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CENTRO DE CIÊNCIAS BIOLÓGICAS

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FRANCISLAINE APARECIDA DOS REIS LÍVERO

ESTEATOSE HEPÁTICA ALCOÓLICA: CORRELAÇÕES COM RECEPTOR NUCLEAR FXR E ESTRESSE OXIDATIVO

CURITIBA

2012

FRANCISLAINE APARECIDA DOS REIS LÍVERO

ESTEATOSE HEPÁTICA ALCOÓLICA: CORRELAÇÕES COM RECEPTOR NUCLEAR FXR E ESTRESSE OXIDATIVO

Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Profª. Dra. Alexandra Acco

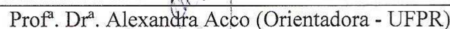
CURITIBA

2012

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Programa de Pós-Graduação em Farmacologia

PARECER

A Comissão Examinadora da Dissertação de Mestrado "Esteatose Hepática Alcoólica: Correlações com Receptor Nuclear e Estresse Oxidativo", de autoria da pós-graduanda **FRANCISLAINE APARECIDA DOS REIS LÍVERO,** sob orientação da Profª. Drª. Alexandra Acco e composta pelos professores: Profª. Drª. Alexandra Acco (UFPR), Profª Drª Emy Luiza Ishii Iwamoto (UEM) e Profª Drª Joice Maria da Cunha (UFPR). De acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, a pós-graduanda foi Aprovada. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas pela sua orientadora. Em Curitiba, 14 de fevereiro de 2011.



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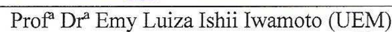

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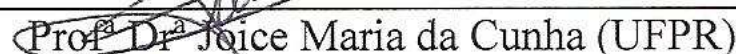

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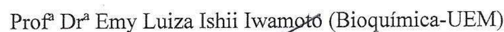
1 **ATA DO JULGAMENTO DA DEFESA DE DISSERTAÇÃO DE MESTRADO**

2 Ao décimo quarto dia do mês de fevereiro do ano de dois mil e doze, às quatorze
3 horas, no Anfiteatro 14 do Setor de Ciências Biológicas da Universidade Federal do
4 Paraná, reuniu-se a Comissão Examinadora da Dissertação de Mestrado de autoria da pós-
5 graduanda em Farmacologia **FRANCISLAINE APARECIDA DOS REIS LÍVERO**,
6 intitulada: "**ESTEATOSE HEPÁTICA ALCOÓLICA: CORRELAÇÕES**
7 **COM RECEPTOR NUCLEAR E ESTRESSE OXIDATIVO**", sob
8 orientação da Prof[a]. Dr[a]. Alexandra Acco e composta pelos membros: Prof[a] Dr[a] Emy Luiza
9 Ishii Iwamoto (Bioquímica-UEM) e Prof[a] Dr[a] Joice Maria da Cunha (Farmacologia-
10 UFPR). A Banca Examinadora iniciou os trabalhos. A candidata teve quarenta e cinco
11 minutos para expor oralmente seu trabalho, sendo em seguida argüida durante quinze
12 minutos por cada um dos membros da Banca, e tendo trinta minutos para responder a cada
13 uma das argüições. No final a Comissão Examinadora emitiu o seguinte parecer:
14 Aprovada. De acordo com o Regimento Interno do Programa de Pós-
15 Graduação em Farmacologia, a pós-graduanda foi aprovada. Para a publicação, o trabalho
16 deverá sofrer as modificações sugeridas, que serão conferidas pelo seu orientador. Nada
17 mais havendo a tratar, a Presidente deu por encerrada a sessão, da qual foi lavrada a
18 presente ata, que será assinada pela Presidente e pelos demais Membros da Banca
19 Examinadora, em Curitiba, 14 de fevereiro de 2012.

Prof[a]. Dr[a]. Alexandra Acco (Orientador- UFPR)



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NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e um artigo científico abordando os experimentos realizados, com resultados e discussão, além da conclusão. O artigo foi formatado conforme as normas propostas pelo periódico *Liver International*, ao qual o artigo foi submetido.

À Maria Cavalcante Lívero, exemplo de mulher, de avó...
Exemplo de honestidade, paciência, fé, generosidade, incentivo,
perseverança e principalmente de muito amor...

AGRADECIMENTO

Agradeço a Deus que me permitiu tudo isso. É a Ele que dirijo minha maior gratidão. Deus, mais do que me criar, deu propósito à minha vida. Vem Dele tudo o que sou, tenho e espero. Agradeço a Ele por ter me oferecido tantas oportunidades, por me oferecer pais maravilhosos, amorosos, pacientes e incentivadores; por me permitir conviver com irmãos tão especiais, intensos... Por colocar em meu caminho o amor em forma de pessoa e fazer dele meu maior companheiro, meu marido gentil, doce, perfeito... Por ter enviado anjos em forma de avós, tios, primos e amigos sempre tão dispostos a ajudar, a compartilhar, a somar, seja no trabalho, seja na vida pessoal... Por me apresentar mestres e doutores, em especial orientadores, que sempre tiveram comprometimento, sabedoria e carinho sem igual... Por me proporcionar estar durante todo o período do Mestrado em um ambiente repleto de funcionários e amigos extremamente solidários e solícitos... Mas agradeço principalmente por plantar em meu coração a semente da curiosidade e do desejo de descobrir coisas novas, o amor pela pesquisa, pela novidade. Agradeço-te meu Senhor por tudo isso que contribuiu para que eu pudesse vencer mais essa etapa de minha vida.

“Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muito conhecimento, que se sintam humildes. É assim que as espigas sem grãos erguem desdenhosamente a cabeça para o Céu, enquanto que as cheias a baixam para a terra, sua mãe”.

Leonardo da Vinci

RESUMO

Objetivos: Esteatose hepática refere-se ao acúmulo de triglicerídeos nos hepatócitos, e pode ser atribuída ao consumo excessivo de etanol. O fígado é o maior órgão de biotransformação do etanol e por isso pode sofrer com o estresse oxidativo gerado pelo etanol. Uma vez que a ativação do receptor FXR governa a homeostase lipídica, o objetivo desse trabalho é avaliar a participação do estresse oxidativo nas lesões hepática induzidas pelo etanol e testar os efeitos desse agonista contra o desenvolvimento da esteatose hepática.

Metodologia: Camundongos machos adultos (8-10 semanas) foram separados em 2 grupos (n=24) que receberam dieta líquida contendo etanol 10% (grupo controle) ou água, bem como ração hipoproteica (6%) ou normoproteica (23%), durante 6 semanas. Nas duas últimas semanas de dieta, os camundongos foram novamente divididos em 8 grupos (n=6) para o início do tratamento. Desses grupos, 4 receberam o agonista FXR 6ECDCA (3 $mg.kg^{-1}$) e 4 receberam tween 1% (veículo), por via oral (gavagem). Ao final do tratamento, os animais foram anestesiados para colheita de amostras (sangue e fígado), com o objetivo de avaliar: bioquímica plasmática (AST, ALT, colesterol e triglicerídeos), estresse oxidativo hepático (catalase, superóxido dismutase, glutationa-S-transferase, glutationa reduzida e peroxidação lipídica), histologia hepática (colorações de Hematoxilina-Eosina, Sudam Black e Azul do Nilo) e expressão gênica de *Srebp1f, FAS, PPARα, CYP7a1, HMGCoA reductase, ApoB, Scd1, p53* and *Bax*.

Resultados: O etanol associado à ração hipoproteica induziu estresse oxidativo hepático, aumentou os níveis plasmáticos de ALT e AST e induziu acúmulo de lipídeos no fígado. A ativação do receptor FXR pelo agonista 6ECDCA teve efeitos diretos: diminuição da expressão relativa de *SREBP1f* diminuição dos níveis plasmáticos de triglicerídeos e colesterol, bem como efeitos indiretos, dependentes do consumo de etanol, como diminuição dos níveis plasmáticos de AST e ALT, aumento da atividade da catalase e da superóxido dismutase, bem como diminuição da taxa de lipoperoxidação e da expressão relativa da *FAS.* Além disso, observou-se diminuição significativa da esteatose hepática. **Conclusão:** Avaliando o papel do estresse oxidativo e do receptor FXR na patogênese da doença hepática alcoólica em camundongos demonstrou-se que o 6ECDCA reverte o acúmulo de lipídeos no fígado e diminui parâmetros envolvidos no desencadeamento do estresse oxidativo. Assim, especulamos uma possível ação terapêutica desse agonista na doença hepática alcoólica, prevenindo a progressão dessa doença a estágios mais severos, como fibrose, cirrose e hepatocarcinoma.

Palavras-chave*:* etanol, esteatose, estresse oxidativo, FXR, 6ECDCA, fígado.

ABSTRACT

Background: Hepatic steatosis refers to the accumulation of triglycerides in hepatocytes, and it can be attributed to excessive ethanol consumption. The liver is the main organ of ethanol biotransformation and therefore, it can suffer with oxidative stress generated by the ethanol. Since the activation of the FXR receptor regulates adipose cell function, the aim of this work is to evaluate the participation of oxidative stress in ethanol-induced liver lesions and test the effects of FXR agonist against alcoholic liver steatosis development.

Experimental approach: Swiss male mice (8-10 weeks) were separated in 2 groups (n=24) which received liquid diet containing 10% ethanol or water (control group) for 6 weeks, as well as a low-protein diet (6%) or norm-protein diet (23%). In the last 15 days of the diet, mice that received ethanol or water were separated again for oral treatment, performing 8 groups (n=6) in the total. From these groups, 2 received FXR agonist 6ECDCA (3 $mg.kg^{-1}$) and 2 received 1% tween (vehicle), through oral treatment (gavage). Following this treatment, animals were anesthetized for sample collections (hepatic tissues and blood), in order to perform: plasma biochemistry assays (AST, ALT, cholesterol and triglycerides), hepatic oxidative stress (catalase, superoxide dismutase, glutathione-S-transferase, reduced glutathione and lipid peroxidation), liver histology (Hematoxylin-Eosin, Sudam Black and Nile Blue staining) and gene expression of *Srebp1f, FAS, PPARα, CYP7a1, HMGCoA reductase, ApoB, Scd1, p53* and *Bax.*

Results: Ethanol associated with low-protein diet induced hepatic oxidative stress, increased plasmatic ALT and AST, and induced hepatic lipid accumulation. The FXR activation by the agonist 6ECDCA had direct effects: decreased relative expression of *SREBP1f* and decreased the plasma levels of triglycerides and cholesterol; as well as it had indirect effects, dependent on ethanol consumption, such as reduced plasma levels of AST and ALT, increased on activity of catalase and superoxide dismutase and decreased rate of lipid peroxidation and the relative expression of *FAS*. Furthermore, we observed a significant decrease in hepatic steatosis. **Conclusion:** Evaluating the role of oxidative stress and FXR in the pathogenesis of alcoholic fatty liver disease in mice it was demonstrated that 6ECDCA reverses the accumulation of lipids in the liver and decreases the oxidative stress in mice. Thus, we speculate a possible therapeutic action of FXR agonists in alcoholic liver disease, aiming to prevent the progression of this disease to more severe stages such as fibrosis, cirrhosis and hepatocellular carcinoma.

Key words: ethanol, steatosis, oxidative-stress, FXR, 6ECDCA, liver

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LISTA DE ABREVIATURAS

MEOS – Sistema microssomal de oxidação do etanol

NAD – Nicotinamida adenina dinucleotídeo

NADH – Nicotinamida adenina dinucleotídeo hidreto

EROS – Espécies reativas de Oxigênio

SOD – Superóxide dismutase

Cat – Catalase

GPx – Glutationa peroxidase

ROOH – Radical peróxido

GSH – Glutationa reduzida

NADPH - Nicotinamida adenina dinucleotídeo fosfato-oxidase

GST – Glutationa-S-transferase

MEC – Matriz extracelular

SREBP1 - Sterol regulatory element-binding protein-1

Srebp1f – *Sterol regulatory element-binding protein-1 factor*

PPAR-α - Peroxisome proliferator-activated receptor-α

FFAs – Ácidos graxos livres

FXR – *Farnesoid X Receptor*

CDCA - Acido quenodeoxicólico

LXR – *Liver X Receptor*

SREBP1C - *Sterol regulatory element-binding protein-1C*

SHP - *Small heterodimer partner*

FAS – Ácido graxo sintase

DCA - Ácido deoxicólico

LCA - Ácido litocólico

UDCA – Ácido ursodeoxicólico

6ECDCA – Ácido 6-etilquenodeoxicólico

GW4064 - 3-(2,6-diclorofenill)-4-(3'-carboxi-2-clorostilbeno-4-il)oximetil-5-isopropilisoxazole.

