. 1. The DRUG-FREE-NPs and DEC-NANO 05 nanoparticles presented solid and consistent spherical shapes, while the DEC-NANO 12.5 nanoparticles exhibited a denser central structure, probably due to the excess of DEC. It was noted that the nanoparticles of both concentrations of DEC were successfully prepared in terms of morphology.

3.2. DEC-NANO improves lipid profile and reduces transaminase levels

In biochemical analysis of the lipid profile, the CCl_4 group exhibited a significant increase in total cholesterol (Fig. 2A) and triglyceride (Fig. 2D) content compared to the control group. CCl_4 + DEC 25 and CCl_4 + DEC-NANO 05 groups did not significantly reduce these lipid markers. However, CCl_4 + DEC 50 and CCl_4 + DEC-NANO 12.5 groups reduced the circulating lipid content when compared to the CCl_4 group.

HDL cholesterol was significantly reduced in all the groups in comparison with the control (Fig. 2B). However, the CCl_4 + DEC-NANO 12.5 group significantly increased the HDL cholesterol content compared with the CCl_4 group. LDL provided opposite results (Fig. 2C), with all groups exhibiting a significant increase compared to the control group and only the CCl_4 + DEC-NANO 12.5 group showing a significant reduction in comparison with the CCl_4 group.

In the assessment of liver damage markers (Fig. 2E and F), AST and ALT transaminases exhibited a significant increase in the CCl_4 group in comparison with the control group. The CCl_4 + DEC 25 and CCl_4 + DEC-NANO 05 groups also presented a significant increase in both enzymes. The CCl_4 + DEC 50 and CCl_4 + DEC-NANO 12.5 groups, however, exhibited a reduction in liver damage markers in comparison with the inflamed group.

3.3. DEC-NANO preserves liver histology

Histological analysis of the liver identified standard morphology in the control group (Fig. 3A), with well-preserved hepatocytes. Several areas of perilobular necrosis of the hepatocytes were observed in the CCl_4 group (Fig. 3B), along with steatosis and inflammatory cells. The CCl_4 + DEC 25 group presented the same inflammatory characteristics as the CCl_4 group (Fig. 3C), with several areas of damaged hepatocytes.

The CCl_4 + DEC 50 and CCl_4 + DEC-NANO 05 groups exhibited a small reduction hepatocyte death, including in necrotic regions, but inflammatory alterations were still present (Fig. 3D and E). The group treated with DEC-NANO 12.5 mg/kg exhibited lower inflammatory disorders, with greater preservation of the hepatic stroma and the reduction of steatosis with fewer necrotic areas (Fig. 3F).

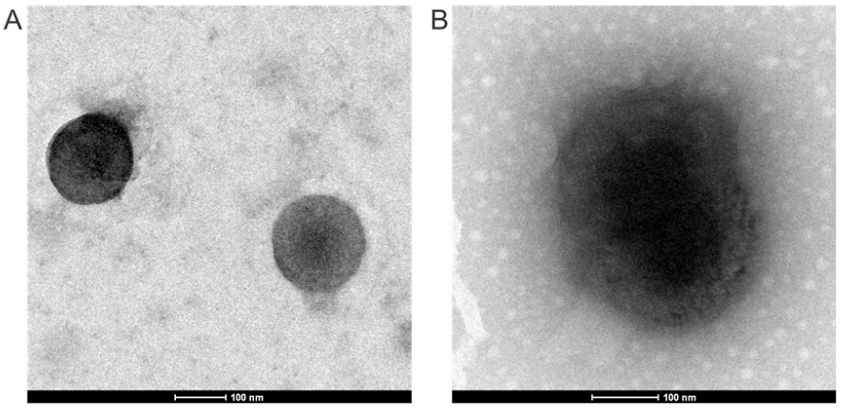


Fig. 1. Images of the nanoencapsulated DEC by transmission electron microscopy. (A) DEC-NANO 05. (B) DEC-NANO 12.5. Bar = 100 nm.

3.4. DEC-NANO reduces nitric oxide levels

In the hepatic evaluation of NO_2^- levels (Fig. 4), all groups showed a significant increase in concentration in comparison with the control group. However, only the CCl_4 + DEC-NANO 12.5 group was able to significantly reduce the concentration of NO_2^- in relation to the CCl_4 group.

3.5. DEC and DEC-NANO increase the activity of glutathione reductase in the liver

In the evaluation of hepatic glutathione reductase activity (Fig. 5), the inflamed group (CCl_4) presented a significant reduction in the production of the enzyme substrate in comparison with the control group. All the groups treated with DEC exhibited an increase in glutathione reductase activity in comparison with the CCl_4 group. The results suggest dose-dependent activity, where the groups treated with nanoencapsulated DEC exhibited greater enzyme activity in the liver.

3.6. DEC-NANO reduces the expression of inflammatory markers

To assess the hepatotoxic effects of CCl_4, immunohistochemistry for TNF-α was performed (Fig. 6 – TNF-α). The inflammatory group demonstrated elevated TNF-alpha positivity, as did the CCl_4 + DEC 25, + CCl_4 + DEC 50 and CCl_4 + DEC-NANO 05 groups. The CCl_4 + DEC-NANO 12.5 group exhibited significantly reduced TNF-α positivity in comparison with the inflammatory group.

DEC is a known inhibitor of the COX-2 enzyme. The expression of PGE_2, a product of cyclooxygenase 2, was evaluated (Fig. 6 – PGE_2). The CCl_4 group exhibited high PGE_2 expression, as did the CCl_4 + DEC 25 and CCl_4 + DEC-NANO 05 groups. The groups treated with DEC 50 mg/kg and DEC-NANO 12.5 mg/kg significantly reduced PGE_2 expression.

All groups exhibited elevated labelling of the inflammatory enzyme iNOS in comparison with the control group (Fig. 6 – iNOS). Only the CCl_4 + DEC-NANO 12.5 group reduced iNOS labelling when compared to the CCl_4 group.

In keeping with the PGE_2 results, the CCl_4, CCl_4 + DEC 25 and CCl_4 + DEC-NANO 05 groups exhibited high expression of the inflammatory enzyme COX-2 (Fig. 6 – COX-2). The CCl_4 + DEC 50 and CCl_4 + DEC-NANO 12.5 groups significantly reduced the expression of cyclooxygenase-2 when compared with the inflammatory group.

In Western blot analysis (Fig. 7), the inflammatory group exhibited high IL-1β cytokine expression, as did the CCl_4 + DEC 25 and

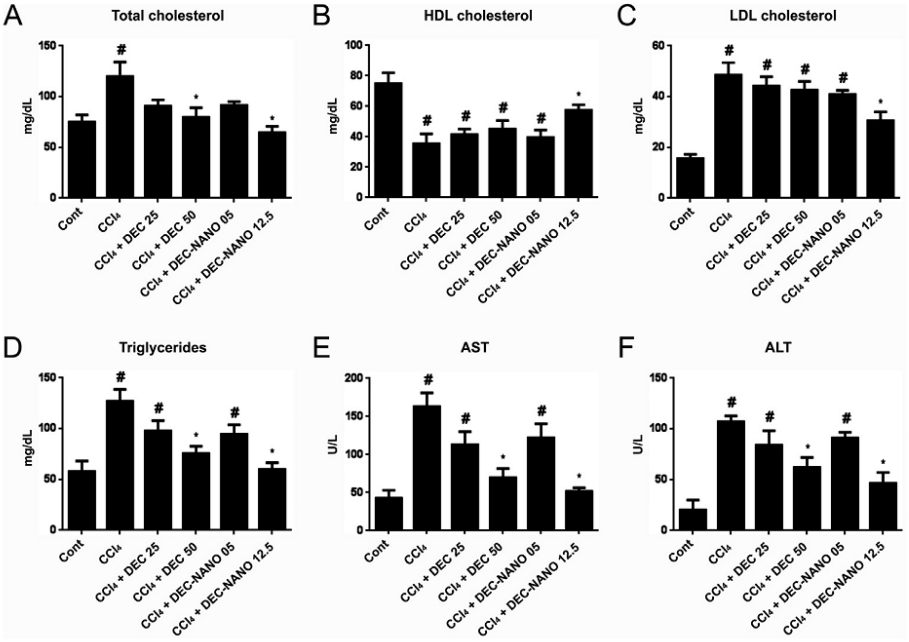


Fig. 2. Biochemical parameters. (A) Total cholesterol. (B) HDL cholesterol (HDL) cholesterol. (C) LDL cholesterol. (D) Triglycerides. (E) Aspartate aminotransferase (AST). (F) Alanine aminotransferase. The results are expressed as mean ± SD of three mice per group. $^*p < 0.05$ vs CCl_4; $^{\#}p < 0.05$ vs control. CCl_4, carbon tetrachloride; Cont, control; DEC, diethylcarbamazine.

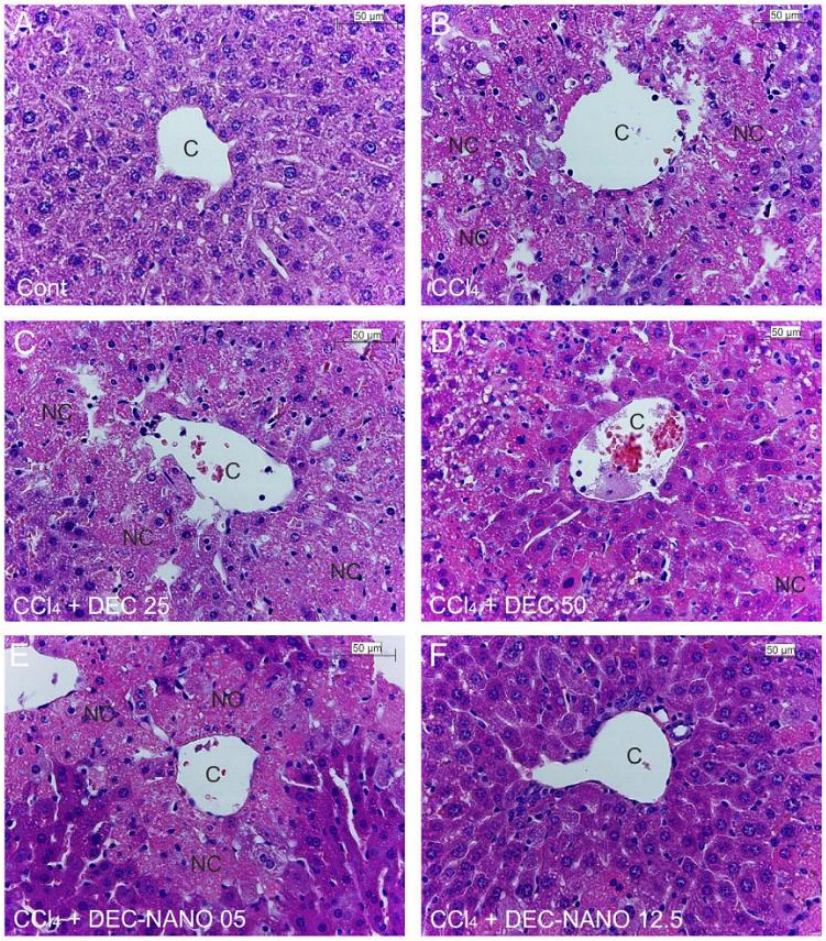


Fig. 3. Histological analysis of liver fragments in haemotoxylin–eosin staining. (A) Control group. (B) CCl_4 group. (C) CCl_4 + DEC 25 mg/kg group. (D) CCl_4 + DEC 50 mg/kg group. (E) CCl_4 + DEC-NANO 5 mg/kg group. (F) CCl_4 + DEC-NANO 12.5 mg/kg group. Bar = 50 µm. Magnification: 400 ×. Liver thin section = 4–5 µm. C, centrilobular vein; CCl_4, carbon tetrachloride; Cont, control; DEC, diethylcarbamazine; NC, necrosis areas.

CCl_4 + DEC-NANO 05 groups. Only the CCl_4 + DEC-NANO 12.5 group significantly reduced the expression of IL-1β in comparison with the CCl_4 group.

In TNF-α expression evaluation, the CCl_4, CCl_4 + DEC 25 and CCl_4 + DEC 50 groups exhibited an increase in cytokine expression when compared to the control group. However, the groups treated with nanoencapsulated DEC 05 mg/kg and 12.5 mg/kg reduced TNF-α expression in comparison with the inflammatory group.

Similar to the results found in immunohistochemistry, all the groups inhibited high iNOS expression in comparison with the control group. Only the CCl_4 + DEC-NANO 12.5 group significantly reduced the expression of the enzyme when compared to the CCl_4 group.

Confirming the results obtained in immunohistochemistry, the CCl_4, CCl_4 + DEC 25 and CCl_4 + DEC-NANO 05 groups exhibited an elevated expression of COX-2. However, the CCl_4 + DEC 50 and CCl_4 + DEC-NANO 12.5 groups efficiently reduced cyclooxygenase expression in comparison with the CCl_4 group.

4. Discussion

Acute hepatitis is an inflammatory condition of the liver. The viral infection is the most common type of hepatitis worldwide. Nevertheless, other forms include autoimmune hepatitis, alcoholic liver disease and other less common types of hepatitis, which occur as a secondary result of toxins, medications and drugs [13–17].

The aim of the present study was to define DEC nanoencapsulation parameters and evaluate the efficacy of this drug with half the treatment time and a quarter and one tenth of the dose utilized in previous studies.

Diethylcarbamazine-PCL nanoparticles were prepared by the double emulsion method. This approach is indicated for the incorporation of hydrophilic drugs due to the formation of an aqueous inner core that can improve the encapsulation of drugs such as DEC [18]. First-generation polymeric nanoparticles were employed in this study to induce a passive accumulation in the liver. This means that no hydrophilic corona was formed, as PEGylated surface. It is well known that the liver acts as a biological filtration system that sequesters 30–99% of administered nanoparticles of first-generation from the bloodstream [19]. Another parameter used to improve the liver accumulation of the nanoparticles was the particle size. The literature data supports that nanoparticles with larger diameter tend to accumulate in RES (Reticuloendothelial System) organs, including the liver [20–22].

Hepatic steatosis involves the accumulation of fat in the liver and is a common and highly prevalent condition. Despite this, it can evolve to steatohepatitis and fibrosis in inflammation processes [23]. The first parameter evaluated was therefore the biochemical measurements. Nanoencapsulated DEC at a concentration of 12.5 mg/kg improved the lipid profile by reducing the total cholesterol and triglyceride content and increasing the HDL and lower LDL cholesterol levels. Concentrations of 12.5 mg/kg of DEC and DEC-NANO 05 mg/kg did not effectively improve the lipid parameters while DEC 50 mg/kg only reduced the content of triglycerides. DEC 50 mg/kg and DEC-NANO 25 mg/kg were effective at reducing the serum transaminases ALT and AST, markers of liver damage, in comparison with the CCl_4 group.

