UNIVERSIDADE FEDERAL DO PARANÁ

SETOR DE CIÊNCIAS BIOLÓGICAS

DEPARTAMENTO DE FARMACOLOGIA

CARACTERIZAÇÃO DE UM MODELO DE ESTEATOSE HEPÁTICA ALCOÓLICA INDUZIDA POR ETANOL E DIETA HIPERLIPÍDICA EM RATOS

CARLOS EDUARDO ALVES DE SOUZA

CURITIBA
2013

CARLOS EDUARDO ALVES DE SOUZA

CARACTERIZAÇÃO DE UM MODELO DE ESTEATOSE HEPÁTICA ALCOÓLICA INDUZIDA POR ETANOL E DIETA HIPERLIPÍDICA EM RATOS

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: Prof[a]. Dr[a]. Alexandra Acco

Co-Orientadora: Prof[a]. Dr[a]. Sílvia Maria Suter Correia Cadena

CURITIBA
2013

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 "CARACTERIZAÇÃO DE UM MODELO DE ESTEATOSE HEPÁTICA ALCOÓLICA INDUZIDA POR ETANOL E DIETA HIPERLIPÍDICA EM RATOS", de autoria do pós-graduando **CARLOS EDUARDO ALVES DE SOUZA**, sob orientação da Prof.ª Dr.ª Alexandra Acco e composta pelos professores: Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR); Anderson Joel Martino Andrade (Fisiologia – UFPR) e Prof. Dr. Jurandir Fernando Comar (Bioquímica - UEM), reuniu-se e, de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, o pós-graduando foi Aprovado. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas pela sua orientadora. Em Curitiba, 21 de fevereiro de 2013.

Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR)

Prof. Dr. Anderson Joel Martino Andrade (Fisiologia – UFPR)

Prof. Dr. Jurandir Fernando Comar (Bioquímica - UEM)

Centro Politécnico – Caixa Postal 19031 – CEP: 81531-990 – Curitiba/PR
Telefones: (41) 3361-1693 – Fax (41) 3266-2042 – pgfarmacologia@ufpr.br
http://farmacologia.bio.ufpr.br/posgraduacao

Dedico este trabalho à minha família e amigos, pelo
apoio incondicional e a todos que estão presentes em minha vida.

Agradecimentos

A Deus, Pai amado que me concedeu a vida e a oportunidade de realizar este sonho e que sempre me amparou e consolou em todos os momentos de minha vida e por iluminar meu caminho.

À minha amada família, em especial, minha mãe Rejane José Alves e ao meu irmão Cesar Vinícius Alves de Souza que sempre deram o melhor, com carinho e dedicação, além de seu amor incondicional... Com carinho e atenção me ensinaram a lutar por um futuro melhor fortalecendo meu caráter.

À minha amada noiva Helen de Morais, pelo amor restaurador, pela paciência consoladora e pelo apoio motivador. TE AMO muito e você tem grande parte nesta conquista, pois foi com o seu incentivo que eu persisti e persistirei sempre.

Aos meus sogros Jair de Morais e Helena Maria, por todo carinho, cuidado, dedicação e atenção durante este período de estudos para a produção deste trabalho.

Às queridas professoras Alexandra Acco e Sílvia Maria Suter Correia Cadena, que são exemplos de pessoas e profissionais, que sempre se dedicaram muito na orientação deste trabalho, com carinho e atenção. Sou muito grato pela paciência, carinho, entusiasmo, apoio e pela possibilidade de conviver estes dois anos com estas pessoas maravilhosas, com as quais tenho muito a aprender e a me espelhar.

Aos colegas de laboratório de Farmacologia & Metabolismo Hepático pelo auxílio e paciência nos experimentos, e aos colegas do Departamento de Bioquímica, do Laboratório de Oxidações Biológicas e Cultivo Celular, especialmente a Lyvia Lintzmaier Petiz pelo auxílio, paciência e dedicação no isolamento de mitocôndrias e nos experimentos com as mesmas.

Agradeço também à CAPES/DS e à Fundação Araucária pelo apoio financeiro.

“Aprender é a única coisa de que
a mente nunca se cansa, nunca
tem medo e nunca se arrepende”.

Leonardo da Vinci

NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo, como artigo científico para publicação, de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná. A dissertação consta de uma revisão bibliográfica, objetivos gerais e específicos do trabalho e um artigo científico com os experimentos realizados, resultados e discussão, além da conclusão sintetizando os achados do mesmo.

RESUMO

O consumo abusivo de álcool é umas das principais causas das doenças hepáticas, como a esteatose hepática alcóolica (EHA), a qual pode evoluir clinicamente para cirrose e hepatocarcinoma. O comprometimento hepático ocorre porque o álcool é metabolizado no fígado, preferencialmente pela enzima álcool desidrogenase, ou pela citocromo P450 2E1, com formação de acetaldeído, um produto menos tóxico. O metabolismo do etanol altera vias bioquímicas hepáticas, o que leva a uma alteração nos lipídeos hepáticos, carboidratos, proteínas, lactato e ácido úrico. Concomitantemente, o metabolismo do etanol favorece o acúmulo de espécies reativas de oxigênio, que contribuem, por sua vez, para o aumento de espécies pró-oxidantes, que têm grande potencial lesivo para os hepatócitos, favorecendo, assim, a instalação de doenças hepáticas como a EHA. Esta doença, fisiopatologicamente, é a primeira mudança morfológica no fígado e caracteriza-se pelo acúmulo intracelular de lipídios e aumento do volume do órgão. Considerando que ainda não há tratamentos farmacológicos eficientes para esta doença, modelos de estudo são necessários para que a patogenia e a terapêutica da EHA sejam melhores estudadas. Este trabalho objetivou estabelecer um modelo de estudo de EHA de baixo custo, através da associação de etanol com sementes de girassol (*Helianthus annuus*) na dieta de ratos. **Material e métodos**: Ratos machos (Wistar) (*Rattus norvegicus*) foram separados em gaiolas individuais com água e ração *ad libitum*. Os animais receberam água ou etanol 10%, e como dieta sólida receberam: ração regular; dieta rica em lipídeos (DRL), representada unicamente por sementes de girassol; ou estes dois alimentos combinados. Durante 30 dias de dieta, o consumo de líquido, de alimento e o peso corporal foram monitorizados. Ao final deste período, os animais foram anestesiados e amostras de fígado e sangue foram colhidas para análise histológica hepática, estresse oxidativo hepático, atividade enzimática mitocondrial e para bioquímica plasmática. **Resultados**: A associação de etanol 10% e DRL induziu ao acúmulo de lipídeos hepáticos (macroesteatose e microesteatose), tumefação de hepatócitos, redução do nível de glutationa e a atividade glutationa-S-transferase, e ao aumento da taxa de peroxidação lipídica e da atividade da enzima superóxido dismutase. Ainda, a oxidação mitocondrial do NADH e do succinato foi parcialmente inibida. **Conclusões**: A combinação de etanol 10% e sementes de girassol na dieta de ratos produziu um modelo de estudo de EHA interessante e de baixo custo, culminando com a instalação desta doença com sucesso após quatro semanas da dieta. A função hepática foi alterada, sendo detectada através de análises morfológicas, nos biomarcadores de estresse oxidativo e no transporte de elétrons mitocondriais (complexos I e II).

Palavras Chave: etanol, dieta hiperlipídica, semente de girassol, esteatose hepática alcoólica, estresse oxidativo, mitocôndria, cadeia respiratória, fígado.

ABSTRACT

The alcohol abuse is one of the main causes of liver disease, such as alcoholic hepatic steatosis (AHS), which can clinically progress to cirrhosis and hepatocellular carcinoma. The liver impairment occurs because the alcohol is metabolized in the liver, preferably by the enzyme alcohol dehydrogenase or by the cytochrome P450 2E1, with formation of acetaldehyde, a less toxic product. The metabolism of ethanol alters hepatic biochemical pathways, which leads to an alteration in hepatic lipids, carbohydrates, proteins, lactate and uric acid. Concomitantly, the metabolism of ethanol favors the accumulation of reactive oxygen species, which contribute for increased pro-oxidant species. These reactive molecules have great harmful potential for the hepatocytes, thus favoring the installation of liver diseases. This disease, is the first morphological change in liver and is characterized by the accumulation of intracellular lipids and enlargement of the organ. Whereas there are no effective pharmacological treatments for this disease, study models are required for the establishment of the pathogenesis and the treatment of AHS. This study aimed to establish and characterize an inexpensive animal model of AHS, through the association of ethanol with sunflower seeds (*Helianthus annuus*) in the diet of rats. **Material and methods:** Male Wistar rats (*Rattus norvegicus*) were kept in a single cage with food and liquid *ad libitum* The rats were fed to water or 10% ethanol and regular chow diet and/or high fat diet (HFD), represented by sunflower seeds. Throughout thirty days on diets, the food consumption, liquid intake and the body weight were monitored. After this period, the animals were anesthetized and blood and liver samples were collected for evaluation of liver histology, hepatic oxidative stress, hepatic mitochondria enzymes activity, and plasma biochemistry. **Results:** The association of 10% ethanol and HFD induced hepatic lipid accumulation (macrostetosis and microsteatosis), hepatocytes tumefaction, decreased levels of glutathione and reduction of glutathione-S-transferase, and increased the level of lipoperoxidation and the activity of superoxide dismutase. Also, the mitochondrial oxidation of NADH and succinate were partially inhibited. **Conclusions:** The combination of 10% ethanol with sunflower seeds as the diet for rats produced an interesting and cheap model to study AHS. The installation of liver steatosis was successfully obtained after 4 weeks of diet. The liver function was modified and the alterations were found in the morphological analysis, oxidative stress biomarkers and in the mitochondrial electron transport (complexes I and II).

Key Words*:* ethanol, high-fat diet, sunflower seed, alcoholic hepatic steatosis, oxidative stress, mitochondria, respiratory chain, liver.