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FXR – Farnesoid X receptor

6ECDCA - 6-Ethylchenodeoxycholic acid

AST – Aspartate aminotransferase

ALT – Alanine aminotransferase

SREBP1f - Sterol regulatory element binding protein 1 factor

FAS - Fatty acid synthase

PPARα - Peroxisome proliferator-activated receptor alpha

CYP7a1- Cholesterol 7-alpha-monooxygenase

HMGCoA reductase - 3-hydroxy-3-methyl-glutaryl-CoA reductase

ApoB - Apolipoprotein B

Scd1 - Stearoyl-CoA desaturase

p53 - Protein 53

Bax - Bcl-2–associated X protein

AFLD – Alcoholic fatty liver disease

UDCA - Ursodeoxycholic Acid

GW4064 - 3-(2,6-Dichlorophenyl)-4-(3'-carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole

SHP – Small heterodimer partner

Apo AI – Apolipoprotein AI

APO CII – Apolipoprotein CII

BSEP – Bile Salt Export Pump

NAFLD - Non-alcoholic fatty liver disease

CHO - Cholesterol

TG – Triglycerides

DPPH - 2,2-diphenyl-1picrylhydrazyl

Cat – Catalase

GST – Glutathione-S-transferase

SOD – Superoxide dismutase

LPO – Lipid peroxidation

GSH – Reduced glutathione

HE – Hematoxylin eosin

ROS – Reative oxygen species

ApoB – Apolipoprotein B

NADH – Nicotinamide adenine dinucleotide hydride

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1. INTRODUÇÃO

1.1 Etanol: problemas sociais e orgânicos decorrentes do consumo

O etanol é uma das substâncias psicoativas mais utilizadas em todo o mundo devido a diversos fatores. Dentre eles o fato de ser uma droga lícita, socialmente aceita, de fácil acesso e ter baixo custo. As conseqüências diretas e indiretas do consumo do etanol, como custos ao sistema de saúde, acidentes, violência e queda de produtividade geram significativo dano econômico (WHO, 2002). O consumo excessivo e a dependência de etanol são problemas que afetam milhares de pessoas e representam o maior problema de saúde pública em todo o mundo (CARLINI *et al.*, 2007). Concomitante com os problemas sociais desencadeados pelo abuso do etanol, complicações médicas ocorrem em indivíduos que o usam constantemente. Muitos mecanismos atuam em conjunto, refletindo a resposta do organismo a uma miríade de ações diretas e indiretas do etanol (WU e CEDERBAUM, 2009).

O etanol induz efeitos deletérios em diversos sistemas, como o gastrintestinal, nervoso, cardiovascular, hematológico e reprodutivo, incluindo disfunção sexual. Ele também pode levar a complicações psiquiátricas como sintomas psicóticos, depressão, síndrome de abstinência, demência, distúrbios de ansiedade e síndrome fetal alcoólica quando consumido por gestantes (SILVEIRA e MOREIRA, 2006). Têm-se estimado que 4% das ocorrências de doenças e, 3,2% de óbitos em todo o mundo podem ser atribuídos ao uso do etanol (ADRIAN, 2006).

O etanol não é armazenado no organismo e é totalmente oxidado no fígado (BAGNARDI *et al.*, 2001). Solúvel tanto em água quanto em lipídeos, esse composto é rapidamente difundido nas mucosas do esôfago e estômago. Assim, há aumento no risco de desenvolvimento de cânceres na cavidade oral, faringe e laringe (BAGNARDI *et al.*, 2001; CORRAO *et al.*, 1999). Há uma forte relação entre a quantidade consumida de etanol e o risco de desenvolvimento de câncer. Até o momento não foi constatado nenhum limite de consumo de

etanol que não aumente o risco de desenvolvimento de lesões cancerosas (BAGNARDI *et al.*, 2001; CORRAO *et al.*, 1999).

Contudo, muitos estudos têm demonstrado que o consumo moderado de etanol promove melhora da saúde: certas quantidades e freqüências previnem o desenvolvimento de diabetes mellitus (RIMM *et al.*, 1995; CONIGRAVE *et al.*, 2001), reduzem o risco de doenças coronárias cardíacas (MUKAMAL *et al.*, 2003; TOLSTRUP *et al.*, 2006) e elevam os níveis de lipoproteínas de alta densidade e de adiponectinas (FACCHINI, CHEN e REAVEN,1994; PISCHON *et al.*, 2005).

1.2 Etanol e nutrição

A redução da ingestão dietética é um dos principais componentes etiológicos da desnutrição, particularmente em pacientes alcoolistas. Como fatores agravantes nesta situação há má absorção intestinal de gorduras e o hipermetabolismo associado ao alcoolismo agudo (MAIO, DICHI e BURINI, 2000).

O abuso crônico do etanol afeta o estado nutricional e está associado com deficiências nutricionais e má-nutrição. O apetite e a qualidade da dieta dos indivíduos expostos a esse tipo de consumo são prejudicados e, além disso, há uma grande predisposição a fatores de risco nutricionais e deficiência de micronutrientes (ROSS *et al.*, 2012).

A doença hepática crônica resulta em grande impacto nutricional, independente de sua etiologia (SARIM *et al.*, 1997), pelo fato do fígado responsabilizar-se por inúmeras vias bioquímicas na produção, modificação e utilização de nutrientes e de outras substâncias metabolicamente importantes (NOMPLEGGI e BONKOVSKY, 1994). A presença do álcool constitui por si só um agravante, por promover desvios de vias metabólicas aumentando o consumo energético, a produção de H^+ e das formas ativas do oxigênio (DICHI *et al.*, 1993).

Há ainda que se ressaltar os hábitos alimentares distorcidos (má qualidade da dieta) dentre os alcoolistas crônicos e indivíduos com hepatopatia (MAIO, DICHI e BURINI, 2000). Paralelamente, pode existir o déficit de micronutrientes, independente da redução da ingestão energética, pela simples adição de álcool à ingestão energética usual (SARIN *et al.*, 1997), de modo independente da presença de doença hepática (GLÓRIA *et al.*, 1997). Assim, o etanol pode ser causa tanto de desnutrição primária, pelo fato de deslocar os nutrientes da dieta, como de desnutrição secundária, por ser responsável pela má absorção e agressão celular decorrentes de sua citotoxicidade direta (LIEBER, 1994).

Durante décadas, as deficiências dietéticas foram consideradas o maior fator responsável pelo desenvolvimento de doença hepática em alcoolistas. Tem sido reportado que uma dieta com pouco carboidrato associada ao etanol induz hepatotoxicidade mais severa, com intensa esteatose (KOROURIAN, 1999). Em contraste, a suplementação com proteína de soja parece melhorar o acúmulo de lipídeo induzido pelo etanol (YANG *et al.*, 2012).

Há pouca informação a respeito do efeito da quantidade, qualidade e composição protéica na patofisiologia da doença hepática alcoólica e não alcoólica; entretanto, a deficiência de proteína ou má-nutrição pode causar ou contribuir para a instalação da esteatose (MEGHELLI-BOUCHENAK *et al.*, 1989; LOCKWOOD *et al.*, 1977). A interação do etanol com nutrientes e/ou seu metabolismo pode contribuir significantemente para a instalação das doenças hepáticas alcoólicas (KHARBANDA, 2009).

1.3 Metabolismo do etanol e estresse oxidativo

A administração de etanol leva a diversas mudanças estruturais, funcionais e interrompe processos metabólicos em humanos e animais de experimentação. O fígado é um dos tecidos mais susceptíveis aos efeitos tóxicos do etanol (KUSUM e KHARBANDA, 2009) visto que é o primeiro local de metabolismo e alvo dos seus próprios metabólitos reativos, especialmente o acetaldeído (HENZEL *et al.*, 2004).

Três principais vias de metabolização do etanol são muito bem descritas em humanos e envolvem as seguintes enzimas: *(a)* álcool desidrogenase, *(b)* sistema microssomal de oxidação do etanol (MEOS) e *(c)* catalase. Durante o consumo de quantidades moderadas há maior envolvimento da enzima álcool desidrogenase. Essa é a via de metabolização clássica, catalisada por essa enzima para formar acetaldeído e tendo como resultado a geração de radicais livres (CEDERBAUM, 1991; DAS, NAYAK e VASUDEVAN, 2005; KUKIELKA, DICKER, e CEDERBAUM, 1994; MANTLE e PREEDY, 1999). Uma das maiores conseqüências da metabolização do etanol por essa via é a produção excessiva de NADH e alteração na razão $NADH/NAD^+$, o que altera o estado redox e reduz a síntese de glicose e a oxidação de ácidos graxos (CRABB e LIANGPUNSAKUL, 2006; SALASPURO e LIEBER, 1979).

O sistema microssomal de metabolização do etanol participa na oxidação através de catálise realizada pelas isoenzimas do Citocromo P450, (LIEBER e DECARLI, 1970) como as isoformas 2E1, 1A2 e 3A4, que variam na sua capacidade oxidativa (SALMELA *et al.*, 1998). A isoforma 2E1 é induzida pelo consumo crônico de etanol, de modo que esse mecanismo torna-se mais importante quantitativamente durante o abuso do etanol. Assim, a CYP2E1 pode ser uma catalisadora significante da formação de espécies reativas de oxigênio (EROS), pois tem sido apontada como geradora de uma grande quantidade de H_2O_2 (NORDSBLOM e COON, 1997). Além da geração de espécies reativas de oxigênio em conseqüência da metabolização, muitos outros passos são descritos como contribuintes fundamentais para a geração de estresse oxidativo durante o consumo de etanol (NORDMANN, RIBIERE e ROUACH, 1992). Dentre eles, cita-se a produção do metabólito reativo acetaldeído, danos mitocondriais, efeitos diretos à membrana, hipóxia, efeitos no sistema imune, alteração na produção de citocinas, mobilização de ferro e efeitos em componentes antioxidantes, particularmente a glutationa citosólica e mitocondrial. Muitas destas vias não são independentes uma da outra e várias contribuem para a geração excessiva de radicais livres. (DEY e CEDERBAUM, 2006; TSUKAMOTO e LU, 2001; ARTEEL, 2003). O resultado desta condição é o estresse oxidativo, no qual os níveis de EROS excedem a capacidade neutralizadora de antioxidantes enzimáticos e não enzimáticos (CZAJA, 2007).

As lesões por estresse oxidativo, portanto, podem resultar da produção excessiva de EROS, diminuição dos níveis de antioxidantes ou ainda de uma combinação dos dois efeitos (CZAJA, 2007).

O papel dos radicais livres no desenvolvimento da doença hepática alcoólica é apontado desde a década de 1960 (ALBANO[1] citado por SHERMAN, PREEDY e WALSON, 2002). Apesar do oxigênio ser essencial para todos os organismos aeróbios, o gás e especialmente seus metabólitos reativos, tais como ânion superóxido, peróxido de hidrogênio e radical hidroxila, são altamente tóxicos para todo o organismo (NISHIKAWA, HASHIDA e TAKAKURA, 2009). EROS podem danificar ou causar degradação completa (peroxidação, por exemplo) de moléculas complexas essenciais nas células, incluindo lipídios, proteínas e DNA (DEY e CEDERBAUM, 2006; TSUKAMOTO e LU, 2001; ARTEEL, 2003). Estas reações químicas resultam em anormal funcionamento das moléculas, organelas subcelulares, células, tecidos, órgãos ou mesmo organismos, dependendo da gravidade do dano oxidativo e da atividade dos sistemas endógenos de reparo (NISHIKAWA, HASHIDA e TAKAKURA, 2009).

