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ANÁLISES DOS EFEITOS DA DIETILCARBAMAZINA (DEC) SOBRE A FIBROSE HEPÁTICA EM CAMUNDONGOS C57BL/6J WILD TYPE

Recife

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Dissertação apresentada ao Programa de Pós Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito final exigido para a obtenção do título de Mestre em Ciências Biológicas, área de concentração: Biotecnologia.

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“Palavra fiel é esta: que, se morrermos com Ele, também com Ele viveremos; Se sofrermos, também com Ele reinaremos; se o negarmos, também Ele nos negará; Se formos infiéis, Ele permanece fiel; não pode negar-se a si mesmo.” II Timóteo 2:11-13.

Resumo

Estudos farmacológicos mostram que a Dietilcarbamazina (DEC) interfere no metabolismo do ácido araquidônico atuando como um fármaco anti-inflamatório. O objetivo deste estudo foi examinar o efeito da DEC sobre a fibrose hepática induzida pelo tetracloreto de carbono (CCl_4). Quarenta camundongos machos da linhagem C57BL/6J foram divididos em 4 grupos experimentais (n=10/grupo): (1) grupo controle, (2) grupo DEC 50 mg/kg (3) grupo CCl_4 e (4) grupo CCl_4+DEC 50mg/kg. A solução de DEC (50mg/kg) foi diluída nos bebedouros dos animais em volume total de 150 ml, por 12 dias. A fibrose foi induzida pelo CCl_4 (0,5µl/g) por 8 semanas (2 injeções por semana). Após o esquema terapêutico, os animais foram eutanasiados e fragmentos hepáticos foram processados para histopatologia (HE), histoquímica para colágeno (Sirius red), ultraestrutura, imunohistoquímica, western blot e RT-qPCR. Nos resultados histopatológicos do grupo controle e grupo DEC 50mg/kg não apresentaram alterações em sua morfologia padrão. No grupo dos animais expostos ao CCl_4 foi observada marcante degeneração citoplasmática e nuclear, com a presença de fibrose e infiltrados inflamatórios. Através da microscopia eletrônica foi possível observar mitocôndrias em degeneração, rompimento do retículo endoplasmático e grande presença de lipídeos. Os animais tratados com CCl_4+DEC, mostraram uma diminuição de todas as lesões observadas no grupo CCl_4. Na marcação para colágeno do grupo CCl_4, observou-se intensa marcação nas áreas fibróticas. No entanto, o grupo CCl_4+DEC apresentou redução da marcação de colágeno, semelhantemente ao grupo controle. Resultados da imunohistoquímica revelaram aumento da expressão de COX-2, α-SMA, TGF-β, p-JNK e p-p38 nas áreas fibróticas e nos infiltrados mononucleares, principalmente em áreas perivenulares no grupo CCl_4. O tratamento com 50mg/kg de DEC promoveu a redução da imunoreatividade desses marcadores. Análises realizadas por western blot e RT-qPCR mostraram aumento da expressão dos marcadores fibróticos como α-SMA, TGF-β, colágeno-1, MMP2 e TIMP1, bem como das proteínas da via das MAPKs como p-JNK e p-p38 no grupo CCl_4 e uma significativa redução da expressão destas proteínas após tratamento com DEC 50mg/kg. De acordo com o presente estudo, a DEC é uma possível alternativa terapêutica para a fibrose hepática.

Palavras-chave: Fibrose-hepática, Dietilcarbamazina (DEC), Tetracloreto de carbono

Abstract

Pharmacological studies show that DEC interferes in the arachidonic acid metabolism, acting as an anti-inflammatory drug. The aim of the study was to examine the effect of DEC on liver fibrosis induced by carbon tetrachloride (CCl_4). Forty male mice C57BL/6J strain were divided into 4 groups (n = 10/group): (1) control group, (2) DEC 50mg kg group (3) CCl_4 group and (4) CCl_4 + DEC 50mg/kg group. The solution of DEC (50mg/kg) was administered to the animals in the drinking water in total volume of 150 ml for 12 days. The induction of fibrosis was made by CCl_4 (0.5mL/g) for 8 weeks (2 injections per week). After the treatment, the animals were euthanized and liver fragments were processed for histological (HE), staining for collagen (Sirius red), ultrastructure, immunohistochemistry, western blot and RT-qPCR. The control and DEC 50mg/kg groups showed no change in their morphology pattern. The group of animals exposed to CCl_4 a striking cytoplasmic and nuclear degeneration were observed, besides the presence of fibrosis and inflammatory infiltration. By electron microscopy several damage were observed, such as, mitochondria degeneration, rupture of the endoplasmic reticulum and large presence of lipids. The animals treated with CCl_4 + DEC showed a decrease of all lesions observed in CCl_4 group. In staining specific for collagen the CCl_4 group showed intense staining in fibrotic areas. In contrary, CCl_4 + DEC group showed reduced collagen labeling, similar to the control group. Results of immunohistochemistry revealed increased expression of as COX-2, α-SMA, TGF-β, p-JNK and p-p38 in fibrotic areas and mononuclear infiltrates, especially in areas perivenulares in CCl_4 group. Treatment with DEC 50 mg / kg promoted a reduction of immunoreactivity of these markers. Western blot and RT-qPCR analyzes showed increased expression of fibrotic markers such as α-SMA, TGF-β, collagen-1, MMP2 e TIMP1, as well as the proteins pathway of MAPKs such as p-JNK and p-p38 in the CCl_4 group and there was a significant decrease in expression of these proteins after treatment with DEC 50mg/kg. According to the present results, DEC is a possible alternative treatment for liver fibrosis induced by CCl_4.

Keywords: Liver fibrosis, Diethylcarbamazine (DEC), Carbon tetrachloride

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Artigo: Diethylcarbamazine (DEC) attenuates liver fibrosis carbon tetrachloride (CCl_4)-induced by downregulating TGF-β and MAPK signaling pathways

LISTA DE SIGLAS E ABREVIATURAS

Ac - Anticorpo

α –SMA – Alfa actina do músculo liso

CEH – Células Estreladas hepáticas

C57BL/6J – Linhagem de camundongo isogênico

CCl_4 – Tetracloreto de carbono

CCl_3 – Triclorometil

Col 1α - Colágeno tipo 1 alfa

COX-2 - Cicloxigenase-2

DEC – Dietilcarbamazina

HE – Hematoxilina Eosina

IHQ – Imunohistoquímica

IL-6 - Interleucina – 6

IL-10 - Interleucina 10

kg – kilograma

MAPK - Proteínas kinase ativadas por mitógeno

MEC – Matriz Extracelular

MMP – Metaloproteinase de matriz

mRNA – Ácido ribonucleico mensageiro

NF-kB - fator de transcrição nuclear kB

OMS – Organização Mundial da Saúde

OOCCl - Triclorometil peroxil

PCR – Reação em Cadeia de Polimerase

JNK - cJun N-terminal kinase

p-JNK - cJun N-terminal kinase fosforilada

p38 - p38 proteínas quinases ativadas por mitógenos

p-p38 - p38 proteínas quinases ativadas por mitógenos fosforilada

P450- Citocromo da família 450

RL – Radicais livres

ROS - Espécies reativas de oxigênio

TGF-β - Fator de crescimento transformante beta

TIMP – Inibidor tecidual de Metaloproteinase

TNF-α - Fator de Necrose Tumoral–alfa

Sumário

1. INTRODUÇÃO

As doenças hepáticas representam um grave problema de saúde pública, comprometendo não só o bem estar social do indivíduo como também a economia do país. Oriundas de diferentes etiologias, as doenças crônicas do fígado têm gerado uma curva ascendente de morbidade, sendo responsável por um considerável número de atendimentos e internações hospitalares com um índice crescente de risco de morte. Dentre as causas mais comuns das doenças do fígado estão o consumo excessivo de álcool e as hepatites virais, principalmente pela infecção com os vírus B e C (CORRAO, et al., 1998; TSUI et al., 2006).

Os tratamentos existentes para as hepatopatias são limitados e diferenciados, a depender da etiologia e/ ou persistência do estímulo. Em geral as terapias atuais tentam deter ou atrasar a agressão tecidual, conseguindo apenas minimizar os danos nas células para reduzir as complicações associadas à doença. Quando as medidas terapêuticas não são eficientes, os pacientes podem evoluir para a cirrose (SCHALM, 1997). O transplante de fígado passa a ser o tratamento mais eficaz disponível para os pacientes com insuficiência hepática crônica (IREDALE et al., 2003). Diante disso, há uma grande necessidade de desenvolver novas estratégias terapêuticas para tratamento de pacientes com fibrose hepática.

O Fator de crescimento transformante beta (TGF-β) é uma das mais importantes citocinas envolvida na fibrose e cirrose hepática, tendo um papel crucial na ativação das células estreladas hepáticas (CEHs) (JARCUSKA e JANICKO, 2010). Uma vez estimuladas, as células estreladas são diferenciadas em miofibroblastos, passando a expressar filamentos intermediários de colágeno (especialmente tipo I e III) e alfa actina do músculo liso (α-SMA), bem como secretam outros componentes da matriz extracelular (MEC) (MARRA, 1999; STALNIKOWITZ et al., 2003).

No fígado, a estabilidade dos componentes da MEC é regulada pelas metaloproteinases (MMP) e por seus inibidores específicos (TIMPs). Quando ocorre dano ao tecido, o equilíbrio funcional é prejudicado e há alterações nos processos de fibrinogênese e fibrinólise, resultando em uma exacerbação do tecido fibroso (HEMMANN, et al. 2007). No entanto, a degradação da MEC

pode ser melhorada através da regulação da MMP e TIMP, promovendo a reversão da fibrose hepática (MOHAMMED et al., 2005).

A indução da fibrose pelo Tetracloreto de carbono (CCl_4) é um dos modelos mais antigos e mais utilizados experimentalmente para indução da fibrose. Este modelo é eficaz no desencadeamento da fibrose e, a longo prazo, da cirrose. Seu mecanismo de ação envolve a metabolização pelo citocromo P450, que estimula a produção de radicais livres (RLs). Estes provocam necrose nos hepatócitos, induzem inflamação e promovem uma maior progressão da fibrose (BASU, 2003). Além disso, ligam-se a macromoléculas, aumentando a lipoperoxidação (LPO) e alterando a homeostase do cálcio intracelular (RECKNAGEL et al., 1989). O modelo de indução da fibrose com CCl_4, apresenta vantagens, pois tem sido claramente caracterizado e, em muitos aspectos, assemelha-se ao padrão da doença observada na fibrose em humanos e na cirrose associada a danos tóxicos (TAMAYO, 1983 e TSUKAMOTO, 1990). Existe uma vasta experiência com este modelo em relação à caracterização das alterações histológicas, bioquímicas e alterações associadas com a inflamação, lesões e fibrose (MAHER, 1990).

A Dietilcarbamazina (DEC) é um derivado da piperazina utilizado eficazmente há mais de 50 anos no tratamento da filariose bancroftiana (FREEDMAN et al., 2001). Além disso, ela também apresenta propriedades anti-inflamatórias, possivelmente devido a alterações no metabolismo do ácido araquidônico (NORÕES et al., 1997; MAIZELS e DENHAM, 1992).

Em alguns estudos foram relatados que a DEC reduziu os níveis de mediadores inflamatórios em hepatócitos de camundongos expostos ao uso crônico de etanol (ROCHA et al., 2012b). Além de atuar como um fármaco hepatoprotetor, diminuindo lesões celulares em camundongos desnutridos e em camundongos submetidos à injúria hepática crônica (ROCHA et al., 2012a; ROCHA et al., 2014).

Desta forma, o presente trabalho teve como objetivo investigar a ação fibrolítica da DEC sobre a fibrose hepática em camundongos C57BL/6J wild type através de análises morfológicas, imunohistoquímicas e moleculares.

2. JUSTIFICATIVA

As doenças hepáticas representam um grave problema de saúde pública mundial, comprometendo não só o bem estar social do indivíduo com também a economia do país. Diante disso, há necessidade de desenvolvimento de novas estratégias terapêuticas a fim de melhorar a função do fígado em pacientes com doenças hepáticas crônicas.

Estudos farmacológicos mostraram que a DEC interfere no metabolismo do ácido araquidônico, atuando como um fármaco anti-inflamatório. Existem informações substanciais de que a DEC bloqueia etapas nas vias da ciclooxigenase e lipoxigenase, incluindo a inibição da quimiotaxia de leucócitos, degranulação de granulócitos e vasodilatação periférica (MAIZELS e DENHAM, 1992; MCGARRY et al, 2005).

Recentemente em nosso laboratório, demonstrou-se que a DEC atua reduzindo os níveis de mediadores inflamatórios, tais como NF-ĸB, TNF-α e outras citocinas inflamatórias em hepatócitos de camundongos expostos ao uso crônico de etanol, além de atuar como um fármaco hepatoprotetor, diminuindo lesões celulares em camundongos desnutridos (ROCHA et al, 2012a,b).

Entretanto, os possíveis efeitos da DEC sobre o processo fibrótico hepático ainda necessita ser elucidado. Este estudo visa contribuir com a caracterização da ação da DEC sobre os mecanismos regulatórios do desenvolvimento da fibrose hepática.

3. OBJETIVOS

3.1 Objetivo geral:

Analisar o mecanismo de ação da DEC sobre ativação das células estreladas, sobre a expressão de marcadores fibróticos e das enzimas da MEC, bem como sobre a expressão das MAPK no processo da fibrose hepática de camundongos C57BL/6J wild type.

3.2 Objetivos específicos:

- Caracterizar o efeito e o mecanismo de ação da DEC (50mg/kg) em modelo de fibrose hepática (induzida por tetracloreto de carbono- CCl_4) em camundongos wild type;
- Analisar os aspectos histopatológicos através da microscopia óptica (Hematoxilina e Eosina; Sirius Red);
- Analisar a ultraestrutura dos hepatócitos através da microscopia eletrônica;
- Caracterizar através da imunohistoquímica a expressão dos principais marcadores fibróticos como (TGF-β e α-SMA) bem como a enzima COX-2 após o tratamento com DEC;
- Quantificar os marcadores fibróticos (TGF-β, α-SMA, colágeno tipo 1α), bem como as principais enzimas da MEC (MMP2 e TIMP1) através de Western Blot;
- Avaliar os níveis de mRNA dos marcadores envolvidos na fibrose hepática e das principais enzimas da MEC e seus inibidores através de Transcrição-Reversa em PCR e Real- Time qPCR, a fim de confirmar a expressão destes genes.

4. REVISÃO BIBLIOGRÁFICA

4.1 O Fígado

O fígado, um dos maiores órgãos do corpo humano, está situado na cavidade abdominal, abaixo do diafragma. É um órgão hematopoiético com capacidade regenerativa e que possui microambientes imunológicos exclusivos (WATANABE et al., 2008).

A posição ocupada pelo fígado na cavidade abdominal favorece a captura, transformação, acúmulo e neutralização de substâncias (JUNQUEIRA e CARNEIRO, 2013). Esse órgão desempenha importantes funções para o organismo, tais como: síntese de substâncias (proteínas e açúcares); secreção de sais e ácidos biliares; armazenamento (lipídios e vitaminas) e metabolismo (lipídios, proteínas e carboidratos), filtragem, armazenamento de sangue, ferro e formação de fatores de coagulação (GUYTO, 2002).