LISTA DE FIGURAS

REVISÃO LITERÁRIA

ARTIGO CIENTÍFICO

LISTA DE TABELAS

ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS

REVISÃO LITERÁRIA

6ECDCA – Ácido 6-etilquenodeoxicólico

AB - Ácidos biliares

ADH - Álcool desidrogenase

AdipoRs – Receptores de adiponectina

ALDH - Aldeído desidrogenase

AMPK – Quinase ativada por AMP

Bax – Proteína X associada à Bcl-2

Cat - Catalase

CYP2E1 - Citocromo P450 2E1

EHA - Esteatose hepática alcoólica

EROS - Espécies reativas de oxigênio

FA - Ácidos graxos livres

FXR - *Farnesoid X receptor*

GPx - Glutationa peroxidade

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

GST - Glutationa S-transferase

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

HSP - Proteína de choque térmico

IBGE - Instituto Brasileiro de Geografia e Estatística

MEOS - Sistema microssomal de oxidação do etanol

NADH - Nicotinamida adenina dinucleotídeo hidreto

NAFLD - *Non-alcoholic fatty liver disease* (Esteatose hepatica não-alcoólica)

NF-κB - Fator nuclear kappa B

Pense - Pesquisa Nacional da Saúde do Escolar

PPARγ - Receptores ativados por proliferadores de peroxissoma γ

PPARα - Receptores ativados por proliferadores de peroxissoma α

PTP - Proteína tirosina fosfatase

PXR - *Pregnane X receptor*

SOD - Superóxido dismutase

SREBP-1 - *Sterol regulatory element binding proteins* 1

TG - Triglicerídeos

TNF-α - Fator de necrose tumoral

Trd - Tiorredoxina

TUDCA - Ácido ursodeoxicólico conjugado

UDCA - Ácido ursodeoxicólico

WHO - Organização Mundial de Saúde

ARTIGO CIENTÍFICO

AHS - Alcoholic hepatic steatosis

ALD - Alcoholic liver disease

ALT - Alanine aminotransferase

AST - Aspartate aminotransferase

BSA - Bovine serum albumin

Cat - Catalase

CHO - Cholesterol

CYP2E1 - Cytochrome P2E1

EDTA - Ethylenediamine tetraacetic acid

EGTA - Ethylene glycol-bis(β- aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid

GSH - Reduced glutathione

GST - Glutathione-S-transferase

H&E - Hematoxylin-eosin

HEPES - 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

HFD - High-fat diet

LPO - Lipid peroxidation

NADH – Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

ROS - Reactive oxygen species

SOD - Superoxide dismutase

TG - Triglyceride

TRIS - Tris(hydroxymethyl)-aminomethane

SUMÁRIO

1. INTRODUÇÃO

1.1 Consumo de Álcool e Epidemiologia Brasileira

O abuso no consumo de álcool e a sua dependência afetam milhões de brasileiros e representam um dos maiores problemas de saúde pública, tanto no Brasil como no resto do mundo (CARLINI *et al.* 2007). As consequências diretas e indiretas do consumo de álcool, como acidentes, violência e perda de produtividade, geram grandes prejuízos econômicos (WHO, 2002). O álcool é a substância psicoativa de maior uso no Brasil, devido a vários fatores, principalmente ao fato de ser uma droga lícita, socialmente aceita, ser de fácil acesso e baixo preço. Em nosso país são observados mais dependentes de álcool no sexo masculino, via de regra em idade produtiva, de 12 a 65 anos (CARLINI *et al.* 2007).

De acordo com a Organização Mundial de Saúde (WHO, 2001), cerca de 10% das populações dos centros urbanos de todo o mundo consomem abusivamente substâncias psicoativas, independentemente da idade, sexo, nível de instrução e poder aquisitivo. Dados fornecidos por estudos realizados pela Universidade de Harvard indicam que, das dez doenças mais incapacitantes em todo o mundo, cinco são de origem psiquiátrica, sendo uma delas o alcoolismo, além de depressão, transtorno afetivo bipolar, esquizofrenia e transtorno obsessivo-compulsivo (MURRAY & LOPEZ, 1996; BRASIL, 2003).

Uma ampla pesquisa foi realizada pelo Instituto Brasileiro de Geografia e Estatística (IBGE, acesso em 16/08/11), intitulada Pesquisa Nacional da Saúde do Escolar (Pense), apresentando informações sobre as condições de vida de 618.555 mil estudantes de escolas particulares e públicas, que cursam o 9º ano do ensino fundamental, nas capitais e no Distrito Federal, e que correspondem à faixa etária de 13 a 15 anos. O estudo demonstrou que o consumo de bebida alcoólica é mais disseminado do que o do fumo, sendo que 71,4% destes alunos já haviam experimentado álcool alguma vez e que 27,3% disseram ter consumido no mês anterior à pesquisa. Quase 20% dos alunos que experimentaram a droga declararam ter obtido a bebida em supermercados ou bares e 12,6% em suas

próprias casas. Dentre esta população estudada, foi identificado que 22,1% dos escolares já haviam se embriagado.

O consumo abusivo de álcool é enquadrado como um problema de saúde pública, pois o alcoolismo desencadeia diversas complicações clínicas provenientes de seu uso constante e cada vez mais precoce. Pode levar a complicações psiquiátricas, como quadros psicóticos, depressão, síndrome de abstinência, síndromes demencias, distúrbios de ansiedade e à Síndrome Fetal Alcoólica, quando consumido por gestantes (SILVEIRA & MOREIRA, 2006). Ainda, o etanol induz efeitos deletérios sobre o sistema cardiovascular, hematológico, reprodutor e sobre o trato gastrintestinal, no qual o fígado é o órgão que sofre as maiores agressões.

1.2 Esteatose Hepática Relacionada à Exposição ao Etanol

O aumento no conteúdo intrahepático de triglicerídeos (TG), a esteatose hepática, tem sido definida como um aumento maior do que 5% do volume ou peso do fígado, ou histologicamente, quando 5% ou mais dos hepatócitos contêm TG intracelulares (HOYUMPA *et al*. 1975). O excessivo acúmulo destes lipídeos está, normalmente, associado com alterações no metabolismo de glicose, ácidos graxos livres (FA) e de lipoproteínas, ou com algum processo inflamatório. A esteatose hepática pode ter diversas etiologias, sendo uma das mais comuns a decorrente do uso abusivo de álcool (HENZEL *et al.* 2004), denominada Esteatose Hepática Alcoólica (EHA). Quando a esteatose não está relacionada ao consumo de álcool, mas a infecções virais, alterações congênitas ou doenças autoimunes, é definida como esteatose hepática não-alcoólica (NAFLD - *non-alcoholic fatty liver disease*). A esteatose hepática alcoólica tem alta prevalência entre a população adulta devido à elevada taxa de pessoas que apresentam uso abusivo do álcool, representadas em nosso país por mais de 25 milhões de brasileiros (CARLINI *et al.* 2007).

O risco, a gravidade e o prognóstico das doenças hepáticas provocadas pelo consumo abusivo do álcool são geralmente dependentes da quantidade consumida, frequência com que a droga é utilizada e duração do consumo de álcool, desencadeando um contínuo processo inflamatório no fígado. Evidências sugerem

que o consumo excessivo de etanol, cerca de 40-80 g/dia para homens e 20-40 g/dia para mulheres, deve levar à EHA (GYAMFI & WAN, 2010). Esta doença é a primeira mudança morfológica no fígado, seguida pela fibrose hepática e cirrose. A EHA é considerada clinicamente benigna, uma vez que pode ser revertida se o acúmulo de lipídeos for removido (MENDEZ *et al.* 2010) por eliminação da causa primária. No entanto, até o momento não há fármacos de uso clínico eficientes para eliminar este acúmulo de lipídeos em hepatócitos.

Existe uma clara correlação existente entre os efeitos cumulativos do álcool e doenças hepáticas. Alguns bebedores pesados de álcool, mas nem todos, desenvolvem sinais de injúrias hepáticas. Isto acontece devido a suscetibilidades destes indivíduos para o efeito tóxico do álcool em seus organismos, que pode envolver interações complexas entre genes e o ambiente (KONISHI *et al.* 2003; CRABB *et al.* 2004; KONISHI *et al.* 2004; ZAKHARI *et al.* 2007). Outros fatores que afetam a suscetibilidade à esteatose alcoólica e não-alcoólica incluem etnia, nutrição, obesidade, infecção com vírus da hepatite C e polimorfismo genético do citocromo P450 2E1 (CYP2E1), glutationa S-transferase (GST) e o fator de necrose tumoral α (TNF-α) (MANDAYAM *et al.* 2004; CRABB *et al.* 2004; LADERO *et al.* 2005; WILFRED DE ALWIS *et al.* 2007; GYAMFI & WAN, 2010).

O etanol produz um amplo espectro de lesões celulares, levando à interferência no metabolismo de lipídeos. A homeostase dos lipídeos hepáticos é mantida pelo balanço na síntese de lipídeos, catabolismo (β-oxidação) e sua secreção, porém o metabolismo do álcool muda o estado redox do fígado, que leva à alteração da homeostase nos lipídeos hepáticos, carboidratos, proteínas, lactato e também do ácido úrico (GYAMFI & WAN, 2010). Os mecanismos moleculares do acúmulo de lipídeos no fígado de consumidores de álcool são também múltiplos e complexos. Este acúmulo parece resultar da inibição de dois processos, o ciclo dos ácidos tricarboxílicos e a oxidação de lipídeos, em parte devidos à geração excessiva de NADH, produzido pela ação da enzima álcool desidrogenase (HOBBS *et al.* 1996; PESSAYRE & FROMENTY, 2005). Os níveis aumentados de NADH no fígado estimulam a síntese de ácidos graxos e sua incorporação aos triglicerídeos (SANAL, 2008). Acredita-se que ocorra aumento da lipogênese e redução da oxidação dos ácidos graxos por inibição de reguladores transcricionais hepáticos tais

como a AMPK (*AMP-activated kinase*), PPARγ (*Peroxisome proliferator-activated receptors* ¥), PPARα (*Peroxisome proliferator-activated receptors* ~) e SREBP-1 (*Sterol regulatory element binding proteins*). PPAR e SREBP1 são receptores nucleares que respectivamente controlam as enzimas responsáveis pela oxidação e síntese de ácidos graxos, o que contribui para a sobrecarga lipídica em fígados alcoólicos (YOU *et al.* 2002). Uma produção alterada de adiponectinas no tecido adiposo com alteração de seus receptores (AdipoRs) tem sido também sugerida (ROGERS *et al.* 2008).

1.3 Estresse Oxidativo Diante do Etanol

O álcool é essencialmente metabolizado no fígado, e sua principal via de biotransformação é representada pela metaloenzima (Zn) álcool desidrogenase (ALD) (COMPORTI *et al.* 2010). A oxidação do etanol ocorre também pela enzima CYP2E1, sendo que ambas catalisam a oxidação do substrato (etanol) para a produção de acetaldeído (LIEBER *et al.* 2004; CURRY-McCOY *et al.* 2010). Ainda há a via da catalase, na presença de peróxido de hidrogênio (H_2O_2) e de radicais livres, que permitem a oxidação do etanol pela presença do radical hidroxila (OH^-). O acetaldeído é então oxidado a acetato pela aldeído desidrogenase (ALDH), e grandes quantidades de acetato são liberadas na circulação sistêmica e oxidadas a CO_2 e H_2O em tecidos extra-hepáticos (BERR *et al.* 2001; HENZEL 2004). O consumo crônico de etanol aumenta a produção de ânions superóxidos ($O_2^{\bullet}$) e óxido nítrico ($NO^{\bullet}$), induzido pela óxido nítrico sintase (CURRY-McCOY *et al.* 2010). Portanto, o metabolismo do álcool no fígado é estritamente relacionado a enzimas envolvidas no estresse oxidativo e geração de EROS, como superóxido ($^{\bullet}OH$) e peróxido de hidrogênio (H_2O_2), que desencadeiam danos celulares (Figura 1). O radical $^{\bullet}OH$ é a molécula oxidante mais reativa, capaz de se ligar ao DNA, lipídios e proteínas, oxidando-os e desencadeando as lesões (VALKO *et al.* 2006). Outra hipótese para as injúrias hepáticas induzidas pelo álcool é a formação do H_2O_2, que atua como um segundo mensageiro que estimula a cascata de quinases em proteínas acopladas à expressão de genes pró-inflamatórios (DAS & VASUDEVAN, 2007). Ainda, segundo Gabbita *et al*. (2000), também envolve o aumento na

produção de H_2O_2, que pode resultar na inativação de fosfatase. A oxidação inicial da GSH resulta na formação da glutationa oxidada (GSSG) a partir do H_2O_2 e da glutationa peroxidase (GPx) (GABBITA *et al.* 2000). Com isso, a GSH e a tiorredoxina (Trdx) podem causar a redução das pontes dissulfeto de volta para a forma ativa da proteína tirosina fosfatase (PTP), o qual ocorre quando a taxa de restauração do GSH é ineficiente, como no caso do aumento do estresse oxidativo no tecido lesionado.