Pequenas quantidades de EROS são normalmente geradas no fígado como produto da respiração mitocondrial (CZAJA, 2007). Para tanto, os hepatócitos possuem sistemas antioxidantes enzimáticos e não-enzimáticos para proteger o orgão contra lesões oxidativas (MCVICKER *et al.*, 2009). As principais enzimas antioxidantes são a superóxido dismutase (SOD), a catalase (Cat) e a glutationa peroxidase (GPx) (KATHIRVEL *et al.*, 2010). Existem duas isoformas da enzima SOD. A SOD cúprica-zinco está presente no citosol e no espaço entre as duas membranas que envolvem as mitocôndrias. Já a SOD contendo manganês está presente na matriz mitocondrial. Ambas as enzimas são fundamentais para a prevenção da toxicidade induzida pelas EROS (FRIDOVICH, 1997). A Cat e o sistema da glutationa ajudam a remover o peróxido de hidrogênio. Cat é encontrada principalmente em peroxissomos e catalisa a reação entre duas moléculas de peróxido de hidrogênio, resultando na formação de água e O_2. Além disso, a catalase pode promover a interação

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[1] ALBANO E. Free Radicals and alcohol-induced liver injury.

do peróxido de hidrogênio (H_2O_2) com doadores de hidrogênio para que o H_2O_2 possa ser convertido em uma molécula de água e o doador reduzido possa ser oxidado (atividade peroxidativa da catalase) (FRIDOVICH, 1997). Compostos que podem fornecer esses átomos de hidrogênio incluem o etanol e o metanol, que são oxidados em acetaldeído e formaldeído, respectivamente. O sistema da glutationa peroxidase é composto por vários componentes, incluindo as enzimas glutationa peroxidase e glutationa redutase, além do NADPH e glutationa (GSH) como co-fatores. Juntas, essas moléculas removem efetivamente o H_2O_2 (FRIDOVICH, 1997). GSH é um componente essencial deste sistema e serve como um co-fator para a enzima glutationa transferase (GST) que ajuda a remover certas drogas e produtos químicos, bem como outras moléculas reativas das células. Por causa de todas as suas funções, GSH é provavelmente o antioxidante não-enzimático mais importante presente nas células. Portanto, as enzimas que ajudam a gerar GSH são críticas para a capacidade do organismo em se proteger contra o estresse oxidativo (FRIDOVICH, 1997). Assim, a apoptose induzida como conseqüência do metabolismo do etanol não é completamente dependente do *status* das EROS, mas é substancialmente dependente dos níveis de GSH (MCVICKER, 2009).

GSTs humanas são uma grande família de enzimas de detoxificação que catalisam a conjugação da GSH com eletrófilos reativos (HAYES, FLANAGAN e JOWSEY, 2005). GSTs podem ser encontradas nas membranas microssomais, no citosol e na mitocôndria da maioria dos tecidos (HAYES, FLANAGAN e JOWSEY, 2005). As isoformas citosólicas de GST são constituídas por várias famílias, dentre elas as classes α, φ e μ, cada uma com vários membros (HAYES, FLANAGAN e JOWSEY, 2005; PASTORE *et al.*, 2003). Como co-fator para as isoformas de GST, alterações na síntese de GSH têm o potencial de perturbar gravemente a capacidade de desintoxicação hepática da GST (LU, 2008).

Adicionalmente, estudos revelam que o estresse oxidativo também pode mediar lesões em hepatócitos e morte celular, alterando vias de transdução de sinais através da oxidação de quinases e fosfatases que regulam os níveis de atividade do sinal (CZAJA, 2002). Sabe-se também que o etanol modula a expressão e a atividade de várias moléculas de sinalização intracelular e fatores de transcrição em monócitos e células de Kupffer (WHEELER e

THURMAN, 2003). A ativação das células de Kupffer por endotoxinas está envolvida na lesão hepática induzida por etanol. Enquanto a tolerância dessas células é observada nas fases iniciais de consumo de etanol, a sensibilização das mesmas é observada mais tarde. Tanto a tolerância quanto a sensibilização das células de Kupffer são induzidas por endotoxinas derivadas do intestino. A ingestão de grandes doses de etanol de forma aguda causa sensibilização por endotoxinas através de mecanismos dependentes de estresse oxidativo (YAMASHINA *et al.*, 2005). O consumo crônico de etanol torna as células de Kupffer hipersensíveis às endotoxinas, o que resulta na produção de citocinas inflamatórias e do fator de necrose tumoral α (TNFα), via receptor toll-like 4 (TLR4), levando à inflamação e necrose hepática (NATH e SZABO, 2009).

1.4 Doença Hepática Alcoólica

O estresse oxidativo induzido pelo etanol desempenha um papel importante nos mecanismos pelos quais o etanol produz lesão hepática (DEY e CEDERBAUM, 2006). A doença hepática alcoólica, considerada uma enfermidade "de estilo de vida", com sua patogênese e predisposição individual regidas por interações gene-ambiente (TSUKAMOTO *et al.*, 2009), ocorre em etapas:

(a) Esteatose hepática: acúmulo de lipídios no fígado ou "fígado gorduroso", é uma característica patológica comum da metabolização hepática comprometida decorrente do consumo crônico de etanol (BREITKOPF *et al.*, 2009; ARTEEL *et al.*, 2009). Embora considerada muitas vezes benigna e reversível, a esteatose é um fator de risco para o desenvolvimento de patologias hepáticas avançadas, incluindo esteatohepatite e fibrose. As alterações nos hepatócitos que acompanham o início de esteatose ainda não estão claramente definidas (ORLICKY *et al.*, 2011). Essa fase ocorre de forma assintomática e os pacientes têm um ligeiro aumento das transaminases, bilirrubina e fosfatase alcalina (BREITKOPF *et al.*, 2009; ARTEEL *et al.*, 2009);

(b) Esteatohepatite: forma aguda de lesão hepática induzida por etanol cujas manifestações são mal-estar, anorexia, dor abdominal, perda de peso e bioquímica anormal após um longo período de consumo excessivo de álcool.

Uma pequena porcentagem de pacientes com esteatohepatite pode progredir para cirrose alcoólica, mesmo após a interrupção do uso (BREITKOPF *et al.*, 2009; ARTEEL *et al.*, 2009);

(c) Fibrose: resposta de cicatrização de lesões recorrentes, que leva ao acúmulo excessivo de matriz extracelular (MEC), principalmente colágeno tipo I (BREITKOPF *et al.*, 2009; ARTEEL *et al.*, 2009);

(d) Cirrose: estágio final e a mais grave forma de doença hepática alcoólica, marcada por fibrose, alteração da arquitetura do fígado, remodelação insuficiente da MEC, hipertensão portal, ascite, esplenomegalia, encefalopatia hepática e insuficiência hepática (BREITKOPF *et al.*, 2009; ARTEEL *et al.*, 2009). Estas doenças podem ocorrer de forma independente, em combinação ou sequencial, com a progressão de esteatose para esteatohepatite e para o estabelecimento de cirrose (ARTEEL *et al.*, 2009). A esteatose hepática alcoólica foi considerada uma condição pouco importante por um longo tempo. No entanto, evidências crescentes sugerem que ela parece ser um estado potencialmente patológico que progride para estados mais severos da doença hepática alcoólica na presença de outros co-fatores, como infecção hepática por vírus e diabetes ou drogas (OTANI *et al.*, 2005; LIEBER, 2004).

Os mecanismos pelos quais o etanol produz esteatose são multifatoriais, envolvendo efeitos no metabolismo hepático de lipídios, hipóxia e peroxidação lipídica (LIEBER, 2004; FRENCH, 1989; SOZIO e CRABB, 2008). As lesões hepáticas alcoólicas envolvem uma complexa série de distúrbios na sinalização de células do sistema imunológico, células parenquimatosas e não parenquimatosas, e múltiplos sistemas sinalizadores são afetados para promover o acúmulo de lipídios nos hepatócitos expostos ao etanol (NATH e SZABO, 2009). A princípio, a esteatose nos hepatócitos resulta do desequilíbrio no metabolismo de lipídeos, com diminuição da oxidação lipídica mitocondrial e síntese de triglicerídeos aumentada. Diversos mecanismos subjacentes tem sido sugeridos, podendo ser o aumento da razão $NADH/NAD^+$ (CRABB, 1993; FROMENTY, BERSON e PESSAYRE, 1997), aumento da atividade da S*terol Regulatory Element-Binding Protein-1* (SREBP-1) (YAHAGI *et al.*, 2002; YOU *et al.*, 2004), diminuição da atividade do *Peroxisome Proliferator-Activated Receptor-α* (PPAR-α) (IP *et al.*, 2003) e diminuição da atividade da proteina quinase dependente do AMP cíclico (YOU *et al.*, 2004). Ainda, a mobilização

de ácidos graxos do plasma para a oxidação é estimulada pelo glucagon e inibida pela insulina, o que favorece o acúmulo de ácidos graxos livres (FFAs) no fígado e predispõe ao estresse oxidativo, estimulando as peroxidases lipídicas microssomais. Algumas das enzimas-chave envolvidas nestes sistemas de oxidação hepáticos são reguladas pelo fator de transcrição PPAR-α. Em estudos *in vivo*, animais PPAR-α *knockout* desenvolveram esteatose hepática grave imediatamente após o nascimento (HASHIMOTO *et al.,* 2000; REDDY e HASHIMOTO, 2001).

O diagnóstico de esteatose é feito quando o conteúdo de lipídios no fígado excede 5 a 10% do seu peso (ADAMS *et al.*, 2005). Histologicamente, a esteatose é caracterizada pelo acúmulo de gotículas lipídicas compostas principalmente de triacilgliceróis no citoplasma dos hepatócitos (CAIRNS e PETERS, 1983). O tamanho e a distribuição lobular dessas gotículas lipídicas citoplasmáticas são variáveis, refletindo a etiologia, gravidade e duração da esteatose (ORLICKY *et al.*, 2011). Esteatose associada ao etanol instala-se inicialmente nas áreas centrolobulares (zona 3), com avanço para áreas intermediárias (zona 2) e áreas periportais (zona 1), o que ocorre com a progressão da doença (BRUNT, 2007; LEFKOWITCH, 2005). Detalhes sobre os mecanismos subdjacentes de iniciação zona-dependente e progressão da esteatose são limitadas (STEWART, JONES e DAY, 2001). Sabe-se no entanto, que o acúmulo de FFAs é problemático, pois são importantes mediadores da lipotoxicidade celular (UNGER, 2002). Os mecanismos exatos de seus efeitos citotóxicos não são claros mas podem envolver múltiplas e independentes vias de regulação e sinalização intimamente ligadas. Dentre elas está a disfunção mitocondrial secundária à superprodução de EROS, peroxidação lipídica e estresse oxidativo (LISTENBERGER *et al.*, 2003; SANYAL, 2002), anteriormente comentados.

1.5 Receptor Nuclear FXR e seus agonistas

Receptores nucleares são reguladores-chave de vários processos incluindo a reprodução, o desenvolvimento e o metabolismo de xeno- e endobióticos, tais como ácidos biliares e drogas (ZOLLNER, WAGNER e TRAUNER, 2010). Pesquisas nas últimas duas décadas permitiram uma

compreensão detalhada da regulação desses processos e, mais importante, levaram ao desenvolvimento de novas drogas visando especificamente os receptores nucleares para o tratamento de uma variedade de doenças (ZOLLNER, WAGNER e TRAUNER, 2010). Alguns agonistas de receptores nucleares já são utilizados em fase clínica, mas muitos ainda estão sendo testados para o tratamento de diabetes, dislipidemia, esteatose hepática, câncer, hepatotoxicidade por drogas e colestase (ZOLLNER, WAGNER e TRAUNER, 2010).

O receptor nuclear FXR (*Farnesoid X Receptor)* é um membro bem caracterizado da subfamília "metabólica" de receptores nucleares, altamente expresso no fígado, intestino, rins e glândulas supra-renais (GROBER *et al.*, 1999). FXR foi isolado originalmente do cDNA do fígado de ratos e foi nomeado *Farnesoid X Receptor* porque foi ativado por concentrações suprafisiológicas de farnesol (FORMAN *et al.,* 1995), no entanto, o farnesol não se ligava ao FXR em concentrações fisiológicas. Em 1999, alguns grupos de pesquisadores apontaram os ácidos biliares como ligantes naturais do receptor e, desde então se sabe que o FXR é um receptor de ácidos biliares (MAKISHIMA *et al.*, 1999; PARKS *et al.*, 1999). No mesmo ano, o FXR foi reconhecido como um sensor de transcrição para ácidos biliares e, portanto, ocorreu seu "renascimento" como moléculas de sinalização depois de 20 anos de pesquisas, o que culminou com o uso clínico do ácido quenodeoxicólico (CDCA) e ácido ursodeoxicólico (UDCA) para o tratamento de cálculos na vesícula biliar e desordens colestásicas hepáticas. Ácidos biliares, oxisteróis e ácido colestanóico pertencem a distintas classes de moléculas esteróides derivadas do colesterol. Todos atuam como moléculas de sinalização e participam de uma complexa rede de interações que por fim governam a homeostase de colesterol, lipídeos, esteróides e estão envolvidos em processos como utilização de glicose, inflamação e câncer (PELLICCIARI, COSTANTINO e FIORUCCI, 2005).