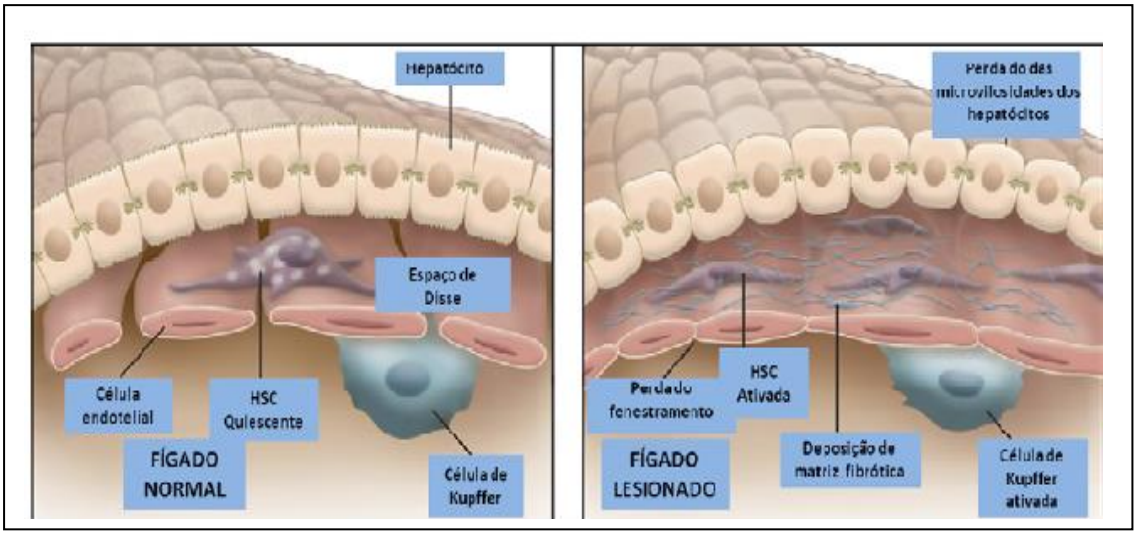
No processo de biotransformação, muitos compostos são metabolizados pelo fígado que altera a sua toxicidade, reduz sua atividade e os elimina. A exposição crônica a substâncias tóxicas geralmente resulta em alteração da função orgânica, diminuindo o tamanho do órgão e aumentando o tecido conjuntivo, causando a fibrose intra-hepática (RAMAIAH et al., 2001; FRIEDMAN e ARTHUR, 2002).

Nos lóbulos, as células hepáticas ou hepatócitos dispõe-se em placas orientadas radialmente a partir de uma veia central e entrelaçadas de forma ordenada por sinusóides. Os sinusóides são condutos de sangue, que não possuem parede estruturada e são revestidos por dois tipos celulares: a) células endoteliais típicas dos capilares sanguíneos e b) os macrófagos que no fígado são denominados como células de Kupffer (FRIEDMAN e ARTHUR, 2002; FRIEDMAN, 2003, 2008a).

As células de Kupffer são macrófagos altamente móveis inseridos no revestimento do sinusóide, sobretudo na área periportal. Estas células apresentam intensa atividade fagocitária como a fagocitose de hemácias em via de desintegração, a consequente digestão de hemoglobina e produção de bilirrubina. Como todos os macrófagos, apresentam grande quantidade de lisossomos, que em seu interior contém enzimas necessárias para a digestão intracelular das substâncias fagocitadas, remoção por endocitose de bactérias, vírus, parasitas e células tumorais (TOTH e THOMAS, 1992; SHERLOCK e DOOLEY, 2004).

Entre os hepatócitos e os sinusóides encontra-se um espaço estreito, denominado espaço de Disse, onde são localizadas as células estreladas hepáticas (CEHs), conforme apresenta a Figura 1. (FRIEDMAN, 2003) As célula estrelada hepática (CEHs). Estas células são extremamente versáteis, vitais para a função hepatocelular e a resposta do fígado à lesão (FRIEDMAN, 2008). Estas células representam cerca de um terço da população de células não parenquimatosas e 15% do número total de residentes no fígado normal.

Figura 1: Papel das células residentes do fígado na lesão hepática. As mudanças no espaço perisinusoidal de Disse durante o desenvolvimento da fibrose em resposta a lesões no fígado incluem alterações, tanto no comportamento celular, quanto na composição da Matriz Extracelular (MEC). A ativação das CEHs leva a deposição de matriz fibrótica e perda das microvilosidades dos hepatócitos precedendo a falência hepática. A ativação das células de Kuppfer tem ação parácrina sobre as CEHs.

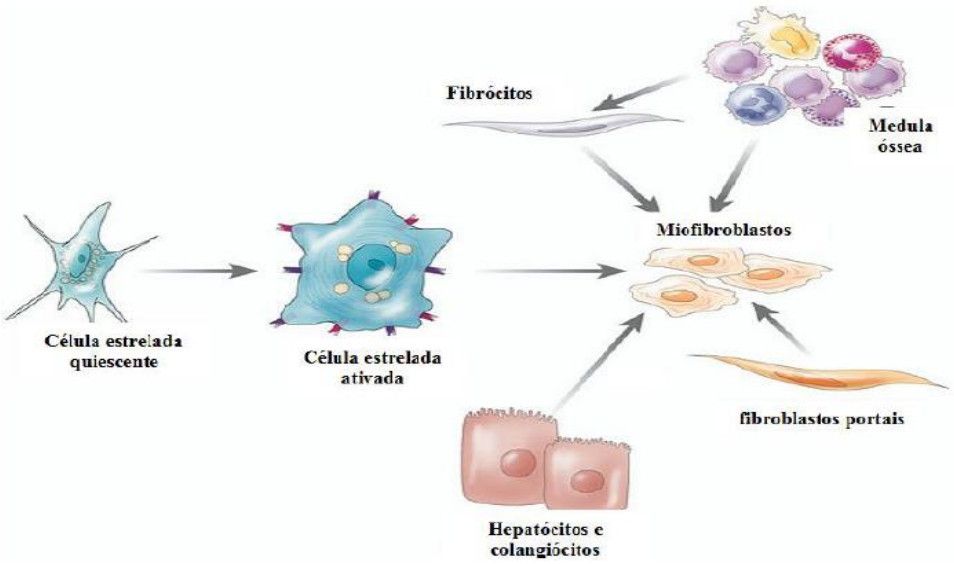


Fonte: Adaptado de Iredale (2008).

O seu traço mais característico normal é o armazenamento citoplasmático de gotas de vitamina A (retinóides). Em condições fisiológicas, a CEH mostra um retículo endoplasmático rugoso moderadamente desenvolvido, um complexo de Golgi pequeno e longos processos citoplasmáticos que envolvem os sinusóides no espaço de Disse (ATZORI et al., 2009).

A ativação das CEHs refere-se à transformação de uma célula rica em vitamina A em repouso para um estado proliferativo, fibrogênico e contrátil. A fibrogênese deve-se, principalmente à ativação fenotípica das células estreladas, que em estado normal, encontram-se quiescentes no espaço perinusoidal de Disse (FRIEDMAN, 2008), conforme ilustrado na Figura 2. Embora seja cada vez mais claro que outras populações de células mesenquimais também possam contribuir para o acúmulo de matriz extracelular (MEC), a ativação de CEHs continua a ser a via mais dominante levando à fibrose hepática, pois representam o principal local de produção de MEC (BATALLER et al., 2000; FRIEDMAN, 2008a). É bem conhecido o papel das CEHs como as principais células produtoras de colágeno em casos de lesão hepática crônica o que permite relacioná-las estritamente ao aparecimento da fibrose hepática. A ativação consiste de duas grandes fases: a iniciação e perpetuação, seguida pela resolução da fibrose caso a injúria seja retirada. (FRIEDMAN, 2008; LI et al., 2008).

Figura 2: Célula estrelada, forma quiescente e ativada



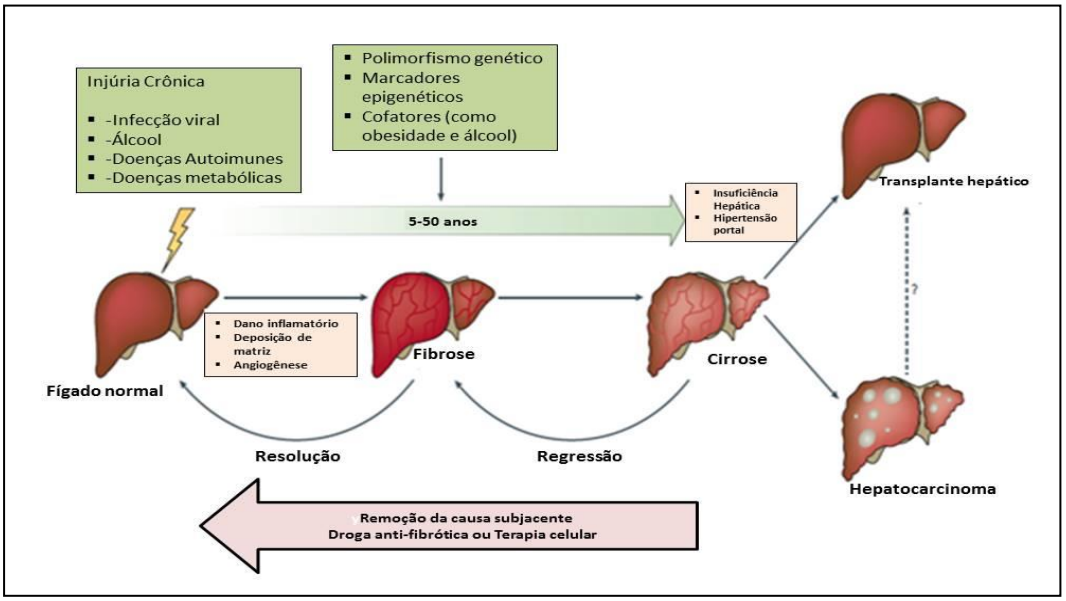
Fonte: FRIEDMAN, 2008b (Imagem adaptada).

4.2 Lesão Hepática Crônica

As doenças hepáticas crônicas são de alta prevalência em todo o mundo. A fibrose hepática é a resposta de cicatrização do fígado de múltiplas causas de lesão crônica e as suas causas mais frequentes relacionam-se aos vírus das hepatites B e C, ao alcoolismo, a doenças auto-imunes, a alterações metabólicas, tóxicas ou colestáticas. Na lesão hepática crônica, uma série de eventos mediados por citocinas leva à ativação da célula estrelada hepática, à deposição de colágeno e à fibrose do tecido, a qual pode evoluir para cirrose (PARSONS et al., 2007).

Independentemente da causa subjacente, a lesão causa danos inflamatórios, deposição de matriz, morte de células do parênquima e angiogênese levando a fibrose progressiva. Os componentes da matriz normalmente acumulam-se muito lentamente, mas assim que a fibrose é estabelecida o potencial para reverter este processo é diminuído e desenvolve complicações. Se a causa da fibrose é eliminada, a resolução (isto é, completa reversão à arquitetura hepática quase normal) de fibrose hepática precoce pode ocorrer (Figura 3).

Figura 3. História natural da doença hepática crônica. Após progressão da fibrose, a cirrose é estabelecida e o potencial para reverter este processo é diminuído. A fibrose hepática pode ser revertida se a sua causa for eliminada. Na cirrose, embora a resolução não seja possível, regressão (ou seja, a melhoria, mas não reversão) de fibrose melhora os resultados clínicos. As terapias anti-fibróticas podem retardar a progressão da fibrose. O transplante de fígado torna-se o único tratamento para a insuficiência hepática ou para carcinoma hepatocelular, o qual está crescendo em incidência em todo o mundo.



Fonte: PELLICORO, 2014 (Imagem adaptada)

Entretanto, as terapias antifibróticas estão surgindo e podem retardar, deter ou reverter a progressão da fibrose. Atualmente, o transplante de fígado é o único tratamento disponível para a insuficiência hepática ou para alguns casos de câncer primário de fígado. O transplante, por sua vez, apresenta algumas complicações, tais como o baixo número de órgãos disponíveis para o transplante, complicações nas técnicas cirúrgicas envolvendo estruturas vasculares e biliares e uso de imunossupressores para o resto da vida. Sendo necessária a busca de novas alternativas terapêuticas que minimize os danos decorrentes de agentes lesivos ao fígado.

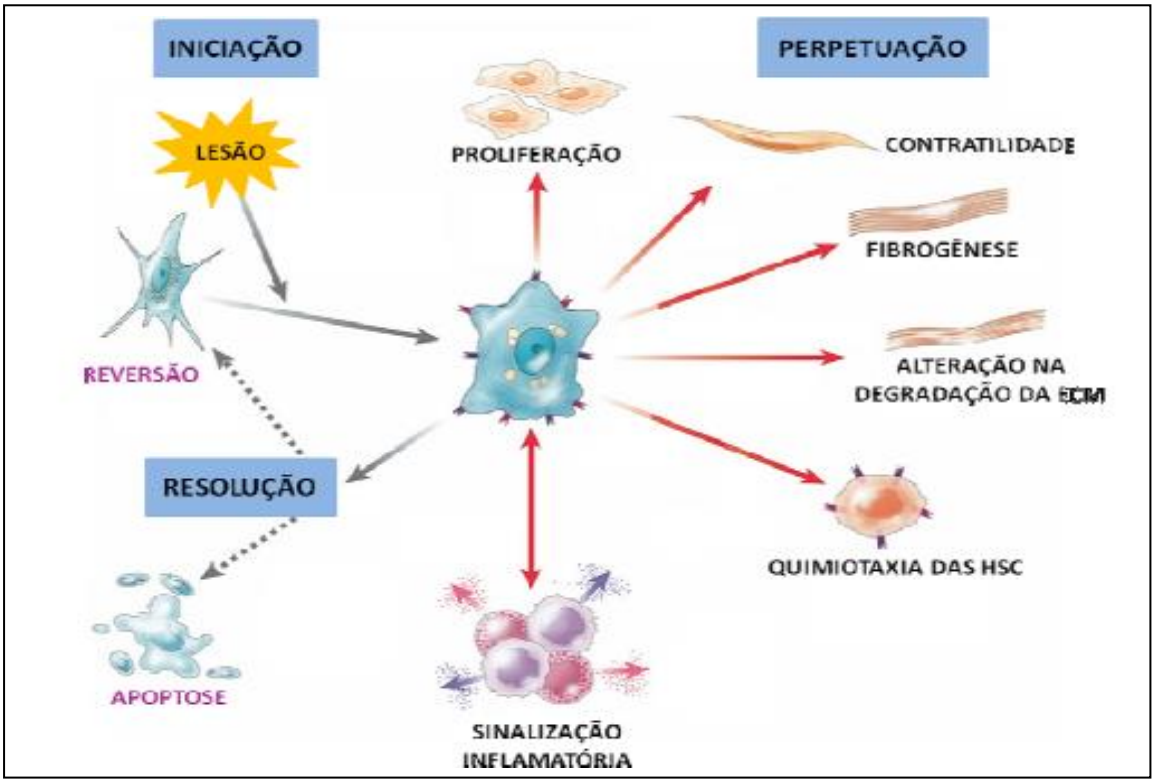
4.3 Fibrose hepática

Lesões hepáticas persistentes ou necroses hepatocelulares repetidas oriundas de diferentes causas resultam em um processo de reparo que tenta restabelecer o funcionamento normal do fígado. Quando o fígado sofre algum dano, como por exemplo, no estresse oxidativo, células inflamatórias e residentes, a exemplo das células de Kupffer, podem ser diretamente ativadas pelo tetracloreto de carbono (WEILER-NORMANN et al., 2007). Essas células são recrutadas e passam a liberar citocinas, tais como Fator de Crescimento Tumoral-β (TGF-β), desencadeando a ativação de várias alterações morfológicas como a expressão de alfa actina do músculo liso (α-SMA), liberam Fator de Necrose Tumoral-α (TNF-α) e outros agentes quimiostáticos que regulam em conjunto a resposta inflamatória e estimulam a fibrogênese (PINZANI et al., 1989; GRESSNER et al., 2002). Esta última deve-se, principalmente à ativação fenotípica das células estreladas, que em estado normal, encontram-se quiescentes no espaço perinusoidal de Disse (FRIEDMAN, 2008).