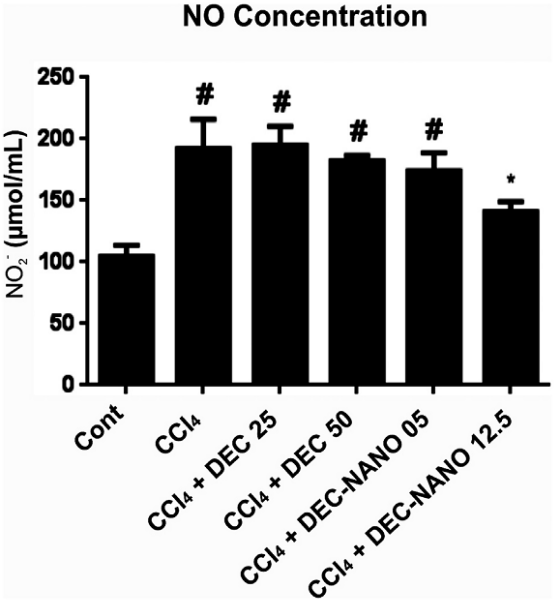


Fig. 4. Assessment of hepatic NO concentration through the measure of total nitrite metabolites. The results are expressed as mean ± SD of three mice per group. $^{*}p < 0.05$ vs CCl4; $^{\#}p < 0.05$ vs control. CCl4, carbon tetrachloride; Cont, control; DEC, diethylcarbamazine.

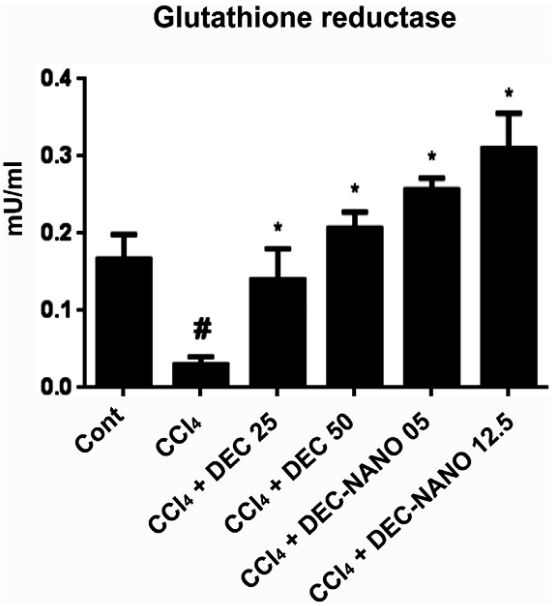


Fig. 5. Assessment of hepatic glutathione activity. Unit definition (U): one unit is defined as the amount of enzymes that generates 1.0 µmol of 5-thionitrobenzoic acid (TNB) per minute at 25 °C. The results are expressed as mean ± SD of three mice per group. $^{*}p < 0.05$ vs CCl4; $^{\#}p < 0.05$ vs control. CCl4, carbon tetrachloride; Cont, control; DEC, diethylcarbamazine.

Liver inflammation is usually associated with hepatocyte necrosis and apoptosis [24]. This hepatocyte death is the main driver of chronic liver inflammation and fibrosis [25]. Additionally, apoptotic bodies activate quiescent hepatic stellate cells and Kupffer cells, promoting inflammatory and fibrogenic responses [26]. In the histological analysis of the CCl_4 group, the high death of hepatocytes was observed, with remarkable perilobular necrosis. The CCl_4 + DEC 50 and CCl_4 + DEC-NANO 05 groups exhibited a minor reduction in cell death. However, the CCl_4 + DEC-NANO 12.5 group effectively reduced hepatocyte death and areas of necrosis, suggesting a hepatoprotective effect.

The interaction of nitric oxide and oxygen results in the generation of reactive nitrogen species (RNS). Together, RNS and reactive oxygen species (ROS), participate in redox balance, essential for liver homeostasis. When this balance is disrupted, oxidative stress results in cell damage with subsequent inflammation [27]. In the results of the present study, carbon tetrachloride induced an increase in hepatic NO in all the treated groups. Only the CCl_4 + DEC-NANO 12.5 group reduced nitric oxide concentration.

Glutathione, which is present in the liver, is a major antioxidant that protects cells against exogenous and endogenous toxins, including ROS and RNS [28]. Reduced (GSH) and oxidized (GSSG) glutathione forms operate in concert with one another, with GSSG produced from the consumption of GSH restored by the action of glutathione reductase [29]. Thus, hepatic glutathione reductase was used as a parameter for the antioxidant activity of glutathione. In the groups treated with DEC, the results suggested dose-dependent glutathione activity. The DEC encapsulated groups exhibited greater enzyme activity, probably due to the greater retention time of the drug in the liver. These findings, together with the NO results, indicate the antioxidant action of DEC-NANO in the liver, most probably due to the stimulation of glutathione activity.

Carbon tetrachloride induces the production of trichloromethyl radicals in hepatocytes due to the action of the cytochrome *p*450 2E1 enzyme (CYP2E1). These radicals lead to lipid peroxidation, with the production of free radicals, thus activating the Kupffer cells, which produce pro inflammatory cytokines such as TNF-α and IL-1β [30]. Immunohistochemical and Western blot analysis identified elevated levels of TNF-α and IL-1β in animals that received CCl_4. Treatment with DEC-NANO 12.5 mg/kg was able to prevent the increase of these inflammatory markers.

Expressed in all liver cells (hepatocytes, Kupffer cells, vascular endothelial cells, and stellate cells), iNOS is associated with the development and propagation of inflammation [31,32]. CCl_4 treated groups demonstrated elevated levels of iNOS in both Western blot and immunohistochemical analysis. Only the CCl_4 + DEC-NANO 12.5 group significantly reduced the concentration of iNOS in comparison with the CCl_4 group.

Cyclooxygenase-2 is up-regulated by growth factors and different cytokines such as IL-1β, TNF-α or IL-6, and is among the most important proinflammatory mediators. COX-2 can induce the nuclear factor kappa B (NF-κB), a transcription factor responsible for the transcription of inflammatory cytokines, with the subsequent recruitment of macrophages. Moreover, PGE_2, the product of arachidonic acid conversion, also has an inflammatory effect, regulating cytokine production in leukocytes [33,34].

Diethylcarbamazine is a known inhibitor of COX-2. The present study evaluated the expression of PGE_2 in the liver. The DEC 50 mg/kg and DEC-NANO 12.5 mg/kg groups reduced the labelling of the liver sections in comparison with the inflammatory group. We previously reported the inhibition of COX-2 by DEC at a concentration of 50 mg/kg for 12 days [6]. In the present study, the same concentration of DEC with only 6 days of treatment was able to reduce the expression of COX-2 in comparison with the CCl_4 group. Strikingly, DEC-NANO 12.5 mg/kg exhibited greater inhibition of the enzyme than its non-encapsulated form.

The present study reports for the first time the use of a nanoencapsulated formulation of diethylcarbamazine in an inflammation model. The findings demonstrate the efficacy of nanoencapsulation, with a significant reduction in the dose and treatment time widely used in literature. The DEC-NANO 12.5 presented the best results between the two nanoformulations evaluated. These results could be explained by the dose administered, but also could be influenced by the difference in the size of the two nanoparticles formulations evaluated in this study. The DEC-NANO 12.5 presented the larger nanoparticles (see Table 1)

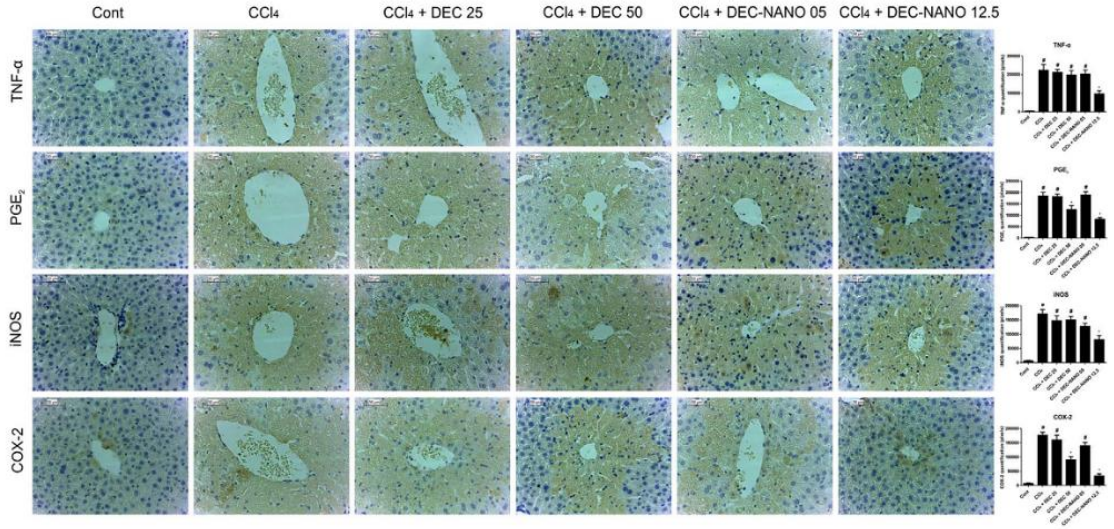


Fig. 6. Immunohistochemistry for TNF-α, PGE_2, iNOS and COX-2 in the liver. Quantification (seven areas arbitrarily selected per animal) was performed using GIMP 2.8 image analysis software ($n = 3$ animals per group). The results are expressed as mean ± SD of three mice per group. $^{*}p < 0.05$ vs CCl4; $^{\#}p < 0.05$ vs control. Bar = 50 μm. Magnification: 400 ×. Liver thin section = 4–5 μm. CCl4, carbon tetrachloride; Cont, control; COX-2, cyclooxygenases-2; DEC, diethylcarbamazine; iNOS, inducible nitric oxide synthase; PGE_2, prostaglandin E2; TNF-α, tumor necrosis factor alpha.

and this may display increased avidity due to their greater individual surface area of nanoparticle for interaction with the cell membrane and adjacent receptors [19].

The results indicate the remarkable anti-inflammatory effect of DEC-NANO through the reduction of cytokines and inflammatory enzymes, as well as increased glutathione activity.

In conclusion, the nanoencapsulation of DEC exhibited a greater effect than its conventional formulation, even with only a quarter of the

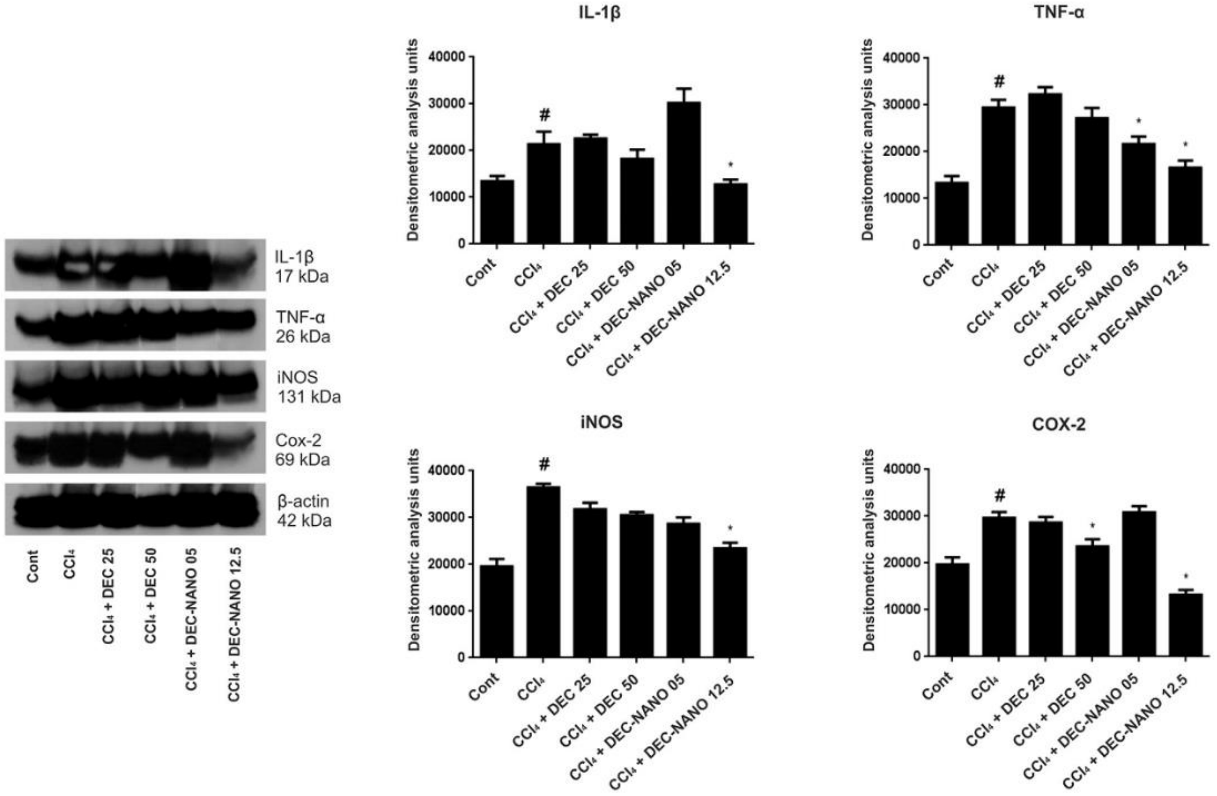


Fig. 7. Immunoblot for IL-1β, TNF-α, iNOS and COX-2. The densitometric analysis was performed with IMAGEJ 1.38 software. The results are expressed as mean ± SD of five mice per group. $^{*}p < 0.05$ vs CCl4; $^{\#}p < 0.05$ vs control. CCl4, carbon tetrachloride; Cont, control; COX-2, cyclooxygenases-2; DEC, diethylcarbamazine; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor alpha.

dose, possibly resulting in reduced side effects and a longer action time, and representing a promising formulation in the treatment of inflammatory disorders.

Author disclosure

The authors declare no conflicts of interest.

Acknowledgements

None.