Recentemente, experimentos *in vitro* e em modelos animais vêm sugerido que o FXR desempenha um papel central no metabolismo de lipídeos nas células hepáticas. Ele pode afetar dois outros receptores nucleares*: Liver X Receptor* (LXR) e o *Sterol Regulatory Element Binding Protein 1C* (SREBP-1C), que está intimamente relacionado com a regulação de lipídeos (YANG, SHEN e

SUN, 2010). Uma vez ativado, o FXR regula a expressão gênica de alvos ligando-se ao elemento de resposta ao FXR (FXRE) após heterodimerização com o *Retinoid X Receptor* (RXR) (WANG *et al.*, 2008), conforme mostra a figura 1.

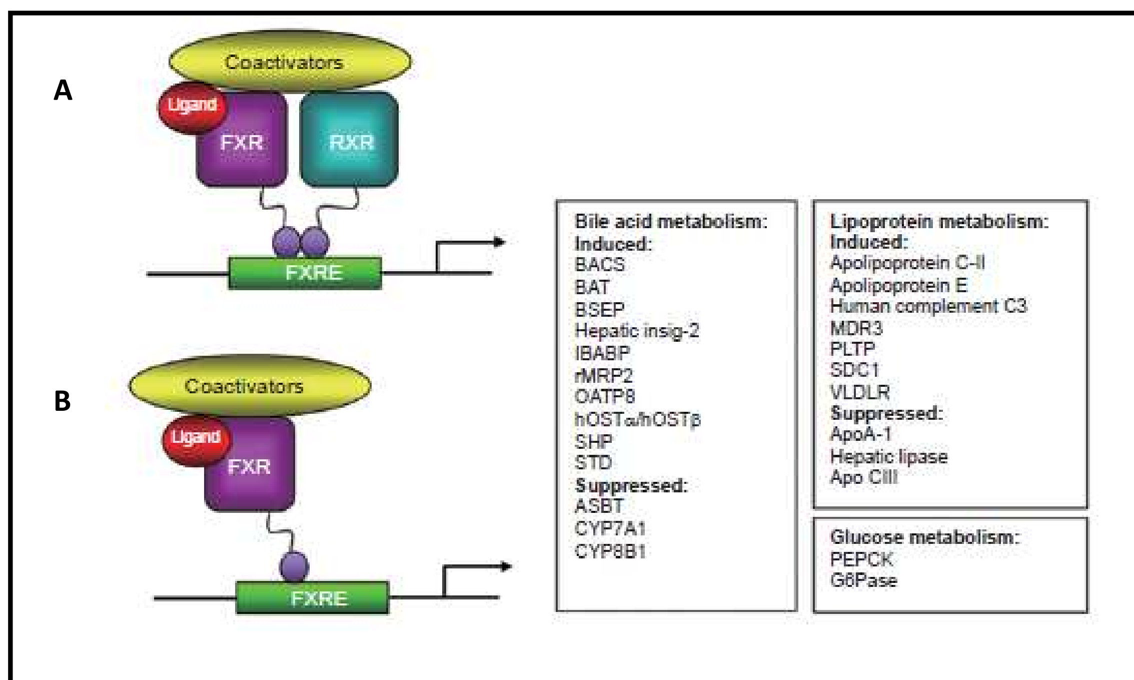

Figura 1. FXR regula um vasto número de genes-alvo envolvidos no metabolismo de ácidos biliares, lipoproteínas e glicose. Para isto, liga-se ao DNA tanto como (A) heterodímero com o RXR quanto como (B) monômero, para regular a expressão de diversos genes mostrados nos quadros. Legenda: FXR: Farnesoid X Receptor; RXR: Retinoid X Receptor; FXRE: elemento de resposta ao FXR (adaptada de WANG *et al.*, 2008).

Estudos têm demonstrado que o FXR está envolvido na regulação de muitas vias metabólicas através da regulação de genes-alvo relacionados (WANG *et al.*, 2008). A ativação do FXR pelo *Small Heterodimer Partner (*SHP) reduz LXR e seus genes-alvo *SREBP-1C* e ácido graxo sintase (*FAS*), o que inibe a síntese e promove a degradação de triglicerídeos bem como oxidação de ácidos graxos por ativação da lipase e do PPARα. O receptor FXR, além disso, tem importante função regulatória no controle da disposição e circulação entero-hepática de ácidos biliares (GROBER *et. al.*, 1999). Ele regula não somente uma ampla variedade de genes envolvidos na síntese, transporte e metabolismo de ácidos biliares, mas também medeia criticamente o

metabolismo de glicose, colesterol e triglicerídeos (LEE *et al.*, 2006; FIORUCCI *et al.,* 2007; LEFEBVRE *et al.*, 2009). Somadas a essas funções, o receptor FXR modula a regeneração hepática, carcinogênese, inflamação, resposta imune inata hepática e intestinal, superexpressão bacteriana intestinal, bem como replicação do vírus da hepatite C (HUANG *et al.*, 2006; INAGAKI *et al.*, 2006; YANG *et al.*, 2007; VAVASSORI *et al.*, 2009).

Tanto ácidos biliares conjugados quanto não conjugados são capazes de ativar FXR em concentrações fisiológicas. O ácido biliar hidrofóbico quenodeoxicólico (CDCA) é o maior e mais efetivo ativador do FXR. O ácido deoxicólico e o ácido litocólico (LCA) podem ativar FXR, porém em uma extensão muito menor que o CDCA. Já o ácido hidrofílico UDCA e o ácido muricólico não podem ativar o FXR (MAKISHIMA *et al.*, 1999). Alguns ácidos graxos poliinsaturados como o ácido araquidônico, ácido decosaexanóico (ZHAO *et al.*, 2004) e metabólitos de ácidos biliares, como os álcoois biliares 25 ou 26-hidroxilase (NISHIMAKI-MOGAMI *et al.*, 2004), também foram identificados como fracos ligantes do FXR. Além disso, alguns agonistas FXR sintéticos foram gerados, como o GW4064 e o AGN29 (DUSSAULT *et al.*, 2003). O ligante FXR mais utilizado é o análogo isoxazólico não-esteroidal GW4064 (MALONEY *et al.*, 2000). Porém, a potencial citotoxicidade e a biodisponibilidade incerta restringem seu uso futuro. Em contrapartida, o ácido 6-etilquenodeoxicólico (6ECDCA), um novo composto derivado do agonista natural CDCA, tem-se mostrado uma boa alternativa para ativação do FXR (COSTANTINO *et al.*, 2003; PELLICCIARI *et al.,* 2002).

Devido a esta significante regulação gênica, agonistas FXR têm sido sugeridos como terapia para colestase e fibrose hepática (ZHANG *et al.*, 2009) e também como drogas potenciais para o tratamento de resistência à insulina e esteatose hepática não alcoólica (NAFLD) (WEI *et al.*, 2009), bem como para o tratamento de várias doenças hepatobiliares e gastrintestinais (HUANG *et al.*, 2006; INAGAKI *et al.*, 2006; YANG *et al.*, 2007; VAVASSORI *et al.*, 2009). Apesar dos dados mostrando o envolvimento do FXR na regulação do metabolismo lipídico e o possível uso terapêutico em NAFLD, não há estudos correlacionando este receptor nuclear nem com esteatose hepática alcoólica, nem com mecanismos de estresse oxidativo, razões que motivaram o desenvolvimento deste trabalho.

2. OBJETIVOS

2.1. Objetivo Geral

Investigar a influência do estresse oxidativo e das vias dependentes do receptor nuclear FXR no desenvolvimento da esteatose hepática alcoólica, e se o tratamento com o agonista FXR 6ECDCA reduz o acúmulo de lipídeos em hepatócitos.

2.2. Objetivos Específicos

a) Avaliar a extensão dos danos hepáticos na presença do etanol, em ratos tratados ou não com o agonista FXR, através de marcadores plasmáticos da função hepática (ALT, AST) e análise histológica;
b) Verificar a extensão dos efeitos hepáticos do etanol em combinação com diferentes níveis de proteínas na dieta;
c) Qualificar o acúmulo de lipídeos em hepatócitos, através de histologia pelas técnicas de Hematoxilina-Eosina, Sudam Black e Azul do Nilo;
d) Avaliar parâmetros do estresse oxidativo e suas implicações na EHA e no metabolismo hepático, através de medidas de peroxidação lipídica (LPO), glutationa (GSH) e atividades enzimáticas da superóxido dismutase (SOD), glutationa S-transferase (GST) e catalase (Cat);
e) Avaliar se o etanol ou o tratamento com o agonista FXR alteram os níveis plasmáticos de triglicerídeos e colesterol;
f) Investigar a expressão de genes-alvo da síntese, oxidação e transporte de lipídeos que são regulados por FXR, como ácido graxo sintase (*FAS*), estearoil-CoA desaturase (*Scd1*), 3-hidroxi-3-metil-glutaril-CoA redutase (*HMG-CoA redutase*), Sterol regulatory element binding protein (*SREBP1*) e Peroxisome proliferator-activated receptor alpha (*PPARα*).

3. ARTIGO CIENTÍFICO

FXR AGONIST 6ECDCA REDUCES HEPATIC STEATOSIS AND OXIDATIVE STRESS INDUCED BY ETHANOL IN MICE

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Abstract

Background: Hepatic steatosis refers to the accumulation of triglycerides in hepatocytes, which can be attributed to excessive ethanol consumption. The liver is the main organ of ethanol biotransformation; therefore, it can suffer from oxidative stress generated by ethanol. Since the farnesoid X receptor (FXR) agonist 6-ethylchenodeoxycholic acid (6ECDCA) regulates adipose cell function, the aim of this work was to evaluate the participation of oxidative stress in ethanol-induced liver lesions and to test the effects of the FXR agonist on alcoholic liver steatosis development.

Experimental approach: Swiss male mice (8–10 weeks old) were separated into 2 groups (n=24) which received a liquid diet containing 10% ethanol or water (control group) for 6 weeks, as well as a low-protein diet (6%) or normal-protein diet (23%). In the last 15 days of the diet, mice that received ethanol or water were separated again for oral treatment, with a total of 8 groups (n=6). From these groups, 4 received the FXR agonist 6ECDCA (3 $mg \cdot kg^{-1}$) and 4 received 1% tween (vehicle), through oral treatment (gavage). Following this treatment, animals were anesthetized for sample collection (hepatic tissues and blood), to perform serum biochemistry assays (aspartate aminotransferase [AST], alanine aminotransferase [ALT], cholesterol, and triglycerides), hepatic oxidative stress (catalase, superoxide dismutase, glutathione-S-transferase, reduced glutathione, and lipid peroxidation), liver histology (hematoxylin-eosin, Sudam Black, and Nile Blue staining) and gene expression of *Srebp1f*, *FAS*, *PPARα, CYP7a1*, *HMGCoA reductase*, *ApoB*, *Scd1*, *p53*, and *Bax*.

Results: Ethanol associated with low-protein diet induced hepatic oxidative stress, increased plasmatic ALT and AST, and induced hepatic lipid accumulation. The FXR activation by the agonist 6ECDCA had direct effects: decreased the relative expression of *SREBP1f* and decreased the plasma levels of triglycerides and cholesterol; as well as it had indirect effects, dependent on ethanol consumption, such as reduction in the plasma levels of AST and ALT, increase in the activity of catalase and superoxide dismutase and decreased rate of lipid peroxidation and the relative expression of *FAS*. Furthermore, we observed a significant decrease in hepatic steatosis. **Conclusion:** By evaluating the role of oxidative stress and FXR in the pathogenesis of alcoholic fatty liver disease in mice, we demonstrated that 6ECDCA reverses the accumulation of lipids in the liver and decreases oxidative stress. Thus, we speculate a possible therapeutic action of FXR agonists in alcoholic liver disease, which may prevent the

progression of this disease to more severe stages such as fibrosis, cirrhosis, and hepatocellular carcinoma.

Key words: ethanol, steatosis, oxidative stress, FXR, 6ECDCA, liver

3.1. Introduction

Ethanol is one of the most widely used psychoactive substances worldwide, most notably due to it being a legal drug, socially acceptable, easily accessed, and inexpensive. The direct and indirect consequences of ethanol consumption, such as financial costs to the healthcare system, accidents, violence, and loss of productivity, generate significant economic damage (1). Excessive ethanol consumption and dependence are problems that affect millions of people and represent a major public health problem around the world (2). Concomitant to the social problems triggered by ethanol abuse, medical complications affect individuals who constantly use ethanol. This drug induces deleterious effects in several systems, including the gastrointestinal, nervous, cardiovascular, hematological, and reproductive systems, along with sexual dysfunction. Ethanol abuse can also lead to psychiatric complications, such as psychotic symptoms, depression, withdrawal syndrome, dementia, anxiety disorders, and fetal ethanol syndrome (when consumed by pregnant women) (3).