O desenvolvimento de fibrose hepática é baseado na ativação das CEHs. Essa ativação é um processo patológico que se caracteriza pela perda das gotículas de gordura onde a vitamina A é armazenada, pelo aumento do tamanho da célula, proliferação e pela diferenciação das CEHs em células proliferativas, fibrogênicas, contráteis, muito similares aos miofibroblastos, passando a expressar filamentos intermediários de colágeno e α-SMA e a secretar componentes da MEC (FRIEDMAN et al., 1985). (Friedman, 2008a; Friedman, 2008b; Saren et al., 2006).

A ativação das células estreladas consiste em duas fases: iniciação e perpetuação. A iniciação, também chamada de estágio pré-inflamatório, refere-se a alterações iniciais na expressão gênica e no fenótipo, que tornarão as células aptas a responder às citocinas e outros estímulos. A segunda fase resulta dos efeitos desses estímulos sobre a manutenção do fenótipo ativado e a produção de fibrose. A fase de perpetuação compreende, contratilidade, fibrogênese, perda de retinóides, infiltração de células inflamatórias e degradação de MEC. A figura 4 resume o processo fibrótico.

Figura 4: Vias de ativação das CEHs. Características da ativação das CEHs podem ser distinguidas entre aquelas que estimulam a iniciação e as que contribuem para a perpetuação. A iniciação e provocada por estímulos solúveis que incluem: estresse oxidativo (intermediários reativos de oxigênio), corpos apoptóticos, lipopolissacarídeos bacterianos e estímulos parácrinos de células vizinhas envolvendo macrófagos hepáticos (Células de Kuppfer), células do endotélio sinusoidal e hepatócitos. Após, vem a perpetuação, caracterizada por um número de mudanças fenotípicas específicas incluindo proliferação, contratilidade, fibrogênese, degradação de ECM alterada, quimiotaxia e sinalização pro-inflamatória. A resolução da fibrose culmina ou com a reversão do fenótipo ou com a apoptose das células lesadas.



Fonte: Adaptado de Friedman, 2008b.

Na fibrose, ocorre uma substituição da MEC normal por tecido cicatricial, com efeitos deletérios para a função celular (FRIEDMAN, 2008b). Essa

ativação é fonte de mediadores de moléculas da matriz, proteases e seus inibidores que juntos levam à formação da cicatriz hepática. As células estreladas, bem como as de Kupffer e as plaquetas, secretam TGF-β, o fator fibrogênico mais potentes para as células estreladas (ALBANIS e FRIEDMAN, 2006).

Produtos da lipoperoxidação e as espécies reativas de oxigênio (ROS) são estímulos importante na ativação das CEHs, assim como no recrutamento de células inflamatórias. Uma vez ativadas, as células estreladas secretam substâncias inflamatórias que levam à geração de um ciclo vicioso, no qual células fibrogênicas e inflamatórias estimulam-se umas às outras, fazendo perpetuar o processo hepático de dano e reparo (GUIMARAES et al., 2006).

As células estreladas hepáticas (CEHs) são um alvo atrativo para o estudo de novos agentes antifibróticos, na tentativa de tratar a fibrose em seus diferentes estágios. A redução do processo inflamatório e resposta imune, a inibição da ativação das CEHs, a indução da apoptose, a interrupção das atividades fibrogênicas, contráteis, proliferativas e pro-inflamatórias dessas células, a diminuição da síntese dos componentes da MEC ou o aumento de sua degradação são algumas das possíveis hipóteses para a interrupção do processo fibrótico (FRIEDMAN, 2008b).

4.4 Metaloproteinase da matriz

No fígado saudável, a homeostase da MEC é caracterizada por um remodelamento permanente regulado especialmente pelas Metaloproteinase da matriz (MMPs) e suas inibidoras específicas, os inibidores teciduais de metaloproteinase (TIMPs) (RODERFELD et al., 2007). As MMPs constituem uma grande família de endopeptidases cálcio e zinco dependentes que são responsáveis pela degradação de proteínas da MEC (CONSOLO et al., 2009). São desta forma enzimas proteolíticas que, embora exibam um amplo espectro de substrato, são divididas de acordo com seus principais substratos em: colagenases, gelatinases, estromelisinas, matrilisinas, metaloelastase, MMPs tipo membrana e outras (HEMMANN et al., 2007). Assim como outras proteases, são produzidas como formas inativas, zimogênios, sendo ativadas após liberação das células. A estrutura modular das MMPs permite que as

mesmas interajam com seus inibidores, os TIMPS. Estas últimas se ligam às MMPs pelo reconhecimento de dois diferentes locais: um localizado nos domínios tipo hemopexina e o outro no sítio catalítico. Neste controle de remodelamento constante da MEC, as MMPs são reguladas em vários níveis, sendo secretadas como pró-enzimas inativas e moduladas pelos TIMPs (HERNANDEZ-GEA, et al., 2011), levando a degradação da matriz extracelular.

Quatro TIMPs foram identificados até o momento TIMP1, TIMP2, TIMP3 e TIMP4 e todas as MMPs conhecidas podem ser inibidas por pelo menos uma delas (HEMMANN et al., 2007). Desses inibidores, os mais avaliados no processo de fibrose hepática são o TIMP-1 principalmente, e o TIMP-2. O TIMP-1 apresenta um papel importante na fibrose hepática, pois além de inibir a degradação da matriz pelas MMPs, também tem um efeito anti-apoptótico nas CEHs, prevenindo a depuração das CEHs ativadas durante a lesão, promovendo a sua sobrevida por indução da "*B cell lymphoma 2*" (Bcl-2) (MURPHY et al., 2002). O papel dos TIMPs é, de fato, regular as MMPs, tanto na atividade como na localização espacial, assegurando o término da atividade de protease (CONSOLO et al., 2009). As baixas concentrações de TIMPs e a razão MMPs/TIMPs são críticas na determinação da real atividade de protease: baixas concentrações de TIMP ou alta razão MMPs/TIMPs permitem a ativação das MMPs, enquanto altas concentrações de TIMPs levam à inibição da ativação das MMPs. Diversos estudos demonstraram variação na expressão destas enzimas ao longo da deposição dos componentes da MEC.

A apoptose da CEH ativada somente não é suficiente para a resolução da fibrose, sendo necessária também a degradação da matriz pela ação das MMPs. A clivagem inicial do colágeno tipo I parece ser crucial para a regressão global da fibrose hepática (BENYON et al., 2001). O TIMP-1, em particular, é rapidamente infra-regulada, com aumento da atividade de colagenase, degradação da matriz e regressão da fibrose hepática.

4.5 Sinalização da via MAPK

As proteínas kinase ativadas por mitógeno (MAPK) representam uma outra via de sinalização intracelular que é estimulada na ativação de CEHs. Membros da família MAPK como cJun N-terminal kinase (JNK) e p38 são

ativados por vários fatores de crescimento e por estresse, são submetido a fosforilação e translocados para o núcleo, onde há subsequente ativação de fatores de transcrição (ROBINSON et al., 1997), resultando nas várias respostas celulares, como proliferação, diferenciação e regulação de vias metabólicas específicas (LAPADAT et al., 2002).

JNK é um regulador positivo da proliferação célular na CEHs (SCHNABL et al., 2001). Múltiplos estresses estimulam ao aumento da atividade da JNK, incluindo citocinas, drogas citotóxicas e espécies reativas de oxigênio (DENT et al., 2003). JNK e p38 parecem ter efeitos divergentes na proliferação de CEHs. O boqueio da atividade da JNK nas CEHs, previne a proliferação celular. Por outro lado, o tratamento com inibidores de p38, aumentam a proliferação celular, sugerindo que p38 é um regulador negativo da proliferação das CEHs. Ambos JNK e p38 participam da regulação da expressão da α-SMA (TOCK et al., 2003). O TGF-β também pode induzir a ativação da sinalização da p38 MAPK, no entanto JNK não é ativada pela estimulação de TGF-β (HANAFUSA et al., 1999; TSUKADA et al., 2005). Este fator de crescimento induz a expressão do gene do colágeno tipo I, que é parcialmente mediada pela sinalização da p38 nas células estreladas (CAO et al., 2002).

4.6 Tetracloreto de Carbono

Existem várias drogas tóxicas capazes de reproduzir a lesão hepática experimentalmente, entre elas encontra-se o Tetracloreto de carbono (CCl_4)(JIMENEZ et al., 1992; PAVANATO et al., 2003; PEREIRA-FILHO et al., 2008), dimetilnitrosamina (ALA-KOKKO et al., 1989) e a tioacetamida (NAKAMURA et al., 1975).

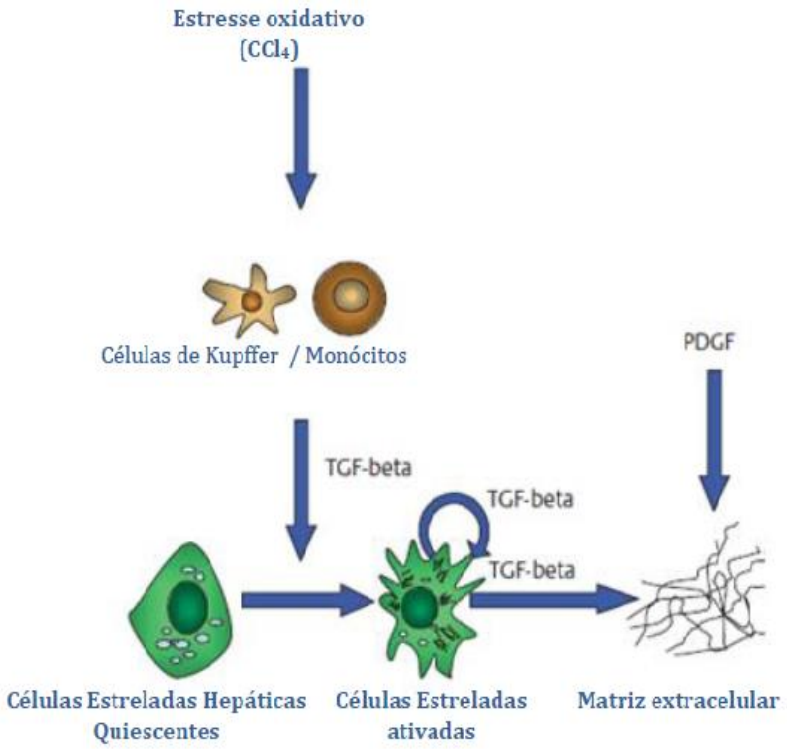
O CCl_4 é uma potente droga hepatotóxica que causa lesão ao fígado, mediada pelo aumento de radicais livres (RLs) e quando administrado repetidamente, induz fibrose hepática e posteriormente cirrose (MURIEL e ESCOBAR, 2003; WEBER et al., 2003).

O CCl_4 é o modelo mais utilizado experimentalmente para indução de fibrose. Apresenta vantagens, pois tem sido claramente caracterizado e, em muitos aspectos, é similar ao padrão da doença observada na fibrose em humanos e na cirrose associada a danos tóxicos (TAMAYO, 1983; TSUKAMOTO, 1990). Além disso, existe uma vasta experiência com este

modelo em relação à caracterização das alterações histológicas, bioquímicas e alterações associadas com a inflamação, lesões e fibrose (MAHER, 1990).

Conforme demonstrado na figura 5, o estresse oxidativo leva à ativação de células do sistema imune no fígado como as células Kupffer, monócitos e trombócitos. Estas células podem ser diretamente ativadas pelo tetracloreto de carbono. Qualquer ativação dessas células leva à secreção de citocinas como o TGF-β, entre outros. TGF-β conduzirá para uma ativação de células estreladas hepáticas que estavam quiescentes, desencadeando a ativação de várias alterações morfológicas como a expressão de alfa actina de músculo liso (α-SMA). Com as células estreladas ativadas, ocorre produção de matriz extracelular, contribuindo para o processo de fibrose hepática (WEILER-NORMANN et al., 2007).

Figura 5: Estresse oxidativo oriundo da hepatotoxicidade do CCl_4 , levando à ativação de células do sistema imune no fígado.

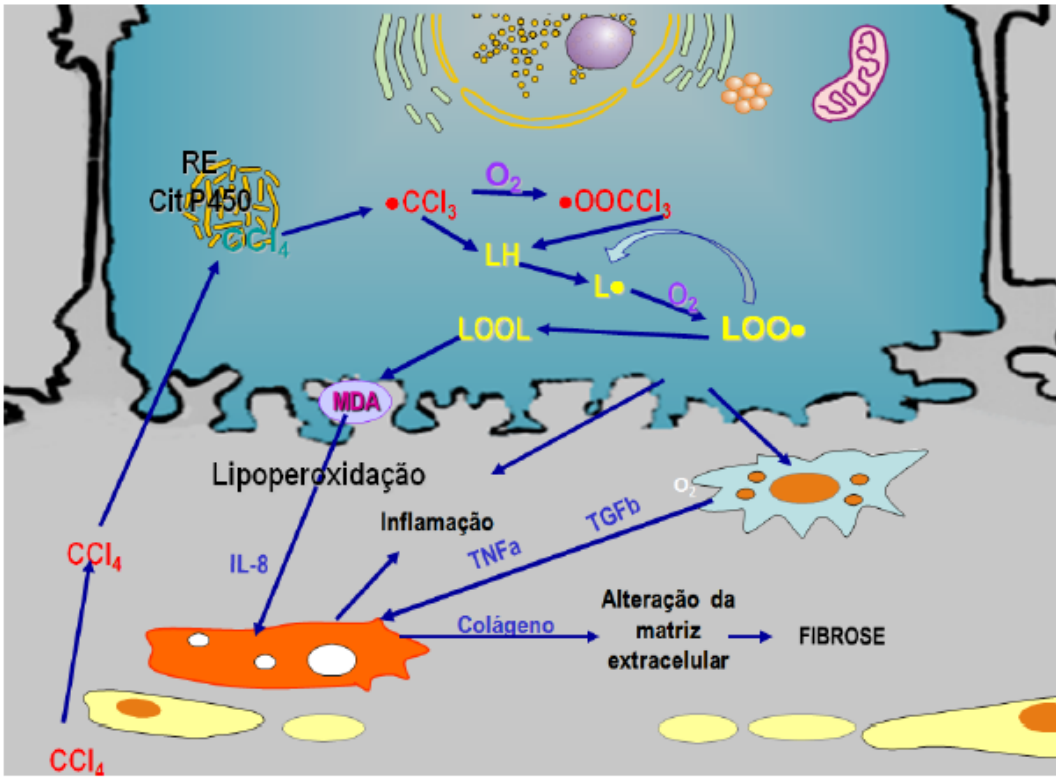


Fonte: WEILER-NORMANN et al., 2007 (Imagem adaptada).

4.6.1 Metabolismo do tetracloreto de carbono

O mecanismo de toxicidade do tetracloreto de carbono (CCl_4) se dá pela sua conversão em metabólitos tóxicos reativos, por um complexo enzimático oxidativo, o citocromo P450 presente no retículo endoplasmático liso do fígado (RECKNAGEL et al., 1989; JIMENEZ et al., 1992; SHERLOCK e DOOLEY, 2004). O citocromo P450 é o principal produtor de espécies reativas de oxigênio nas células hepáticas (BAYOL-DENIZOT et al., 2000). O metabolismo do CCl_4 no fígado pelo complexo P450, resulta na produção de RLs como o triclorometil ($\bullet CCl_3$) e triclorometil peroxil ($OOCCl\bullet$) que provocam necrose nos hepatócitos, induzem inflamação e promovem uma maior progressão da fibrose (JIMENEZ et al., 1992; BASU, 2003) (Figura 6).