References

[1] F. Saliba, D. Samuel, Acute liver failure: current trends, J. Hepatol. 59 (1) (2013) 6–8.
[2] C. Panackel, R. Thomas, B. Sebastian, S.K. Mathai, Recent advances in management of acute liver failure, Indian J. Crit. Care Med. 19 (1) (2015) 27–33.
[3] K. Kiso, et al., The role of Kupffer cells in carbon tetrachloride intoxication in mice, Biol. Pharm. Bull. 35 (6) (2012) 980–983.
[4] G. Malaguarnera, E. Cataudella, M. Giordano, G. Nunnari, G. Chisari, M. Malaguarnera, Toxic hepatitis in occupational exposure to solvents, World J. Gastroenterol. 18 (22) (2012) 2756–2766.
[5] C.A. Peixoto, B.S. Silva, Anti-inflammatory effects of diethylcarbamazine: a review, Eur. J. Pharmacol. 734C (Apr. 2014) 35–41.
[6] G.B. Rodrigues, et al., Diethylcarbamazine: possible therapeutic alternative in the treatment of alcoholic liver disease in C57BL/6 mice, Clin. Exp. Pharmacol. Physiol. 42 (4) (2015) 369–379.
[7] R. Wang, P.S. Billone, W.M. Mullett, Nanomedicine in action: an overview of cancer nanomedicine on the market and in clinical trials, J. Nanomater. (April) (2013) 87–2013.
[8] A.P. Nikam, P.R. Mukesh, S.P. Chaudhari, Nanoparticles – an overview, Int. J. Res. Dev. Pharm. L. Sci. 3 (5) (2014) 1121–1127.
[9] S.R. Mudshinge, A.B. Deore, S. Patil, C.M. Bhalgat, Nanoparticles: emerging carriers for drug delivery, Saudi Pharm. J. 19 (3) (2011) 129–141.
[10] M. Allman, L. Gaskin, C.A. Rivera, CCl4-induced hepatic injury in mice fed a western diet is associated with blunted healing, J. Gastroenterol. Hepatol. 25 (3) (2010) 635–643.
[11] P. Chetoni, et al., Solid lipid nanoparticles as promising tool for intraocular tobramycin delivery: pharmacokinetic studies on rabbits, Eur. J. Pharm. Biopharm. 109 (2016) 214–223.
[12] F. Lince, D.L. Marchisio, A.A. Barresi, Strategies to control the particle size distribution of poly-ε-caprolactone nanoparticles for pharmaceutical applications, J. Colloid Interface Sci. 322 (2) (2008) 505–515.
[13] E.-C. Shin, P.S. Sung, S.-H. Park, Immune responses and immunopathology in acute and chronic viral hepatitis, Nat. Rev. Immunol. 16 (8) (2016) 509–523.
[14] H. Devarbhavi, An update on drug-induced liver injury, J. Clin. Exp. Hepatol. 2 (3) (2012) 247–259.
[15] A.J. Czaja, Diagnosis and management of autoimmune hepatitis: current status and future directions, Gut Liver 10 (2) (2016) 177–203.
[16] B. Sid, J. Verrax, P.B. Calderon, Role of AMPK activation in oxidative cell damage: implications for alcohol-induced liver disease, Biochem. Pharmacol. 86 (2) (Jul. 2013) 200–209.
[17] E.S. Orman, G. Odena, R. Bataller, Alcoholic liver disease: pathogenesis, management, and novel targets for therapy, J. Gastroenterol. Hepatol. 28 (Suppl. 1) (Aug. 2013) 77–84.
[18] M. Iqbal, N. Zafar, H. Fessi, A. Elaissari, Double emulsion solvent evaporation techniques used for drug encapsulation, Int. J. Pharm. 496 (2) (2015) 173–190.
[19] Y.N. Zhang, W. Poon, A.J. Tavares, I.D. McGilvray, W.C.W. Chan, Nanoparticle–liver interactions: cellular uptake and hepatobiliary elimination, J. Control. Release 240 (2016) 332–348.
[20] J.-B. Coty, E. Eleamen Oliveira, C. Vauthier, Tuning complement activation and pathway through controlled molecular architecture of dextran chains in nanoparticle corona, Int. J. Pharm. (2017) (in press), http://www.sciencedirect.com/science/article/pii/S0378517317303587?via%3Dihub.
[21] R. Kumar, H. Korideck, W. Ngwa, R.I. Berbeco, G.M. Makrigiorgos, S. Sridhar, Third generation gold nanoplatform optimized for radiation therapy, Transl. Cancer Res. 2 (4) (2013) 1–6.
[22] R.M. Williams, et al., Mesoscale nanoparticles selectively target the renal proximal tubule epithelium, Nano Lett. 15 (4) (2015) 2358–2364.
[23] S. Carvalhana, J. Leitão, A.C. Alves, M. Bourbon, H. Cortez-Pinto, How good is controlled attenuation parameter and fatty liver index for assessing liver steatosis in general population: correlation with ultrasound, Liver Int. 34 (6) (2014) 1–7.
[24] A.J. Czaja, Hepatic inflammation and progressive liver fibrosis in chronic liver disease, World J. Gastroenterol. 20 (10) (2014) 2515–2532.
[25] E. Seki, R.F. Schwabe, Hepatic inflammation and fibrosis: functional links and key pathways, Hepatology 61 (3) (2015) 1066–1079.
[26] U.E. Lee, S.L. Friedman, Mechanisms of hepatic fibrogenesis, Best Pr. Res. Clin. Gastroenterol. 25 (2) (2011) 195–206.
[27] P. Muriel, Role of free radicals in liver diseases, Hepatol. Int. 3 (4) (2009) 526–536.
[28] H.L. Martin, P. Teismann, Glutathione—a review on its role and significance in Parkinson's disease, FASEB J. 23 (10) (2009) 3263–3272.
[29] V.I. Lushchak, V.I. Lushchak, Glutathione homeostasis and functions: potential targets for medical interventions, J. Amino Acids 2012 (2012) 1–26.
[30] S. Basu, Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients, Toxicology 189 (1–2) (2003) 113–127.
[31] L. Leifeld, M. Fielenbach, F.-L. Dumoulin, N. Speidel, T. Sauerbruch, U. Spengler, Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) expression in fulminant hepatic failure, J. Hepatol. 37 (5) (2002) 613–619.
[32] Y. Iwakiri, Nitric oxide in liver fibrosis: the role of inducible nitric oxide synthase, Clin. Mol. Hepatol. 21 (4) (2015) 319–325.
[33] C. Sobolewski, C. Cerella, M. Dicato, L. Ghibelli, M. Diederich, The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies, Int. J. Cell. Biol. 2010 (2010) 215158.
[34] E. Ricciotti, G.A. Fitzgerald, Prostaglandins and inflammation, Arterioscler. Thromb. Vasc. Biol. 31 (5) (2011) 986–1000.

ARTIGO II - A new diethylcarbamazine formulation (NANO-DEC) as a therapeutic tool for hepatic fibrosis

International Immunopharmacology 64 (2018) 280–288

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

A new diethylcarbamazine formulation (NANO-DEC) as a therapeutic tool for hepatic fibrosis

Gabriel Barros Rodrigues[a,b], Elquio Eleamen Oliveira[c], Francisco Jaime Bezerra Mendonça Junior[c], Laise Aline Martins dos Santos[a], Wilma Helena de Oliveira[a,b], Maria Eduarda Rocha de França[a,b], Deniele Bezerra Lós[d], Brennda Martins Gabínio[c], Christina Alves Peixoto[a,*]

[a] Laboratório de Ultraestrutura, Instituto Aggeu Magalhães – FIOCRUZ, Recife, Brazil
[b] Programa de Pós-graduação em Ciências Biológicas, Centro de Biociências, Universidade Federal de Pernambuco - UFPE, Recife, Brazil
[c] Laboratório de Síntese e Vetorização de Moléculas (LSVM), Universidade Estadual da Paraíba, João Pessoa, Brazil
[d] Programa de Pós-graduação em Biotecnologia/RENORBIO, Universidade Federal de Pernambuco - UFPE, Recife, Brazil

ARTICLE INFO

Keywords:
Nanoencapsulation
Diethylcarbamazine
Inflammation
Fibrosis

ABSTRACT

The aim of the present study was to assess if the uninterrupted and prolonged administration of nanoparticles containing diethylcarbamazine (NANO-DEC) would cause liver, kidney and heart toxicity and then analyze for the first time its action in model of liver fibrosis. Thus, NANO-DEC was administered in C57BL/6 mice daily for 48 days, and at the end the blood was collected for biochemical analyzes. In the long-term administration assay, the evaluation of serological parameters (CK-MB, creatinine, ALT, AST and urea) allowed the conclusion that NANO-DEC prolonged administration did not cause hepatic, renal and cardiac damage. For fibrosis assays, C57BL/6 mice were divided into six groups: 1) control (Cont); 2) carbon tetrachloride (CCl_4); 3) CCl_4 + DEC 25 mg/kg; 4) CCl_4 + DEC 50 mg/kg; 5) CCl_4 + NANO-DEC 5 mg/kg and 6) CCl_4 + NANO-DEC 12.5 mg/kg. Carbon tetrachloride induced hepatic fibrosis observed through increased inflammatory (TNF-α, IL-1β, COX-2, NO and iNOS) and fibrotic markers (TGF-β and TIMP-1), changes in the hepatic morphology, high presence of collagen fibers and elevated serum levels of AST, ALT and ALP. Treatment with NANO-DEC exhibited a superior anti-inflammatory and anti-fibrotic effects compared to the DEC traditional formulation, restoring liver morphology, reducing the content of collagen fibers and serological parameters, besides decreasing the expression of inflammatory and fibrotic markers. The present formulation of nanoencapsulated DEC is a well tolerated anti-inflammatory and anti-fibrotic drug and therefore could be a potential therapeutic tool for the treatment of chronic liver disorders.

1. Introduction

Chronic liver inflammation has been associated with progressive hepatic fibrosis which can lead to the development of cirrhosis, a global health problem accounting for 1 million deaths each year worldwide [1]. Cirrhosis is also associated with an increased risk of hepatocellular carcinoma, liver failure and portal hypertension, remaining as major causes of morbidity and mortality worldwide associated with increasing economic and social impact [2].

The hepatotoxin carbon tetrachloride (CCl_4) is the most widely used hepatotoxin in the study of liver fibrosis and cirrhosis in mice. In many aspects, it mimics human chronic disease associated with toxic damage. The observed necrosis of hepatocytes in the periphery of the central vein is due to CCl_4 capacity to cause oxidative stress, fatty liver and lipid peroxidation [3,4].

Diethylcarbamazine (DEC), a piperazine derivative [5], that has been used as an antifilarial drug in the treatment and control of lymphatic filariasis since 1947. Despite the effective microfilaricidal action,

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; CCl_4, carbon tetrachloride; CK-MB, creatine kinase-MB; COX-2, cyclooxygenase 2; DEC, diethylcarbamazine; EMC, extracellular matrix; HSC, hepatic stellate cell; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; MMP-2, metalloproteinase 2; NANO-DEC, diethylcarbamazine nanoencapsulated; NF-κB, nuclear factor kappa-B; NO, nitric oxide; TGF-β, transforming growth factor beta; TIMP-1, tissue inhibitor of metalloproteinase 1; TNF-α, tumor necrosis factor alpha

* Corresponding author at: Instituto Aggeu Magalhães, Av Moraes Rego s/n Campus da UFPE, FIOCRUZ, Recife CEP 52171-011, Brazil.
E-mail addresses: gabrielb.rodrigues@hotmail.com (G.B. Rodrigues), peixoto.christina@gmail.com (C.A. Peixoto).

https://doi.org/10.1016/j.intimp.2018.09.010
Received 30 July 2018; Received in revised form 31 August 2018; Accepted 8 September 2018

its mechanism of action is not clear. However, DEC has anti-inflammatory properties result of the inhibition of cyclooxygenase and nuclear factor kappa-B (NF-κB) signaling pathways [6], which regulates a large array of inflammatory genes, such as COX-2, iNOS, TNF-α, IL-1β and IL-6 [7,8]. In a recent study from our laboratory, we observed that during the inflammatory process, diethylcarbamazine was able to inhibit the phosphorylation of NF-κB similarly to pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB [9].

Nanoparticles are defined as particulate dispersions or solid particles ranging in size from 10 to 1000 nm [10]. Nanomedicine has rapidly advanced in recent years with several applications such as drug delivery systems, vaccine development, contrast agents for anatomical and functional imaging, implants production etc. [11]. Nanoencapsulation of therapeutic agents allows changes in their pharmacokinetic properties, protecting the drug from its premature degradation in the biological environment, enhance bioavailability and the concentration in the target tissue. Such drug delivery systems transport the drug to its specific site of action, presenting higher efficacy and specificity over the traditional formulations, with lower systemic toxicity and doses [12,13].

Over the last 8 years, our laboratory has extensively explored the anti-inflammatory properties of DEC. We evaluated the anti-inflammatory action of DEC in models of pulmonary and bone marrow eosinophilia [14], acute pulmonary inflammation induced by carrageenan [15,16] and lipopolysaccharide [17], pulmonary hypertension [18], chronic hepatic inflammation induced by alcohol [9,19,20] carbon tetrachloride [21,22] and liver injury induced by protein malnutrition [23].

All the articles cited were performed with the traditional DEC formulation, and the best results obtained with the dose of 50 mg/kg. In a recent study of our laboratory, we characterized the nanoencapsulation of DEC (NANO-DEC) and its use for the first time in an acute model of inflammation, reducing the standard treatment regimen by half (from 12 to 6 days) with only a quarter (12.5 mg/kg) of the standard dose [24]. This NANO-DEC formulation is under patent submission (BR 10 2017 002700-7 and PCT/BR2018/050027). In face the excellent results obtained in the treatment of acute hepatic inflammation, the present study was realized to evaluate whether prolonged use of this new formulation could cause renal, cardiovascular and hepatic toxicity, and then analyze for the first time its action in model of liver fibrosis, with only a quarter (12.5 mg/kg) and a tenth (5 mg/kg) of the dose of its traditional formulation.

2. Materials and methods

2.1. Animals

The Animal Studies Ethics Committee of the Oswaldo Cruz Institute approved all the experiments reported (no. 72/2014). Eighty-seven male C57BL/6 mice (age: 5 weeks; weight: 15–17 g) were obtained from the Aggeu Magalhães Institute–FIOCRUZ (Pernambuco, Brazil). The health status of the mice was examined and they were acclimated to a laboratory environment of 23 °C and a 12-h light and 12-h dark photoperiod. The animals were housed in metal cages and fed with a standard diet and water ad libitum.

2.2. Diethylcarbamazine solutions

The solutions were composed of distilled water and DEC (Farmanguinhos, FIOCRUZ, Brazil) and were adjusted in accordance with the body weight of each animal. They were administered through intraperitoneal injection (100 μL/day). The control group (Cont) received only distilled water via the same administration route.

2.3. Preparation of DEC-loaded nanoparticles

The nanoparticles were prepared by the double emulsion method. One milliliter of an aqueous solution containing 5 or 12.5 mg/mL of DEC was emulsified in 10 mL of dichloromethane containing 50 μg of poly(ε-caprolactone) (PCL) (Mw = 14,000 g/mol) and 30 μg of Pluronic F68. The first emulsion (W1/O) was obtained using the UltraTurrax (Ultra-Turrax T18 basic, IKA) homogenizer at 25,000 rotations per minute (rpm) for 10 min. The first emulsion was then added to 40 mL of a poly(vinyl alcohol) (PVA) aqueous solution (5 mg/mL) and dispersed by using Ultra-Turrax (Ultra-Turrax T18 basic, IKA) at 25,000 rotations per minute (rpm) for 10 min until the formation of a double emulsion (W1/O/W2). Finally, solvent evaporation was carried out under reduced pressure using a rotary evaporator (RV 10 D, IKA).