Among the serious clinical problems triggered by ethanol abuse, several liver diseases can be identified, because the liver is the primary site of ethanol metabolism and the target of its own reactive metabolites, particularly acetaldehyde (4). Lipid accumulation in hepatocytes, namely steatosis, is the most common and immediate hepatic manifestation of excessive ethanol ingestion (5). While binge drinking typically results in transient steatosis that resolves if drinking ceases, sustained steatosis due to chronic ethanol abuse is frequently related to inflammation and liver damage (i.e., hepatitis) (5). Chronic ethanol consumption is the major etiologic factor in chronic liver diseases that can induce fatty liver, alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma (6). Lipid homeostasis is altered in general by chronic ethanol consumption, inducing not only alcoholic hepatic steatosis (ASH) but also changes in lipids in the blood and other organs (7;8). The elevation of serum triglycerides and certain lipid oxidation products, such as oxidized phospholipids, is a known risk factor for cardiovascular disease. This event may represent endogenous factors capable of triggering the inflammatory cascade, which is responsible for atherosclerosis (9). Thus,

heavy drinkers may develop serious hepatic problems due to changes in lipid metabolism, and this may amplify cardiovascular responses (9). Lipid peroxidation, a component of cellular oxidative stress, is responsible, at least in part, by causing damage to hepatocytes during the process initiated by ethanol.

After decades of research in this field, knowledge of the pathogenesis of the hepatotoxicity of ethanol is still limited (10). Some progress regarding the pathogenesis of liver injuries caused by ethanol has been achieved by establishing the role of oxidative stress and the inflammatory response (10), but there are no effective drugs for the treatment of alcoholic liver disease (4). Although some drugs have been tested, such as steroids (11) and ursodeoxycholic acid (UDCA) or its conjugate (TUDCA) (12), studies in patients have not demonstrated convincing effects (13). Thus, new possibilities for treatment with drugs that can control ASH and its progression to cirrhosis are necessary.

The metabolism of lipids in the liver is regulated by several pathways, which are mediated by the farnesoid X receptor (FXR), a nuclear receptor that functions as a transcription factor, is highly expressed in the liver, and is activated by physiological concentrations of bile acids or by potent agonists, such as 6ECDCA (14). FXR regulates important genes involved in the synthesis and transport of lipoproteins, cholesterol, triglycerides, and bile acids, such as *SHP*, *SREBP1 [Sterol regulatory element binding protein 1]*, *Apo-AI* and -*CII*, *CYP7a1*, and *BSEP (14;15)*. Due to this critical gene regulation, FXR agonists have been suggested as therapy for cholestasis and liver fibrosis (16) and also as potential drugs for the treatment of insulin resistance and non-alcoholic fatty liver disease (NAFLD) (13). Despite data showing the involvement of FXR in the regulation of lipid metabolism and its possible therapeutic use in NAFLD, there are no studies correlating this nuclear receptor with either alcoholic liver steatosis or oxidative stress mechanisms.

For decades, dietary deficiencies were considered to be the major factor responsible for alcoholics developing liver disease. It has been reported that a low-carbohydrate diet associated with ethanol induced more severe hepatotoxicity, with intense steatosis (16). In contrast, supplemented soy protein may ameliorate ethanol-induced lipid accumulation (17). There is little information regarding the effect of protein quantity, quality, and composition on the pathophysiology of alcoholic or non-alcoholic fatty liver disease; however, protein deficiency or malnutrition can cause or contribute to steatosis (18;19). The interaction of ethanol with nutrients and/or their metabolism may contribute significantly to the pathology observed in alcoholic liver injury (20).

Considering the high potential of ethanol to induce oxidative stress and liver diseases, the absence of treatment for ASH, and the evidence of FXR's participation in lipid metabolism, we evaluated the co-participation of oxidative stress and the nuclear receptor FXR in the pathogenesis of ASH. Our hypothesis is that the FXR agonist can revert the accumulation of lipids induced by ethanol.

3.2 Materials and methods

3.2.1 Animals, diet, and treatments

Swiss male mice (8–10 weeks old), weighting 25–35 g, were kept at a controlled temperature (22 ± 2℃), under 12 h light–dark cycle and fed *ad libitum.* All the protocols were approved by the institutional ethics committee for animal research (CEEA) and received the certificate number 438. Two experimental groups were studied, based on diet: (1) low-protein diet (chow with 6% protein[2]) and (2) normal (norm)-protein diet (chow with 23% protein[3]). In both groups, the experimental procedure was exactly the same. Briefly, the animals were separated into individual cages, and food intake, liquid consumption, and the body weight of the animals were controlled weekly. During the first 4 weeks, the animals were divided into 2 groups (n=24), which received a low- or norm-protein diet and liquids containing either 10% ethanol (n=12) or water (n=12). Following this period, the animals were redistributed into 8 groups (n=6) for the initiation of oral treatments (gavage), once a day for 14 days. Thus, the final groups were: G1 (negative control): water + 1% Tween (vehicle), G2: water + 6ECDCA (3 $mg{\cdot}kg^{-1}$), G3 (positive control): ethanol + 1% Tween, and G4: ethanol + 6ECDCA (3 $mg{\cdot}kg^{-1}$), for both the norm-protein diet and low-protein diet. Figure 1 shows the experimental design.

[2] AIN-93G 6% Diet Pelleted, Rhoster Industry and Commerce Ltda. São Paulo – SP - Brazil.
[3] Nuvilab CR1, Nuvital Nutrientes S/A. Colombo – PR - Brazil.

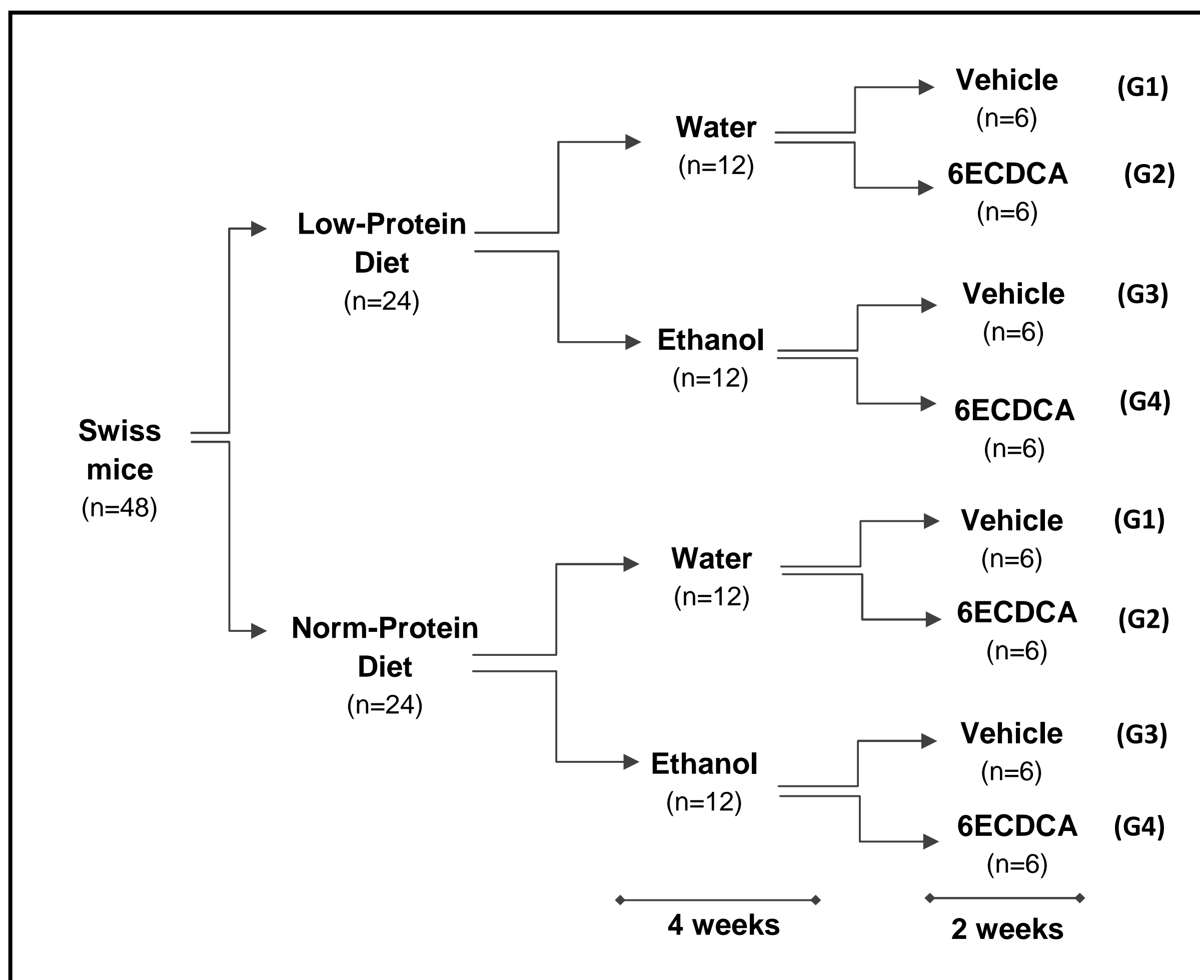

Figure 1. Experimental design over 6 weeks. Mice were assigned to either a low (6%) or norm-protein (23%) diet, and a liquid diet (water or 10% ethanol), and treated with 6ECDCA ($3 mg \cdot kg^{-1}$) or vehicle.

3.2.2 Sample collection

At the end of 6 experimental weeks, the animals were fasted for 12 hours, and then anesthetized with ketamine ($80\ mg \cdot kg^{-1}$) and xylazine ($10\ mg \cdot kg^{-1}$), intraperitoneally. Laparotomy was performed for collection of blood and liver samples. Blood was drawn from the abdominal cava vein with heparinized syringes, and plasma was separated by centrifugation (3,000 rpm for 10 min) and stored at -70℃ for further analysis. The liver was rapidly collected, frozen in liquid nitrogen and also stored at -70ºC. Another section (major lobe) was appropriately stored for histological analysis.

3.2.3 Plasma biochemistry

Plasma was used for determination of the enzymatic activity of aspartate aminotransferase (AST) and alanine aminotransferse (ALT), as well as to determine cholesterol and triglyceride (TG) levels. All of the measurements were performed with commercial kits (Labtest ®, Lagoa Santa, MG - Brazil) in an authomatized system (COBAS 1500®).

3.2.4 In vitro free radical scavenging activity

The reactivity of 6ECDCA (concentrations of 10–1,000 $\mu g \cdot mL^{-1}$) with the stable free radical 2,2-diphenyl-1picrylhydrazyl (DPPH) was determined according to the method previously described (21), with some modifications. The assay consisted of 750 μL of the test solution (6ECDCA) and 250 μL of a methanolic solution of DPPH (1 mg in 25 mL). After 5 min, the decrease in absorbance was measured. Ascorbic acid (50 $\mu g \cdot mL^{-1}$), a reducing agent, was used as the positive control, and distilled water was used as the negative control.

3.2.5 Hepatic oxidative stress

To measure the enzyme activities of catalase (Cat), glutathione-*S*-transferase (GST), and superoxide dismutase (SOD), which are indicators of the redox state of hepatocytes, liver samples were homogenized and centrifuged at 13,000 *rpm* for 20 min. The homogenization was performed on ice and with refrigerated centrifuge at 4°C. The enzymes were analyzed from the supernatant following established methods: Cat following the protocol used in a previous study (22), SOD using the method of pyrogallol oxidation, and GST following the protocol in another study (23). In addition to the enzyme activity measurements, the rate of lipid peroxidation (LPO) was measured by the FOX method following a previously described protocol (24). This technique quantifies the formation of hydroperoxides during lipid peroxidation. The concentration of reduced glutathione (GSH) was measured based on the previously described

technique (25), with modifications. The results were expressed by the amount of protein in the homogenates, determined by a method described elsewhere (26).

3.2.6 Liver histology

Cross-sections of the right lobe of the liver were quickly harvested, stored in buffered 10% formalin for fixation, and stained with hematoxylin and eosin (HE). A further liver sample was stored in buffered formalin for 3 days and then transferred to a 10%, 20%, and 30% sucrose solution, for 24 h at each concentration. After saturation in sucrose, sections were stored in Tissue-Tek® (O.C.T., Sakura®) and rapidly frozen at -80℃, where they remained until the processing of slides for staining with Sudan Black and Nile Blue. These histological techniques were used to confirm the accumulation of lipids in the tissue. The slides were examined under an optical microscope (LeicaDM2500®) to evaluate cellular changes resulting from diet or ethanol treatment. Scores of steatosis and other lesions were analyzed according to a previously described protocol (27).