Associado ao metabolismo propriamente do etanol, há evidências de que em excesso os ácidos graxos também induzem estresse oxidativo, devido à produção de EROS por peroxidação ou através da produção mitocondrial (YANG, 2000), gerando lipotoxicidade. A lipoperoxidação, por sua vez, parece estar ligada a algumas lesões da esteatose e especialmente da esteatohepatite, através da indução de necrose ou apoptose celular (FALDSTEINE *et al.* 2003). O excesso de EROS oxida organelas celulares, resultando em danos letais aos hepatócitos (MITSUYOSHI *et al.* 1999). Este estresse oxidativo parece ser um fator essencial nas lesões secundárias do alcoolismo crônico. Há, no entanto, vários mecanismos adaptativos a esta condição, que assumem importante papel no desenvolvimento e intensidade das lesões teciduais. Os mecanismos celulares de resistência ao estresse oxidativo crônico incluem a indução de enzimas como a superóxido dismutase (SOD), proteína de choque térmico (HSP) e apoptóticas (Bcl2 e Bax), síntese de glutationa (GSH), e fatores de transcrição (BERR *et al.* 2001), sendo alguns destes fatores, como o NF-κB, um amplificador de respostas inflamatórias.

A SOD é uma enzima crucial para manutenção e proteção das células aeróbias devido a sua ação antioxidante. Esta enzima apresenta duas isoformas, Cu/Zn e a Mn-dependente, localizadas no citosol e no espaço extracelular e residente na mitocôndria, respectivamente. A SOD apresenta ação preventiva contra danos teciduais ocasionados pelas EROS, gerados pela respiração aeróbica normal ou provenientes de metabólitos de componentes exógenos, como o etanol (LI *et al.* 2004; CURRY-McCOY *et al.* 2010). A SOD previne a formação do peroxinitrito por catalisar a conversão do superóxido em oxigênio (O_2) e peróxido de hidrogênio (H_2O_2), com a posterior remoção do último produto gerado através da ação das enzimas catalase (Cat) e glutationa peroxidade (GPx) (Figura 1). Assim, a SOD é

importante na prevenção da peroxidação lipídica pela reação com superóxido antes que ocorra reação com as ligações insaturadas dos ácidos graxos intracelulares (OZARAS *et al.* 2003; CURRY-McCOY *et al.* 2010), que podem ser induzidas pelo álcool.

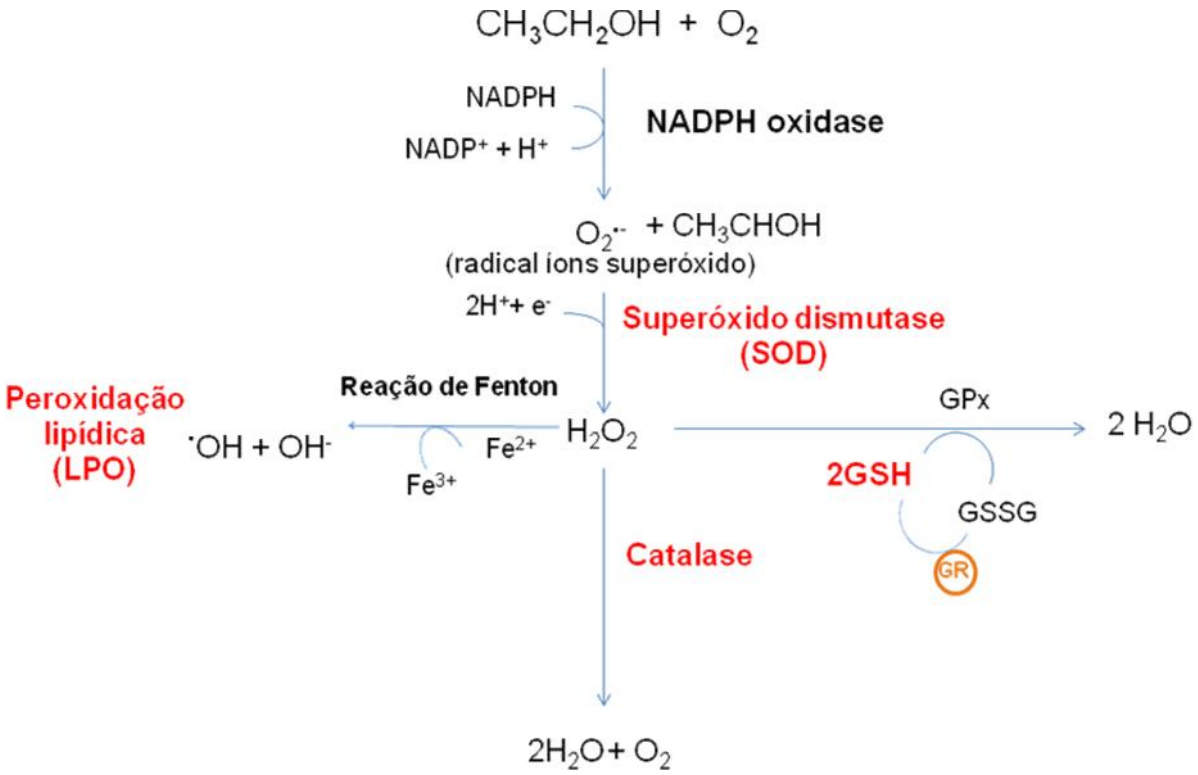

FIGURA 1. Demonstração esquemática da formação de espécies reativas de oxigênio pela ingestão de etanol e a ação de enzimas antioxidantes no combate a estas espécies formadas.
FONTE: (O Autor).

Segundo relatado em literatura, os efeitos do etanol sobre os níveis de GSH hepáticos são variáveis (LIMURO *et al.* 1997; OH *et al.* 1998; FERNANDEZ-CHECA & KAPLOWITZ, 2005). A redução de GSH mitocondrial (mGSH) decorrente da ingestão crônica de etanol, que tem sido observada de forma consistente, o torna um fator lesivo chave para a evolução da lesão hepática (FERNANDEZ-CHECA *et al.* 1991; HIRANO *et al.* 1992; GARCIA-RUIZ *et al.* 1995; FERNANDEZ-CHECA, 2003). A depleção de GSH mitocondrial pela ingestão crônica de etanol ocorre preferencialmente nos hepatócitos pericentrais, onde ocorre a maior parte das lesões em fígado de camundongos (GARCIA-RUIZ *et al.* 1995). Esta depleção pelo etanol é atribuível ao comprometimento no transporte de GSH a partir do citosol para a mitocôndria (GARCIA-RUIZ *et al.* 1995; COLELL *et al.* 1998). Bailey *et al.* (2001),

no entanto, descobriram que os níveis de GSH mitocondrial foram aumentados após a ingestão crônica de etanol no modelo de Lieber-Decarli em cerca de 25%. Estes achados foram sugestivos para uma reflexão referente a uma resposta adaptativa para neutralizar os danos metabólicos provocados pelo etanol relacionados com os aumentos na produção de EROS mitocondrial (DAS & VASUDEVAN, 2007).

Como supracitado, o estresse oxidativo está criticamente envolvido em doenças hepáticas induzidas por álcool, o que leva a um desequilíbrio em prol das espécies pró-oxidantes e/ou desequilíbrio das espécies antioxidantes, culminado em lesões hepáticas (SHUKLA *et al.* 2013). Existem evidências de que as funções das mitocôndrias estão alteradas em humanos com esteatohepatite (CORTEZ-PINTO *et al.* 1999). Como as mitocôndrias são um dos principais sítios de geração de EROS e também onde ocorre a maior parte da oxidação dos ácidos graxos, considerável atenção tem sido dada ao papel das mesmas, especialmente na progressão da esteatose para injúrias hepáticas mais severas (esteatohepatite, fibrose e cirrose). A relevância destas alterações para as funções mitocondriais, peroxisomais e microssomais pode ser averiguada ao se avaliar as vias de oxidação de ácidos graxos, a produção de EROS e a lipoperoxidação em fígados esteatóticos. Verifica-se, entretanto, que são poucos os relatos existentes na literatura. Sabe-se, porém, que o consumo crônico de álcool promove prejuízo das funções mitocondriais e alteração na estrutura das mesmas, produzindo um aumento na produção de EROS e, consequentemente, toxicidade celular (DAS & VASUDEVAN, 2007). A geração de espécies reativas de oxigênio deve ser mais elevada posteriormente ao consumo abusivo de álcool, pois ocorre um decréscimo da atividade enzimática da cadeia respiratória, resultando em acúmulo de transportadores reduzidos no complexo I e II desta cadeia (CEDERBAUM *et al.* 2009). Assim, as mitocôndrias contribuem para o acréscimo nos níveis de oxidação em hepatócitos sob exposição aguda ou crônica ao álcool (VENKATRAMAN *et al.* 2004; WU & CEDERBAUM, 2009).

1.4 Tratamento para a EHA

A elucidação dos mecanismos que levam ao desenvolvimento da EHA propicia um melhor entendimento da enfermidade e de sua progressão, bem como

apontam para possíveis alvos terapêuticos. Alguns fármacos têm sido testados, como esteróides (CHRISTENSEN & GLUUD, 1995), ácido ursodeoxicólico (UDCA) ou seu conjugado (TUDCA) (PLEVRIS *et al.* 1991) e antioxidantes (ARTEEL *et al.* 2003). Ainda, estudos demonstram que o receptor FXR (*Farnesoid X receptor*) exerce um papel central na homeostase dos ácidos biliares, bem como no metabolismo de glicose e lipídeos hepáticos (PAQUETTE *et al.* 2008). Assim, os agonistas FXR, GW4064 e 6ECDCA, têm sido testados em animais visando o tratamento de uma gama de doenças hepáticas, incluindo colestase (LI *et al.* 2009), NAFLD (WEI *et al.* 2009) e esteatose hepática alcoólica (LÍVERO *et al.* 2012). Porém, mesmo com décadas de estudos, ainda não há medicamentos definitivos para seu tratamento (HENZEL *et al.* 2004).

1.5 Associação Entre EHA e Dieta

A completa compreensão da patogenia das doenças hepáticas alcoólicas é impedida pela complexidade das interações que o álcool sofre, como supracitado, bem como pelas variações quanto à quantidade ingerida, tempo de ingestão, tipo de agente agressor e como é administrado (NATH & SZABO, 2009; TSUKAMOTO *et al.* 2009). Progressos significativos foram feitos nos modelos de indução de esteatose hepática alcoólica em animais de diferentes espécies e cultura celular, objetivando investigar os mecanismos de iniciação e progressão da lesão com a fidedignidade da etiologia e da história natural da doença em humanos (MANN *et al.* 2003; SOZIO & CRABB, 2008; NATH & SZABO, 2009; ARTEEL, 2010; SZABO & BALA, 2010). As espécies de animais mais utilizados para a indução de esteatose hepática alcoólica são os roedores (ratos, camundongos e hamsters) e primatas (BATRA *et al.* 1995). Estudos com ingestão de álcool diluído em água por 3-4 anos, em babuínos, levam à evolução de todos os níveis de doenças hepáticas alcoólicas, semelhantemente ao observado em humanos (LIEBER *et al.* 1985; BRANDON-WARNER *et al.* 2012). Entretanto, os custos elevados e o longo tempo desprendido tornam os estudos com primatas pouco viáveis (LI, 2008; DeNUCCI *et al*, 2010; TSUCHIYA *et al.* 2012; MILLER *et al.* 2012).