2.4. Experimental design

2.4.1. Long-term administration model

NANO-DEC (12.5 mg/kg) was administered through intraperitoneal injection (1 injection/day) for 48 days. The animals were anesthetized (xylazine 10 mg/kg and ketamine 100 mg/kg) and sacrificed by cervical dislocation in the days: 0, 6, 12, 24 and 48 (three animals each day). Blood was collected by cardiac puncture and the samples were centrifuged at 1500 rpm for 10 min to obtain the serum. Biochemical determinations were performed as described in item 2.6.

2.4.2. Fibrosis model

Fibrosis was induced by intraperitoneal administration (2 injections/week) of CCl_4 0.5 μL/g of body weight (Sigma-Aldrich, St. Louis, MO, USA) dissolved in olive oil (final volume, 100 μL per mouse) for 8 weeks [3,22]. DEC (25 mg/kg and 50 mg/kg) and DEC-NANO (12.5 mg/kg and 5 mg/kg) were administered through intraperitoneal injection (1 injection/day) for 6 days (last six days of CCl_4 administration). The control groups received distilled water via the same route. The C57BL/6 mice were separated into six groups (n = 6/group): (1) the control group (Cont), which received just distilled water; (2) the CCl_4 group (CCl_4); (3) the CCl_4 plus DEC 25 mg/kg group (CCl_4 + DEC 25); (4) the CCl_4 plus DEC 50 mg/kg group (CCl_4 + DEC 50); (5) the CCl_4 plus DEC-NANO 5 mg/kg group (CCl_4 + DEC-NANO 5) and (6) the CCl_4 plus DEC-NANO 12.5 mg/kg group (CCl_4 + DEC-NANO 12.5). At the end of the experiment the animals were anesthetized (xylazine 10 mg/kg and ketamine 100 mg/kg) and euthanized by cervical dislocation.

For a better comprehension of the experimental procedures, a schematic design was included (Fig. 1).

2.5. Biochemical determinations

Serum was assessed and the following determinations were performed: aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase-MB (CK-MB), creatinine and urea. Serum concentrations were measured by the spectrophotometric method with an Integra 400 (Roche, Basel, Switzerland). Data were compared by one-way ANOVA and Dunnett's or Tukey's tests, with GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA).

2.6. Histopathology

2.6.1. Hematoxylin–eosin staining

Liver fragments were fixed in 10% formalin for 24 h, processed, and embedded in paraffin. Sections of 4–5 μm were cut and mounted on glass slides. Slices were stained with hematoxylin–eosin and assessed by inverted microscopy (Observer Z1; Zeiss MicroImaging, Jena, Germany), a camera, and 4.7.4 image analysis software (AxionCam MRm; Zeiss, Jena, Germany) at a magnification of ×400.

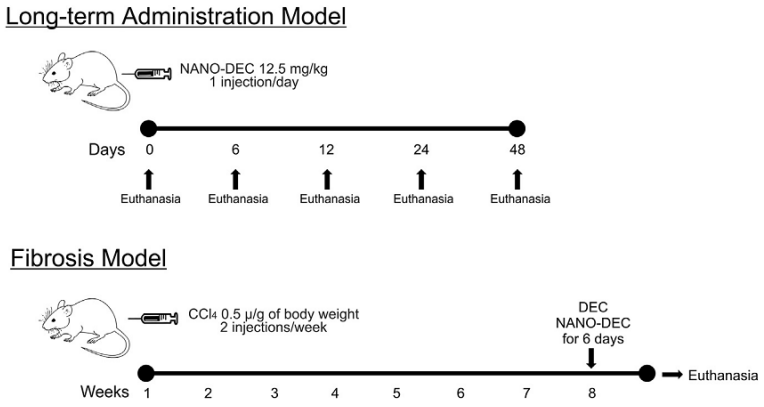


Fig. 1. Schematic drawing of the experimental models.

2.6.2. Immunohistochemical assays

Five sections (5 µm thickness) from each group were cut and adhered to slides treated with 3-amino-propyl-triethoxy-silane (Sigma-Aldrich, St. Louis, MO, USA). The sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). The sections were then heated for 30 min in a sodium citrate buffer (0.01 M, pH 6.0) and treated with 0.3% (v/v) H_2O_2 in water for 5 min. The sections were washed with 0.01 M PBS (pH 7.2) and then blocked with 1% BSA and 0.2% Tween 20 in PBS for 1 h at room temperature. The sections were incubated for 12 h at 4 °C with the following antibodies: anti-IL-1β antibody (1:100 dilution; ab9722; Abcam, Cambridge, UK), anti-TNF-α (1:100 dilution; 500-P64; PrepoTech, London, UK), anti-iNOS (1:250 dilution; ab3523; Abcam) and anti-COX-2 (1:400 dilution; ab15191; Abcam). The antigen-antibody reaction was visualized with avidin-biotin peroxidase (DakoUniversal LSAB + Kit, Peroxidase), using 3.3-diaminobenzidine as the chromogen. The slides were counter-stained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, with the exception of the first antibody, which was omitted. Five images at the same magnification were quantitatively analyzed using GIMP 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.6.3. Sirius red/fast green staining

Liver fragments were fixed in 10% formalin for 24 h, processed, and embedded in paraffin. Sections of 2.5 lm were cut and mounted on glass slides. Slices were stained with picrosirius red solution of 1%, rehydrated in water, and stained with Fast Green 0.1% solution in ethanol. They were then assessed by inverted microscopy (Observer Z1; Zeiss MicroImaging), a camera and 4.7.4 image analysis software (AxionCam MRm; Zeiss) at a magnification of ×400.

2.7. Measurement of nitric oxide

The Griess colorimetric reaction was used for the measurement of nitric oxide (NO), which involved the detection of nitrite (NO_2^-) and the oxidation of NO in the liver as previously described [24].

2.8. Western blot

Livers were quickly dissected and then homogenized in a Wheaton Overhead Stirrer (model 903,475; Wheaton Science Products, Millville, NJ, USA) using an extraction cocktail of 10-mmol/L ethylenediamine tetraacetic acid, 2-mmol/L phenylmethylsulfonyl fluoride, 100-mmol/L sodium fluoride, 10-mmol/L sodium pyrophosphate, 10-mmol/L sodium orthovanadate, 10-mg aprotinin, and 100-mmol/L Tris(hydroxymethyl) aminomethane (pH 7.4). Western blot assays were then performed according to the previously described methodology [9]. The antibodies used were anti-COX-2 (1:1000 dilution; ab15191; Abcam), anti-iNOS (1:1000 dilution; ab3523; Abcam), anti-IL-1β antibody (1:1000 dilution; ab9722; Abcam), TNF-α (1:1000 dilution; 500-P64; PrepoTech), anti-TGF-β (1:1000 dilution; ab66043; Abcam), anti-TIMP-1 (1:1000 dilution.; ab86482; Abcam), anti-MMP-2 (1:1000 dilution; ab110186; Abcam) and anti-β-actin (1:1000 dilution; ab8227; Abcam).

2.9. Statistical analysis

GraphPad Prism version 6.01 was used for statistical analysis. Data were expressed as mean ± SD. Differences between the control and treated groups were analyzed by ANOVA and either Dunnett's test, Tukey's test, or the *t*-test as a post hoc test. Probability values lower than 0.05 were considered significant.

3. Results

3.1. Serological biochemical analyzes in long-term administration model

In order to evaluate if the long-term administration of the nanoparticles containing DEC could cause toxicity, the formulation at the concentration of 12.5 mg/kg (NANO-DEC 12.5) was administered for 48 consecutive days. Biochemical analyzes were performed on days 0, 6, 12, 24 and 48 to evaluate cardiac (CK-MB), hepatic (AST and ALT) and renal function (creatinine and urea). In all parameters evaluated, no significant differences were observed between groups and when compared to day zero (no treatment) (Fig. 2).

3.2. Serological biochemical analyzes

In biochemical analysis of liver damage markers, the CCl_4 group exhibited a significant increase in AST (Fig. 3A) and ALT (Fig. 3B) when compared to the control group. CCl_4 + DEC 25 and CCl_4 + DEC-NANO 5 groups did not significantly reduce these markers. However, CCl_4 + DEC 50 was able to reduce ALT significantly, and only CCl_4 + DEC-NANO 12.5 reduced both transaminases.

In the evaluation of ALP (Fig. 3C), the inflammatory group (CCl_4) presented a high value in relation to the control group, however, all groups treated with DEC formulations, significantly reduced the dosages of ALP when compared to the CCl_4 group, suggesting a strong effect of the DEC on the enzyme activity.

3.3. Determination of hepatic nitric oxide

In the evaluation of hepatic NO_2^- levels (Fig. 4), similar to the

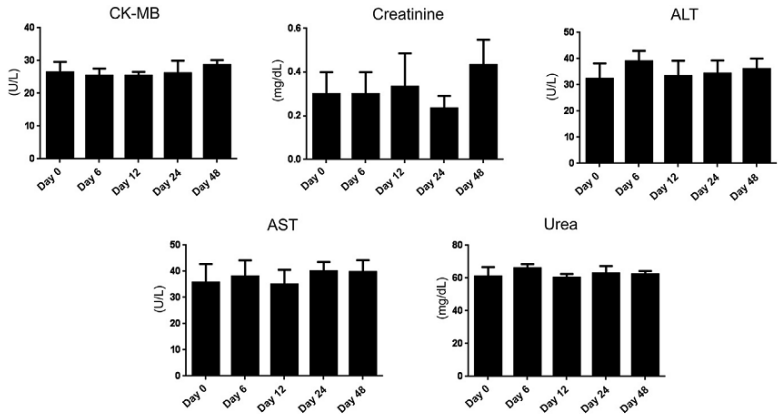


Fig. 2. Serum biochemical parameters (CK-MB, creatinine, AST, ALT and Urea) of the long-term administration model. Day 0 represents the group of animals that did not receive treatment. Days 6, 12, 24 and 48 represent groups of dosing intervals over the 48-day daily administration of NANO-DEC 12.5 mg/kg. The results are expressed as mean ± SD of three mice per group. No statistical differences were observed between groups ($p > 0.05$).

results obtained in the biochemical dosage of the ALP, the CCl_4 group showed a significant increase in the NO_2^- levels, but all the groups treated with DEC and NANO-DEC, were able to significantly reduce hepatic oxide nitric content.

3.4. Histopathological analysis of the hepatic tissue

Histological analysis of control group confirmed standard hepatic morphology, with well-preserved hepatocytes (Fig. 5A). In the group of animals exposed to CCl_4, various histopathological characteristics of persistent inflammation were identified such as scattered lobular inflammation, macrovesicular steatosis, hepatocellular ballooning, inflammatory infiltrates and several areas of necrosis (Fig. 5B, C and D).

The CCl_4 + DEC 25, CCl_4 + DEC 50 and CCl_4 + DEC-NANO 5 groups exhibited the same histological alterations found in inflammatory group (CCl_4) (Fig. 6A, B and C). However, the group CCl_4 + DEC-NANO 12.5 presented a reduction in all of the histological alterations. There was a noticeable decrease in inflammatory infiltrate, macrovesicular steatosis and necrosis areas, furthermore the hepatocytes returned to the cord morphology (Fig. 6D).

3.5. Evaluation of collagen fibers content

Tissue collagen accumulation represents an important marker of the liver fibrosis. In order to evaluate the collagen content, we performed the Sirius Red/Fast Green that is a histochemical staining for quantification type I and III collagens [25]. The control group exhibited little staining of collagen fibers (red coloring), which was restricted to the periphery of the central veins, in addition to presenting a greenish background (fast green dye) characteristic of low or absent presence of collagen (Fig. 7A). The CCl_4, CCl_4 + DEC 25, CCl_4 + DEC 50 and CCl_4 + DEC-NANO 5 groups presented very intense labelling for collagen fibers, dispersed throughout all hepatic stroma, confirming the

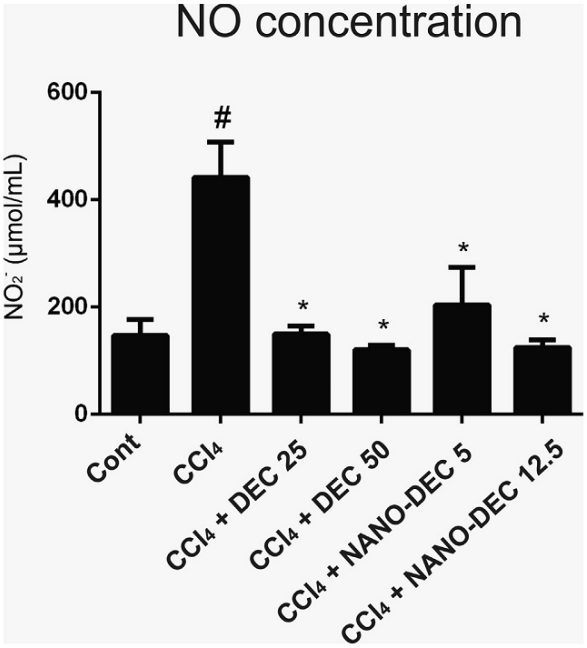


Fig. 4. Assessment of hepatic NO concentration through the measure of total nitrite metabolites (NO_2^-). The results are expressed as mean ± SD of three mice per group. *$p < 0.05$ vs CCl4; #$p < 0.05$ vs control.

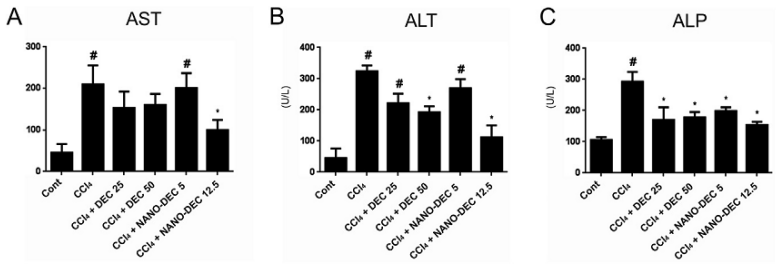


Fig. 3. Biochemical parameters of the fibrosis model. (A) Aspartate aminotransferase (AST). (B) Alanine aminotransferase (ALT). (C) Alkaline phosphatase (ALP). The results are expressed as mean ± SD of three mice per group. *$p < 0.05$ vs CCl_4; #$p < 0.05$ vs control.