Figura 6: Geração de radicais livre e a injúria hepática pela ação do CCl_4



Fonte: PAVANATO, 2004.

Estes metabólitos tóxicos reativos danificam as células através de ligação covalente direta às proteínas e aos lipídeos de membrana, ou mais comumente

pela formação de RLs e espécies reativas de oxigênio (ROS), os quais causam auto-oxidação dos ácidos graxos presentes dentro dos fosfolipídeos da membrana (CREMONESE et al., 2001). Em seguida, inicia-se uma série de processos bioquímicos e fisiológicos secundários, que serão as últimas causas para o desdobramento das conseqüências patológicas do metabolismo do CCl_4 (CREMONESE et al., 2001; LEE et al., 2001). Essas alterações lesam a célula, provocando sua morte e consequente fibrose tecidual (LEE et al., 2001).

O CCl_4 pode ser administrado via inalação, gavagem, injeção subcutânea ou injeção intraperitoneal (TAMAYO, 1983 e TSUKAMOTO, 1990).

O desenvolvimento e estabilização da fibrose ocorre com a administração do CCl_4 durante 6 a 8 semanas (IREDALE et al., 1996; 2007). Já o desenvolvimento da cirrose reversível ocorre em 8 semanas de intoxicação pelo CCl_4 e o desenvolvimento da cirrose parcialmente reversível ocorre após 12 semanas de intoxicação (VARGA, et al., 2005).

4.7 Dietilcarbamazina

A dietilcarbamazina (DEC) é o filaricida amplamente utilizado no tratamento da filariose bancroftiana, é um derivado da piperazina sintetizada como 1-dietilcarbamil-4-metilpiperazina e preparada na forma de cloridrato, citrato ou fosfato. A partir de 1950, foi distribuída como sal citratado por inúmeras companhias farmacêuticas sob diferentes nomes. 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 e NORÕES, 1997).

A DEC é rapidamente absorvida pelo trato gastrointestinal, atingindo um pico da sua concentração plasmática entre uma a três horas após a ingestão oral, não se concentrando em nenhum órgão específico, sendo metabolizado no fígado e sua excreção basicamente renal (OTTESEN, 1985; DREYER; NORÕES, 1997; ILONDU et al., 2000).

Para o tratamento da filariose linfática, a Organização Mundial de Saúde (OMS), preconiza a administração via oral do citrato de dietilcarbamazina, utilizando uma posologia padrão de 6mg/kg/dia durante um período de 12 dias

(WHO, 1992) e no caso de eosinofilia pulmonar tropical (EPT), ele deve ser prolongado por até 30 dias (DREYER et al., 1996).

4.7.1 Mecanismo de ação da DEC

Atualmente, têm-se realizado alguns estudos sobre o mecanismo de ação desta droga, que apesar de mais de 50 anos de uso, teve o seu potencial farmacológico pouco explorado. Sabe-se, até o momento, que parte dos efeitos atribuídos à DEC, deve-se a sua interferência no metabolismo do ácido araquidônico (NORÕES et al., 1997). Esta alteração confere a dietilcarbamazina propriedades anti-inflamatórias (MAIZELS e DENHAM, 1992). Sabe-se que a via do ácido araquidônico inclui as enzimas lipoxigenase e ciclooxigenase (COX). A via da COX apresenta similaridade com a via do óxido nítrico, uma vez que ambas possuem isoformas constitutivas e induzíveis de suas enzimas e controlam as respostas inflamatórias (CLANCY e ABRAMSON, 1995; MCGARRY, et al., 2005).

A DEC possui outro papel terapêutico como droga anti-inflamatória para condições asmáticas (SALAZAR-MALLÉM, 1971; SRINIVAS e ANTANI, 1971; THIRUVENGADAM et al., 1974). Além disso, umtrabalho de Queto et al., 2010, realizado em colaboração com o nosso laboratório, mostraram que a DEC tem importante ação no bloqueio da inflamação eosinofílica pulmonar em camundongos sensibilizados com ovalbumina. Foi observado que a DEC bloqueia a hiper-reatividade pulmonar, a produção de citocinas, na ativação e migração de eosinófilos e a eosinofilopoiese *in vivo* e *in vitro*.

Estudos recentes comprovam que a DEC, na concentração de 50mg/kg, atua diminuindo a esteatose hepática, infiltrados inflamatórios, atividade das transaminases e citocinas inflamatórias em camundongos desnutridos e expostos ao uso crônico do etanol, atuando como um fármaco hepatoprotetor e anti-inflamatório. A DEC também reduziu a dosagem de malondialdeído, fator de necrose tumoral e a interleucina – 6 (IL-6), além de reduzir a expressão do fator de transcrição nuclear kB (NF-kB) em camundongos submetidos a injúria hepática induzida pelo etanol (ROCHA et al., 2012 a,b). Em modelo de inflamação hepática crônica induzida por CCl_4, a DEC reduziu a expressão da enzima envolvida no processo inflamatório como a ciclooxigenase (COX-2), e

interleucina 1 (IL-1), além de aumentar da expressão da citocina anti-inflamatória interleucina 10 (IL-10) (ROCHA et al., 2014).

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Capítulo 1

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Diethylcarbamazine (DEC) attenuates liver fibrosis carbon tetrachloride (CCl_4)-induced by downregulating TGF-β and MAPK signaling pathways

Diethylcarbamazine (DEC) attenuates liver fibrosis carbon tetrachloride (CCl_4)-induced by downregulating TGF-β and MAPK signaling pathways

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Abstract

Diethylcarbamazine (DEC) presents important anti-inflammatory effects on experimental models of liver injury. However, the mechanisms of its action are poorly understood. The aim of the study was to investigate fibrolitic potential of DEC. Mice were administered with CCl_4 together with or without DEC for 8 weeks (n = 10 per group). We assessed the expression of markers of HSC activation, including smooth muscle α-actin (a-SMA), collagen I, transforming growth factor-β 1 (TGF-β1), metalloproteinases-2 (MMP-2) and tissue inhibitors of metalloproteinases (TIMPs). The influence of DEC on HSCs intracellular MAPK pathways (JNK and p38 MAPK) was also estimated. DEC inhibited HSC activation measured as the production of a-SMA and collagen I. In addition, it

down regulated the production of TGF-β and TIMP-1, and concomitantly increased the activity of MMP-2. Furthermore, DEC significantly inhibited the activation of the JNK and p38 MAPK signaling pathways. In conclusion, DEC significantly attenuated the severity of CCl_4-induced liver injury and the progression of liver fibrosis, exerting a strong fibrolytic effect in the CCl_4 - induced model of fibrosis.

1. Introduction

Liver fibrosis is a serious public health problems worldwide because of their life-threatening complications, which include cirrhosis, portal hypertension and liver failure, and the risk of hepatocellular carcinoma (Friedman, 2008; Iredale, 2007; Lee, 2011). Liver fibrosis is a wound healing response to chronic liver injury arising from different etiologies, including alcohol and drug abuse, virus, autoimmunity diseases and metabolic syndrome (Bosserhoff, 2011; Cubero, 2006; Probst, 2011; Xu, 2001).

Upon liver injury, a variety of factors such as cytokines, chemokine or reactive oxygen species (ROS) induce the activation of stellate cells (HSCs), which have a crucial role in liver fibrosis (Kong, 2012). The TGF-β is one of the most important cytokines involved in this process (Jarcuska, 2010). The blockade of TGF-b signaling by various methods prevents the progression of liver fibrosis in experimental animal models (Yata, 2002). Once stimulated, stellate cells are trans-differentiated into myofibroblasts, passing to express intermediate filaments of collagen (especially type I and III) and alpha smooth muscle actin (α-SMA), as well as secrete components extracellular matrix (ECM) (MARRA, 1999; STALNIKOWITZ, 2003), in addition of matrix degradation proteins, collectively known as matrix metalloproteinases (MMPs) (Arthur, 1989). The activity of the MMPs is regulated by their inhibitors, tissue inhibitor of metalloproteinases (TIMPs) (Iredale, 1997). However the overproduction of TIMPs abolishes the matrix degradation process and result in fibrosis due to ECM accumulation in the liver (McCrudden, 2006).

One of the intracellular signaling pathways that are stimulated by activation of HSCs by growth factors and stress are the mitogen-activated protein kinase (MAPK) (Raman, 2007; Tsukada, 2006). When members of the

MAPK family as cJun N-terminal kinase (JNK) and p38 are activated, they are subjected to phosphorylation and translocated to the nucleus where there is activation of transcription factors (Robinson, 1997). Thus resulting in various cellular responses such as proliferation and differentiation (Johnson and Lapadat, 2013). The interaction of MAPKs and α-SMA in HSCs must be elucidated in fibrosis investigation.

Although understanding of the mechanisms underlying the pathogenesis of liver fibrosis has increased, no specific treatments are yet available (Yao and Tu, 2013). Therefore, the development of new therapeutic strategies for treating liver fibrosis is required.

Diethylcarbamazine (DEC) is a piperazine derivative presenting exhibits anti-inflammatory properties and antioxidant (Dreyer and Norões, 1997; Maizels, 1992; Rocha et al, 2012a). Despite being used for more than 50 years, it has little explored their therapeutic potential. DEC is thought to treating acute lung injury by reducing inflammatory mediators and the production of NO (Ribeiro et al, 2014). In our previous studies, we showed that DEC is effective to attenuate the pro-inflammatory cytokines, oxidative stress and necrosis in chronic inflammation of the liver induced by CCl_4 (Rocha et al, 2014), suggesting a potential therapeutic use in chronic inflammation (Peixoto and Silva, 2014). In addition, DEC has also a role in reducing cell damage hepatic in malnourished mice (Rocha et al, 2012b).

However, little is known on the exact effect and mechanism of DEC in the progression of fibrosis in chronic liver injury. We proposed that administration of DEC could attenuate the progression of liver fibrosis by reducing the expression of pro-fibrogenic markers and cellular proliferation in the CCl_4-induced liver fibrosis.

2. Materials and Methods

2.1. Animals and experimental design.

Forty male 5-week-old C57BL/6 mice, weighting 15-16g, were used in all experiments. Mice were examined to determine their health status and acclimated to the laboratory environment of 23-24∘C. They were kept in a 12/12 h day/night cycle photoperiod. The animals were housed in metal cages and fed a standard diet and water *ad libitum*. The Ethics Committee of the Oswaldo

Cruz Institute approved all the experiments reported herein under protocol number 11/2010 Liver fibrosis was induced by i.p. administration of CCl_4 0.5 μL/g of body weight (Sigma-Aldrich, St. Louis, MO, USA) dissolved in olive oil (final volume, 0.1mL per mouse) (Zhao, 2003). Two CCl_4 injections were administered by week for 8 weeks (Iredale, 2007; Seki et al, 2009). Treatments were composed of distilled water and DEC (Farmanguinhos, FIOCRUZ, Brazil). DEC 50 mg/kg of body weight was administered through the drinking water during the last 12 days of the liver injury (Rocha, 2012b; Zhao, 2003). Body weights were recorded every day and the drug concentration in the water was adjusted to maintain the dose. The control group received only water using the same procedure as described above. The C57BL/6 mice were separated into four groups (n = 10): (1) the control group; (2) the DEC-treated group (DEC); (3) the CCl_4 group (CCl_4); and (4) the CCl_4 plus DEC group (CCl_4+ DEC).

2.2. Histopatology.

Liver fragments were fixed in 10% formalin for 24 hours, before being processed and embedded in paraffin (Rocha et al, 2012b). Five sections of 4-5 μm from each group were cut and mounted on glass slides. The slices were stained with hematoxylin-eosin and examined by an inverted microscope (Observer Z1, Zeiss MicroImaging, GmbH) equipped with a camera and 4.7.4 image analysis software (AxionCam MRm Zeiss) at a magnification of 400x. The fibrosis areas were quantified in five random fields on each slide using GIMP 2.6 imaging software (Rocha et al, 2012a).

2.3. Measurement of Hepatic Collagen Content.

The hepatic collagen content was also assessed by the Picrosirius red staining of five paraffin-embedded sections. Sirius-red positive áreas were analyzed in five random fields (magnification ×400) on each slide and quantified using GIMP 2.6 imaging software (Rocha et al, 2012a).

2.4. Electron Transmission Microscopy Assays.

The fragments of liver were fixed in a solution containing 2.5% glutaraldehyde and 4% formaldehyde in 0.1M cacodylate buffer. After fixation, the samples were washed twice in the same buffer and then postfixed in a solution

containing 1% osmium tetroxide, 2mM calcium chloride, and 0.8% potassium ferricyanide in 0.1M cacodylate buffer with a pH of 7.2, dehydrated in acetone and embedded in Epon 812 resin (Sigma Company, St. Louis, MO). Polymerization was carried out at 60◦C for 2 days. Ultrathin sections were collected on 300- mesh copper grids, counterstained with uranyl acetate and lead citrate, and examined with a Morgani FEI transmission electron microscope (Rocha et al, 2012b).

2.5. Immunohistochemistry (IHC).

Five sections (5 μm in thickness) from each group were cut and adhered to slides treated with 3-amino-propyl-triethoxy-silane (APES) (Sigma, USA). The sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). To increase epitope exposure, the sections were heated for 30 minutes in a sodium citrate buffer (0.01 M, pH 6.0). To minimize endogenous peroxidase activity, the slides were treated with 0.3% (v/v) H_2O_2 in water for five minutes. The sections were washed with 0.01M 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 then incubated for 12 hours at 4◦C with a antibody against COX-2 (Abcam, ab15191), TGF-β (Santa Cruz, sc-109), α-SMA (Abcam, ab5694) and p-JNK (Santa cruz, sc-6254). The optimal concentration used was 1:100 for these antibodies. 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 with 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 pictures at the 40x magnification were quantitatively analyzed using GIMP 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.6. Western Blot.

The livers were quickly dissected and then homogenized in a Wheaton Overhead Stirrer (no.903475) using the following extraction cocktail: 10mM ethylenediamine tetraacetic acid (EDTA); 2mM phenylmethylsulfonyl fluoride (PMSF); 100mM sodium fluoride; 10mM sodium pyrophosphate; 10mM sodium orthovanadate (NaVO4); 10 mg aprotinin and 100mM Tris (hydroxymethyl)

aminomethane (pH 7.4). Homogenates were centrifuged at 3000 ×g for 10min and the supernatant was collected and stored at −70◦C until its use in the immunoblotting. Protein levels were determined using the Bradford method, with bovine serum albumin as the standard (Bradford, 1976). The proteins (40mg) were separated with 12% (α-SMA, p38, p-p38, JNK e p-JNK) e 14% (TGF-β) sodium dodecyl sulfate-polyacrylamide by gel electrophoresis under reduced conditions and were electrophoretically transferred onto nitrocellulose membranes (Bio Rad, CA, USA, Ref. 162-0115). After blocking overnight at 4◦C with 5% non fat milk in TBS-T (Tris buffered saline 0.1% plus 0.05% Tween 20, pH7.4), the membranes were incubated at room temperature for 2h with rabbit polyclonal antibodies anti-α-SMA (1 : 1000 dilution; Abcam, USA), anti-p38 and anti-p-p38 (1:1000 dilution, Cell signaling, USA), anti-TGF-β, anti-JNK and anti-p-JNK (both 1:1000 dilution, Santa cruz Biotechnology, CA, USA), diluted in buffer solution TBS-T containing 3% non-fat milk. After washing (six times, 10 min each) in TBS-T, the membranes were further reacted with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:3000 (Ref. ab6721) dilution, Abcam, USA), diluted in TBS-T with 1% non fat milk, for 1 h 30 min, at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used to visualize the labeled protein bands and the blots were developed on X-ray film (Fuji Medical, Kodak, Ref. Z358487-50EA). For quantification, the density of pixels of each band was determined by the Image J 1.38 program (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD). The results were confirmed in three sets of experiments for each protein investigated. The immunoblot for β-actin was used as a control for the above protein blots. After protein blot visualization with enhanced chemiluminescence, the protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-β-actin antibody (1:1000 dilution, Sigma, USA). Protein densitometry was subsequently carried out.