3.2.7 Gene expression

Measurement of the expression of target genes involved in lipogenesis and apoptosis was performed for each of the liver samples. The mRNA levels were determined for the following genes: *Srebp1f*, *FAS* [Fatty acid syntase], *PPARα* [Peroxisome proliferator-activated receptor alpha], *CYP7a1* [Cholesterol 7-alpha-monooxygenase], HMGCoA reductase [3-hydroxy-3-methyl-glutaryl-CoA reductase], *ApoB* [Apolipoprotein B], *Scd1* [Stearoyl-CoA desaturase], *p53* [protein 53], and *Bax* [Bcl-2–associated X protein]. The complementary DNA (cDNA) was synthesized from 2.0 µg of RNA, while following all of the reaction steps in a specific program for the PCR-thermocycler. The quality of the synthesized cDNA was evaluated by quantification of the housekeeping gene *36b4*, using LightCycler 480 System (Roche®). The expression of the above genes was described subsequently as mRNA relative expression. For this purpose, specific primers for murine genes were used, and the sequences (5'→3') were prepared by Biolegio® or Interprise® (the Netherlands).

3.2.8 Statistical Analysis

Data were analyzed for homogeneity of variance and normal distribution. Two-way ANOVAs followed by Bonferroni *post hoc* tests were used for plasma biochemistry, hepatic oxidative stress and gene expression. The DPPH assay was analyzed by a parametric test, using 1-way ANOVA and Newman–Keuls test. Body weight gain, consumption of water or ethanol, and chow consumption were analyzed using one-way ANOVA and Bonferroni *post hoc* tests. The level of significance was set at 95% ($p<0.05$). Statistical analysis and the preparation of graphs were performed with Graphpad Prism version 5.0. The results were expressed as mean ± standard error of the mean.

3.3. Results

3.3.1 Chow intake, liquid consumption, and body weight of the mice

Greater changes in ethanol or water consumption and body weight gain were observed in the group of mice that received the low-protein diet. In addition, we observed a decrease in ethanol consumption relative to water consumption, in both the norm-protein and low-protein groups. Groups that were fed a low-protein diet showed an increase in body weight gain in animals consuming an alcoholic liquid diet, regardless of the consumption of food. As an example of this measurements, table 1 shows body weight gain in the ethanol and vehicle group that was fed a low-protein diet, measured over 6 weeks.

Table 1 Body weight, ethanol intake, and food intake of the G3 group (low-protein, 10% ethanol, and vehicle)

	WEEK				
	1st	2st	4th	5th	6th
Body Weight (g)	29.73 ± 2.78	29.18 ± 3.91	34.67 ± 1.89*#	35.18 ± 2.34*##	36.84 ± 2.28**##
Ethanol Consumption (mL)	22.17 ± 4.45	24.33 ± 7,94	21.17 ± 10.72	27.83 ± 18.89	27.17 ± 11.99
Food Intake (g)	23.61 ± 4.62	30.92 ± 10.13	33.67 ± 3.84	28.98 ± 4.17	32.03 ± 5.51

Values are expressed as mean ± standard error of the mean and were analyzed by 1-way ANOVA and Bonferroni tests. Symbols: * comparison with 1st week; # comparison with 2nd week; * and # $p<0.05$, ** and ## $p<0.01$.

3.3.2 Plasma biochemistry

When 10% ethanol was the only beverage offered to mice during 6 weeks, an increase of plasma TG was induced only in animals fed a norm-protein diet. However, the FXR agonist reduced this elevation, normalizing the TG to basal levels. Interestingly, 6ECDCA significantly decreased the plasma TG levels in the mice fed a low-protein diet, independent of the presence of ethanol (Figure 2A). Similar results were also observed for cholesterol (Figure 2B). The FXR agonist decreased cholesterol levels when compared with the positive and negative control groups that were fed a low-protein diet. Under the norm-protein diet, cholesterol levels were lower in both groups that received ethanol, independent of the treatment with 6ECDCA. In addition, both the diet and treatment parameters showed statistical significance for differences in plasma lipids, as well as the interaction between them.

Regarding the activity of transaminases (Figure 2C, 2D), ethanol increased the plasma concentrations of AST and ALT in mice fed a low-protein diet. This increase was in the order of 50% and 350% for AST and ALT, respectively, when compared with the negative control (water + vehicle). However, these elevations were reversed by administration of the FXR agonist. Although the effects on both transaminases were

subtle under the norm-protein diet, an elevation in plasma AST was observed when ethanol was used in combination with the FXR agonist.

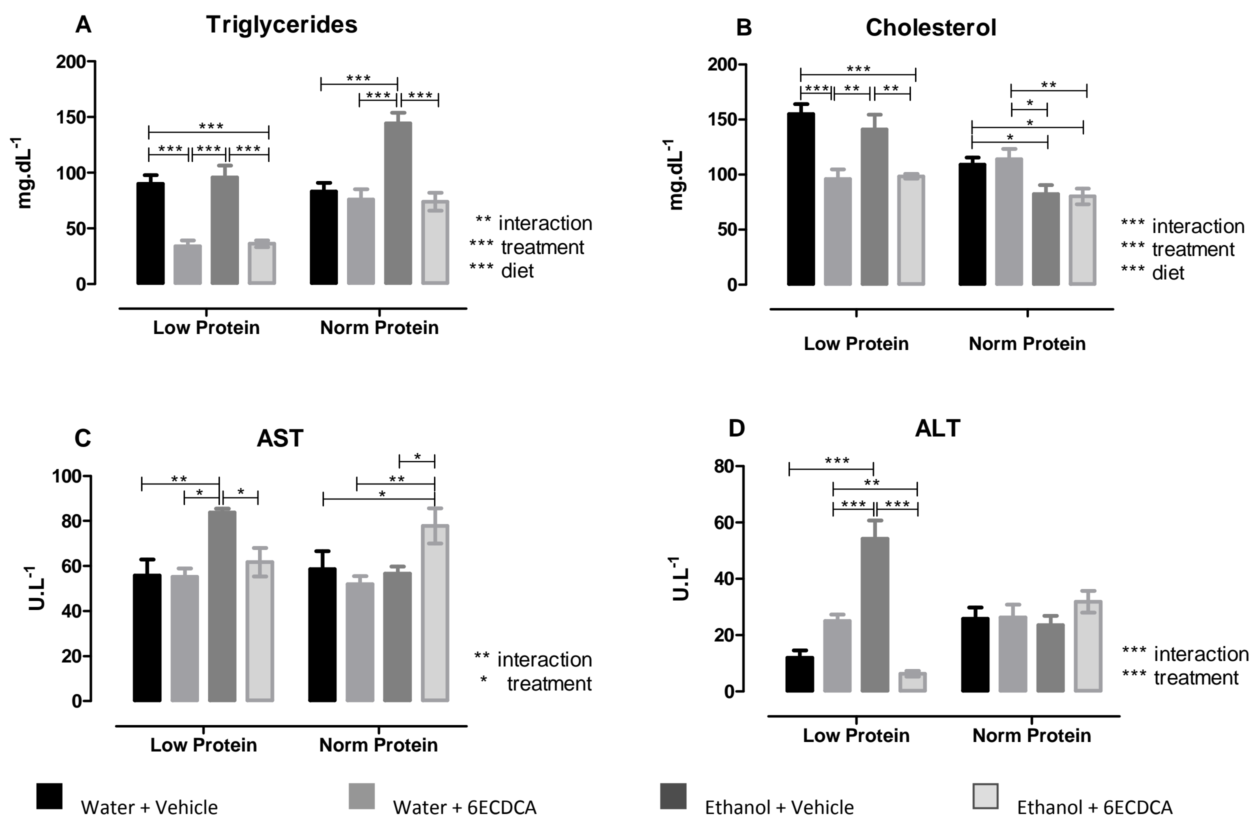

Figure 2. Plasma concentrations of (A) triglycerides ($mg \cdot dL^{-1}$), (B) cholesterol ($mg \cdot dL^{-1}$), (C) Aspartate aminotransferase (AST) ($U \cdot L^{-1}$), and (D) Alanine aminotransferase (ALT) ($U \cdot L^{-1}$) in mice fed a low- or norm-protein diet, liquid diet (water or 10% ethanol), and treatment with 6ECDCA or vehicle (n=6). Values are expressed as mean ± standard error of the mean and were analyzed by 2-way ANOVA and Bonferroni tests. Symbols: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.3.3 In vitro antioxidant activity of 6ECDCA

Considering that ethanol can potentially cause oxidative stress and induce liver damage by reactive oxygen species, we investigated whether the molecule of 6ECDCA had antioxidant activity. This hypothesis was tested *in vitro* and later on *in vivo*. The first results are shown in Figure 3. 6ECDCA showed antioxidant activity from concentrations of 10 μg/mL and sustained until 1000 μg/mL. The antioxidant activity of

6ECDCA was lower than that of ascorbic acid, which can efficiently scavenge the free radical DPPH.

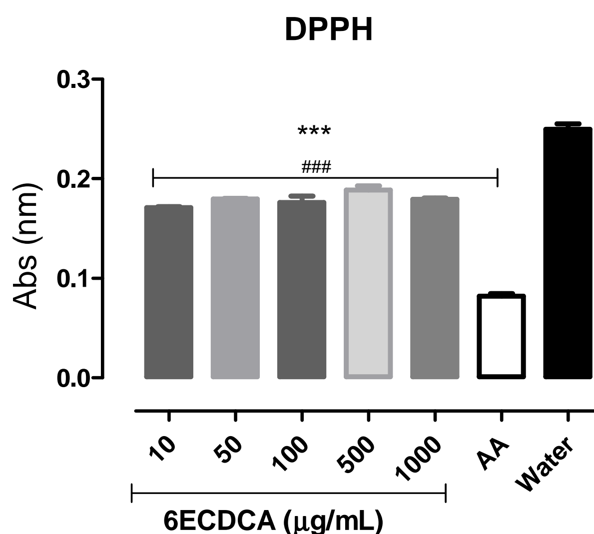

Figure 3. *In vitro* evaluation of free radical scavenging activity of 6ECDCA at concentrations ranging between 10 and 1000 $\mu g.mL^{-1}$. Ascorbic acid (AA, 50 $mg.mL^{-1}$) was used as a positive control and water was used as a negative control. One-way ANOVA and Newman–Keuls tests were used for statistical analysis. Symbols: * comparison with water; $^{\#}$ comparison with AA; *** and $^{\#\#\#}$ $p<0.001$.

3.3.4 Hepatic oxidative stress

6ECDCA was able to partially reverts the ethanol-induced alterations in plasma enzymes. Since oxidative stress can be an important factor in the induction of these changes, we investigated the antioxidant effect of 6ECDCA *in vivo*. Once more, the largest effects of ethanol were found in mice fed a low-protein diet. Ethanol decreased the activity of catalase (Cat) in mice that received less protein (Figure 4A). This effect was reversed by the administration of the FXR agonist. The activity of superoxide dismutase (SOD) remained the same between the groups that received a low-protein diet. In contrast, there was an increase of SOD in the animals fed a regular diet and ethanol when compared with the negative control group. The test drug was able to normalize hepatic SOD to basal activity levels (Figure 4B).

No significant difference was observed in the activity of hepatic GST among the groups of mice. However, the factor of diet showed some influence, owing to the ability

of the low-protein diet to reduce the activity of this enzyme relative to mice fed a regular diet (Figure 4C). The effect of diet was also important for the hepatic level of glutathione, as the low-protein diet increased GSH levels, while the presence of the FXR agonist tended to reduce them, as shown in Figure 4D.

Ethanol elevated the rate of hepatic lipoperoxidation (LPO) in 48%, but this effect happened exclusively when ethanol was ingested in combination with a low-protein diet. However, the FXR agonist treatment reversed this effect, and further reduced the LPO (22%) of mice when compared with control (water + vehicle) under the same diet condition (Figure 4E).