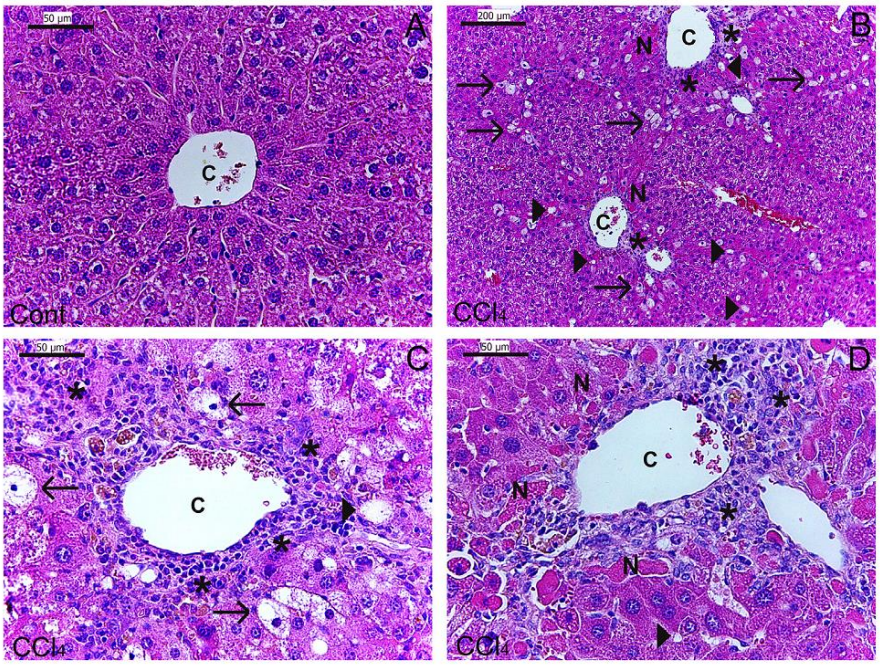


Fig. 5. Histopathological analysis of the hepatic tissue after CCl_4 administration. (A) Control group, (B, C and D) CCl_4 group. Bar = 200 µm (B), Bar = 50 µm (A, C and D) showing several histological changes characteristic of a persistent inflammatory process. (C) Central vein; (asterisk) inflammatory infiltrate; (N) necrosis areas; (arrowheads) lipid macrovesicle; (arrows) hepatocellular ballooning.

fibrous feature of the model (Fig. 7B–E). Only the CCl_4 + DEC-NANO 12.5 group exhibited a noticed reduction of collagen fibers labelling, presenting a light greenish background tone (Fig. 7F).

3.6. Immunohistochemistry analysis

Liver tissue sections from mice in the CCl_4 group demonstrated elevated staining in the periphery of the central veins for the inflammatory cytokines IL-1β and TNF-α, and for the inflammatory enzymes COX-2 and iNOS when compared with the control group (Fig. 8). CCl_4 + DEC 25 and CCl_4 + DEC-NANO 5 also presented elevated specific immunolabelling for those markers.

The CCl_4 + DEC 50 group was only able to reduce the TNF-α and COX-2 labelling. However, the CCl_4 + DEC-NANO 12.5 group significantly reduced the labelling of IL-1β, TNF-α, COX-2 and iNOS when compared to the inflammatory group (CCl_4).

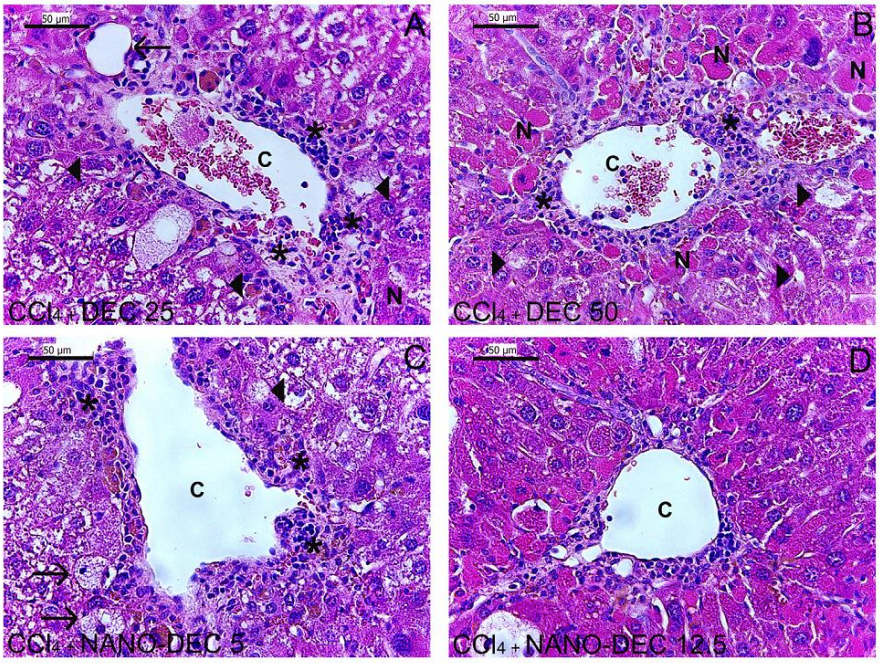


Fig. 6. Histopathological analysis of hepatic tissue after DEC and NANO-DEC treatments. Observe that only CCl_4 + NANO-DEC 12.5 treatment decreased inflammatory infiltrate, macrovesicular steatosis and necrosis areas. Bar = 50 µm. (C) central vein; (asterisk) inflammatory infiltrate; (N) necrosis areas; (arrowheads) lipid macrovesicle; (arrows) hepatocellular ballooning.

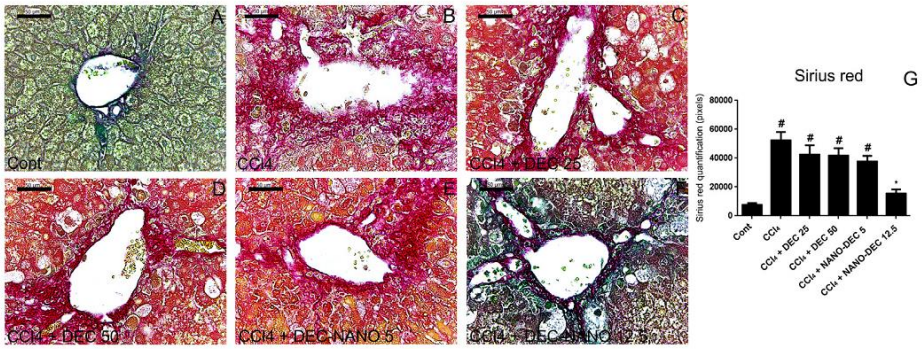


Fig. 7. Assessment of the content of collagen fibers by Sirius red/fast green staining. Quantification (five arbitrarily selected areas per animal) was performed using GIMP 2 image analysis software (n = 3 animals per group). The results are expressed as mean ± SEM of four mice per group. Bar = 50 μm. Magnification: 400×. *p < 0.05 vs CCl_4; #p < 0.05 vs control.

3.7. Western blot analysis

The western blot analysis of the livers fragments revealed a significant high expression of all inflammatory and fibrotic markers (COX-2, iNOS, IL-1β, TNF-α, TGF-β and TIMP-1) in the CCl_4, CCl_4 + DEC 25, CCl4 + DEC 50 and CCl_4 + DEC-NANO 5 groups relative to the control group, with the exception of the CCl_4 + DEC 50 group that reduced the expression of COX-2 (Fig. 9A–F). In addition, all groups mentioned also exhibited a reduction in the expression of the matrix metalloproteinase-2 (MMP-2) (Fig. 9G).

Interestingly, the group that had DEC nanoencapsulated at the concentration of 12.5 mg/kg was able to significantly reduce the expression of all markers, and it was the only group capable of increasing the expression of the metalloproteinase-2, in accordance to the results obtained in the analysis of immunohistochemistry and collagen fibers content.

4. Discussion

Current medical management for chronic liver disease, a term comprising several persistent pathological conditions of the liver, are limited and differentiated, depending on the etiology and/or persistence of the stimulus. When therapeutic measures are ineffective, patients may progress to cirrhosis. Liver transplantation remains the only treatment for end-stage cirrhosis, being responsible for than 60% of all liver transplantations [26–28].

Due to the recent results obtained with the administration of NANO-DEC for the first time in a model of acute inflammation, the aim of this study was to evaluate whether the continued use of this formulation could be toxic and would be effective in a model of severe hepatic injury. Therefore, NANO-DEC was daily administered (at the concentration of 12.5 mg/kg) for 48 days and then two NANO-formulations with a quarter (12.5 mg/kg) and a tenth (5 mg/kg) of the usual doses were evaluated and compared with the traditional formulations at doses of 50 and 25 m/kg in a model of hepatic fibrosis.

Transaminases are enzymes found in high concentrations in the liver and also present in the kidney, heart and muscle. Any type of liver cell injury can cause the increase of ALT levels, and greatly elevated ALT levels are specific to hepatocellular diseases. Besides, ALT and AST are elevated in chronic liver diseases like liver tissue degeneration and necrosis [29]. Elevated levels of alkaline phosphatase are observed in cirrhosis, hepatitis and congestive cardiac failure, besides indicating higher risk of cardiovascular diseases [29,30]. Creatine kinase-MB (CK-MB) is a specific myocardial necrosis marker [31] and elevated serum CK-MB values represent it leakage from damaged membranes of cardiomyocites into circulation and is an indicator of cardiotoxicity [32].

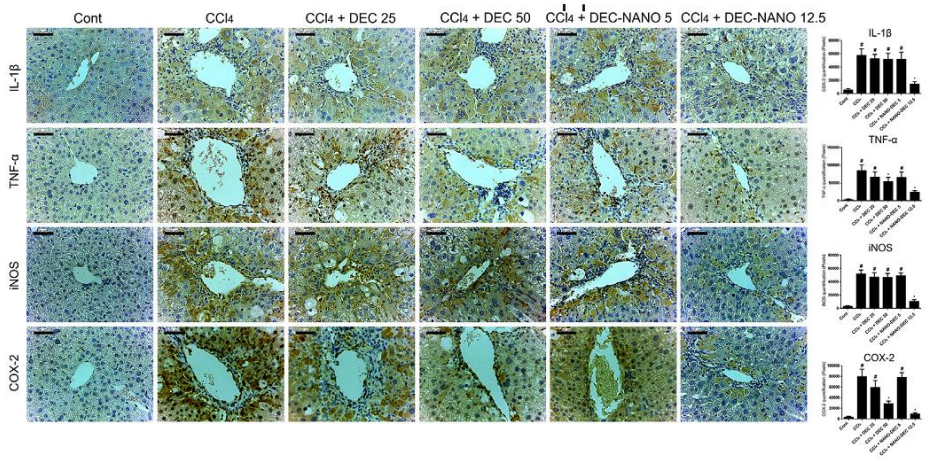


Fig. 8. Immunohistochemistry for IL-1β, TNF-α, iNOS and COX-2 in the liver. Quantification (seven areas arbitrarily selected per animal) was performed using GIMP 2.8 image analysis software (n = 3 animals per group). The results are expressed as mean ± SD of three mice per group. *p < 0.05 vs CCl_4; #p < 0.05 vs control. Bar = 50 μm. Magnification: 400×.

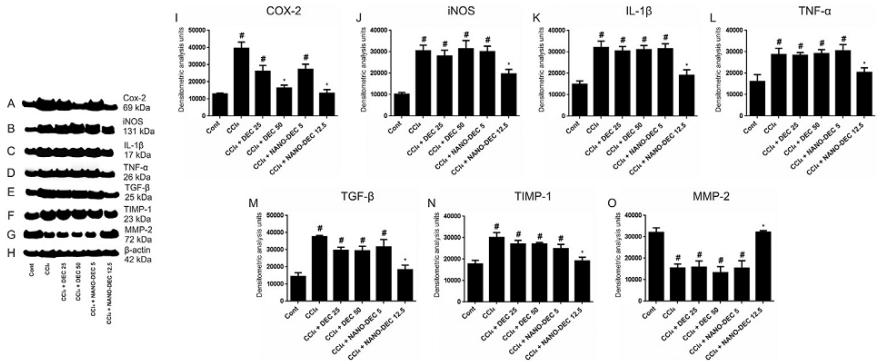


Fig. 9. Immunoblot for COX-2, iNOS, IL-1β, TNF-α, TGF-β, TIMP-1 and MMP-2 with the liver homogenate. The densitometric analysis was performed with IMAGEJ 1.38 software. The results are expressed as mean ± SD of five mice per group. *$p < 0.05$ vs CCl_4; #$p < 0.05$ vs control.

The continued use of NANO-DEC 12.5 did not promote increase of the CK-MB, AST and ALT dosages. These data suggest that prolonged NANO-DEC administration does not cause damage to the liver and heart.

The administration of CCl_4 promoted elevated serological values of ALT, AST and ALP, confirming the potent hepatotoxic action of this toxin. Although DEC in its original formulation of 50 mg/kg reduced the dosage of ALT, only DEC-NANO 12.5 was able to lower the values of both transaminases. In addition, DEC seems to have a strong inhibitory effect on ALP, reducing the expression of the enzyme in all formulations and dosages containing DEC.

Serum creatinine allows establish empiric dosing for medication of potentially toxic drugs whose elimination is dependent on renal function [33], and elevated serum creatinine often results in reduction of drug dose and may lead to discontinuation of the use of the medication [34]. Urea is an indicator of renal function where a decrease glomerular filtration rate results in rise of plasma concentrations of serum creatinine and urea [35]. Besides, high levels of serum urea are associated with kidney disease or failure and blockage of the urinary tract by a kidney stone [36]. In the present study, the use of NANO-DEC for 48 days did not increase the serum creatinine. In addition, none of the days analyzed showed significant variation in serum urea values when compared to day zero. These data suggest that the long-term use of the NANO-DEC formulation does not interfere with function nor cause renal damage.

In the liver, small amounts of NO generated by endothelial NO synthase (eNOS) maintains liver homeostasis and protect against pathological conditions. The inducible NO synthase (iNOS) expression is absent in resting cells, but profibrotic stimuli such as TNF-α and IL-1β cytokines activate Kupffer cells to produce large amounts of NO, which is a major source of reactive nitrogen species (RNS) and peroxynitrite (ONOO−), through an induction of iNOS. These radicals (RNS and ONOO−) can oxidize DNA bases, tyrosine residues of proteins and glutathione, causing hepatocyte death besides activate hepatic stellate cells (HSCs), promoting liver fibrosis [37,38]. All formulations containing DEC reduced hepatic NO levels. However, only the nanoencapsulated DEC at 12.5 mg/kg concentration was efficient in reduce the iNOS expression in immunohistochemistry and western blot assays.

The extracellular matrix (ECM), formed by a complex network of proteins and proteoglycans, is present in all tissues providing a physical scaffold and structural support for cells. ECM also plays an important role in maintaining homeostasis by regulating important cellular functions such as differentiation, migration, adhesion and proliferation [39]. In healthy liver the integrity of the EMC is maintained by a permanent balance between the secretion of metalloproteinases (MMPs), a family of endopeptidases capable of degrading virtually any component of the ECM, and their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), a family of proteins capable of inhibit activated MMPs as well as the conversion of pro-MMPs to activated MMPs [40].

In chronic inflammation, the liver express high levels of TIMP-1 leading to the inhibition of MMPs activity and subsequent accumulation of matrix proteins, in particular the types I, III and IV of collagens. Hepatic fibrosis is characterized by the excessive deposition ECM proteins [41,42]. Furthermore, in patients with hepatitis C, TIMP-1 concentrations were used as non-invasive parameters of liver fibrosis [43]. MMP-2, also known as gelatinase A, MMP-2 (gelatinase A) can cleave collagen type I [44] and IV [45]. Radbill et al., 2011 [46] have demonstrated that MMP-2−/− mice developed increased liver fibrosis compared with MMP-2+/+ mice after chronic exposure to CCl_4 and that exogenous MMP-2 suppresses collagen expression in culture of HSC-like cells.