2.7. RNA Isolation, RT-PCR, and Real-Time Quantitative PCR.

Total RNA from mouse tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was treated with RNase-free DNase I and amplified with oligo (dT) primer, using the SuperScript First-Strand Synthesis

System for RT-PCR (Invitrogen). Then, 1 μg of total RNA was reverse-transcribed using the QuantiTec Reverse Transcription kit (Qiagen, Hilden, Germany), using random hexameric primers, according to the manufacturer's instructions. Quantitative real-time PCR was performed with the SYBR Green PCR system (Applied Biosystems, Foster City, CA, USA), using GAPDH as an internal control for normalization. RTqPCR was carried out with an ABI PRISM 7500 instrument (Applied Biosystems, CA, USA). The forward and reverse primers used for each gene were as follows: 5'-GAACGGTCCACGATTGCATG -3' and 5'-GGCATGTTGCTAGGCACGAAG-3' for Col-1; 5-AAAATCAAGTGTGGAGCAAC- 3' and 5'-CCACGTGGAGTTTGTTATCT-3' for TGF-β; 5'-ATCTGGCACCACTCTTTCTA-3' and 5'-GTACGTCCAGAGGCATAGAG-3' for α-SMA; 5'-GCATCTGGCATCCTCTTGTT-3' and 5'-AAGAAGCTGCAGGCATTGAT-3' for TIMP1; 5'-GVTGATACTGACACTGGTACTG-3' and 5'-CAATCTTTTCTGGGAGCTC-3' for MMP2; and 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3' for GAPDH (endogenous control). All reactions were performed in triplicate and included the following: 1μL of cDNA; 5 μM of each primer; 2x SYBRGreen PCRMasterMix (Applied Biosystems); and water added to a final volume of 25 μL. The relative amount of mRNA was determined using the comparative threshold (Ct) method by normalizing target cDNA Ct values to that of GAPDH. Fold increase ratios were calculated relative to the control (basal conditions) for each group using the formula $2e$ − ΔΔCt.

2.8. Statistical Analyses.

For statistical analysis GraphPad Prism software (version 5) was used. Data were expressed using mean ± standard deviation. Differences between the control and treated groups were analyzed by analysis of variance (ANOVA), followed by Dunnett's test, Tukey's test, or the t- test (post hoc). The values less than 0.05 were considered significant.

3. Results

3.1 Histopathological findings

The control group showed the liver pattern characteristics (Figure 1A). The hepatocytes were well preserved and arranged uniformly. Similarly, the DEC group no showed histological alteration (Figure 1B). The group exposed to carbon tetrachloride presented a striking cytoplasmic degeneration, vacuolization and nuclear disorganization. Besides, several fibrosis areas were observed in the centrilobular areas (Figure 1C). Animals exposed to CCl_4 and treated with DEC 50 mg/kg showed an improvement in the architecture of hepatocytes when compared to the CCl_4 group. Besides, DEC reduced the fibrosis and attenuated the inflammatory infiltrates (Figure 1D). Fibrosis areas were quantified by image Gimp 2.6 program (Figure 1E).

3.2 DEC reduced CCl_4-induced collagen liver deposition

Hepatic fibrosis was assessed by Picrosirius red staining to visualize collagen fibers and through collagen-1α mRNA expression. The control and DEC groups showed collagen labelling only surrounding the endothelium, which is the standard feature of this tissue (Figure 2A and 2B). In contrast, the CCl_4 group showed deposition of collagen around the portal spaces and in fibrotic areas (Figure 2C). Treatment with DEC resulted in decreased deposition of collagen in the liver compared to the CCl_4 group (Figure 2D and 2F). Sirius red labelling were quantified by image Gimp 2.6 program (Figure 2E). RT-qPCR analysis confirmed this result (Figure 2F).

3.3 *Ultrastructural analysis*

The hepatocytes of the control group showed well-preserved organelles such as rough endoplasmic reticulum (rER), mitochondria and nucleus showing homogeneous euchromatin (Figure 3A and 3B). Contrary, the CCl_4 group revealed mitochondrial degeneration characterized by several vacuoles. Furthermore, the rER was fragmented and numerous lipids inclusions were

observed (Figures 3 C and 3 D). DEC+CCl4 group showed similar ultrastructure to that observed in the control group (Figure 3E and 3F).

3.4 DEC decreased the COX-2 expression

The control and DEC groups showed basal levels of COX-2 expression (Figures 4A, 4B). However, significantly elevated levels of expression of COX-2 were observed in CCl_4 group, mainly in fibrosis areas and in mononuclear infiltrates. Treatment group with DEC and CCl_4 markedly reduced COX-2 expression near to the basal levels observed in the control group (Figure 4D). The labelling quantification of COX-2 was performed using the Gimp 2.6 image program (Figure 4E).

3.5 Expression of α-SMA (hepatic stellate cell activation)

Hepatic stellate cells are activated in response to liver damage. These cells produce type I collagen leading to hepatic fibrosis and also express α-SMA (Lin and Zhang, 2012). In CCl_4-induced liver fibrosis model mice, expression of α-SMA increased significantly (Figure 5C). In contrast, treatment with DEC significantly decreased the α-SMA immunostaining (Figure 5D). The effect of DEC on the α-SMA expression was confirmed by the western blot analysis (Figure 5F) and RT-qPCR (Figure 5G).

3.6 Effect of DEC on the TGB-β expression

TGF-β is considered as the potent stimulus for the production of ECM in hepatic fibrosis (Bissell et al, 2001). The group CCl_4 (Figure 6C) revealed higher TGF-β staining than the control group (Figure 6A), and treatment with DEC reduced significantly the TGF-β immunoreactivity in hepatic tissue (Figure 6D). Western blot and RT-PCR analysis also showed a decrease of TGF-β expression after DEC treatment (Figure 6E and Figure 6F).

3.7 DEC influences MAPK activation in HSC

To identify the DEC potential anti-fibrogenic molecular mechanisms liver through CCl_4-induced liver fibrosis, several well-documented markers of the fibrosis signaling pathways were investigated. Because the expression of genes

involved in activation and proliferation the hepatic stellate cells are widely regulated through MAPK signal cascades, we assessed the effect of DEC on MAPK activity, including the JNK and p38 pathways. By immunohistochemistry, CCl_4 significantly enhanced the phosphorylation of JNK (Figure 7) and p38 (Figure 8), indicating the participation of both pathways in HSC activation. Treatment with DEC, in contrast, significantly inhibited the phosphorylation of both JNK (Figure 7D) and p38 (Figure 8D), indicating that at least some DEC effects in liver fibrosis are mediated by the inhibition of MAPK signalling pathway. Western blot analysis also confirmed this result (Figure 7E and Figure 8E).

3.7 DEC reduces liver profibrotic factors

The TIMP-1 mRNA expression was significantly higher in the CCl_4 group compared with the other groups, whereas CCl_4+DEC inhibited it significantly (Figure 9A). Conversely, MMP-2 mRNA expression was decreased in the CCl_4 group, and in contrast, DEC treatment increased MMP-2 mRNA expression (Figure 9B).

4. Discussion

The current study shows that the administration of DEC effectively attenuated the progression of CCl_4-induced liver fibrosis. In chronic CCl_4 intoxication, activation of HSC and increased accumulation of collagen in the liver were evident. Treatment with DEC effectively reduced CCl_4-induced the expression levels of various pro-fibrogenic factors. In adition, DEC decreased the level of JNK and p38 occurring during liver injury.

Initially hepatic stellate cells are quiescent under normal conditions. In the presence of injury these cells are activated (Geerts, 2001). The regulation of HSC has been considered as having an important role in the reversal of liver fibrosis (Hong and Par, 2013). The expression of alpha smooth muscle actin (α-SMA) indicates that stellate cells were activated (Akpolat et al, 2005), preceding the deposition of fibrous tissue (Carpino et al, 2005). In this study, activation of HSCs was identified with increased expression of the activation marker α-SMA in CCl_4 treated groups, while treatment of DEC significantly reduced α-SMA

expression. This indicated that DEC might deactivate HSC by preventing the initiation of fibrotic process and the synthesis of excessive connective tissue component. This was accompanied by accumulation of collagen in fibrous areas. The Picrosirius red staining for collagen confirmed this finding (Fig. 2). The progression of liver fibrogenesis is closely related to the activation of stellate cells (Kweon et al, 2001) by the expression of various pro-fibrogenic factors and the production of extracellular matrix (Benyon R.C. and Arthur, 2001; Schuppan and Ruehl, 2001).

During liver injury, hepatic cells produce a number of cytokines and mediators inflammatory such as TGF- β1. TGF- β1 is one of the most important cytokines involved in the fibrotic liver (Jarcuska and Janicko, 2010), it activates the hepatic stellate cells, as well as the subsequent production of extracellular matrix proteins (Hellerbrand et al, 1999). The results of this study showed that DEC treatment significantly decreased the extraordinarily high level of TGF-β1, suggesting that the inhibitory effects of DEC on liver fibrosis might be related to its action on HSC de activation by control of the production of TGF- β1.

Metalloproteinases are a group of enzymes that help to preserve the homeostasis of ECM, therefore, are of crucial importance because in the presence of a hepatotoxic agent, HSC is activated, and the tissue inhibitor of metalloproteinase 1 (TIMP-1) is upregulated, blocking ECM degradation by inhibiting MMPs activity leading to fibrogenesis (Cheung et al, 2009; Schuppan and Ruehl, 2001). MMP-2 (gelatinase A) can cleave collagen type I and IV and their expression is increased and remains elevated during in experimental liver fibrogenesis induced by CCl_4 (Hemmann et al, 2007). It has been reported that TIMP-1 has antiapoptotic effect on activated HSCs, possible decrease in its production could be beneficial for the resolution of liver fibrosis (Yoshiji et al, 2002). Consequently, the ECM degradation can be improved by regulating the activity of MMPs and TIMPs to promote the reversal of hepatic fibrosis (Doll et al, 2005; Mohammed et al, 2005). Our results clearly demonstrate the potential for DEC modulation of expression of both TIMP-1 and MMP-2 in liver tissue.

Cyclooxygenase-2 is associated with chronic liver disease as well as hepatitis C infection (Nunez et al, 2004). Experimental evidence indicate that the use of pharmacological inhibitors COX-2 may be useful in the treatment of liver diseases (Chavez and Segovia, 2010). In fact, in this study, it was shown

that activity enzyme COX-2 was induced selectively by CCl_4. As expected, DEC was capable of inhibiting the activity of COX-2 produced by CCl_4 administration. We demonstrated histologically DEC was able to completely reduce hepatic collagen. On the one hand, DEC is a selective inhibitor of COX-2 and is capable of prevent TGF- β1 expression and ECM accumulation; in the other, DEC induces activity of MMP-2 and so ECM degradation. In studies conducted by Chavez et al., 2010, evaluated the effects of Celecoxib on liver fibrosis induced by chronic administration of CCl_4. This study showed that Celecoxib prevents and reverses hepatic fibrosis produced by CCl_4 intoxication. Furthermore, the antifibrotic effects of Celecoxib can be explained by its ability to decrease the activity of TGF- β1 and COX-2 by increasing the levels of MMP-2 and their anti-oxidant properties. Corroborating these data, one can see similarities to the effects of DEC, since this drug has reducing action of COX-2.

The mitogen-activated protein kinase (MAPK) signaling cascade appears as other intracellular signaling pathway that is stimulated in activated HSCs. Members of the MAPK family, includes extracellular regulated kinase (ERK), c-jun N-terminal kinase 1 (JNK), and p38 MAPK [16]. When these molecules are activated, leads to the transcription of cell-proliferative and profibrogenic factors (Johnson and Lapadat, 2002). The JNK and p38 MAPK pathway plays an important signal in functional regulation of HSCs, regulate the expression of α-SMA, as occurs during activation and proliferation of HSCs, and the production of ECM proteins which contribute to the progression of liver fibrosis (Hong and Park, 2013; Tock et al, 2003). Our results showed that DEC reduced expression of p38 MAPK and JNK phosphorylated, thus reducing HSC activation and progression of liver fibrosis. These data indicate that not only TGF- β1 signaling but also MAPK pathways induced are important in the activation of HSCs.

The current study shows that administration of DEC was effective in attenuating CCl_4-induced liver injury and fibrosis, as confirmed by histological findings and liver fibrogenesis indicators. Moreover, DEC attenuated HSC activation via the downregulation of CCl_4-induced p38 MAPK, JNK and TGF-β signaling activation. The enhancement of MMP-2 production by DEC and the inhibition of TIMP-1 production seem to be additional mechanisms of its antifibrotic activity. Therefore, on the basis of our work, DEC should be

regarded as a promising drug and should be useful for the treatment of liver fibrosis.

Acknowledgements

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Legends to figure

Figure 1. H&E staining of liver sections (x200 and x400): (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC and (e) Quantification fibrosis area (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p< 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group. Fibrotic areas (arrows)

Figure 2. Picrosirius red staining of liver sections (x200 and x400): (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC and (e) Quantification fibrosis area and (f) Relative expression of mRNA Col-1 (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p< 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group. Collagen is stained red, demonstrating the fibrous septae in group (c).

Figure 3. Ultrathin sections of hepatocytes. (a) and (b) Control group; (c) and (d) CCl_4 group; (e) and (f) DEC + CCl_4 group. Note that the chronic cell injury exhibits vacuoles within mitochondria (white arrows). Note also the disruption of rER and to the large presence of lipids. Mitochondria (M), rough endoplasmic reticulum (rER), Stellate cell (S) and lipids (L). Bar: 1, 2 and 5 μm.

Figure 4. Effects of DEC on COX-2 expression. Immunohistochemistry (IHC) for COX-2 (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC, (e) quantification IHQ (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p < 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group.

Figure 5. Effects of DEC on HSC activation. Immunohistochemistry (IHC) for α-SMA (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC, (e) quantification IHQ, (f) western blot analysis and (g) RT-qPCR (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p < 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group.

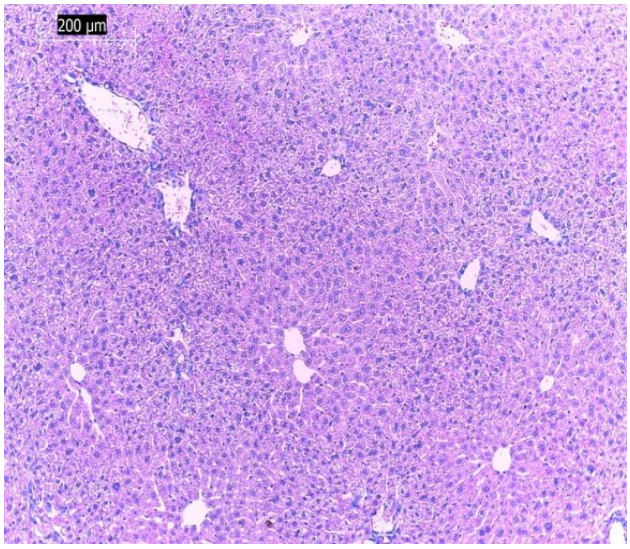
Figure 6. Effects of DEC on TGF-β expression. Immunohistochemistry (IHC) for TGF-β (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC, (e) quantification IHQ, (f) western blot analysis and (g) RT-qPCR (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p < 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group.

Figure 7: Effects of DEC on p-JNK expression. Immunohistochemistry (IHC) for p-JNK (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC, (e) quantification IHQ and (f) western blot analysis (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p < 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group.