A ingestão de álcool por via oral em roedores mimetiza muitos dos efeitos de consumo de álcool em humanos (MANN *et al.* 2003). A quantidade de álcool ingerida e a duração deste consumo são fatores de grande importância para a evolução da disfunção hepática. Contudo, os meios de fornecimento do álcool também desempenham um papel importante na determinação da quantidade de álcool ingerida, o que eleva às consequências patológicas progressivas (GILPIN *et al.* 2008; BRANDON-WARNER *et al.* 2012).

Pessoas com doenças hepáticas alcoólicas substituem calorias dos alimentos por calorias provenientes do álcool (GRIFFITH & SCHENKER, 2006). Esta substituição corresponde a mais de 50% das calorias totais ingeridas/dia. (MENDENHALL *et al.* 1984). Além disso, a proporção de calorias com origem alcoólica parece aumentar a gravidade das disfunções hepáticas, com a redução da qualidade alimentar (MEZEY, 1991; SCHENKER & HALFF, 1993; MEZEY, 1998). Em contrapartida, consumidores crônicos de álcool perdem o desejo por alimentos, o que pode levar à anorexia, que também está correlacionada com a gravidade da lesão hepática (MENDENHALL *et al.* 1995; HIRSCH *et al.* 1999).

Dietas hiperlipídicas também têm sido sugeridas como um fator de risco para o desenvolvimento e agravamento de doenças hepáticas alcoólicas em animais e humanos (MEZEY, 1998). Mais de 90% dos bebedores crônicos que associam álcool à dieta hiperlipídica desenvolvem EHA. Em contrapartida, dados mostram que em países aonde a dieta rica em gorduras saturadas é prevalente, ocorre uma menor incidência de doenças hepáticas, apesar da ingestão de álcool rotineiramente (MCCULLOUGH & O'CONNOR, 1998). Isto também vem sendo observado em pesquisas com animais submetidos a uma ingestão crescente de gordura saturada, levando à reversão das alterações hepáticas, mesmo com a ingestão de álcool continuada. Este efeito, em parte, pode ser atribuído à menor produção de radicais livres a partir de gordura saturada da dieta (YOU *et al.* 2005).

Com esses dados fica clara a interação entre dieta, etanol e o desenvolvimento de enfermidades hepáticas, com diferentes graus de severidade. Baseando-se nisto é que o presente trabalho foi delineado, com a proposta de caracterizar um modelo de EHA de baixo custo, associando o consumo de etanol à dieta rica em lipídeos, representada unicamente por semente de girassol.

2. OBJETIVO GERAL

Este trabalho objetiva propor e caracterizar um modelo de estudo de EHA *in vivo*, que seja fidedigno da enfermidade em humanos e que tenha custo viável para sua execução, com uma dieta hiperlipídica associada ao etanol.

2.1. Objetivos Específicos

2.1.1 Quantificar os lipídeos presentes no fígado dos animais submetidos às diferentes dietas;

2.1.2 Avaliar histologicamente o grau da lesão celular decorrente da indução da esteatose hepática alcoólica *in vivo*;

2.1.3 Avaliar as funções mitocondriais possivelmente envolvidas na evolução da EHA, avaliando a respiração celular;

2.1.4 Avaliar o grau de estresse oxidativo celular através de biomarcadores do estado redox de hepatócitos.

3. ARTIGO CIENTÍFICO

Characterization of an Alcoholic Hepatic Steatosis Model Induced by Ethanol and High-Fat Diet in Rats

Carlos Eduardo Alves de Souza[1], Aline Maria Stolf[1], Arturo Alejandro Dreifuss[1], Francislaine dos Reis Lívero[1], Liana de Oliveira Gomes[1], Lyvia Lintzmaier Petiz[2], Olair Carlos Beltrame[3], Rosangela Locatelli Ditrich[3], José Ederaldo Queiroz Telles[4], Silvia Maria Suter Correia Cadena[2], Alexandra Acco[1*]

[1] Department of Pharmacology, Federal University of Paraná, Curitiba, Brazil

[2] Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, Brazil

[3] Department of Veterinary Medicine, Federal University of Paraná, Curitiba, Brazil

[4] Department of Medical Pathology, Federal University of Paraná, Curitiba, Brazil

*** Corresponding author:**

Alexandra Acco, Federal University of Paraná (UFPR), Biological Science Sector, Department of Pharmacology, Centro Politécnico, Cx. P. 19031, Curitiba – Paraná – Brazil, Zip Code 81531-980

Phone: +55 (41) 3361-1743; Fax: +55 (41) 3266-2042

E-mail: aleacco@ufpr.br

Financial support: CAPES; Fundação Araucária

Running title: Ethanol and sunflower seeds inducing hepatic steatosis

3.1 Abstract

Background: Alcoholic liver disease is characterized by wide spectrum of liver damage, ranging from fatty liver to steatohepatitis and liver fibrosis/cirrhosis. The ethanol induces the accumulation of intracellular lipids, oxidative stress and damage in respiratory chain. However, this damage increases when the ethanol is associated with high-fat diet (HFD). The aim of this work was to establish and characterize a model of alcoholic hepatic steatosis (AHS) through the combination of 10% ethanol and sunflower seeds as the unique source of HFD. **Material and methods:** Male Wistar rats (*Rattus norvegicus*) were kept in a single cage with food and liquid *ad libitum*. The rats were fed to water or 10% ethanol and regular chow diet and/or high fat diet (HFD), represented by sunflower seeds. Throughout thirty days on diets, the food consumption, liquid intake and the body weight were monitored. After this period, blood and liver samples were collected for evaluation of liver histology, hepatic oxidative stress, hepatic mitochondria enzyme activity, and plasma biochemistry. **Results:** The association of 10% ethanol and HFD induced hepatic lipid accumulation, macrostetosis, hepatocytes tumefaction, decreased levels of reduced glutathione and reduction of glutathione-S-transferase, and increased the level of lipoperoxidation and the activity of superoxide dismutase. Also, the mitochondrial oxidation of NADH and succinate were partially inhibited. **Conclusions:** The combination of 10% ethanol with sunflower seeds as the diet for rats produced an interesting and cheap model to study AHS. The installation of liver steatosis was successfully obtained after 4 weeks of diet. The liver function was modified and the alterations were found in the morphological analysis, oxidative stress biomarkers and in the mitochondrial electron transport.

Keywords: steatosis, ethanol, high-fat diet, sunflower seeds, oxidative stress, mitochondrial chain

3.2 Introduction

Alcoholic dependency is considered to be a worldwide public health problem, and a direct causal relationship has been observed between alcohol consumption and more than 60 different types of diseases and injuries, including those in the liver (Miranda-Mendez *et al.* 2010). Alcoholic liver disease (ALD) is characterized by a wide spectrum of liver damage, ranging from liver steatosis (fatty liver) to steatohepatitis and liver fibrosis/cirrhosis. It is believed that the obvious cause for ALD is hepatocyte death induced by alcohol itself (Byun and Jeong, 2010; Curry-McCoy *et al.* 2010). However, the involvement of oxidative injury in ethanol toxicity has emerged from a growing number of reports that showed that alcohol-fed experimental animals, as well as patients with ALD, have increased contents of lipid peroxidation products in the liver and in the blood (Albano, 2008).

Mechanisms implicated in alcohol-induced liver damage involve many biochemical reactions, with different pathways interacting with each other simultaneously. These mechanisms involve enzymes, reactive oxygen species (ROS), endotoxins, cytokines, immune system cells, and genetic predisposition to liver disease (Lu and Cebderbaum, 2008; Miranda-Mendez *et al.* 2010). The intracellular accumulation of lipids is the most frequent liver lesion in heavy drinkers. The impairment of mitochondrial lipid oxidation has been proposed as one of the mechanisms that is responsible for this fat accumulation (Pessayre and Fromenty, 2005; Pessayre, 2012). Oxidative stress associated with alcohol toxicity is mainly caused by ROS generated by the mitochondrial respiratory chain, by the enzyme responsible for the ethanol metabolism (CYP2E1) in hepatocytes, and by the NADPH oxidase of Kupffer cells and liver-infiltrating granulocytes. In addition, the oxidation of ethanol through the alcohol dehydrogenase pathway produces acetaldehyde, which is converted to acetate. Both reactions promote an increase of NADH, which, in excess, results in several metabolic disorders, including the inhibition of fatty acid oxidation and tricarboxylic acid cycle (Tilg, 2011). Therefore, it increases the hepatic fat accumulation.

For decades, dietary deficiencies were considered the major factor responsible for the development of liver disease in alcoholics (Korourian *et al.* 1999), because ethanol displaces normal nutrients, causing malnutrition (Liber *et al.* 2004; Comporti *et al.* 2010). Moreover, it has been experimentally proved that nutritional

deficiencies cause liver damage (Di Pascoli *et al.* 2004; Rautou *et al.* 2008; Caballero *et al.* 2011). Therefore, it was postulated that the combination of these factors cause ALD (Comporti *et al.* 2010). For instance, it has been reported that a low-carbohydrate diet associated with ethanol induced severe hepatotoxicity with intense steatosis in rats (Korourian *et al.* 1999). Despite alcoholic fatty liver has long been considered benign, increasing evidence supports the idea that steatosis may contribute to the progression of other hepatic injuries (Powel *et al.* 2005).

Because the interaction between ethanol and nutrients may contribute significantly to the pathology of an alcoholic liver injury, the aim of this study was to establish and characterize an alcoholic hepatic steatosis (AHS) model induced by ethanol associated with a high-fat diet (HFD). Because specific chow with high fat or low protein contents, both used in combination with ethanol to induce the steatosis model, are expensive, the purpose of this study is to propose a cheaper model of alcoholic steatosis by using sunflower seeds (*Helianthus annuus*) as the unique source of the HFD. This study focused on the hepatic oxidative stress and the mitochondrial dysfunction induced by the combination of the liquid diet (10% ethanol) and HFD (sunflower seeds).

3.3 Material and Methods

3.3.1 Animal care, diets, and sample collection

Male Wistar rats (*Rattus norvegicus*) weighing 200 ± 20 g were used for this study. The experimental study (Figure 1) was approved by the Institutional Animal Ethics Committee of the Biological Sciences Sector of the Federal University of Paraná (certificate #584). Briefly, each animal was housed in a single cage with food and water *ad libitum* (4 weeks) and maintained at room temperature (22°C ± 2°C) on a 12/12-h light/dark cycle. After acclimation for 1 week, the animals were separated into 6 groups (n = 10 each) according to their liquid and solid diets: (1) water and chow diet (WC), (2) water and high-fat diet with only sunflower seeds (WH), (3) water and chow plus sunflower seeds (WCH), (4) ethanol and chow diet (EtC), (5) ethanol and high-fat diet with only sunflower seeds (EtH), and (6) ethanol and chow diet plus

high-fat diet with sunflower seeds (EtCH). Food (chow and/or sunflower seeds) consumption, liquid intake (water or ethanol), and body weight were monitored 3 times per week during a 1-month period. The consumption of sunflower seeds was calculated using only the weight of the peeled seed, which was 48% of the total weight of the seed. The nutritional values of the regular chow and the sunflower seeds are shown in the supplementary material. After 30 days of consumption of the diet, the animals were anesthetized with ketamine (60 $mg \cdot kg^{-1}$- Vetnil, Louveira, SP, Brazil) and xylazine (7.5 $mg \cdot kg^{-1}$- König, Santana de Parnaíba, SP, Brazil) by intraperitoneal injection. Blood from individual rats was collected from the abdominal cava vein to determine the plasma levels of biochemical parameters. The liver was quickly snap-frozen in liquid nitrogen and stored at -80°C for hepatic oxidative stress analysis and quantification of lipids. A portion of the liver was fixed in 10% neutral-buffered formalin for histological analysis. For isolation of liver mitochondria, the animals were euthanized by decapitation.