TGF-β is considered a master regulator of fibrotic pathologies. TGF-β promotes the differentiation of quiescent fibroblasts into extracellular matrix secreting myofibroblasts. Furthermore, TGF-β regulates the expression of several profibrotic genes, including fibronectin, collagens (COL1A1, COL3A1, COL5A2, COL6A1, COL6A3, COL7A1) and protease inhibitors such as plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinase 3 (TIMP-3), key components in the development of liver fibrosis [47,48].

In the Sirius red staining (specific for collagens type I and III) the groups that received carbon tetrachloride exhibited high labelling presented intense positive staining throughout all the hepatic stroma. These results were confirmed by the elevated expression of TGF-β and TIMP-1 and the reduction of MMP-2, observed in the western blot assay, which are factors directly related to the fibrotic process. Nevertheless, confirming the effective anti-fibrotic action of NANO-DEC 12.5, its administration was capable to decrease collagen production, as also decrease the expression of the fibrotic factors TGF-β and TIMP-1, and increase the MMP-2 levels.

Tumor necrosis factor alpha (TNF-α) is a major pro-inflammatory cytokine involved in the development of many chronic inflammatory diseases. Primarily produced by monocytes and Kupffer cells, increased TNF-α production is one of the earliest responses of the liver to injury, and during the chronic liver injury, the hepatocyte damage leads to recruitment of Kupffer cells and neutrophils to produce TNF-α and IL-1β that mediate the inflammatory response and lead to the deposition of extracellular matrix by activation of HSCs [49,50]. The NANO-DEC 12.5 formulation reduced the expression of both cytokines (TNF-α and IL-1β) in all analyzes.

Cyclooxygenase-2 (COX-2), the inducible COX isoform is expressed upon response to cell stressors, such as pro-inflammatory cytokines, growth factors and hormones, represents a major target in the

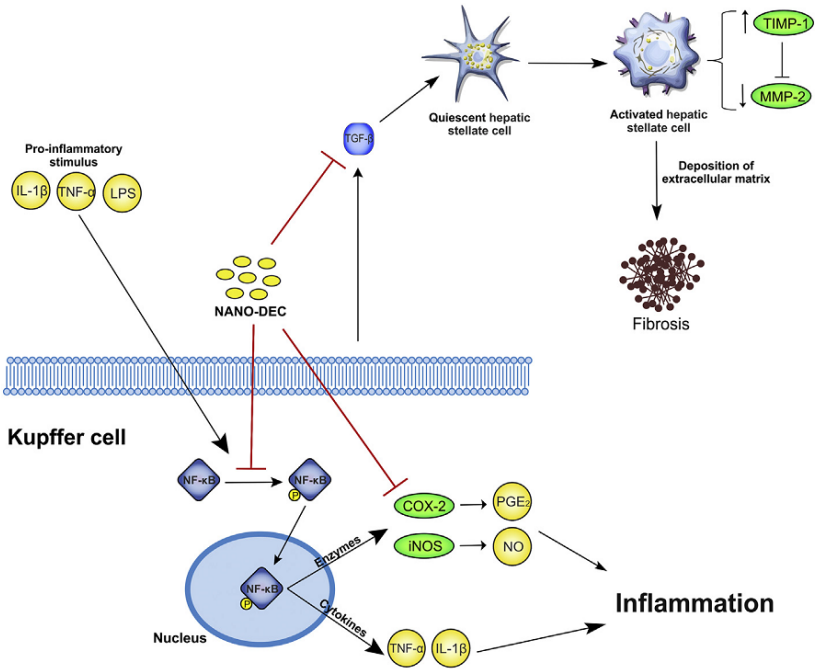


Fig. 10. Schematic diagram showing the possible mechanisms involved in the anti-inflammatory and anti-fibrotic NANO-DEC action. NANO-DEC can direct inhibit COX-2 and the NF-κB pathway, and thereby inhibit the production of inflammatory markers. In addition, NANO-DEC decreases the expression of TGF-β, impairing the activation of stellate cells capable of promote fibrosis through deposition of extracellular matrix.

treatment of inflammatory disorders [51]. COX-2 is involved in the high synthesis of prostaglandins (PGs) under pathological conditions. These PGs, especially the prostaglandin E2 (PGE_2), can elicits a wide range of biological effects associated with inflammation and cancer. Furthermore, PGE_2 produced by COX-2 act as vasodilator to facilitate the tissue influx of neutrophils and macrophages from the bloodstream leading to swelling and edema and promoting the progression of inflammation [52,53].

Diethylcarbamazine is a cyclooxygenase-2 pathway inhibitor [6]. Previous studies from our laboratory have demonstrated the effective inhibition of COX-2 (as well as the reduction of PGE_2 expression) by DEC in its traditional formulation of 50 mg/kg [9,21]. In the present study, this result was confirmed. However, NANO-DEC 12.5 containing only a quarter of the traditional formulation, demonstrated potent inhibition of COX-2 expression similar to that observed in DEC 50 (Fig. 10).

5. Conclusion

The present results demonstrated that the new nanoencapsulated formulation of DEC allowed reduction of the dose and the time of treatment of hepatic fibrosis when compared to the DEC traditional dosage, besides being more efficient to reduce inflammatory markers. Surprisingly, in only 6 days, the NANO-DEC treatment was able to reverse the fibrotic process induced by CCl_4. In addition, this new DEC formulation showed to be safe even in long-term treatments. Therefore, NANO-DEC is a potent and well-tolerated drug for treatment of chronic liver disorders.

Declaration of conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was funding by Fundação de Amparo à Ciência e Tecnologia de Estado de Pernambuco (FACEPE/IBPG - 0163-2.09/14) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 301777/2012-8).

References

[1] Y. Koyama, D.A. Brenner, Liver inflammation and fibrosis, J. Clin. Invest. 127 (2017) 55–64, https://doi.org/10.1172/JCI88881.orders.
[2] R. Xu, Z. Zhang, F.S. Wang, Liver fibrosis: mechanisms of immune-mediated liver injury, Cell. Mol. Immunol. 9 (2012) 296–301, https://doi.org/10.1038/cmi.2011.53.
[3] S.C. Yanguas, B. Cogliati, J. Willebrords, M. Maes, B. Van Den Bossche, C. Pinto, M. Souza, S. Paulo, Experimental Models of Liver Fibrosis, (2016), https://doi.org/10.1007/s00204-015-1543-4.Experimental.
[4] K. Kiso, S. Ueno, M. Fukuda, I. Ichi, K. Kobayashi, T. Sakai, K. Fukui, S. Kojo, The role of Kupffer cells in carbon tetrachloride intoxication in mice, Biol. Pharm. Bull. 35 (2012) 980–983, https://doi.org/10.1248/bpb.35.980.
[5] S.F. Sanchez Bruni, D.G. Jones, Q. a McKellar, Pharmacological approaches towards rationalizing the use of endoparasitic drugs in small animals, J. Vet. Pharmacol. Ther. 29 (2006) 443–457, https://doi.org/10.1111/j.1365-2885.2006.00806.x.
[6] C.A. Peixoto, B.S. Silva, Anti-inflammatory effects of diethylcarbamazine: a review, Eur. J. Pharmacol. 734C (2014) 35–41, https://doi.org/10.1016/j.ejphar.2014.03.046.
[7] T. Liu, L. Zhang, D. Joo, S.-C. Sun, NF-κB signaling in inflammation, Signal Transduction Targeted Ther. 2 (2017) 17023, , https://doi.org/10.1038/sigtrans.2017.23.
[8] R. Khan, A.Q. Khan, A. Lateef, M.U. Rehman, M. Tahir, F. Ali, O.O. Hamiza, S. Sultana, Glycyrrhizic acid suppresses the development of precancerous lesions via regulating the hyperproliferation, inflammation, angiogenesis and apoptosis in the colon of Wistar rats, PLoS One 8 (2013) 1–22, https://doi.org/10.1371/journal.pone.0056020.
[9] G.B. Rodrigues, S.W.S. Rocha, L.A.M. dos Santos, W.H. de Oliveira, F.O. dos S. Gomes, M.E. da R. de França, D.B. Lós, C.A. Peixoto, Diethylcarbamazine: possible therapeutic alternative in the treatment of alcoholic liver disease in C57BL/6 mice, Clin. Exp. Pharmacol. Physiol. 42 (2015) 369–379, https://doi.org/10.1111/1440-1681.12369.
[10] A.P. Nikam, M.P. Ratnaparkhiand, S.P. Chaudhari, Nanoparticles – an overview, Int. J. Res. Dev. Pharm. L. Sci. 3 (2014) 1121–1127.
[11] B. Pelaz, C. Alexiou, R.A. Alvarez-Puebla, F. Alves, A.M. Andrews, S. Ashraf, L.P. Balogh, L. Ballerini, A. Bestetti, C. Brendel, S. Bosi, M. Carril, W.C.W. Chan, C. Chen, X. Chen, X. Chen, Z. Cheng, D. Cui, J. Du, C. Dullin, A. Escudero, N. Feliu, M. Gao, M. George, Y. Gogotsi, A. Grünweller, Z. Gu, N.J. Halas, N. Hampp, R.K. Hartmann, M.C. Hersam, P. Hunziker, J. Jian, X. Jiang, P. Jungebluth, P. Kadhiresan, K. Kataoka, A. Khademhosseini, J. Kopeček, N.A. Kotov, H.F. Krug, D.S. Lee, C.M. Lehr, K.W. Leong, X.J. Liang, M. Ling Lim, L.M. Liz-Marzán, X. Ma, P. Macchiarini, H. Meng, H. Möhwald, P. Mulvaney, A.E. Nel, S. Nie, P. Nordlander, T. Okano, J. Oliveira, T.H. Park, R.M. Penner, M. Prato, V. Puntes, V.M. Rotello, A. Samarakoon, R.E. Schaak, Y. Shen, S. Sjöqvist, A.G. Skirtach, M.G. Soliman,

M.M. Stevens, H.W. Sung, B.Z. Tang, R. Tietze, B.N. Udugama, J.S. VanEpps, T. Weil, P.S. Weiss, I. Willner, Y. Wu, L. Yang, Z. Yue, Q. Zhang, Q. Zhang, X.E. Zhang, Y. Zhao, X. Zhou, W.J. Parak, Diverse applications of nanomedicine, ACS Nano 11 (2017) 2313–2381, https://doi.org/10.1021/acsnano.6b06040.

[12] A. Kumari, R. Singla, A. Guliani, S.K. Yadav, Nanoencapsulation for drug delivery, EXCLI J. 13 (2014) 265–286, https://doi.org/10.17877/DE290R-15592.

[13] S.R. Mudshinge, A.B. Deore, S. Patil, C.M. Bhalgat, Nanoparticles: emerging carriers for drug delivery, Saudi Pharm. J. 19 (2011) 129–141, https://doi.org/10.1016/j.jsps.2011.04.001.

[14] T. Queto, P. Xavier-Elsas, M.A. Gardel, B. De Luca, M. Barradas, D. Masid, P.M.R.E. Silva, C.A. Peixoto, Z.M.F. Vasconcelos, E.P. Dias, M.I. Gaspar-Elsas, Inducible nitric oxide synthase/CD95L-dependent suppression of pulmonary and bone marrow eosinophilia by diethylcarbamazine, Am. J. Respir. Crit. Care Med. 181 (2010) 429–437, https://doi.org/10.1164/rccm.200905-0800OC.

[15] E.L. Ribeiro, K.P.D.S. Barbosa, I.T. Fragoso, M.A.M. Donato, F. Oliveira Dos Santos Gomes, B.S. Da Silva, A.K.S.E. Silva, S.W.S. Rocha, V. Amaro Da Silva Junior, C.A. Peixoto, Diethylcarbamazine attenuates the development of Carrageenan-induced lung injury in mice, Mediat. Inflamm. 2014 (2014) 1–12, https://doi.org/10.1155/2014/105120.

[16] L.A.M. Santos, E.L. Ribeiro, K.P.S. Barbosa, I.T. Fragoso, F.O.D.S. Gomes, M.A.M. Donato, B.S. Silva, A.K.S. Silva, S.W.S. Rocha, M.E.R. França, G.B. Rodrigues, T.G. Silva, C.A. Peixoto, Diethylcarbamazine inhibits NF-κB activation in acute lung injury induced by carrageenan in mice, Int. Immunopharmacol. 23 (2014) 153–162, https://doi.org/10.1016/j.intimp.2014.08.017.

[17] I.T. Fragoso, E.L. Ribeiro, F.O. dos S. Gomes, M.A.M. Donato, A.K.S. Silva, A.C.O.d. Oliveira, S.M. da R. Araújo, K.P.S. Barbosa, L.A.M. Santos, C.A. Peixoto, Diethylcarbamazine attenuates LPS-induced acute lung injury in mice by apoptosis of inflammatory cells, Pharmacol. Rep. 69 (2017) 81–89, https://doi.org/10.1016/j.pharep.2016.09.021.

[18] E.L. Ribeiro, I.T. Fragoso, F.O. dos S. Gomes, A.C. Oliveira, A.K.S. Silva, P.M. Silva, B.T. Ciambarella, I.P.R. Ramos, C.A. Peixoto, Diethylcarbamazine: a potential treatment drug for pulmonary hypertension? Toxicol. Appl. Pharmacol. 333 (2017) 92–99, https://doi.org/10.1016/j.taap.2017.08.015.

[19] S.W.S. Rocha, B.S. Silva, F.O. dos S. Gomes, A.K.S. e Silva, C. Raposo, K.P.S. Barbosa, D. de O. Torres, A.C.O. dos Santos Cipriano, C.A. Peixoto, Effect of diethylcarbamazine on chronic hepatic inflammation induced by alcohol in C57BL/6 mice, Eur. J. Pharmacol. 689 (2012) 194–203, https://doi.org/10.1016/j.ejphar.2012.05.044.

[20] B.S. da Silva, G.B. Rodrigues, S.W.S. Rocha, E.L. Ribeiro, F.O.D.S. Gomes, A.K.S. e Silva, C.A. Peixoto, Inhibition of NF-κB activation by diethylcarbamazine prevents alcohol-induced liver injury in C57BL/6 mice, Tissue Cell 46 (2014), https://doi.org/10.1016/j.tice.2014.06.008.

[21] S.W.S. Rocha, M.E.R.D. França, G.B. Rodrigues, K.P.S. Barbosa, A.K.S. Nunes, A.F. Pastor, A.G.V. Oliveira, W.H. Oliveira, R.L.A. Luna, C.A. Peixoto, Diethylcarbamazine Reduces Chronic Inflammation and Fibrosis in Carbon Tetrachloride- (CCl_4-) Induced Liver Injury in Mice, Mediat. Inflamm. 2014 (2014), https://doi.org/10.1155/2014/696383.