Figure 8: Effects of DEC on p-p38 expression. Immunohistochemistry (IHC) for p-p38 (a) Control, (b) DEC, (c) CCl_4, (d) CCl_4+DEC, (e) quantification IHQ and (f) western blot analysis (mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [b]p < 0.05 when compared with DEC group; [c]p < 0.05 when compared with CCl_4 group; [d]p < 0.05 when compared with CCl_4 + DEC group.

Figure 9: (a) Relative expression of mRNA TIMP1; (b) mRNA expression of MMP2. The results are expressed as the mean ± S.D., n = 5). [a]p < 0.05 when compared with control group; [d]p < 0.05 when compared with CCl_4 + DEC group.

Figure 1



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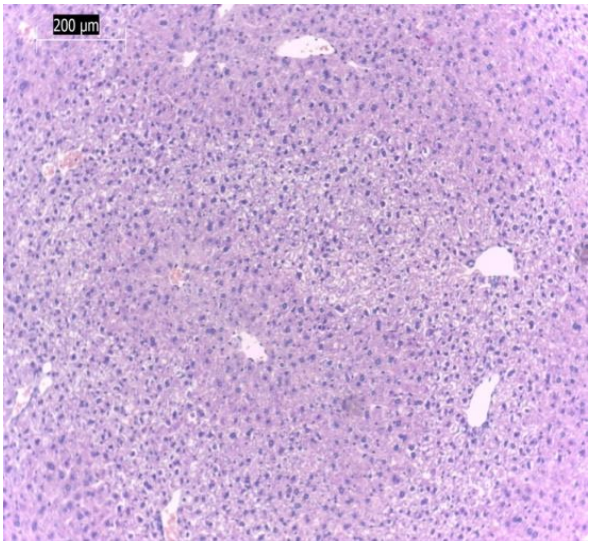

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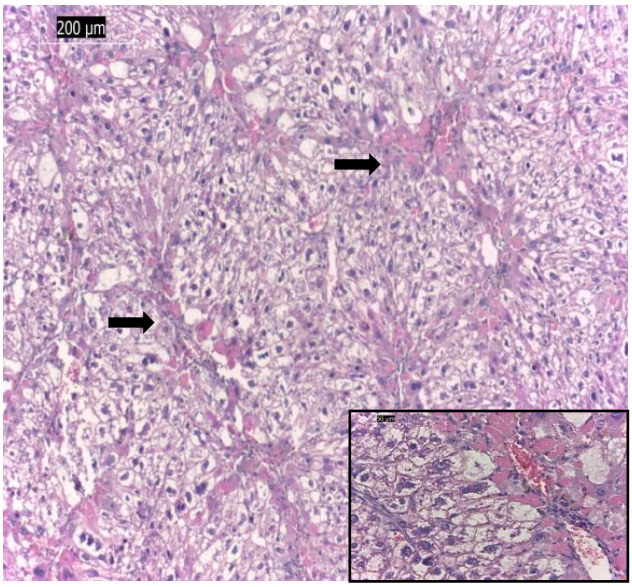

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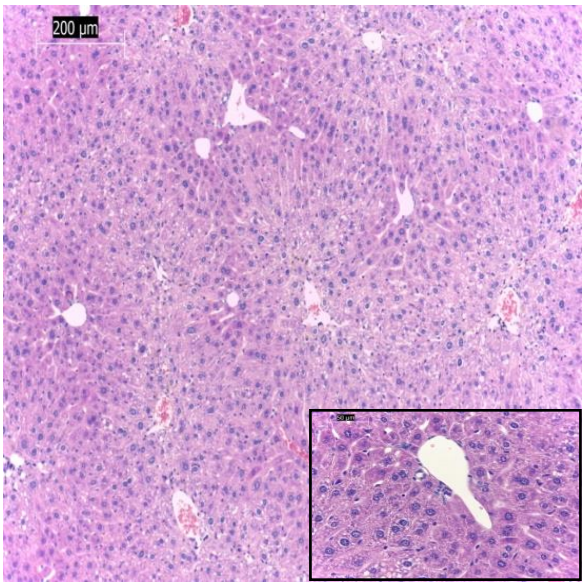

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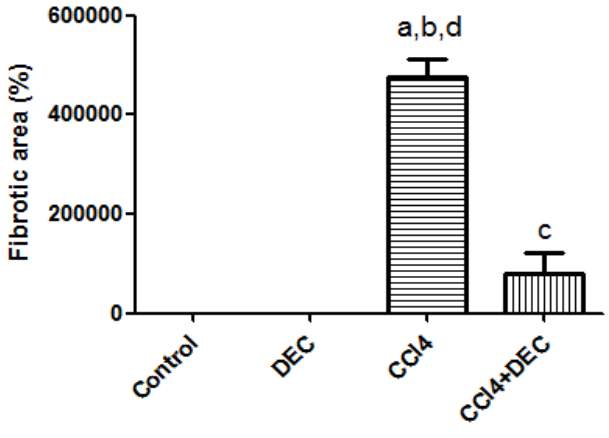

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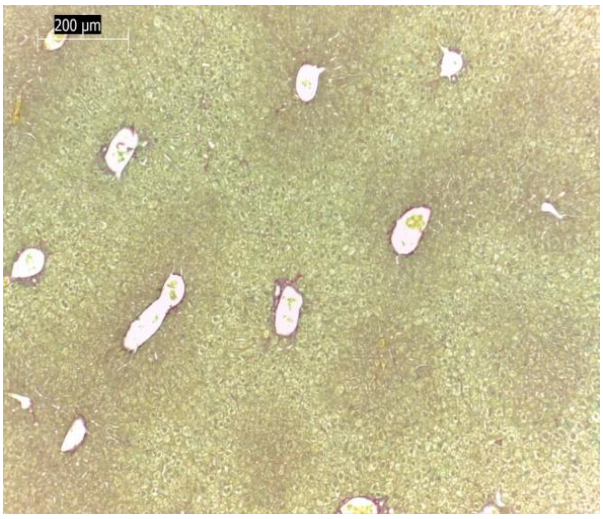
Figure 2



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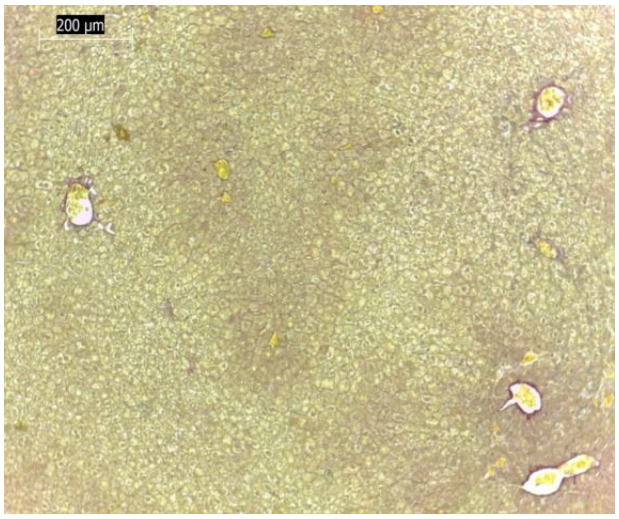

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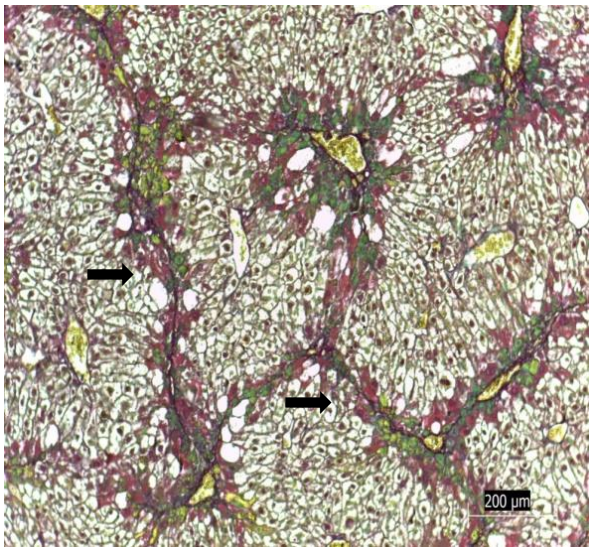

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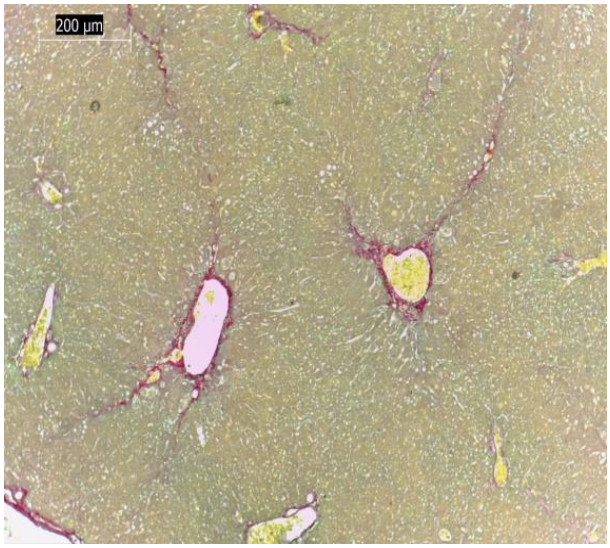

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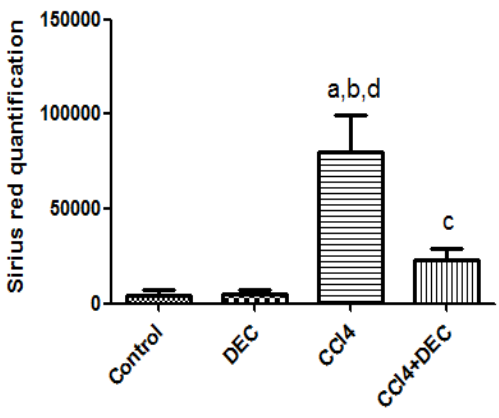

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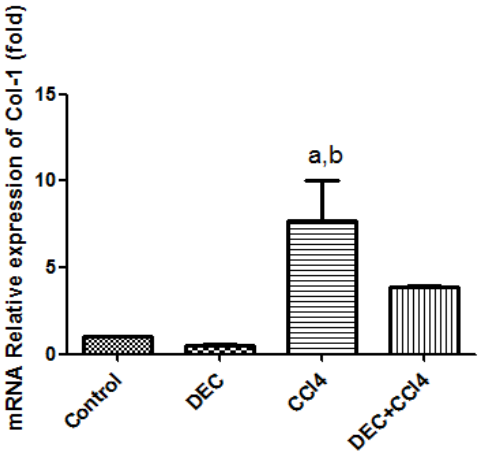

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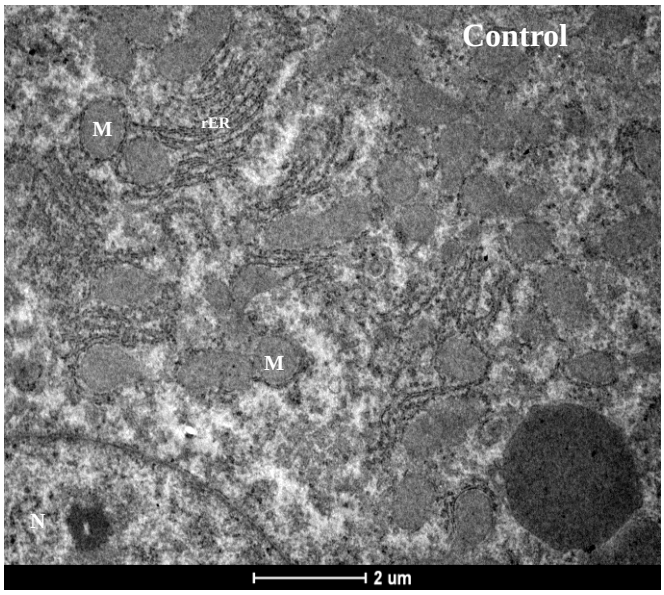
Figure 3



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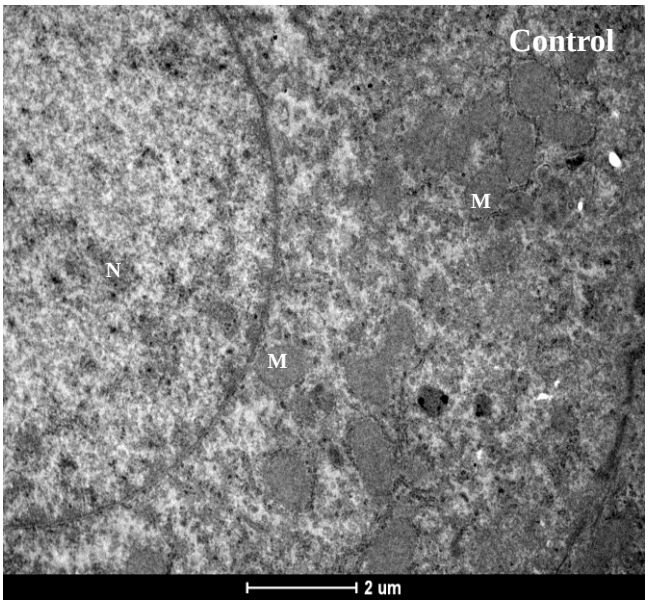

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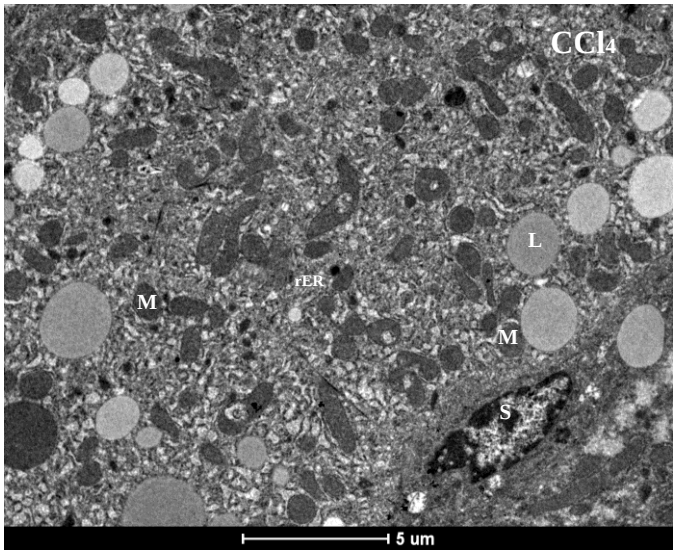

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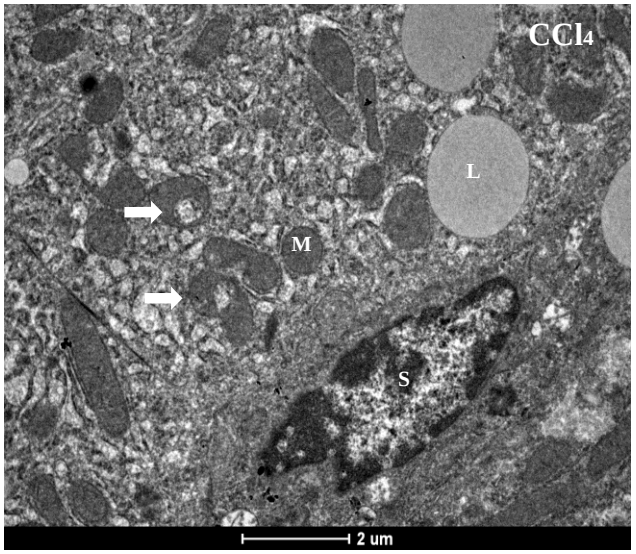