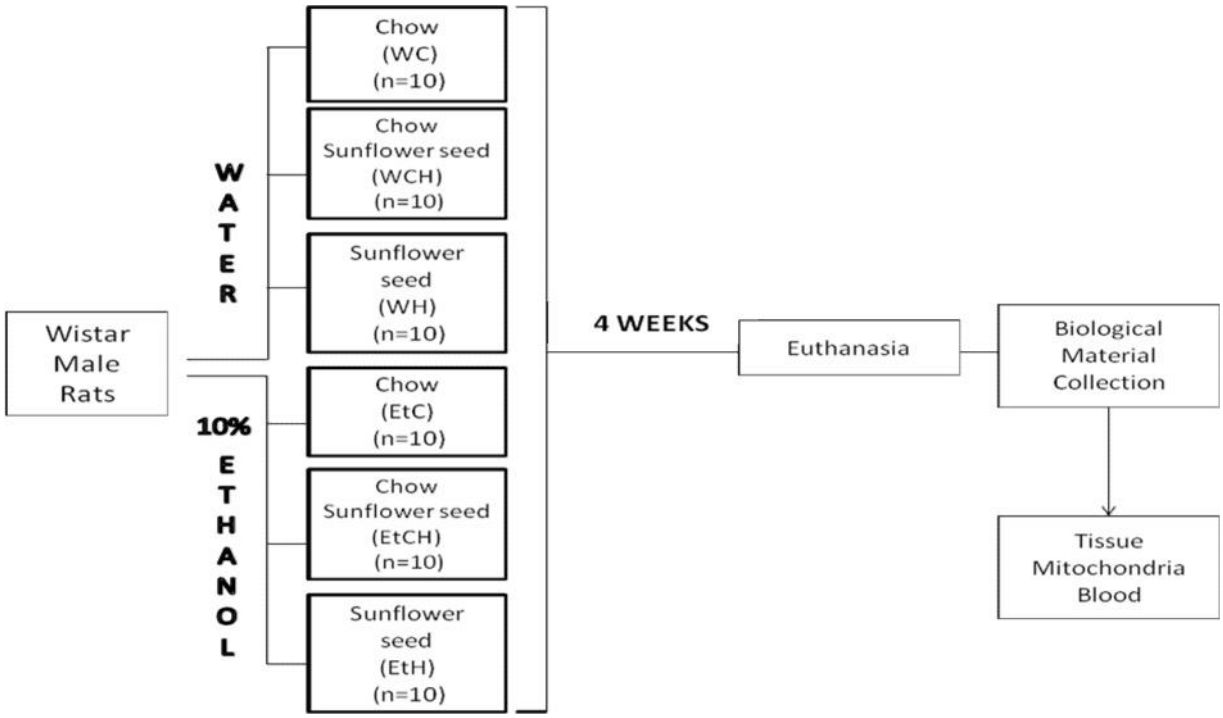

Figure 1. Experimental designed over 4 weeks with rats fed with chow and/or sunflower seeds combined with water or 10% ethanol. Groups: WC (water - chow diet), WCH (water - chow diet and high-fat diet with sunflower seed), WH (water - high-fat diet with sunflower seed), EtC (10% ethanol - chow diet), EtCH (10% ethanol - chow diet and high-fat diet with sunflower seed) and EtH (10% etanol - high-fat diet with sunflower seed).

3.3.2 Hepatic histology

The liver tissue, which was fixed in 10% (v/v) neutral-buffered formalin, was further embedded in paraffin at room temperature. Thin sections (4 µm) from paraffin blocks were processed for histology, and the tissue was stained with hematoxylin-eosin according to the routine technique applied at the Department of Medical Pathology of Federal University of Paraná. Another liver tissue fraction was embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (Sakura, Torrance, CA, USA), processed in a cryostat, and stained with Sudan Black or Nile Blue, both of which are specific stains for lipids. All slides were then analyzed by light microscopy (Leica DM2500, Wetzlar, Germany).

3.3.3 Measurement of liver lipid content

The lipid content in the tissue was determined by the gravimetric method (Oller do Nascimento and Willianson, 1986). All results were expressed as micrograms of triglyceride per milligram of liver tissue ($\mu g \cdot mg^{-1}$ liver).

3.3.4 Plasmatic analysis

Plasma was obtained by centrifugation of the total blood and stored at -80°C. Then, cholesterol (CHO) and triglyceride (TG) levels, as well as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, were analyzed using a biochemical-automated system (Labtest Diagnóstica, Lagoa Santa, MG, Brazil) according to the manufacturer's instructions.

3.3.5 Hepatic oxidative stress measurements

Liver tissue was homogenized in phosphate-buffered saline (pH 6.5) using a homogenizer and centrifuged at 10,000 $\times$ *g* at 4°C for 20 min. The activities of antioxidant enzymes were determined in the supernatant. The supernatant was used to measure the activity of catalase (Cat) (Aebi, 1984), superoxide dismutase (SOD) (Gao, 1998), and glutathione S-transferase (GST) (Habig, 1974). In addition, the rate

of lipid peroxidation (LPO) (Jiang, 1991) and reduced glutathione (GSH) (Sedlak, 1968) were measured. Results were expressed relative to the protein levels in each liver homogenate.

3.3.6 Isolation of rat liver mitochondria

Mitochondria were isolated from the liver by differential centrifugation as described by Voss et al. (1961) by using an extraction medium consisting of 250 mM D-mannitol, 10 mM HEPES (pH 7.2), 1 mM EGTA, and 0.1 g% BSA. All procedures were performed at 4°C, and the mitochondrial suspension was stored in liquid nitrogen until the activity of enzymes linked to the respiratory chain was determined.

3.3.7 Mitochondrial enzymatic activity

Disrupted mitochondria that were obtained by a freeze-thawing treatment were used to determine the activity of the respiratory enzyme chain. NADH and succinate oxidase activities were assayed polarographically (Singer and Gutman, 1971). NADH dehydrogenase (EC 1.6.5.3 NADH:ubiquinone oxidoreductase) and succinate dehydrogenase (EC 1.3.5.1 succinate:ubiquinone oxidoreductase) activities were assayed spectrophotometrically according to Singer (1971). NADH-cytochrome c reductase (EC 1.6.99.3 NADH:cytochrome c oxidoreductase) and succinate cytochrome c reductase (EC 1.3.99.1 succinate:cytochrome c oxidoreductase) activities were measured by cytochrome c reduction at 550 nm as described by Somlo (1965). The activity of cytochrome c oxidase (EC 1.9.3.1 ferrocytochrome c:oxygen oxidoreductase) was determined according to Mason et al. (1973).

3.3.8 Protein determination

Protein concentrations in the liver homogenate for hepatic oxidative stress measurements was determined by the Bradford method (1976), and those from

hepatic mitochondria homogenates were determined by the method described by Lowry et al. (1951). BSA was used as the standard in both methods.

3.3.9 Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey-Kramer test. Two-way ANOVA was used to analyze the liquid consumption, food consumption, and body weight gain, with the time (week) and the diet as the variables. The GraphPad Prism 5.0 (La Jolla, CA, USA) program was used for statistical analysis, and $p < 0.05$ was the value for statistical significance.

3.4 Results

3.4.1 Diet consumption and body weight gain of rats

The consumption of liquid (water or alcohol) and solid food (regular chow and/or sunflower seeds) of each animal was measured thrice per week, while the body weight was monitored once per week. The consumption of liquid over the 4-week period was the highest in animals that were fed the regular diet with water (WC), followed by the group of animals that were fed ethanol and regular chow (EtC) (Figure 2A). Similarly, a higher intake of solid food occurred in animals treated with the water and regular chow diet (WC), which was statistically different from all of the other groups (Figure 2B). With the observed differences in the amount of solid diet that was consumed, we sought to quantify the gross energy present in the diet of animals using a calorimeter ($kcal \cdot kg^{-1}$) that was based on the consumption of each group. We observed that the animals that ingested the ethanol-chow and high-fat diet with sunflower seeds (EtCH) or the ethanol-sunflower seed diet (EtH) proportionately consumed more calories than animals in the other groups (Figure 2C). However, higher calorie intake from a solid diet did not result in increasing body weight; the EtH and WH groups were the groups that gained the least weight over the 4-week period

(Figure 2D). Therefore, the combination of ethanol and sunflower seeds (EtH) reduced the liquid and solid consumption and reduced the body weight gain, but it increased the caloric intake.