[22] M.E.R.D. França, S.W.S. Rocha, W.H. Oliveira, L.A. Santos, A.G.V. de Oliveira, K.P.S. Barbosa, A.K.S. Nunes, G.B. Rodrigues, D.B. Lós, C.A. Peixoto, Diethylcarbamazine attenuates the expression of pro-fibrogenic markers and hepatic stellate cells activation in carbon tetrachloride-induced liver fibrosis, Inflammopharmacology (2017), https://doi.org/10.1007/s10787-017-0329-0.

[23] S.W.S. Rocha, A.C.O. dos Santos, B. dos S. Silva, D. de O.C. Torres, E.L. Ribeiro, K.P.S. Barbosa, F. de O. Gomes, C.A. Peixoto, Effects of diethylcarbamazine (DEC) on hepatocytes of C57BL/6J mice submitted to protein malnutrition, J. Food Drug Anal. 20 (2012) 524–531, https://doi.org/10.6227/jfda.2012200214.

[24] G.B. Rodrigues, E.E. Oliveira, F.J.B.M. Junior, L.A.M. dos Santos, W.H. de Oliveira, M.E.R. de França, D.B. Lós, B.M. Gabínio, F.C.M.L. de Lira, C.A. Peixoto, Characterization and evaluation of nanoencapsulated diethylcarbamazine in model of acute hepatic inflammation, Int. Immunopharmacol. 50 (2017) 330–337, https://doi.org/10.1016/j.intimp.2017.07.014.

[25] C. Segnani, C. Ippolito, L. Antonioli, C. Pellegrini, C. Blandizzi, A. Dolfi, N. Bernardini, Histochemical detection of collagen fibers by Sirius Red/Fast Green is more sensitive than van Gieson or Sirius Red alone in normal and inflamed rat colon, PLoS One 10 (2015) 1–10, https://doi.org/10.1371/journal.pone.0144630.

[26] J.L. Newton, D.E.J. Jones, Managing systemic symptoms in chronic liver disease, J. Hepatol. 56 (2012) S46–S55, https://doi.org/10.1016/S0168-8278(12)60006-3.

[27] M.P. Manns, Liver cirrhosis, transplantation and organ shortage, Dtsch Arztebl Int 110 (2013) 83–84, https://doi.org/10.3238/arztebl.2013.0083.

[28] C.Y. Zhang, W.G. Yuan, P. He, J.H. Lei, C.X. Wang, Liver fibrosis and hepatic stellate cells: etiology, pathological hallmarks and therapeutic targets, World J. Gastroenterol. 22 (2016) 10512–10522, https://doi.org/10.3748/wjg.v22.i48.10512.

[29] S. Gowda, P.B. Desai, V.V. Hull, A. a K. Math, S.N. Vernekar, S.S. Kulkarni, A review on laboratory liver function tests, Pan Afr. Med. J. 3 (17) (2009), https://doi.org/10.11604/pamj.2009.3.17.125.

[30] J.W. Li, C. Xu, Y. Fan, Y. Wang, Y. Bin Xiao, Can serum levels of alkaline phosphatase and phosphate predict cardiovascular diseases and total mortality in individuals with preserved renal function? A systemic review and meta-analysis, PLoS One 9 (2014) 1–14, https://doi.org/10.1371/journal.pone.0102276.

[31] E. Hodzic, Potential anti-inflammatory treatment of ischemic heart disease, Med. Arch. (Sarajevo, Bosnia Herzegovina) 72 (2018) 94–98, https://doi.org/10.5455/medarh.2018.72.94-98.

[32] R. Zilinyi, A. Czompa, A. Czegledi, A. Gajtko, D. Pituk, I. Lekli, A. Tosaki, The cardioprotective effect of metformin in doxorubicin-induced cardiotoxicity: the role of autophagy, Molecules 23 (2018), https://doi.org/10.3390/molecules23051184.

[33] M.P. Ducharme, M. Smythe, G. Strohs, Drug-induced alterations in serum creatinine concentrations, Ann. Pharmacother. 27 (1993) 622–633.

[34] X. Chu, K. Bleasby, G.H. Chan, I. Nunes, R. Evers, The complexities of interpreting reversible elevated serum creatinine levels in drug development: does a correlation with inhibition of renal transporters exist? Drug Metab. Dispos. 44 (2016) 1498–1509, https://doi.org/10.1124/dmd.115.067694.

[35] D. Pandya, A.K. Nagrajappa, K.S. Ravi, Assessment and correlation of urea and creatinine levels in saliva and serum of patients with chronic kidney disease, diabetes and hypertension– a research study, J. Clin. Diagn. Res. 10 (2016) ZC58–ZC62, https://doi.org/10.7860/JCDR/2016/20294.8651.

[36] S. Gowda, P.B. Desai, S.S. Kulkarni, V.V. Hull, A.A.K. Math, S.N. Vernekar, Markers of renal function tests, N. Am. J. Med. Sci. 2 (2010) 170–173.

[37] Y. Iwakiri, M.Y. Kim, Nitric oxide in liver diseases, Trends Pharmacol. Sci. 36 (2015) 524–536, https://doi.org/10.1016/j.tips.2015.05.001.NITRIC.

[38] Y. Iwakiri, Nitric oxide in liver fibrosis: the role of inducible nitric oxide synthase, Clin. Mol. Hepatol. 21 (2015) 319–325, https://doi.org/10.3350/cmh.2015.21.4.319.

[39] C. Frantz, K.M. Stewart, V.M. Weaver, The extracellular matrix at a glance, J. Cell Sci. 123 (2010) 4195–4200, https://doi.org/10.1242/jcs.023820.

[40] S. Duarte, J. Baber, T. Fujii, A.J. Coito, Matrix metalloproteinases in liver injury, repair and fibrosis, Matrix Biol. May–Jun (2015) 147–156, https://doi.org/10.1016/j.coviro.2015.09.001.Human.

[41] S. Hemmann, J. Graf, M. Roderfeld, E. Roeb, Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies, J. Hepatol. 46 (2007) 955–975, https://doi.org/10.1016/j.jhep.2007.02.003.

[42] A. Baiocchini, C. Montaldo, A. Conigliaro, A. Grimaldi, V. Correani, F. Mura, F. Ciccosanti, N. Rotiroti, A. Brenna, M. Montalbano, G. D'Offizi, M.R. Capobianchi, R. Alessandro, M. Piacentini, M.E. Schininà, B. Maras, F. Del Nonno, M. Tripodi, C. Mancone, Extracellular matrix molecular remodeling in human liver fibrosis evolution, PLoS One 11 (2016) 1–14, https://doi.org/10.1371/journal.pone.0151736.

[43] J.S. El-Gindy, A.T. El Rahman, M.A. El-Alim, Diagnostic potential of serum matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 as non-invasive markers of hepatic fibrosis in patients with HCV related chronic liver disease, Egypt J. Immunol. 10 (2003) 27–35.

[44] R.T. Aimes, J.P. Quigley, Matrix metalloproteinase-2 is an interstitial collagenase, J. Biol. Chem. 270 (1995) 5872–5876, https://doi.org/10.1074/jbc.270.11.5872.

[45] J. Kurzepa, A. M, G. Czechowska, J. Kurzepa, K. Celiński, W. Kazmierak, M. Slstrokomka, Role of MMP-2 and MMP-9 and their natural inhibitors in liver fibrosis, chronic pancreatitis and non-specific inflammatory bowel diseases, Hepatobiliary Pancreat. Dis. Int. 13 (2014) 570–579, https://doi.org/10.1016/S1499-3872(14)60261-7.

[46] B.D. Radbill, R. Gupta, M.C.M. Ramirez, A. DiFeo, J.A. Martignetti, C.E. Alvarez, S.L. Friedman, G. Narla, R. Vrabie, R. Bowles, Y. Saiman, M.B. Bansal, Loss of matrix metalloproteinase-2 amplifies murine toxin-induced liver fibrosis by upregulating collagen I expression, Dig. Dis. Sci. 56 (2011) 406–416, https://doi.org/10.1007/s10620-010-1296-0.Loss.

[47] K.L. Walton, K.E. Johnson, C.A. Harrison, Targeting TGF-β mediated SMAD signaling for the prevention of fibrosis, Front. Pharmacol. 8 (2017), https://doi.org/10.3389/fphar.2017.00461.

[48] K.K. Kim, D. Sheppard, H.A. Chapman, TGF-b1 signaling and tissue fibrosis, Cold Spring Harb. Perspect. Biol. 10 (2018) a022293, , https://doi.org/10.1101/cshperspect.a022293.

[49] L.J. Dixon, M. Barnes, H. Tang, M.T. Pritchard, L.E. Nagy, Kupffer cells in the liver, Compr. Physiol. 3 (2013) 785–797, https://doi.org/10.1002/cphy.c120026.

[50] N. Parameswaran, S. Patial, Tumor necrosis factor-α signaling in macrophages, Crit. Rev. Eukaryot. Gene Expr. 20 (2010) 87–103, https://doi.org/10.1615/CritRevEukarGeneExpr.v20.i2.10.

[51] R. Horrillo, A. Planagumà, A. González-Périz, N. Ferré, E. Titos, R. Miquel, M. López-Parra, J.L. Masferrer, V. Arroyo, J. Clària, Comparative protection against liver inflammation and fibrosis by a selective cyclooxygenase-2 inhibitor and a nonredox-type 5-lipoxygenase inhibitor, J. Pharmacol. Exp. Ther. 323 (2007) 778–786, https://doi.org/10.1124/jpet.107.128264.

[52] M. Nakanishi, D.W. Rosenberg, Multifaceted roles of PGE2 in inflammation and cancer, Semin. Immunopathol. 35 (2013) 123–137, https://doi.org/10.1007/s00281-012-0342-8.

[53] P. Martín-Sanz, R. Mayoral, M. Casado, L. Boscá, COX-2 in liver, from regeneration to hepatocarcinogenesis: what we have learned from animal models? World J. Gastroenterol. 16 (2010) 1430–1435, https://doi.org/10.3748/wjg.v16.i12.1430.

4 CONCLUSÃO

Nosso laboratório caracterizou com sucesso a ação anti-inflamatória da formulação tradicional da dietilcarbamazina (DEC) na concentração de 50 mg/kg em diversos modelos de inflamação, em tratamentos de 12 dias. A partir de tais resultados, o objetivo do presente estudo foi melhorar a eficácia e a potência do fármaco como também otimizar seu esquema de tratamento. Dessa forma, foi realizado o nanoencapsulamento da DEC (NANO-DEC), gerando novas formulações de com um quarto (12,5 mg/kg) e um décimo (5 mg/kg) da dosagem usual e avaliado pela primeira vez sua eficácia anti-inflamatória em diversas fases da inflamação hepática (inflamação aguda e fibrose) em camundongos com apenas metade do tempo de tratamento (6 dias). Tais formulação estão sob submissão de patente: BR 10 2017 002700-7 e PCT / BR2018 / 050027 (Anexo B).

Foi possível concluir que a nova formulação na dose de 12,5 mg/kg (NANO-DEC 12.5) permitiu redução da dose (melhora da potência) e do tempo de tratamento quando comparada à dose tradicional de DEC em todas as fases avaliadas. Além disso, a NANO-DEC 12.5 mostrou-se mais eficaz na sua atividade anti-inflamatória e anti-fibrótica. Também foi possível concluir que uso prolongado desta nova formulação (48 dias) não se mostrou tóxico em testes realizados no fígado, pulmão, coração e rins.

Dessa forma, o presente trabalho demonstrou que a NANO-DEC é um medicamento mais potente e eficaz que sua formulação tradicional, sendo bem tolerado para o tratamento de desordens agudas e crônicas do fígado.

5 REFERÊNCIAS

ALVAREZ-LORENZO, C.; CONCHEIRO, A. Smart drug delivery systems: from fundamentals to the clinic. **Chemical Communications**, v. 50, n. 58, p. 7743–7765, 2014.

BANIK, B. L.; FATTAHI, P.; BROWN, J. L. Polymeric nanoparticles: the future of nanomedicine. **Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology**, v. 8, n. 2, p. 271–299, 2016.

BASU, S. Carbon tetrachloride-induced lipid peroxidation: Eicosanoid formation and their regulation by antioxidant nutrients. **Toxicology**, v. 189, n. 1–2, p. 113–127, 2003.

BEERS, M.H.; BERKOW, R. **Manual Merck: Diagnóstico e Tratamento – Edição Centenária**. 17. ed., São Paulo: Roca; p. 478, 2001.

BENEDICT, M.; ZHANG, X. Non-alcoholic fatty liver disease: An expanded review. **World Journal of Hepatology**, v. 9, n. 16, p. 715–732, 2017.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. **Guia de Vigilância Epidemiológica e Eliminação da Filariose Linfática**. Brasília: Ministério da Saúde, 2009. Disponível em: <http:// http://bvsms.saude.gov.br/bvs/publicacoes/guia_vigilancia_filariose_linfatica.pdf>. Acesso em: 02 jun. 2018.

BUXTON, S. K.; ROBERTSON, A. P.; MARTIN, R. J. Diethylcarbamazine Increases Activation of Voltage-Activated Potassium (SLO-1) Currents in Ascaris suum and Potentiates Effects of Emodepside. **PLoS Neglected Tropical Diseases**, v. 8, n. 11, p. e3276, 2014.

CHEN, M. et al. Drug-induced liver injury: Interactions between drug properties and host factors. **Journal of Hepatology**, v. 63, n. 2, p. 503–514, 2015.

CHRISTENSON, J. C.; MANALOOR, J. J. Hepatitis A, B, and C. **Pediatrics In Review**, v. 37, n. 10, p. 426–438, 2016.

CRUCHO, C. I. C.; BARROS, M. T. Polymeric nanoparticles: A study on the preparation variables and characterization methods. **Materials Science and Engineering C**, v. 80, p. 771–784, 2017.

CZAJA, A. J. Hepatic inflammation and progressive liver fibrosis in chronic liver disease. **World Journal of Gastroenterology**, v. 20, n. 10, p. 2515–2532, 2014.

DA SILVA, B. S. et al. Inhibition of NF-κB activation by diethylcarbamazine prevents alcohol-induced liver injury in C57BL/6 mice. **Tissue and Cell**, v. 46, n. 5, p. 363–371, 2014.

DIXON, L. J. et al. Kupffer Cells in the Liver. **Compr Physiol**, v. 3, n. 2, p. 785–797, 2013.

DREYER, G.; NORÕES, J. Dietilcarbamazina no tratamento da filariose bancroftiana. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 30, n. 3, p. 229–240, 1997.

FISCHER, P. U. et al. Potential Value of Triple Drug Therapy with Ivermectin, Diethylcarbamazine, and Albendazole (IDA) to Accelerate Elimination of Lymphatic Filariasis and Onchocerciasis in Africa. **PLoS Neglected Tropical Diseases**, v. 11, n. 1, p. e0005163, 2017.

FRAGOSO, I. T. et al. Diethylcarbamazine attenuates LPS-induced acute lung injury in mice by apoptosis of inflammatory cells. **Pharmacological Reports**, v. 69, n. 1, p. 81–89, 2017.