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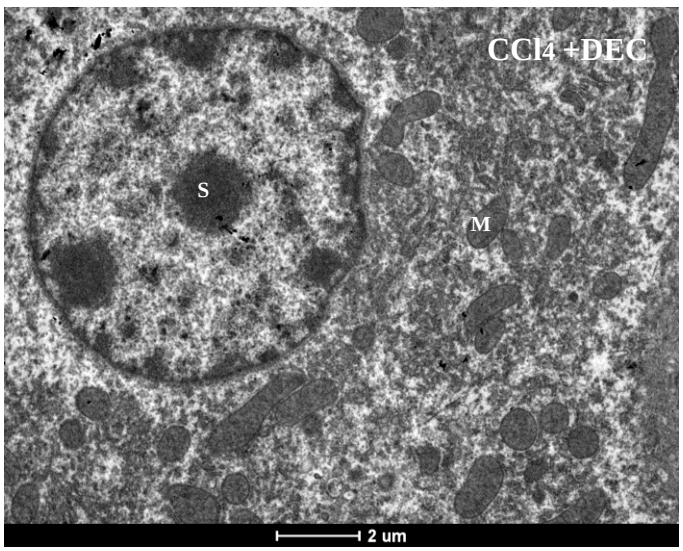

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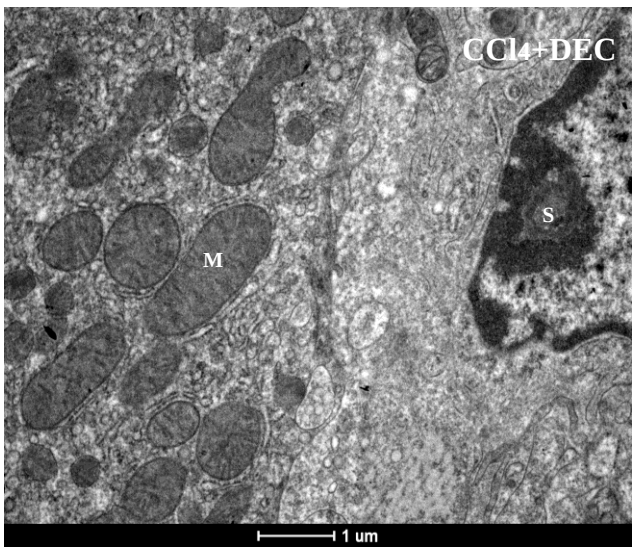

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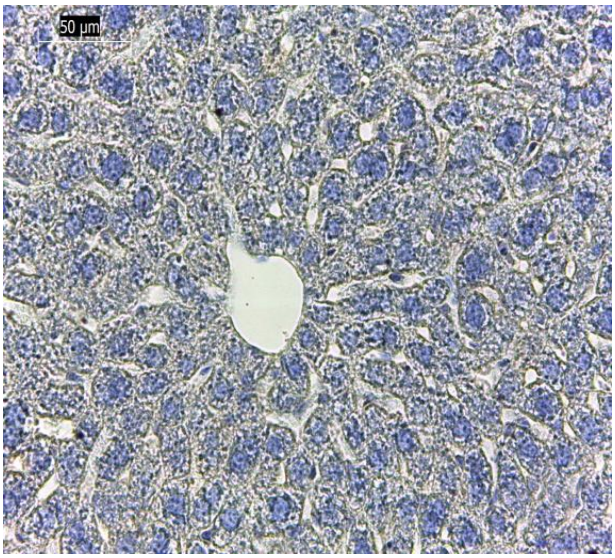
Figure 4



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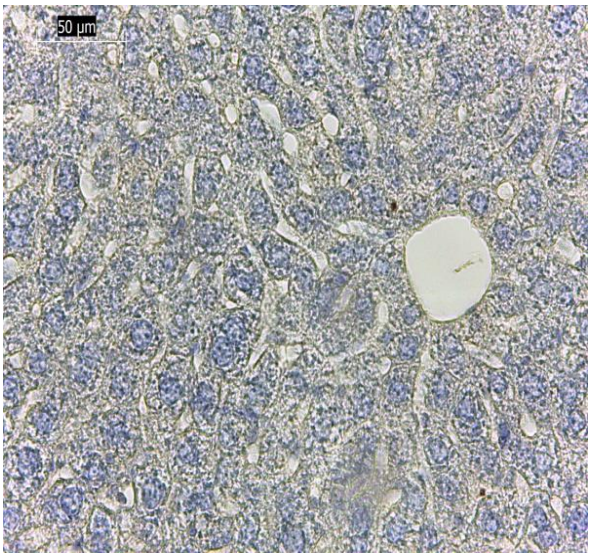

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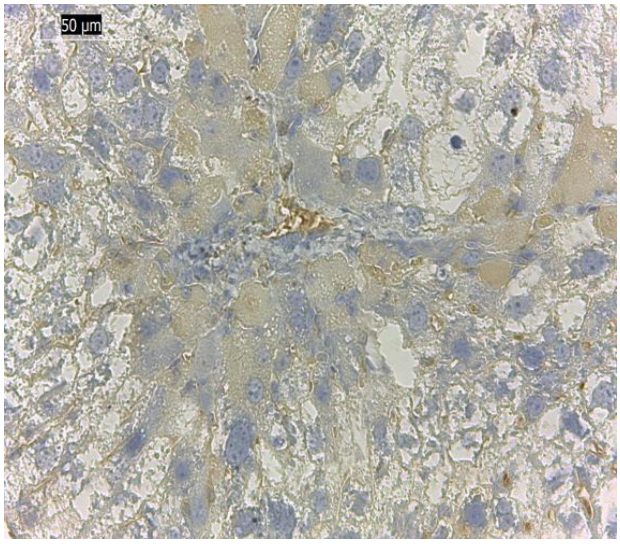

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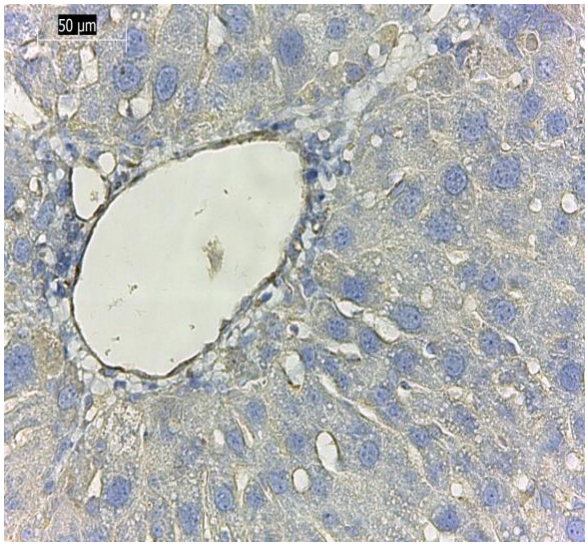

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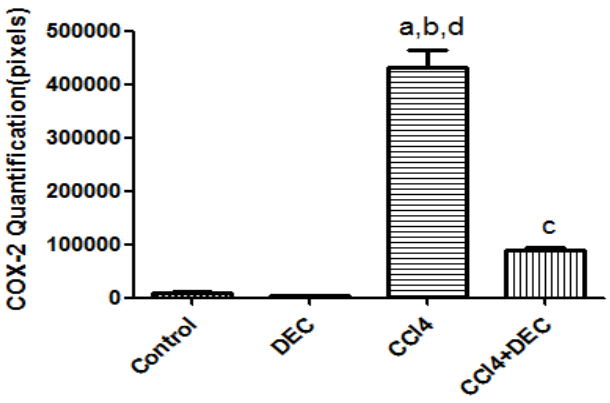

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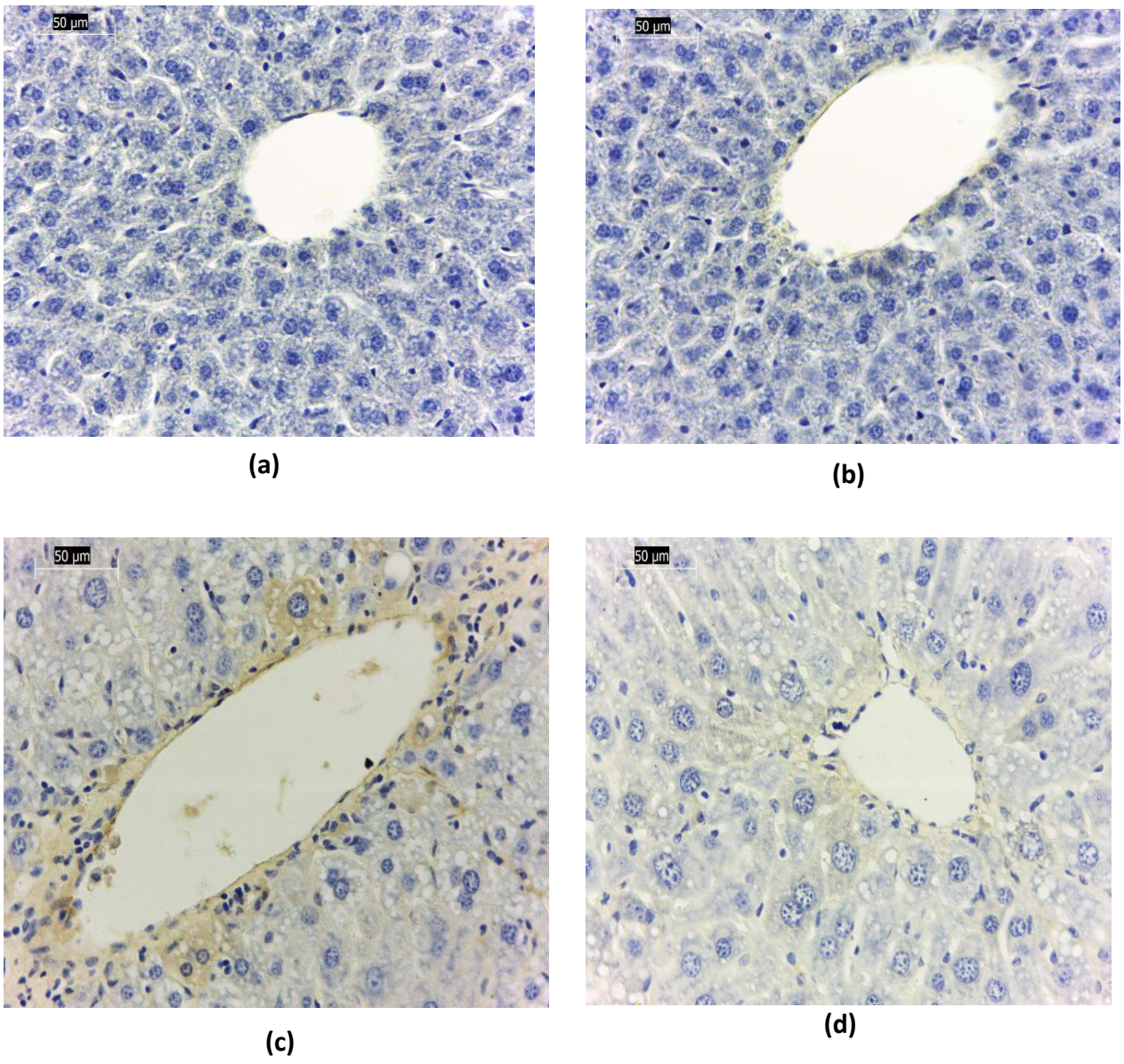
Figure 5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(c)
50 µm
(d)
α-SMA
β-actin
α-SMA Quantification(pixels)
400000
300000
200000
100000
0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC
(e)
α-SMA Quantification(pixels)
50000
40000
30000
20000
10000
0
a,d
c
Control
DEC
CCl4
CCl4+DEC
(f)
mRNA Relative expression ofα-SMA (fold)
4
3
2
1
0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC
(g)

Figure 6

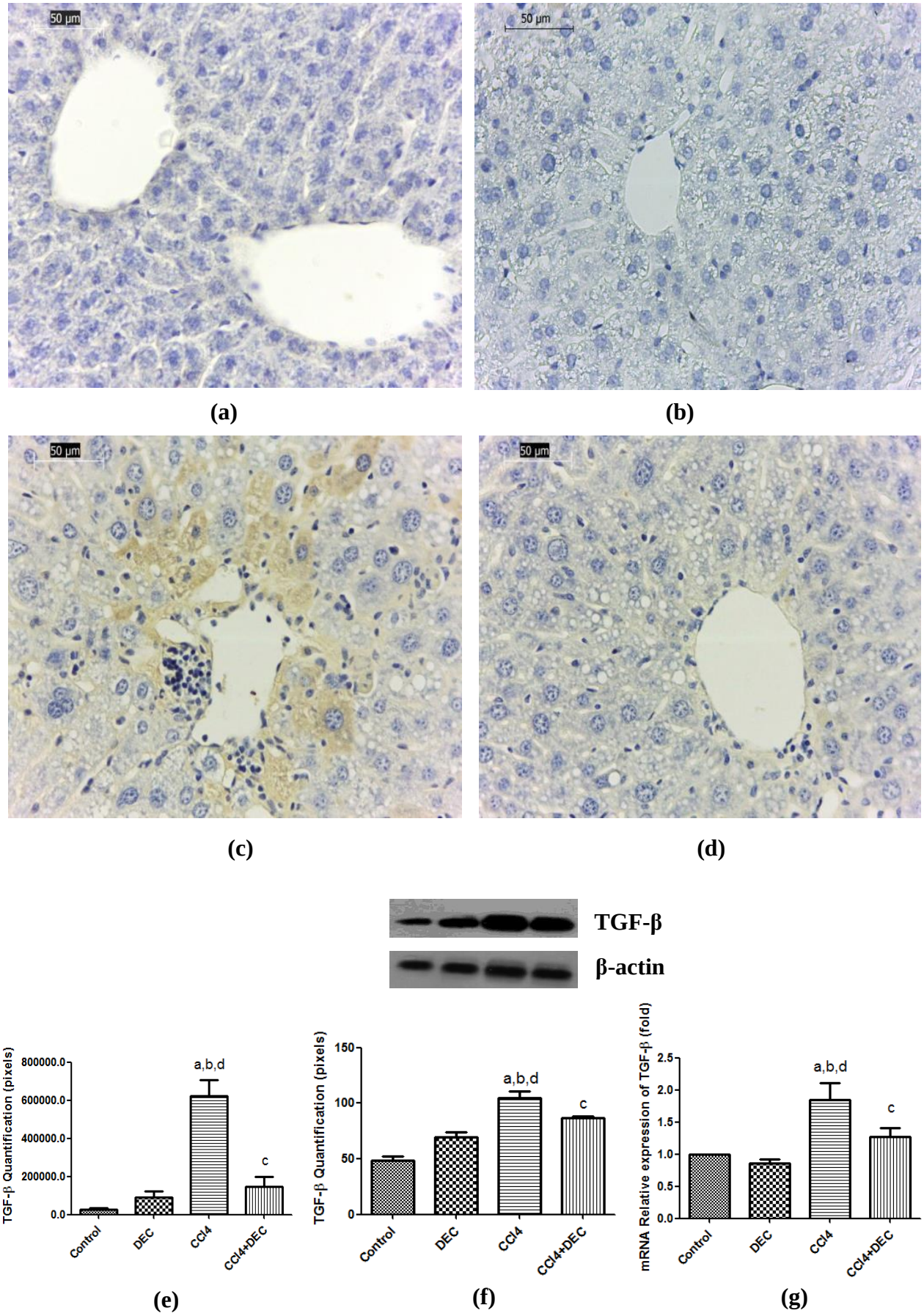

50 µm
50 µm
(a)
(b)
50 µm
50 µm
(c)
(d)
TGF-β
β-actin
TGF-β Quantification (pixels)
800000.0
600000.0
400000.0
200000.0
0.0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC
(e)
TGF-β Quantification (pixels)
150
100
50
0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC
(f)
mRNA Relative expression of TGF-β (fold)
2.5
2.0
1.5
1.0
0.5
0.0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC
(g)