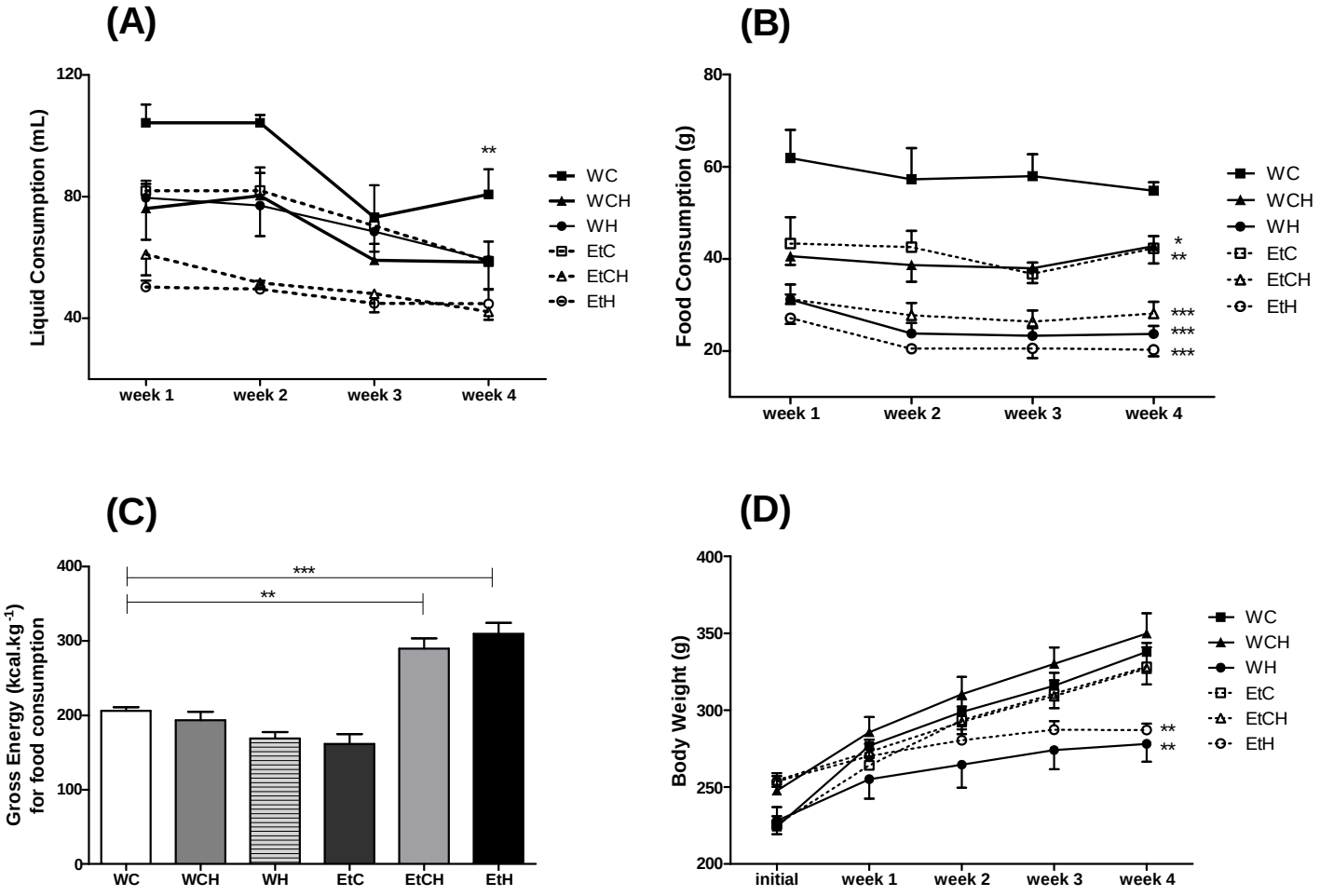

Figure 2. Consumption of the liquids **(A)** and food **(B)** by rats fed with chow and/or sunflower seeds combined with water or 10% ethanol for 4 weeks. The gross energy (kcal·kg^{-1} of food) derived from the consumption of the solid diet **(C)** and the body weight gain in rats **(D)** are also shown. Values are expressed as mean ± standard error of the mean (n=10), and they were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test as a *post-hoc* analysis for gross energy and two-way ANOVA for liquid consumption, food consumption, and body weight gain. Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$ compared to other groups (C) at the same week (A,B,D). Groups: WC (water - chow diet), WCH (water - chow diet and high-fat diet with sunflower seed), WH (water - high-fat diet with sunflower seed), EtC (10% ethanol - chow diet), EtCH (10% ethanol - chow diet and high-fat diet with sunflower seed) and EtH (10% etanol - high-fat diet with sunflower seed).

3.4.2 The diet combination of ethanol and sunflower seeds induced severe hepatic steatosis

At macroscopic observation, the liver of the rats that were fed an HFD and ethanol (EtH and EtCH) was pale and fatty (data not shown). To confirm the lipid accumulation, histopathological analysis of the livers was performed by staining with hematoxylin-eosin. The lesions were classified according to the Rappaport score (1958). Livers of animals from the WH, EtCH, and EtH groups displayed predominantly microvesicular steatosis and less macrovesicular steatosis. However, no steatosis was observed in livers of animals from the EtC group after 4 weeks of the diet, and only a low degree of microsteatosis was found in the livers of animals from the WCH group. Indeed, the histology of the livers from the EtC group was similar to that of the livers from the control group (WC) (Figure 3). Other lesions, such as lymphocytes infiltration, were also observed and quantified as shown in Table 1. The visualization of lipid droplets using Sudan Black and Nile Blue (Figure 3F) staining confirmed the considerable accumulation of lipids. The former stains lipids in intense black, while Nile Blue stains acids and neutral lipids in pink.

The hepatic lipid accumulation was detected using the gravimetric method (Figure 4). The animals in the EtH and EtCH groups had increased intracellular lipids after 4 weeks of the diet, while the WH group presented a moderate increase that was not statistically significant. The EtC group presented the same level of intrahepatic lipid as the control group (WC), which indicates that the ethanol alone did not cause the accumulation of lipids in the liver. These results are consistent with the histological observations (Figure 3).

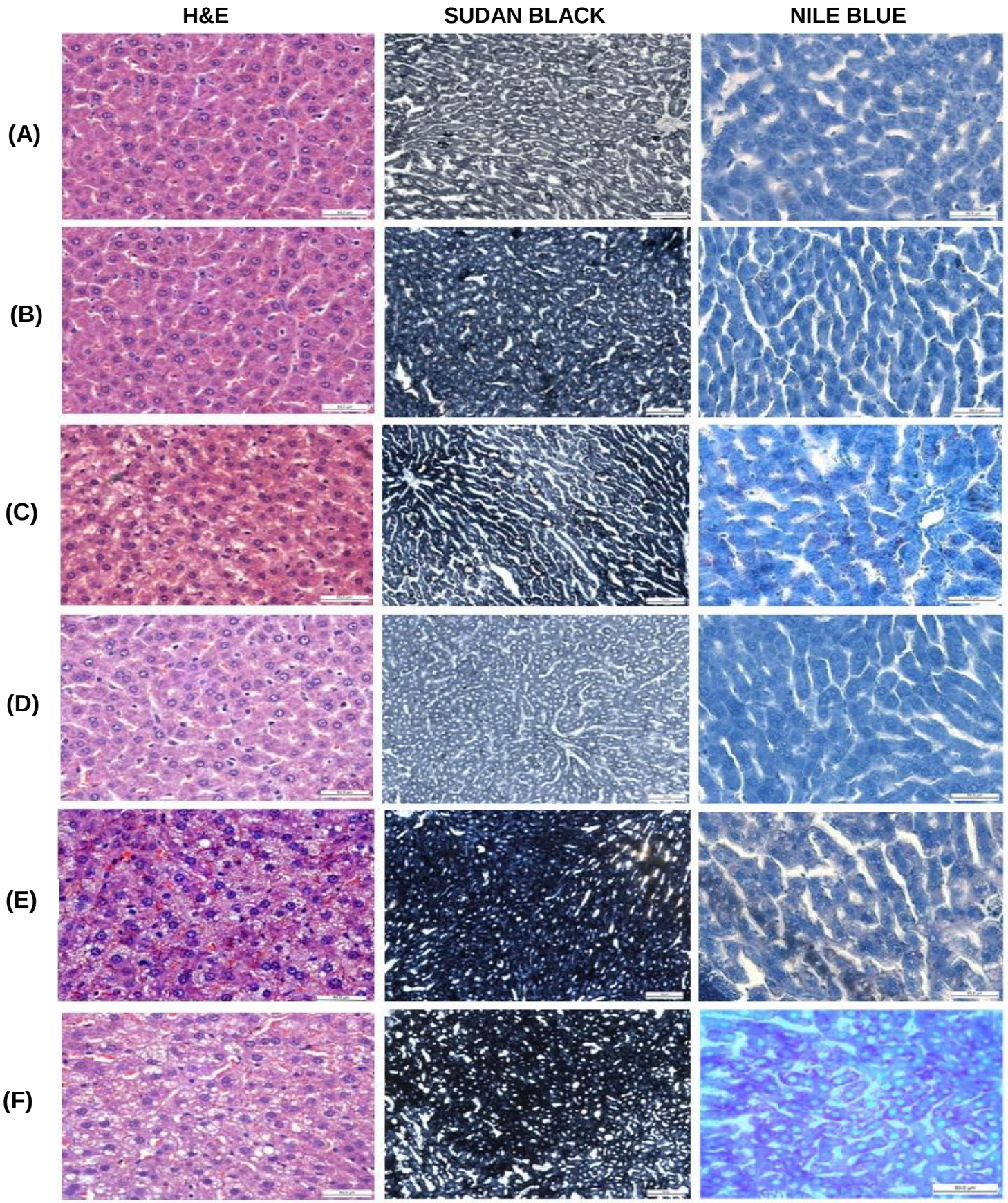

Figure 3. Liver histology demonstrated in rats after 4 weeks under modified solid and/or liquid diet. The staining was performed with hematoxylin-eosin (H&E), Sudan Black and Nile Blue to all experimental groups: **(A)** WC, **(B)** WCH, **(C)** WH, **(D)** EtC, **(E)** EtCH e **(F)** EtH. The morphology was performed in increased 400x or 200x respectively, showing the accumulation of lipids within hepatocytes.

Table 1. Hepatic histology lesions observed in rats after receiving different diets for 30 days to induce steatosis.

	Microsteatosis	Macrosteatosis	Tumefaction	Focus lymphocytosis
WC	-	-	-	-
WCH	+	-	-	-
WH	+	+	-	+
EtC	-	-	-	-
EtCH	++	-	-	+
EtH	++	+	+	+

Groups: WC (water - chow diet), WCH (water - chow diet and high-fat diet with sunflower seed), WH (water - high-fat diet with sunflower seed), EtC (10% ethanol - chow diet), EtCH (10% ethanol - chow diet and high-fat diet with sunflower seed) and EtH (10% etanol - high-fat diet with sunflower seed).The Rappaport score (1958) means: absence of lesion or lesion in less than 10% of the microscopic areas (-); mild lesion, in 10-30% (+); moderate lesion, in 30-60% (++); and severe lesion, in 60-100% of the analyzed areas (+++).

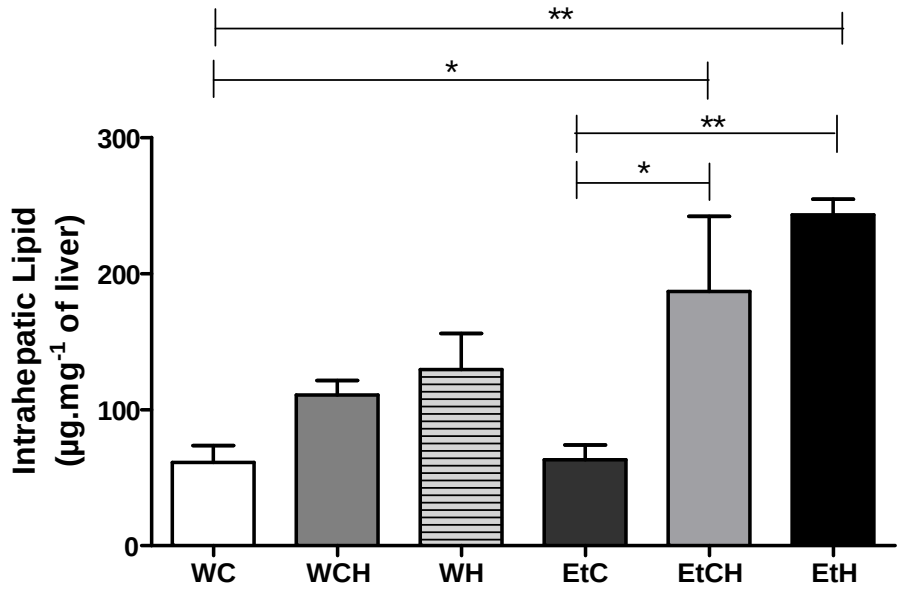

Figure 4. Intrahepatic lipids represented by triglycerides (µg·mg^{-1} liver) in rats receiving different diets. Values are expressed as mean ± standard error of the mean (n=10) and were analyzed by one-way ANOVA followed by the Tukey test as a *post-hoc* analysis. Symbols: *, $p < 0.05$; **, $p < 0.01$. Groups: WC (water - chow diet), WCH (water - chow diet and high-fat diet with sunflower seed), WH (water - high-fat diet with sunflower seed), EtC (10% ethanol - chow diet), EtCH (10% ethanol - chow diet and high-fat diet with sunflower seed) and EtH (10% etanol - high-fat diet with sunflower seed).