GARTNER, L.P.; HIATT, J.L. **Tratato de Histologia em Cores**. 3ª ed., Rio de Janeiro: Saunders Elsevier; p.576, 2007.

GE, P. S.; RUNYON, B. A. Treatment of Patients with Cirrhosis. **New England Journal of Medicine**, v. 375, n. 8, p. 767–777, 2016.

ILONDU, N. et al. Pharmacokinetics of Diethylcarbamazine: Prediction by Concentration in Saliva. **Biological & Pharmaceutical Bulletin**, v. 23, n. 4, p. 443–445, 2000.

JAISHANKAR, M. et al. Toxicity, mechanism and health effects of some heavy metals. **Interdisciplinary Toxicology**, v. 7, n. 2, p. 60–72, 2014.

JIANG, J. X.; TÖRÖK, N. J. Liver Injury and the Activation of the Hepatic Myofibroblasts. **Curr Pathobiol Rep**, v. 1, n. 3, p. 215–223, 2013.

JU, C.; TACKE, F. Hepatic macrophages in homeostasis and liver diseases: from

pathogenesis to novel therapeutic strategies. **Cellular and Molecular Immunology**, v. 13, n. 3, p. 316–327, 2016.

JUNQUEIRA, L.C.; CARNEIRO, J. **Histologia Básica**. 12ª ed., Rio de Janeiro: Guanabara Koogan; p.524, 2013.

KRENKEL, O.; TACKE, F. Liver macrophages in tissue homeostasis and disease. **Nature Reviews Immunology**, v. 17, n. 5, p. 306–321, 2017.

KUMARI, A. et al. Nanoencapsulation for drug delivery. **EXCLI Journal**, v. 13, p. 265–286, 2014.

LANHAM, A.; MWANRI, L. The Curse of Lymphatic Filariasis: Would the Continual Use of Diethylcarbamazine Eliminate this Scourge in Papua New Guinea? **American Journal of Infectious Diseases and Microbiology**, v. 1, n. 1, p. 5–12, 2013.

MAITZ, M. F. Applications of synthetic polymers in clinical medicine. **Biosurface and Biotribology**, v. 1, n. 3, p. 161–176, 2015.

MANNS, M. P. Liver Cirrhosis, Transplantation and Organ Shortage. **Deutsches Arzteblatt International**, v. 110, n. 6, p. 83–84, 2013.

MCGARRY, H. F.; PLANT, L. D.; TAYLOR, M. J. Diethylcarbamazine activity against Brugia malayi microfilariae is dependent on inducible nitric-oxide synthase and the cyclooxygenase pathway. **Filaria journal**, v. 4, n. 4, p. 1-9, 2005.

MOHAMED, J. et al. Mechanisms of diabetes-induced liver damage: The role of oxidative stress and inflammation. **Sultan Qaboos University Medical Journal**, v. 16, n. 2, p. 132–1471, 2016.

MOHANRAJ, V.; CHEN, Y. Nanoparticles – A Review. **Tropical Journal of Pharmaceutical Research**, v. 5, n. 1, p. 561–573, 2006.

MUDSHINGE, S. R. et al. Nanoparticles: Emerging carriers for drug delivery. **Saudi Pharmaceutical Journal**, v. 19, n. 3, p. 129–141, 2011.

NEWTON, J. L.; JONES, D. E. J. Managing systemic symptoms in chronic liver disease. **Journal of Hepatology**, v. 56, n. SUPPL. 1, p. S46–S55, 2012.

NIKAM, A. P.; RATNAPARKHIAND, M. P.; CHAUDHARI, S. P. Nanoparticles – an

overview. **Int. J. Res. Dev.Pharm. L. Sci.**, v. 3, n. 5, p. 1121–1127, 2014.

OSNA, N. A. et al. Alcoholic Liver Disease: Pathogenesis and Current Management. **Alcohol research : current reviews**, v. 38, n. 2, p. 147–161, 2017.

PARK, E. J. et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. **Cell**, v. 140, n. 2, p. 197–208, 2010.

PEIXOTO, C. A.; SILVA, B. S. Anti-inflammatory effects of diethylcarbamazine: A review. **European journal of pharmacology**, v. 734C, n. 2014, p. 35–41, 2014.

PELAZ, B. et al. Diverse Applications of Nanomedicine. **ACS Nano**, v. 11, n. 3, p. 2313–2381, 2017.

QARAH, N.; K, B.; ABDULRAHMAN, S. A. Spectrophotometric Assay of Diethylcarbamazine Citrate in Pharmaceuticals and Human Urine via Ion-Pair Reaction Using Methyl Orange Dye. **Journal of Pharmaceutical Care & Health Systems**, v. 04, n. 04, p. 1–7, 2017.

QUETO, T. et al. Inducible nitric oxide synthase/CD95L-dependent suppression of pulmonary and bone marrow eosinophilia by diethylcarbamazine. **American Journal of Respiratory and Critical Care Medicine**, v. 181, n. 5, p. 429–437, 2010.

RIBEIRO, E. L. et al. Diethylcarbamazine attenuates the development of Carrageenan-induced lung injury in mice. **Mediators of Inflammation**, v. 2014, p. 1–12, 2014.

RICCIOTTI, E.; FITZGERALD, G. A. Prostaglandins and Inflammation. **Arterioscler Thromb Vasc Biol**, v. 31, n. 5, p. 986–1000, 2011.

ROBINSON, M. W.; HARMON, C.; O 'FARRELLY, C. Liver immunology and its role in inflammation and homeostasis. **Cellular & Molecular Immunology**, v. 13, n. 10, p. 267–276, 2016.

ROCHA, S. W. S. et al. Diethylcarbamazine reduces chronic inflammation and fibrosis in carbon tetrachloride- (CCl4) induced liver injury in mice. **Mediators of Inflammation**, v. 2014, n. 2014, p. 1-16, 2014.

ROCHA, S. W. S.; SILVA, B. S.; et al. Effect of diethylcarbamazine on chronic hepatic

inflammation induced by alcohol in C57BL/6 mice. **European journal of pharmacology**, v. 689, n. 1–3, p. 194–203, 2012.

ROCHA, S. W. S.; SANTOS, A. C. O. dos; et al. Effects of diethylcarbamazine (DEC) on hepatocytes of C57BL/6J mice submitted to protein malnutrition. **Journal of Food and Drug Analysis**, v. 20, n. 2, p. 524–531, 2012.

RODRIGUES, G. B. et al. Diethylcarbamazine: Possible therapeutic alternative in the treatment of alcoholic liver disease in C57BL/6 mice. **Clinical and Experimental Pharmacology and Physiology**, v. 42, n. 4, p. 369–379, 2015.

SALIBA, F.; SAMUEL, D. Acute liver failure: Current trends. **Journal of Hepatology**, v. 59, n. 1, p. 6–8, 2013.

SANTOS, L. A. M. et al. Diethylcarbamazine inhibits NF-κB activation in acute lung injury induced by carrageenan in mice. **International Immunopharmacology**, v. 23, n. 1, p. 153–162, 2014.

TACKE, F. Targeting hepatic macrophages to treat liver diseases. **Journal of Hepatology**, v. 66, n. 6, p. 1300–1312, 2017.

TRAUTWEIN, C. et al. Hepatic fibrosis: Concept to treatment. **Journal of Hepatology**, v. 62, n. S1, p. S15–S24, 2015.

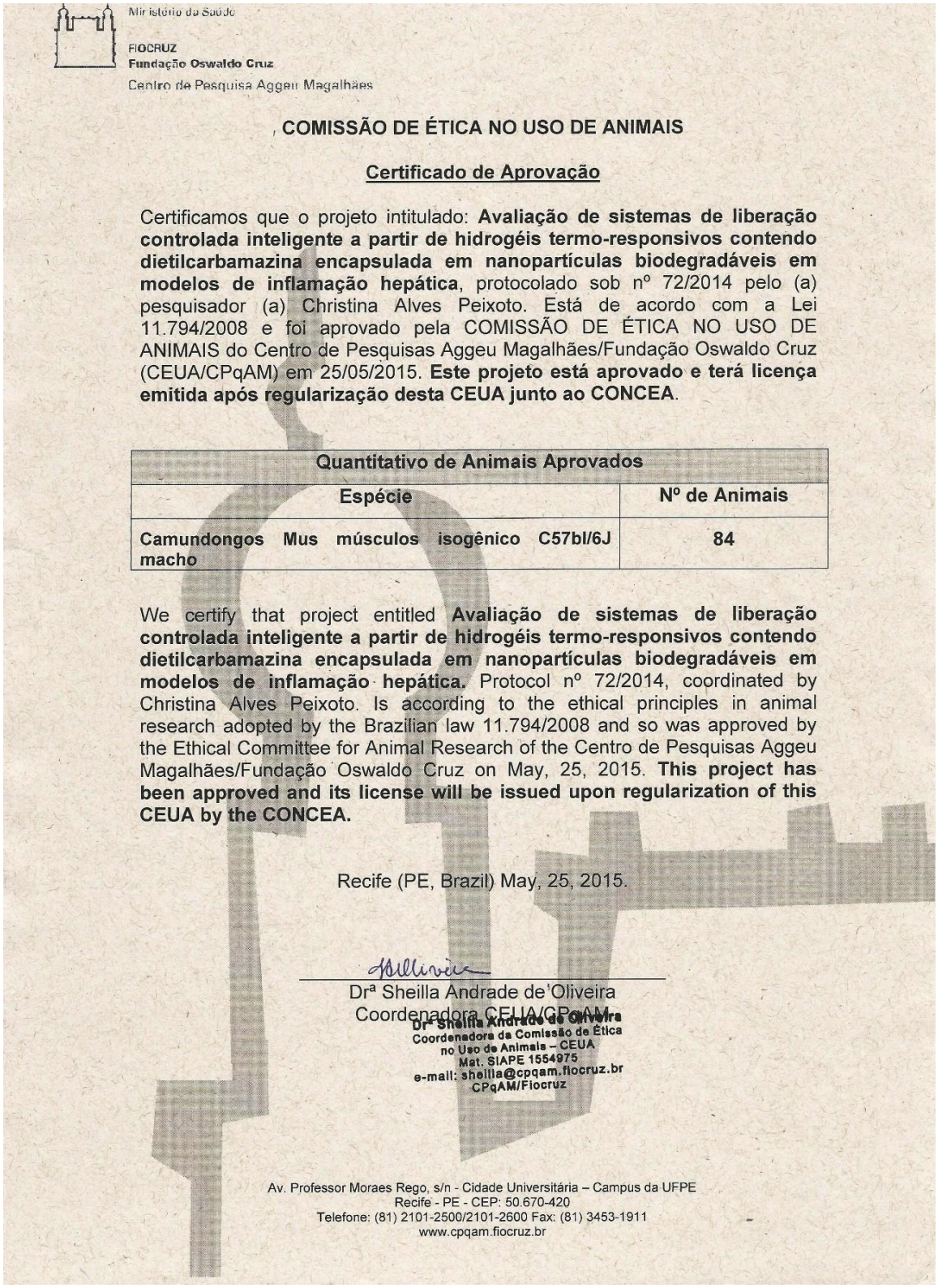
WEBER, L. W. D.; BOLL, M.; STAMPFL, A. Hepatotoxicity and Mechanism of Action of Haloalkanes: Carbon Tetrachloride as a Toxicological Model. **Critical Reviews in Toxicology**, v. 33, n. 2, p. 105–136, 2003.

WELLS, R. G.; SCHWABE, R. Origin and Function of Myofibroblasts in the Liver. **Seminars in Liver Disease**, v. 35, n. 2, p. 97–106, 2015.

YANGUAS, S. C. et al. Experimental models of liver fibrosis. **Arch Toxicol**, v. 90, n. 5, p. 1025–1048, 2016.

YIN, C. et al. Hepatic stellate cells in liver development, regeneration, and cancer. **The Journal of Cinical Investigation**, v. 123, n. 5, p. 1902–1910, 2013.

ANEXO A - Parecer da comissão de ética no uso de animais.



Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado: **Avaliação de sistemas de liberação controlada inteligente a partir de hidrogéis termo-responsivos contendo dietilcarbamazina encapsulada em nanopartículas biodegradáveis em modelos de inflamação hepática**, protocolado sob nº 72/2014 pelo (a) pesquisador (a) Christina Alves Peixoto. Está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/CPqAM) em 25/05/2015. **Este projeto está aprovado e terá licença emitida após regularização desta CEUA junto ao CONCEA**.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Camundongos Mus músculos isogênico C57bl/6J macho	**84**

We certify that project entitled **Avaliação de sistemas de liberação controlada inteligente a partir de hidrogéis termo-responsivos contendo dietilcarbamazina encapsulada em nanopartículas biodegradáveis em modelos de inflamação hepática.** Protocol nº 72/2014, coordinated by Christina Alves Peixoto. Is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz on May, 25, 2015. **This project has been approved and its license will be issued upon regularization of this CEUA by the CONCEA.**

Recife (PE, Brazil) May, 25, 2015.

Drª Sheilla Andrade de Oliveira
Coordenadora CEUA/CPqAM

Drª Sheilla Andrade de Oliveira
Coordenadora da Comissão de Ética
no Uso de Animais – CEUA
Mat. SIAPE 1554975
e-mail: sheilla@cpqam.fiocruz.br
CPqAM/Fiocruz

Av. Professor Moraes Rego, s/n - Cidade Universitária – Campus da UFPE
Recife - PE - CEP: 50.670-420
Telefone: (81) 2101-2500/2101-2600 Fax: (81) 3453-1911
www.cpqam.fiocruz.br

ANEXO B - Comprovante de Depósito de Pedido de Patente.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

10/02/2017 870170008944 10:01

00.000.2.2.16.0411026.5

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 002700 7

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE ESTADUAL DA PARAIBA
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 12671814000137
Nacionalidade: Brasileira
Qualificação Jurídica: Instituição de Ensino e Pesquisa
Endereço: Rua Baraúnas, 351
Cidade: Campina Grande
Estado: PB
CEP: 58429-500
País: Brasil
Telefone: (83) 3315 3383
Fax:
Email: sisilsantos@gmail.com

PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 10/02/2017 às 10:01, Petição 870170008944

Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	FORMULAÇÃO FARMACÊUTICA CONTENDO DIETILCARBAMAZINA PARA USO NA PROFILAXIA E NO TRATAMENTO DE DOENÇAS INFLAMATÓRIAS HUMANAS E VETERINÁRIAS
Resumo:	“FORMULAÇÃO FARMACÊUTICA CONTENDO DIETILCARBAMAZINA PARA USO NA PROFILAXIA E NO TRATAMENTO DE DOENÇAS INFLAMATÓRIAS HUMANAS E VETERINÁRIAS”. A presente invenção refere-se ao uso de formulações farmacêuticas e/ou veterinárias contendo dietilcarbamazina (DEC) para uso com fins terapêutico para tratamento ou profilaxia de patologias inflamatórias agudas ou crônicas em humanos e/ou animais.
Figura a publicar:	1