Figure 7

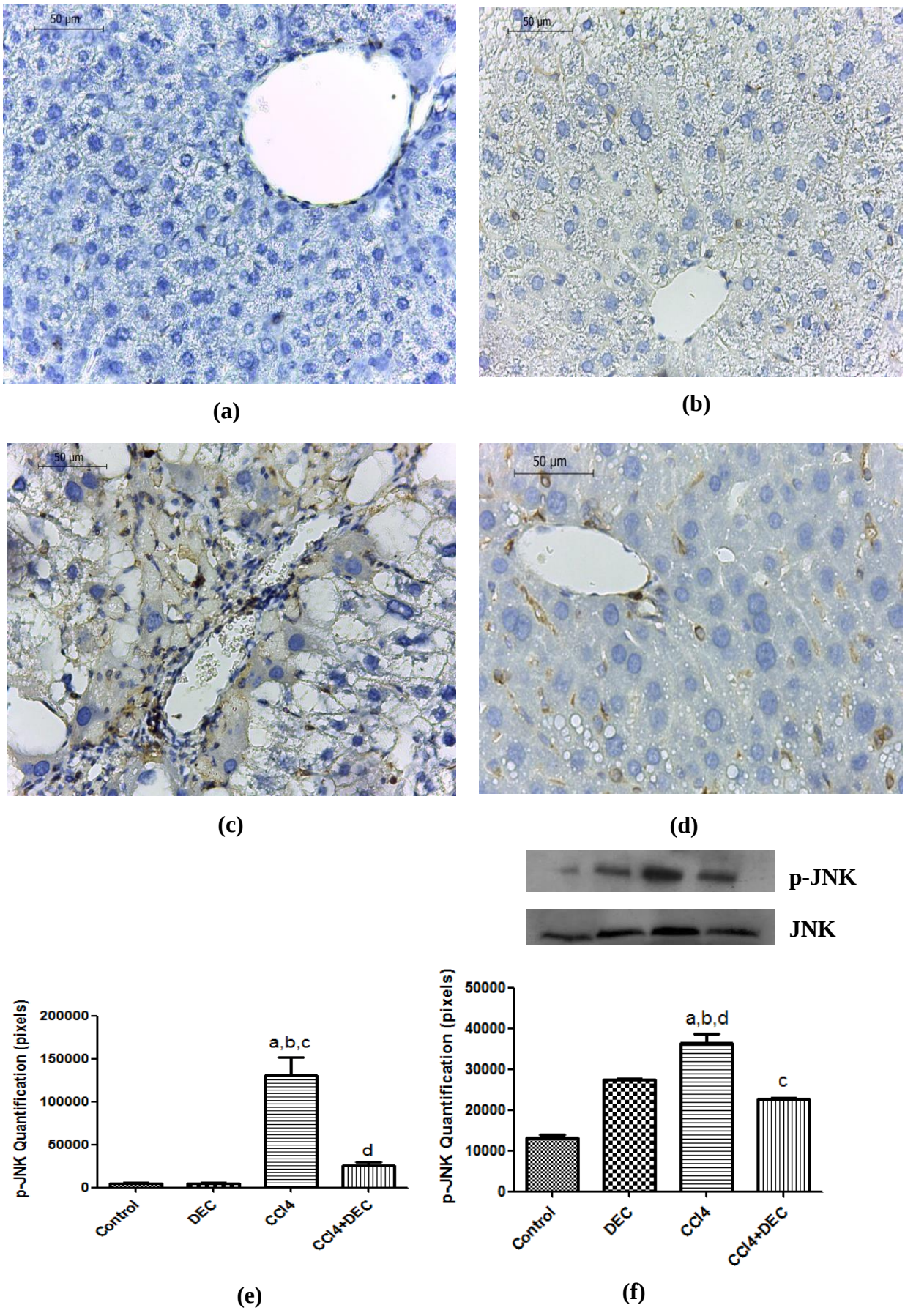
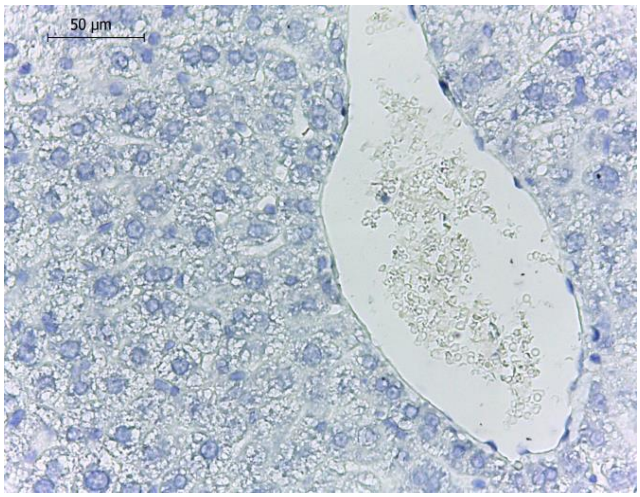
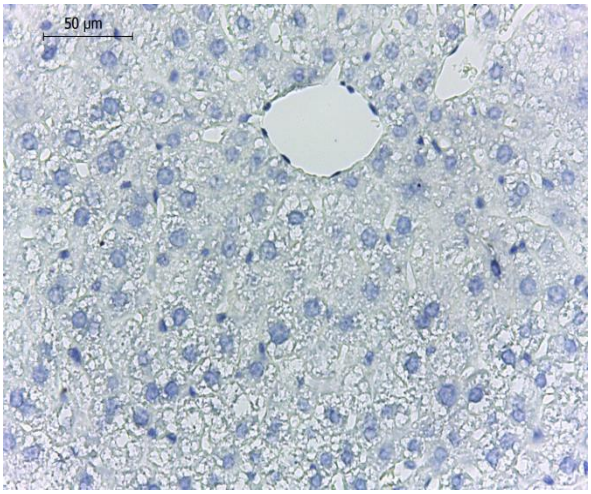

50 µm
(a)
50 µm
(b)
50 µm
(c)
50 µm
(d)
p-JNK
JNK
p-JNK Quantification (pixels)
200000
150000
100000
50000
0
a,b,c
d
Control
DEC
CCl4
CCl4+DEC
(e)
p-JNK Quantification (pixels)
50000
40000
30000
20000
10000
0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC
(f)

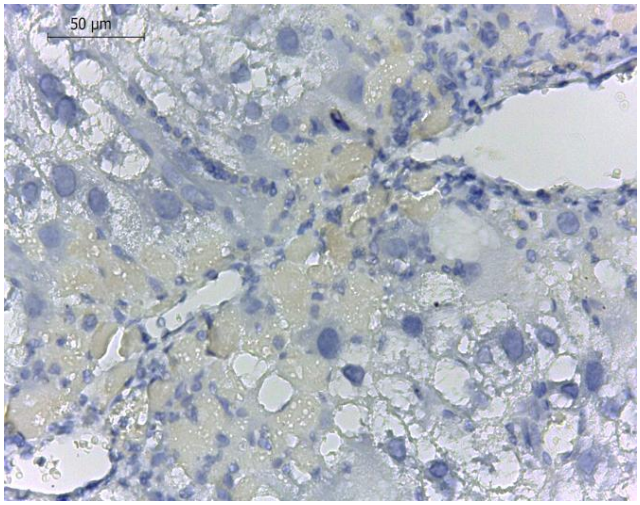
Figure 8



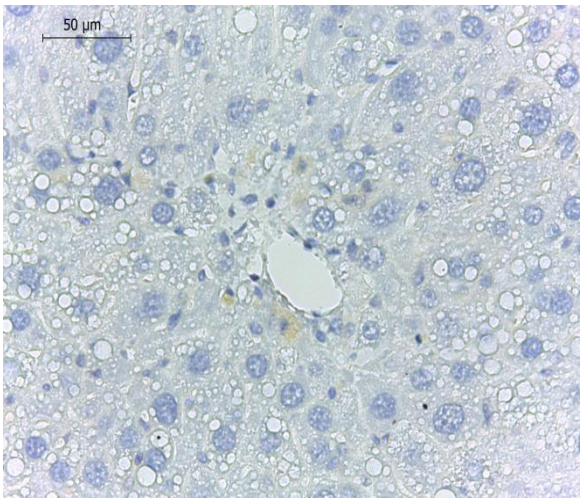
(a)



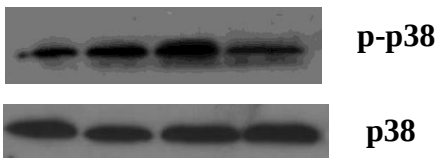
(b)

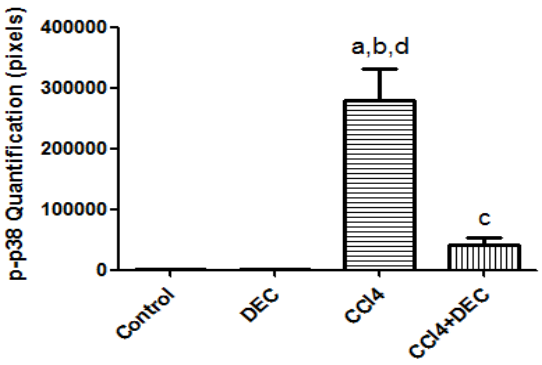


(c)

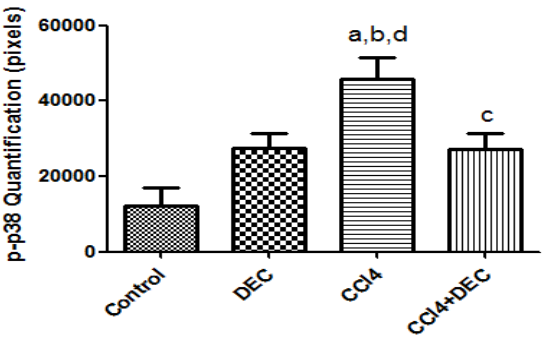


(d)



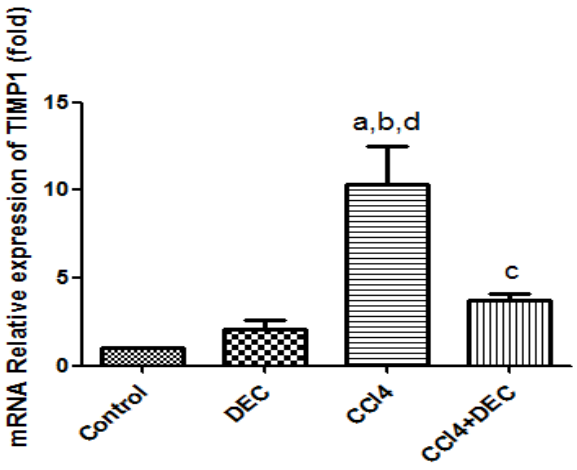


(e)

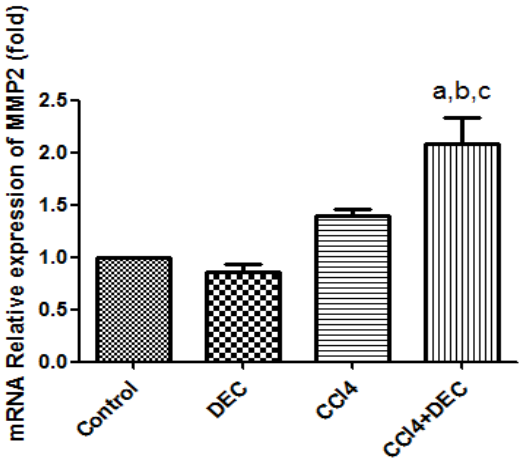


(f)

Figure 9


mRNA Relative expression of TIMP1 (fold)
15
10
5
0
a,b,d
c
Control
DEC
CCl4
CCl4+DEC

(a)


mRNA Relative expression of MMP2 (fold)
2.5
2.0
1.5
1.0
0.5
0.0
a,b,c
Control
DEC
CCl4
CCl4+DEC

(b)

6. Conclusão

1. A Dietilcarbamazina (DEC) promoveu atenuação significante da fibrose hepática em modelo experimental induzida por CCl_4, como foi confirmado pelos achados histológicos e indicadores de fibrogênese hepática.
2. O TGF-ß é uma das citocinas mais importantes envolvidos na fibrose do fígado, esta proteína está relacionada com a ativação das células estreladas hepáticas (CEHs). A DEC foi capaz de reduzir esta citocina e consequentemente a progressão da fibrogênese hepática, que está intimamente relacionada com a ativação das (CEHs). Neste estudo, a ativação das CEHs foi identificada através da elevada expressão de α-SMA nos grupos tratados com CCl_4, enquanto que o tratamento de DEC reduziu significativamente expressão desse marcador. Isto indicou que a DEC pode desativar CEHs inibindo o início de processo fibrótico e a síntese de componentes de tecido conjuntivo excessivo.
3. A DEC aumentou a expressão da metaloproteinase de matriz (MMP2) e diminuiu a expressão gênica do inibidor tecidual de metaloproteinase (TIMP2), do TGF-β, α–SMA e do colágeno 1α neste modelo experimental, demonstrando claramente o potencial de DEC de modulação da expressão dos marcadores fibróticos no tecido hepático;
4. Foi possível observar que a DEC reduziu expressão de JNK e p38 MAPK fosforilada, reduzindo assim, a ativação CEHs e progressão da fibrose hepática. Estes dados indicam que não apenas a sinalização de TGF- β, mas também a sinalização da MAPK é importante na ativação de CEHs e na proliferação celular.
5. Portanto, a DEC apresentou efeito fibrolítico em modelo de fibrose hepática induzida por CCl_4, que apresenta semelhanças com patologias humanas. Assim, a DEC deve ser considerada como uma droga promissora e deve ser útil para o tratamento de fibrose hepática.

7. Anexo

7.1 Comissão de ética no uso dos 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: "**ANÁLISES DOS EFEITOS DA DIETILCARMAZINA SOBRE O PROCESSO INFLAMATÓRIO HEPÁTICO AGUDO E CRÔNICO EM CAMUNDONGOS C57BL/6J WILD TYPE E KNOCKOUT PARA iNOS**" protocolado sob o Nº 11/2010, coordenado 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 reunião 28/04/2011. Na presente versão, este projeto está licenciado e tem validade até mês de outubro 2014.

Quantitativo de Animais Aprovados	
Espécie - linhagem	**Nº de Animais**
CAMUNDONGO MUS MUSCULUS C57BL/6 WILD TYPE	**240**
CAMUNDONGO MUS MUSCULUS C57BL/6 KNOCOUT PARA iNOS	**240**
TOTAL	**480**

We certify that the project entitled **"ANÁLISES DOS EFEITOS DA DIETILCARBAMAZINA SOBRE O PROCESSO INFLAMATÓRIO HEPÁTICO AGUDO E CRÔNICO EM CAMUNDONGOS C57BL/6J WILD TYPE E KNOCKOUT PARA iNOS"** *(CEUA* Protocol Nº 11/2010), coordinated by **CHRISTINA ALVES PEIXOTO** 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 April 28, 2011. In the present version this project is licensed and valid until october 2014.

Recife (PE, Brazil) may 18, 2011.

Sheilla Andrade de Oliveira, Msc, PhD
Pesquisadora Adjunta da FIOCRUZ
Vice-Coordenadora da CEUA/CPqAM
Mat. Siape - 1654975 - Recife - Brasil

Dra Sheilla Andrade de Oliveira
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Centro de Pesquisas Aggeu Magalhães – 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
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7.2 Normas da revista

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The *European Journal of Pharmacology* publishes full-length papers on the mechanisms of action of chemical substances affecting biological systems. The journal also considers short reviews (not exceeding 12 pages in print) intended to debate recent advances in rapidly developing fields that are within its scope.
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