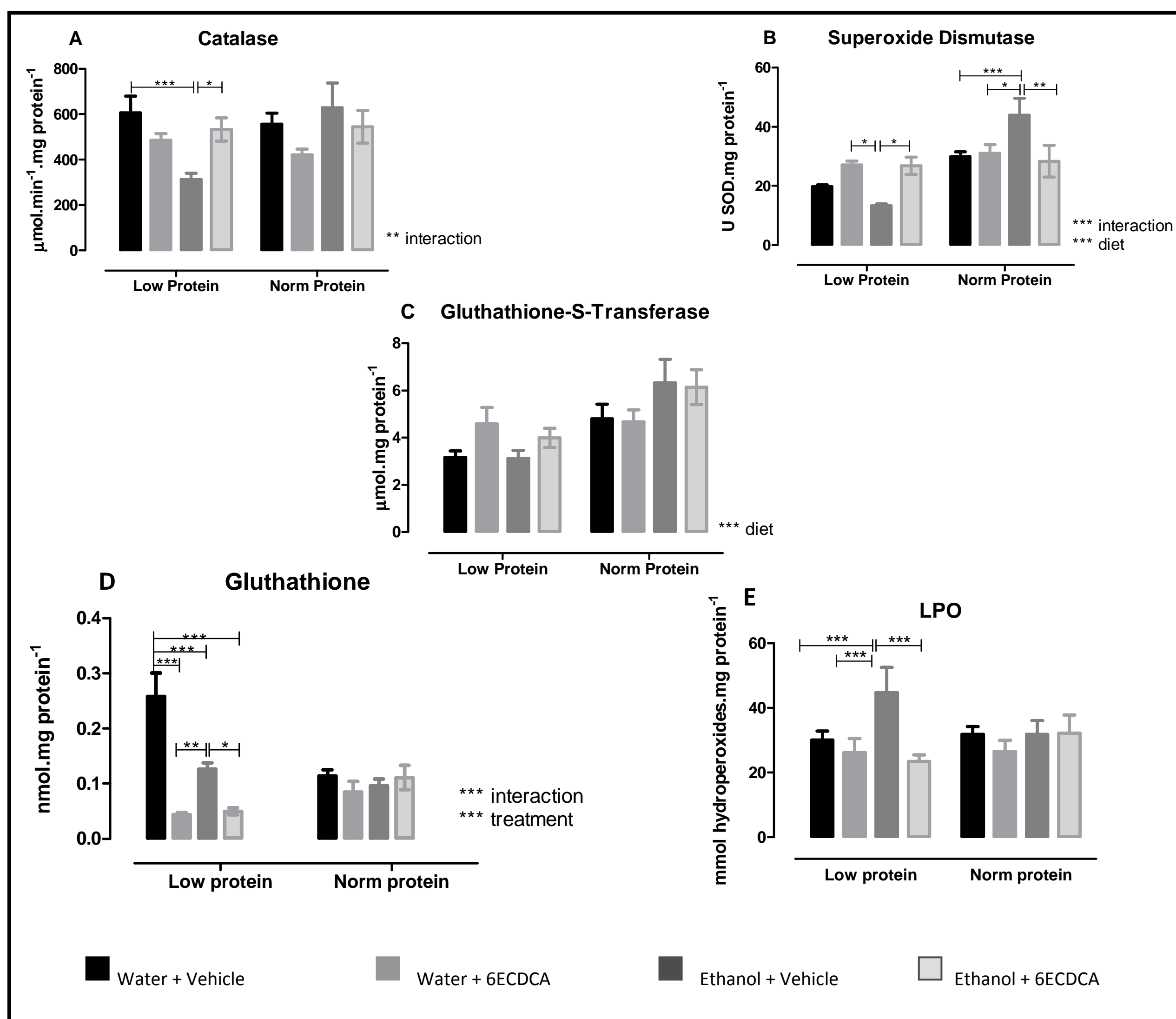

Figure 4. Hepatic (A) catalase (μmol∙min^{-1}∙mg protein^{-1}), (B) superoxide dismutase (U SOD∙mg protein^{-1}), (C) glutathione-*S*-Transferase (μmol∙mg protein^{-1}), (D) glutathione (nmol∙mg protein^{-1}), and (E) lipoperoxidation (LPO, mmol∙mg protein^{-1}) in mice fed a low or norm-protein diet, liquid diet (water or 10% ethanol), and treated with 6ECDCA (3 mg∙kg^{-1}) or vehicle (n=6).

Values are expressed as mean ± standard error of the mean and analyzed by 2-way ANOVA and Bonferroni tests. Symbols: *p<0.05, **p<0.01, ***p<0.001.

3.3.5 Liver histology

Considering the alterations observed in plasma biochemistry, oxidative stress biomarkers, and the macroscopic aspect of the liver in mice that received ethanol (yellow dots suggestive of lipid storages), significant histological changes were expected in the hepatocytes. Cellular changes were observed mainly in the group that was treated with ethanol. No alterations were observed in mice that received a norm-protein diet, independent of whether they received ethanol or 6ECDCA. Thus, the changes described refer specifically to the group of animals treated with a low-protein diet. Steatosis grade 0 and hepatocyte ballooning code 1, localized mainly in zone 3, were observed in the negative control (water and vehicle). The positive control (ethanol and vehicle) mice presented with micro- and macro-steatosis grade 2 and 3, ballooning code 0 and 1, and Mallory's hyaline bodies (diffuse or predominant in zone 3). In addition, multiple necrotic foci were observed. The FXR agonist 6ECDCA was able to reverse these alterations, as no changes were observed in histological sections of mice treated with this drug. The accumulation of lipids revealed by the HE technique (vacuoles in white) was confirmed by Sudan Black (lipids in black) and Nile Blue (TG in pink) stains. These histological findings are shown in Figure 5.

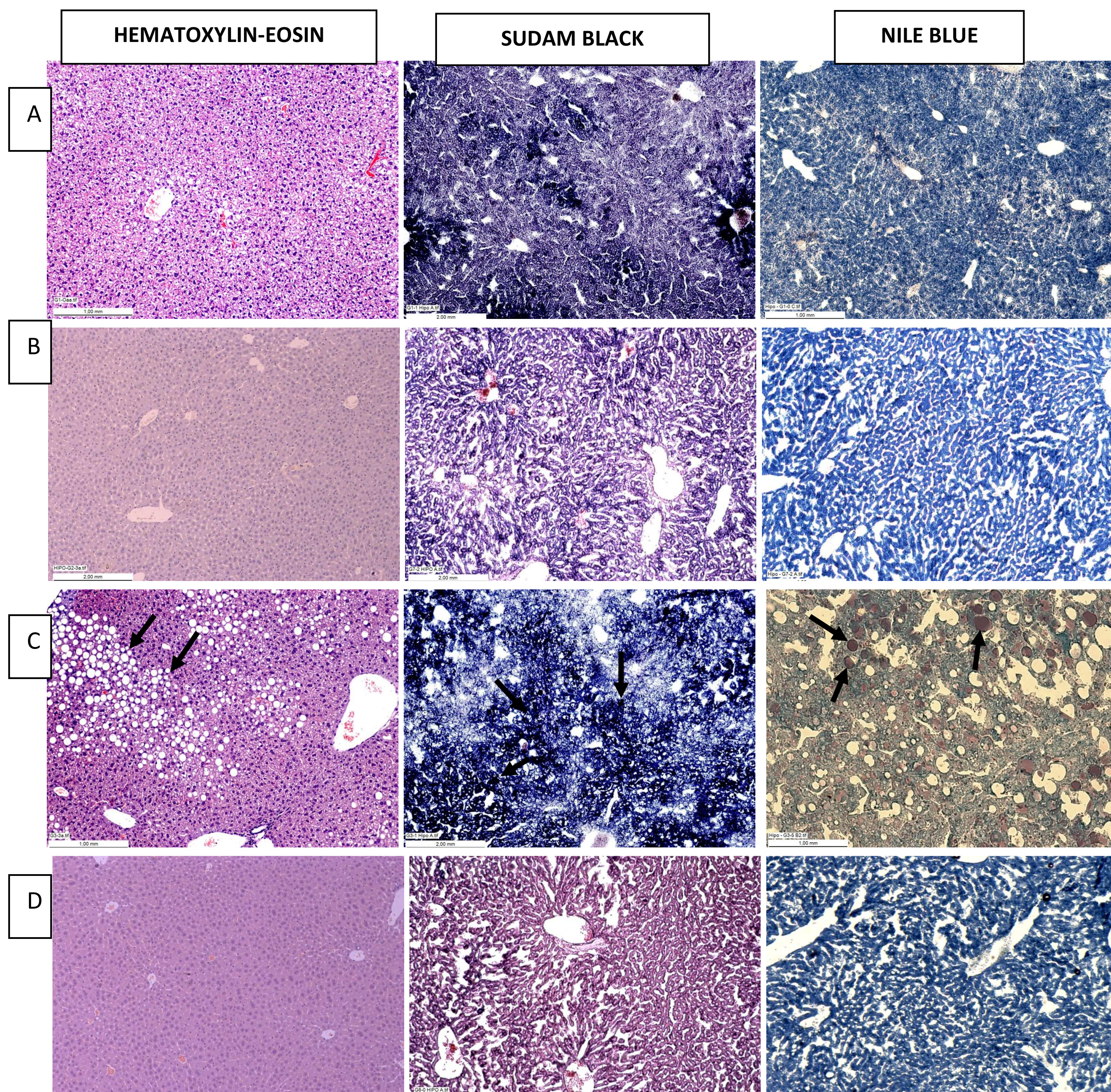

Figure 5. Hepatic histology of mice fed a low-protein diet, showing cellular changes, particularly fatty vacuoles (arrows), indicating steatosis. (A) Water+vehicle, (B) water+6ECDCA (3 mg·kg^{-1}), (C) ethanol+vehicle, and (D) ethanol+6 ECDCA (3mg·kg^{-1}). n=6. Staining: hematoxylin and eosin, Sudan Black, and Nile Blue.

3.3.6 Gene Expression

Since ethanol consumption altered the plasma levels of TG and cholesterol, and also induced hepatic steatosis, the expression of genes that regulate hepatic lipid metabolism was investigated, as well as genes that participate in apoptosis. The most significant alterations were observed in the expression of *Srebp1f* and *FAS*. Both genes showed the same pattern of expression. Ethanol induced the expression of *FAS* only in the mice fed a low-protein diet. 6ECDCA markedly decreased the expression of *FFAS* and *SREBP1f* , even in animals that did not receive ethanol, indicating a direct effect of the agonist, probably in association with low-protein diet. Both diet and treatment and the interaction between these factors were statistically significant for the relative expression of *Srebp1f* and *FAS*, respectively. These data are shown in Figure 6, normalized by the housekeeping gene *36b4*. In addition, the relative expression of other genes linked to lipid metabolism was evaluated: *PPARα,* the enzymes *Cyp7a1*, *Scd1*, *HMGCoA reductase*, and the lipoprotein *ApoB*. However, no significant alteration was observed in these genes, regardless of ethanol or FXR agonist treatment. Absence of significance was also found for expression of the apoptosis-linked genes *Bax* and *p53*. These data are shown in the Table 2.

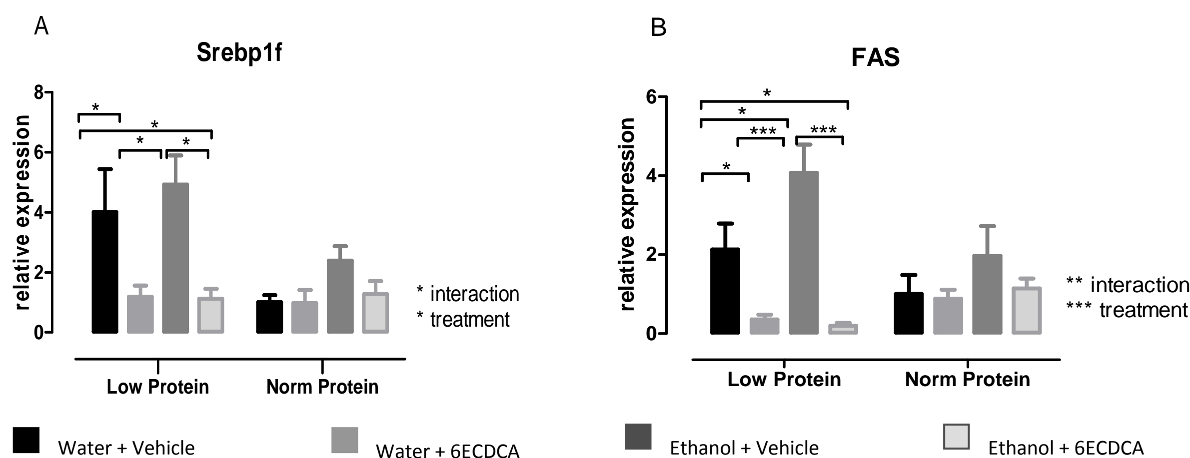

Figure 6. mRNA expression of hepatic A) *Srebp1f*, B) *FAS*, and C) *PPARα* of mice fed a low or norm-protein diet, liquid diet (water or 10% ethanol), and treated with the FXR agonist 6ECDCA (3 mg$\cdot$kg^{-1}) or vehicle. Values are expressed as mean $\pm$ standard error of the mean, normalized by the gene *36b4*. Two-way ANOVA and Bonferroni tests were used for statistical analysis. Symbols: *p<0.05, **p<0.01, ***p<0.001.