3.4.3 The diets influenced the plasmatic levels of ALT and cholesterol

The highest ALT activity was observed in the groups that were fed an HFD, independent of the liquid that was consumed (water or ethanol), clearly showing the influence of the solid diet. However, the association of 10% ethanol with an HFD increased the severity of liver damage, as illustrated by the high ALT level in the EtCH and EtH groups but not in the WCH and EtC groups (Figure 5A). Similar results were observed for the CHO levels in the plasma: rats receiving the sunflower seed diet had increased CHO levels with water or ethanol. However, balanced diets with or without 10% ethanol did not influence the cholesterol levels (Figure 5B). No differences in AST and triglycerides levels were observed among the groups (data not shown).

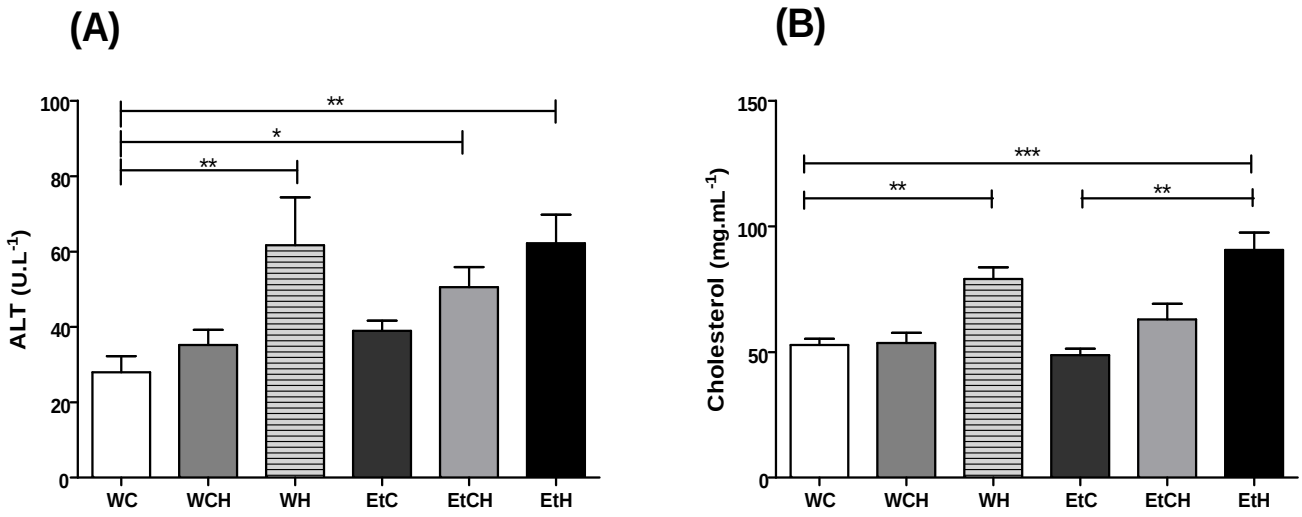

Figure 5. Alanine aminotransferase (ALT) **(A)** and cholesterol **(B)** levels in rat plasma after 4 weeks of different diets. Values are expressed as mean ± standard error of the mean (n=10) and were analyzed by one-way ANOVA followed by the Tukey test as a *post-hoc* analysis. Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Groups: WC (water - chow diet), WCH (water - chow diet and high-fat diet with sunflower seed), WH (water - high-fat diet with sunflower seed), EtC (10% ethanol - chow diet), EtCH (10% ethanol - chow diet and high-fat diet with sunflower seed) and EtH (10% etanol - high-fat diet with sunflower seed).

3.4.4 Diet of ethanol and sunflower seeds induced hepatic oxidative stress

The SOD activity in the EtCH and EtH groups was significantly higher (50%) than that in the control group (WC). However, GST activity (-33%) and GSH level (-59%) were lower in the EtH group than in the WC group (Figure 6A–C). Moreover, the LPO level was higher in the WH, EtCH, and EtH groups, reaching about 62%, 112%, and 137%, respectively, than the level in the WC group (Figure 6D). These data indicate that the ethanol and HFD reduce hepatic GSH levels and GST activity and increase the SOD activity. Furthermore, LPO, despite occurring only in the presence of ethanol, was aggravated by HFD. No differences were observed in the hepatic Cat activity (data not shown).

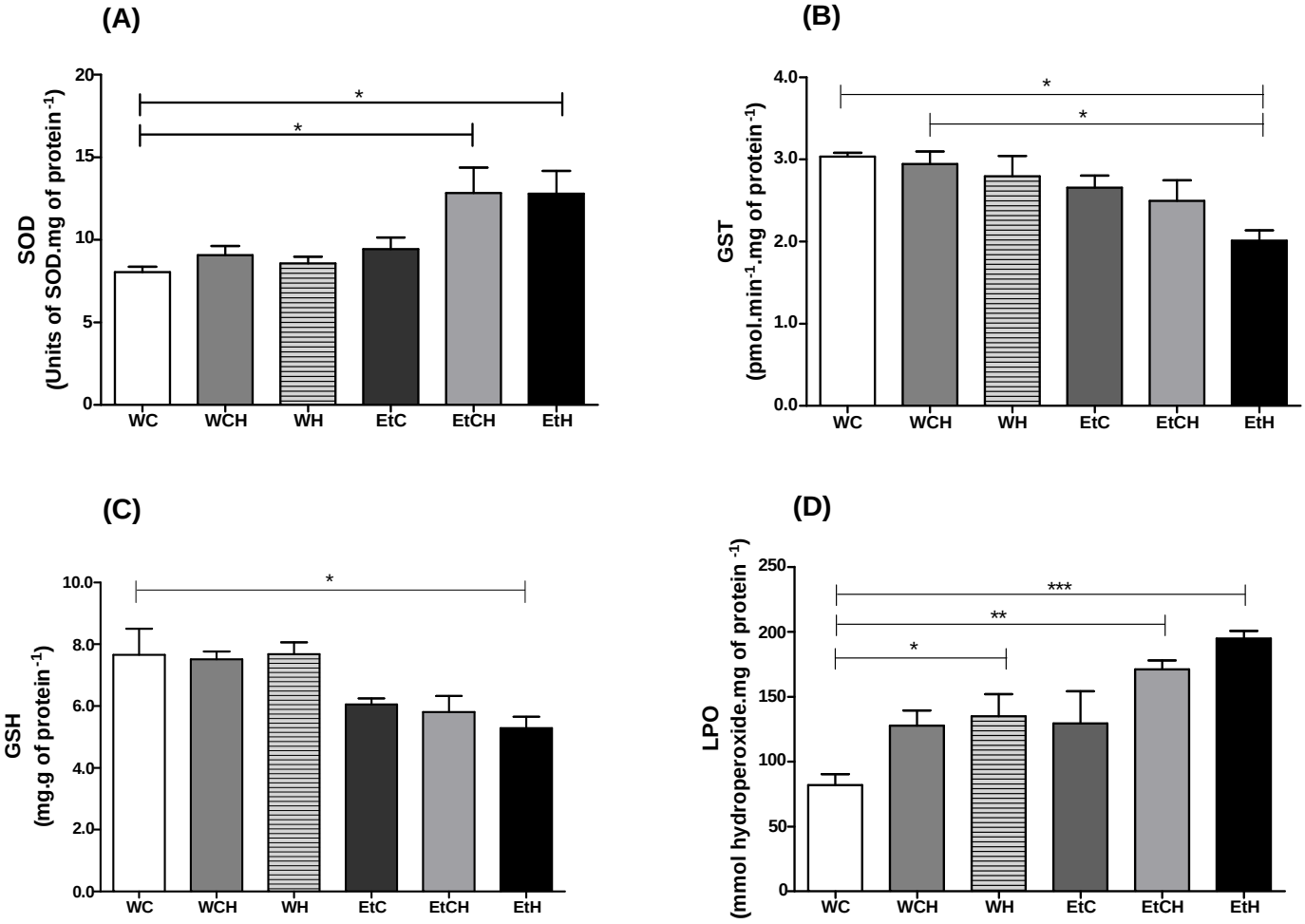

Figure 6. Biomarkers of oxidative stress in rat liver samples collected after 4 weeks of diet. The biomarkers analyzed were superoxide dismutase **(A)**, glutathione S-transferase **(B)**, reduced gluthatione **(C),** and lipid peroxidation **(D)**. Values represent mean ± standard error of the mean (n=10), and were analyzed by one-way ANOVA followed by the Tukey test as a *post-hoc* analysis. Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Groups: WC (water - chow diet), WCH (water - chow

diet and high-fat diet with sunflower seed), WH (water - high-fat diet with sunflower seed), EtC (10% ethanol - chow diet), EtCH (10% ethanol - chow diet and high-fat diet with sunflower seed) and EtH (10% etanol - high-fat diet with sunflower seed).

3.4.5 Both ethanol and diet impaired mitochondrial enzymatic activities

The complete oxidation of NADH (Figure 7A) and succinate (Figure 7D) in the respiratory chain was inhibited by about 25% and 22%, respectively, in the WH, EtC, and EtH groups compared to the control group (WC). We analyzed other enzymatic segments with the aim of identifying the site in the respiratory chain where the inhibition takes place. The activity of NADH dehydrogenase (complex I) was inhibited by about 13%, 22%, and 21% in the WH, EtC, and EtH groups, respectively, relative to the control group (WC) (Figure 7B). The electron transport in the segment containing NADH cytochrome c reductase was reduced by about 14%, 19%, and 16% in the WH, EtC, and EtH groups, respectively (Figure 7C).

The activity of succinate dehydrogenase (complex II) was also inhibited about 23%, 12%, and 29% in the WH, EtC, and EtH groups (Figure 7E), respectively. This inhibition was also observed in the segment containing succinate cytochrome c reductase (Figure 7F). No effect on cytochrome c oxidase (complex IV) was observed (data not shown). Taken together, these results show that the ethanol and/or HFD inhibited electron transport in the respiratory chain, mainly affecting complex I and II.