Table 2. Relative mRNA levels of hepatic genes that mediate lipogenesis or apoptosis

Gene	Norm-protein diet				Low-protein diet			
	G1	G2	G3	G4	G1	G2	G3	G4
ApoB	1.00±0.78	0.86±0.84	2.21±2.53	0.79±0.88	0.90±0.81	0.37±0.32	0.88±0.75	0.28±0.26
Cyp7a1	1.00±0.48	0.98±0.45	0.62±0.55	1.24±0.66	1.27±1.02	1.33±1.05	0.62±0.41	1.00±0.48
HMG-CoA reductase	1.00±1.02	1.44±1.35	1.06±0.34	1.37±1.79	0.37±0.31	0.40±0.44	0.95±0.41	0.69±0.68
PPARα	1.00±0.79	0.73±0.52	0.96±0.52	0.79±0.63	0.61±0.47	0.25±0.11	0.92±0.25	0.30±0.11
Scd1	1.00±0.64	0.71±0.34	2.12±2.05	2.15±2.03	1.54±1.04	1.41±0.60	2.43±1.09	0.82±0.41
Bax	1.00±0.57	1.02±0.95	0.66±0.28	1.44±1.48	1.35±0.22	1.65±0.42	1.08±0.32	1.47±1.20
p53	1.00±0.49	1.06±0.75	0.79±0.60	0.52±0.36	1.19±0.49	0.63±0.44	1.34±0.37	1.02±0.86

Biological material (liver) was collected from mice fed a low- or norm-protein diet, liquid diet (water or 10% ethanol) and treated with the FXR agonist 6ECDCA or vehicle. Values are expressed as mean ± standard error of the mean, normalized by the gene *36b4* and considering G1 norm as the basal value. Legend: G1 = water + vehicle, G2 = water + 6ECDCA (3 mg·kg^{-1}), G3 = 10% ethanol + vehicle, G4 = 10% ethanol + 6ECDCA (3 mg·kg^{-1}).

3.4. Discussion

The choice of the correct experimental model was extremely important for the study of alcoholic liver disease. The combination of a low-protein diet with administration of ethanol for 6 weeks induced considerable changes in the livers of mice. Our initial hypothesis that the nuclear receptor FXR and oxidative stress acted concomitantly in the pathogenesis of alcoholic liver steatosis was confirmed. The activation of FXR receptor by 6ECDCA reversed the increase in AST and ALT that was induced by ethanol, and decreased plasma levels of TG and cholesterol, independent of the ethanol administration. Moreover, the agonist was effective in normalizing the activity of liver Cat and SOD, and decreased lipid peroxidation. These effects appear to be mediated by *FAS*, since its gene expression was decreased with the use of the agonist.

Several mechanisms operate in a synchronized manner and reflect the body's response to the direct or indirect effects of ethanol (28). The influence of a low-protein diet in aggravating the hepatic injuries induced by ethanol is very clear in the data presented. Ethanol induced the most prominent biochemical and genetic alterations when it was consumed together with 6% protein chow. The combination of ethanol + low-protein diet increased plasma ALT and AST levels, increased hepatic levels of LPO and accumulation of lipids, decreased Cat activity and GSH concentrations, as well as elevated the gene expression of *FAS*. Our data are similar to other studies recently conducted on non-alcoholic steatosis, in which protein malnutrition (8% protein) induced hepatic steatosis in lactating mothers and infants during breast-feeding (29).

Hepatic up-regulation of genes linked to oxidative stress supports the development of steatohepatitis (29). A central factor in the development of ethanol-induced damage is the excessive generation of free radicals, which can damage or cause complete destruction of essential molecular complexes in cells, including lipids, proteins, and DNA (30). The data showed that LPO was 2-fold higher in ethanol consuming mice, but treatment with 6ECDCA significantly reduced this LPO elevation, thus indirectly reducing damage to hepatocytes. This mechanism may be responsible, at least in part, for the reduction in both plasma transaminases (ALT and AST) in mice treated with the FXR agonist. Transaminase release can be caused by both cell lysis and increases in plasma membrane permeability, and both phenomena are induced by lipid peroxidation. It was previously reported that the absence of hepatic FXR (FXR-null mice) spontaneously generates oxidative stress (31), so it was expected that an FXR agonist would reduce oxidative stress, as we observed in 6ECDCA-treated animals. We reported an intermediary antioxidant effect of 6ECDCA in the DPPH *in vitro* test, however a considerable antioxidant effect was observed in hepatic tissue, which mainly reduced LPO and affected enzymes.

The production of reactive oxygen species (ROS) is a naturally occurring process, and a variety of enzymatic and non-enzymatic mechanisms are involved in cellular protection from ROS (32). Importantly, some of these mechanisms are broken after long periods of exposure to ethanol. Antioxidant molecules involved in the elimination of ROS include SOD, Cat, GSH, and GST. The mitochondrial antioxidant enzyme known as manganese superoxide dismutase (Mn-SOD) is induced consistently in experimental animals after acute and chronic ethanol administration (33). Chronic ethanol ingestion causes an up-regulation of Mn-SOD at the mRNA level, which seems to be a protective mechanism (33;34). With repeated ethanol administration the increased level of Mn-SOD is gradually diminished. Thus, the adaptive response of Mn-

SOD is blunted, leading to increased toxicity with prolonged ethanol exposure (34). Nevertheless, the effects of chronic exposure to ethanol in the cellular contents or activity of SOD are controversial, with reports of increased, decreased, or no change in activity, depending on the model, diet, amount, and timing of ethanol exposure (30). Our results indicate an increase in the hepatic activity of SOD in animals that received only ethanol and a norm-protein diet when compared with the negative control group. In the group fed the same diet and ethanol, but treated with 6ECDCA, SOD activity was reduced to basal levels, underscoring the critical influence of diet.

Cat is another important antioxidant and cytoprotective enzyme in hepatocytes that are exposed to ethanol (35). Cat can promote the interaction of hydrogen peroxide with hydrogen donors, such as ethanol and methanol (36). Cat activity was decreased in the group of mice that received a low-protein diet and ethanol, compared with the control group, which may be explained by the influence of Cat in ethanol oxidation. This effect was reversed by the administration of 6ECDCA. We believe that the present work is the first to report the influence of this FXR agonist in hepatic Cat and SOD activities. These data suggest the recognition of 6ECDCA as an antioxidant drug.

Ethanol has been shown to decrease the levels of GSH, particularly in the mitochondria, which usually require high levels of GSH to eliminate ROS generated during mitochondrial respiratory chain activity (36). Nevertheless, the effects of ethanol on the levels of GSH are controversial: one study (37) found an increase in GSH levels in rats fed with ethanol. Other authors reported decreases in GSH levels after ethanol intake for prolonged periods and reported no change in hepatic GSH levels after 7 weeks of ethanol consumption (38;39). Our data are compatible with the previous studies, as no statistical differences were found between the groups treated with 6ECDCA or vehicle and fed a norm-protein diet. Regarding the GSH levels of animals fed a low-protein diet, an increase in GSH concentration was found only in the negative control group, possibly indicating a diet-linked effect, independent of ethanol or 6ECDCA treatment. From the data presented, the exact mechanism for this effect cannot be inferred. GSH is an essential component of the antioxidant system and acts as a cofactor for GST, a group of polymorphic enzymes that help to remove certain drugs, chemicals, and other reactive molecules of the cells, and GST is important in the protection against oxidative stress (30;40). Following the same tendency as the substrate GSH, the enzyme GST did not present significant differences of activity among the groups of mice, even in those treated with FXR agonist. However, down-regulation of GST expression was previously reported in FXR-null mice (31).

Steatosis due to chronic ethanol abuse is largely attributed to two main causes. First, as mentioned above, ethanol metabolism leads to oxidative stress, which results in cellular damage and lipid peroxidation. Second, lipid synthesis is induced by the activation of the sterol response element binding proteins (SREBP) (41-43). Activation of FXR down-regulates SREBP, which inhibits the synthesis of TG and promotes the degradation of TG and fatty acid oxidation (43). In our study, the relative gene expression of *Srebp1f,* independently of ethanol administration, was decreased with administration of the FXR agonist 6ECDCA, which also reduced the accumulation of lipids in the liver. The SREBP1 protein regulates genes involved in the synthesis of TG, such as FAS, which catalyzes the final step in the biosynthesis of fatty acids. Thus, our gene expression results are complementary with previous data, since the activation of FXR by 6ECDCA reduced the expression of the genes of both the factor *SREBP* and the enzyme *FAS*.

The intake of ethanol for prolonged periods has effects on lipid metabolism in extra-hepatic tissues, leading to the development of hypertriglyceridemia and hypercholesterolemia (7;44). Hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase is the major enzyme that controls cholesterol biosynthesis. Prolonged exposure to ethanol leads to several changes in the rate-limiting steps of cholesterol biosynthesis, as shown in a previous study (45), which indicated that prolonged consumption of ethanol leads to a significant increase in mRNA expression of low-density lipoprotein receptors and HMGCoA reductase. Our results do not corroborate those data, since we did not observe alterations in plasma cholesterol or in the gene expression of *HMGCoA reductase* and *ApoB* of animals treated with ethanol. Interestingly, in agreement with a previously reported study (46) with the FXR agonist GW4064, 6ECDCA was able to decrease plasma levels of cholesterol and TG in mice that received ethanol associated with a low or norm-protein diet. The decreased levels of circulating lipids are also reflected in the lipids observed by liver histology, mainly in samples stained with Sudan Black (specific for lipids evidence) and Nile Blue (specific for TG evidence) techniques. Additionally, ethanol consumption increased the body weight of animals. This effect was not observed in mice that received a combination of ethanol + 6ECDCA. This increase in body weight gain was not concomitant with an increase in food intake, which confirms that body weight gain was due to the intake of ethanol, an important caloric source, and not the result of diet.

Taken together, the effects of ethanol on gene expression involved in lipid accumulation in the liver can be summarized as follows: ethanol metabolism increases the levels of NADH in the liver and stimulates the synthesis of fatty acids and their

incorporation into TG. Ethanol mediates the decrease or inhibition of PPARα and stimulates SREBP, both receptors that control the response to enzymatic oxidation and fatty acid synthesis, respectively, which may contribute to hepatic lipid accumulation in alcoholic liver disease (43). The receptor FXR is involved in gene regulation of these mediators of lipogenesis, which regulates ethanol-induced alterations at the biochemical, genetic, and plasma levels.

Finally, we conclude that ethanol is able to induce hepatic oxidative stress and the accumulation of lipids in the livers of mice. These effects are more evident when ethanol is combined with a low-protein diet. This damage is reversed by the oral administration of 3 $mg \cdot kg^{-1}$ 6ECDCA, an FXR agonist. Importantly, 6ECDCA administration is effective in ameliorating hepatic lesions and metabolism disturbances induced by ethanol, and protects hepatocytes from oxidative stress. The FXR agonist 6ECDCA is promising as a potential therapy for alcoholic liver steatosis. However, further studies are necessary to validate these results, including long-term 6ECDCA treatment and a toxicity investigation.

3.5. Acknowledgments

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4. CONSIDERAÇÕES FINAIS

Diante dos resultados obtidos neste trabalho, conclui-se que:

- ✓ A escolha do modelo correto para estudo da esteatose hepática alcoólica é de extrema importância, visto que os efeitos do etanol sobre o sistema enzimático antioxidante, expressão gênica, bioquímica plasmática e histologia foram mais evidentes quando houve associação com uma dieta com baixo nível protéico;
- ✓ O etanol administrado por 42 dias em uma concentração de 10% associado à dieta contendo 6% de proteína foi capaz de induzir estresse oxidativo hepático e acúmulo de lipídeos no fígado de camundongos;
- ✓ As lesões hepáticas foram revertidas pela administração oral do agonista FXR 6ECDCA, na dose de $3mg.Kg^{-1}$;
- ✓ A administração do 6ECDCA foi efetiva em melhorar o distúrbio lipídico induzido pelo etanol e proteger os hepatócitos do estresse oxidativo;
- ✓ O agonista FXR tem potencial terapêutico para esteatose hepática alcoólica;
- ✓ Mais estudos são necessários para corroborar esses resultados, incluindo tratamento mais prolongado e investigação da toxicidade de agonistas FXR.

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