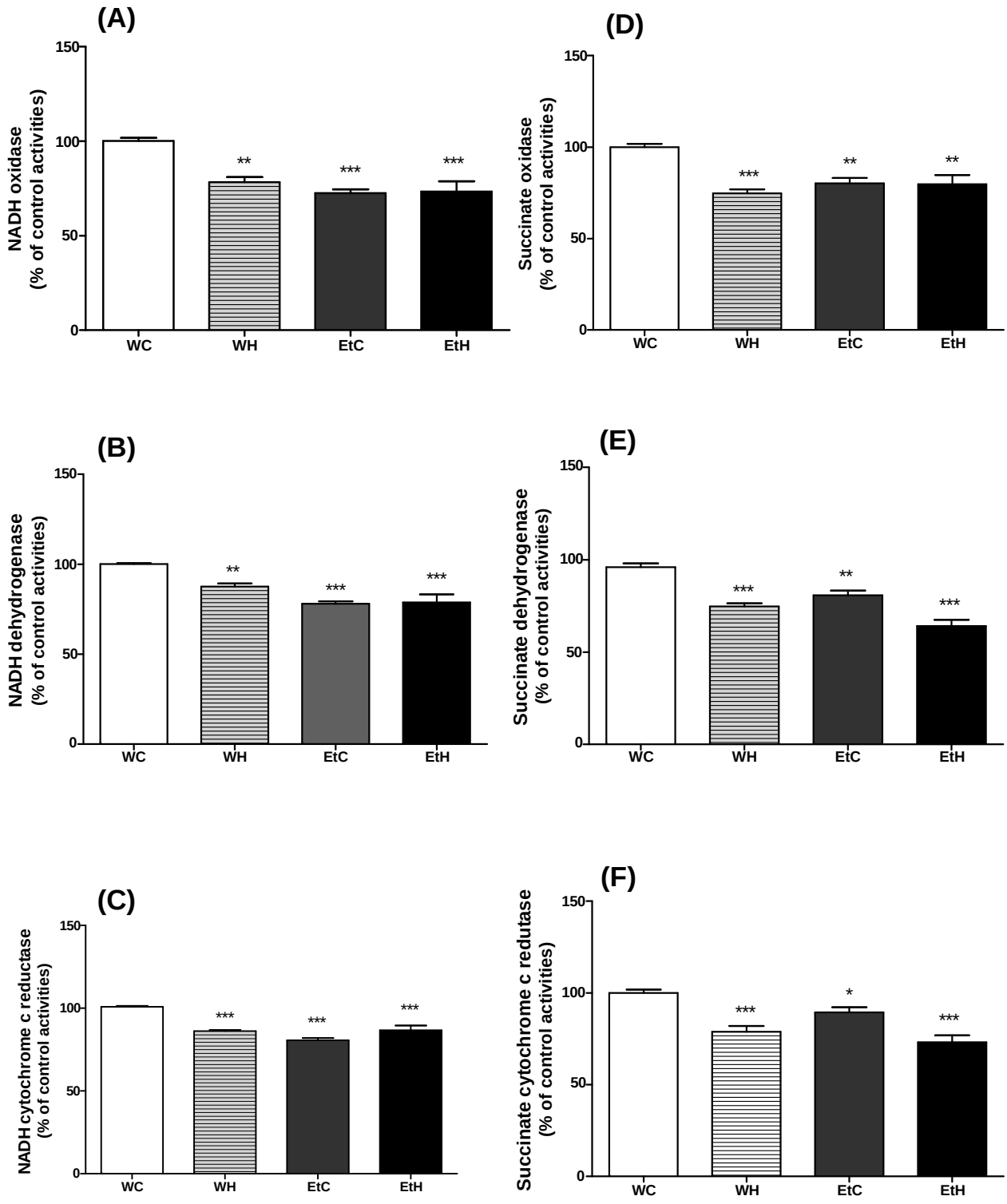

Figure 7. Effect of high-fat diet and ethanol on the enzymatic activities of the hepatocyte respiratory chain in rats. Control values (100%) were: **(A)** NADH oxidase: 73 ± 19 nmol O_2 consumed $min^{-1}{\cdot}mg^{-1}$ mitochondrial protein; **(B)** NADH dehydrogenase: 78 ± 13 nmol of ferricyanide reduced in $min^{-1}{\cdot}mg^{-1}$ mitochondrial protein; **(C)** NADH cytochrome c reductase: 86 ± 9 nmol of cytochrome c reduced $min^{-1}{\cdot}g^{-1}$ mitochondrial protein; **(D)** succinate oxidase: 80 ± 18 nmol O_2 consumed $min^{-1}{\cdot}mg^{-1}$ mitochondrial protein; **(E)** succinate dehydrogenase: 64 ± 13 nmol of DCPIP reduced $min^{-1}{\cdot}mg^{-1}$ mitochondrial protein; and **(F)** succinate cytochrome c reductase: 73 ± 12 nmol of cytochrome c reduced $min^{-1}{\cdot}mg^{-1}$ mitochondrial protein. Results (mean ± SEM of 4 independent experiments) are expressed as % of control activities. ANOVA followed by the Tukey test as a *post-hoc* analysis was used for statistical comparison. Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.5 Discussion

Hepatic steatosis is a primary response to the chronic consumption of ethanol in over 90% of individuals (Schwabe and Wiley, 2011). In accordance with this, our data showed lipid accumulation in hepatocytes of the animals that developed AHS by consuming an HFD with 10% ethanol (Figure 3-4). The HFD, which was represented by sunflower seeds, was selected because of the palatable taste of these seeds for rodents (Abdel-Gawad and Taha, 2011; Abbas *et al.* 2008; Jabbar et al, 2008), and because its protein amount is similar to that of the regular chow (supplementary material), allowing a comparison between both diets. Another aspect that should be considered for the establishment of this model is the low cost of this diet (only 5%) compared with the cost of a low-protein diet, which is also used in combination with ethanol in a steatosis model (Lívero, 2012).

The accumulation of hepatic triglycerides is closely related to the development of liver injury (Monsénégo, 2012). Both morphological and gravimetric methods confirmed the accumulation of intrahepatic lipids, which were mainly triglycerides (Figures 3-4). We observed discrete microsteatosis when supplied HFD and water to the group WH. However, the severity of microsteatosis was intensified when the HFD was combined with ethanol, which demonstrates the involvement of ethanol in the development of steatosis (Dey and Cederbaum, 2006; Ronis et al*,* 2010; Bharrhan *et al.* 2011; Kirpich *et al.* 2012). In contrast, the macrosteatosis seems to be associated with HFDs, but it does not appear to be associated with ethanol (Table 1). Hepatic steatosis was present in at least 5% of histological sections, which already characterizes the liver injury (Hoyumpa *et al.* 1975; Albano, 2008).

The characterization of the steatosis model investigated in this work can also be seen in the gross energy present in the food consumed by the animals over 4 weeks of receiving the diet. The rats that had a higher gross energy intake also tended to have an increased prevalence of AHS, which was confirmed in the histopathological observations (Figure 3). It is clear that the combination of 10% ethanol and an HFD, which accelerated microsteatosis, macrosteatosis, and hepatocyte tumefaction, caused the worsening of steatosis and the deployment of the liver disease. It is important to emphasize that steatosis or other liver injuries were not induced with 10% ethanol with regular chow (EtC). The plasma levels of

ALT, which is an important indicator of cellular hepatic lesions (Sathaye *et al.* 2011; Chacko *et al.* 2011), were increased in the WH, EtCH, and EtH groups, but it was not in EtC group (Figure 5A). ALT is very active in the liver; thus, it can be easily detected in small quantities in the plasma after liver injuries. Enzyme release can be caused by hepatocyte lysis and by increases in plasma membrane permeability (Babcock *et al.* 1981; Christoff *et al.* 2008). Our data, which suggest that the plasma ALT is more influenced by the HFD than by the ethanol, corroborate those of Demori et al. (2006).

Hochgraf *et al*. (1997) demonstrated that oxidized linoleic acid, which is present in sunflower seeds, promotes a significant increase in the plasma cholesterol levels in rats. In contrast, some other studies reported reduced levels of plasma cholesterol and triglycerides in rats and pigs in response to the consumption of oxidized dietary oil (Eder, 1990; Eder and Kirchgessner, 1999; Eder, 1999; Eder and Stangl, 2000; Eder, 2003; Acikgoz *et al.* 2011). In this context, our results demonstrated increased cholesterol levels with HFD, which agrees with the proposal of Hochgraf et al. (1997). This increase was not associated or intensified by the presence of ethanol in the diet (Figure 5B). The higher plasma cholesterol levels might be related to impaired liver uptake of cholesterol (Hochgraf *et al.* 1997).

Considering the liver injuries observed, we measured biomarkers of hepatic oxidative stress, because increases of oxidative stress are an essential factor in the development of secondary lesions of chronic alcoholism (Berr *et al.* 2001; Henzel, 2004). Chronic ethanol consumption diminishes the level of cellular antioxidants such as reduced glutathione and renders hepatocytes more susceptible to free radical–induced injury by means of unimpeded lipid peroxidation. Our results corroborate this idea because the hepatic GSH level was reduced in the EtH, EtC, and EtCH groups (Figure 6C). According to these findings, the activity of GST, which is involved in the metabolism of xenobiotics and also has an important antioxidant function, was decreased in the EtH and EtCH groups (Figure 6B), showing a partial reduction in the detoxification capacity of those livers (Lu, 2010; Tiwari and Chopra, 2012). Also, the enzymatic activity of SOD increased in the presence of ethanol and the HFD (Figure 5A). SOD is highly efficient in the catalytic removal of $O_2^{\bullet -}$ through its dismutation to H_2O_2 (Tiwari and Chopra, 2012). Increased SOD activity can lead to the production of toxic levels of H_2O_2 because it is generated from $^{\bullet}OH$, which is more reactive than

$O_2^{\bullet-}$. The increased $^{\bullet}OH$ levels could be prevented by Cat, which reduces H_2O_2 to water. Thus, simultaneous to SOD role, a rise in Cat activity is essential for an overall beneficial effect and an increased SOD activity (Sathaye *et al.* 2011). In our study, the higher SOD activity was not accompanied by changes in Cat activity, which could promote the accumulation of H_2O_2 and, consequently, an increase in the generation of $^{\bullet}OH$. This radical can attack hepatocyte membranes, resulting in a significant lipoperoxidation. The increased level of LPO that we observed in the livers from both the EtH and EtCH groups confirmed this hypothesis (Figure 6D).

A larger attack on free fatty acids in the membrane of hepatocytes reduces mitochondrial activity (Yang, 2000), as observed in our study. Fat accumulation in hepatocytes is the result of imbalanced fat metabolism, such as decreased mitochondrial lipid oxidation and enhanced synthesis of triglycerides. Therefore, the development of hepatic steatosis is associated with increased values of oxidative stress and structural defects in mitochondria (Sanyal, 2001; Carabelli *et al.* 2011), and it impacts mitochondrial respiration (Carabelli *et al.* 2011). In this work, the activity of segments of the mitochondrial respiratory chain was analyzed in isolated hepatic mitochondria, thus accessing the sites in the respiratory chain on which inhibitions in response to diet occur. Our results show that the oxidation of NADH and succinate was partially inhibited in the WH, EtCH, and EtH groups. Since differences among the groups were observed, we suggest that both factors, ethanol and HFD, can influence the inhibition, but with different intesity. This result is consistent with that of Chacko et al. (2011). Also, the combination of dietary ethanol and HFD seems to increase this inhibition. In fact, the activities of complexes I and II were impaired, and complex I was the most affected. These results suggest that the combination of ethanol and sunflower seeds in the diet impairs the mitochondria in AHS, contributing to decreased functioning of the oxidative phosphorylation system and depressed rates of ATP synthesis.

Despite of the forced ethanol consumption in drinking water of the rats, what can be pointed as a limitation of the proposal method (Brandon-Warner et al, 2012), this model was efficient for investigating the AHS. Liver steatosis was successfully induced in rats after 4 weeks of receiving a diet with 10% ethanol and HFD. The liver function was modified and the alterations were identified by morphological analysis, oxidative stress biomarkers, plasmatic parameters, and mitochondrial activity. Thus,

the combination of ethanol and sunflower seeds produced an interesting and inexpensive model to study ALD, what can be used as a pathological or pharmacological tool in this field of investigation.

3.6 Acknowledgements

The authors are grateful to Isabella Aviles Fabosi and Dr. Lauro Mera de Souza (UFPR) for the inestimable help in the experiments, and to CAPES and Fundação Araucária (Protocols # 18292 and 18781) for the financial support.

3.7 References

Abbas, T.E.E. and Yagoub, Y.M. (2008). Sunflower cake as a substitute for groundnut cake in commercial broiler chick diets. Pakistan J Nut. *7*:782–784.

Abdal-Gawad, H. and Taha, H. (2011). Bioavailability and toxicological potential of sunflower-bound residues of 14 C-chlorpyrifos insecticide in rats. J Environ Sci Health B Part B. *46*:683–690.

Acikgoz, Z., Bayraktar, H., Altan, O., Akhisaroglu, S.T., Kırkpınar, F. and Altun, Z. (2011). The effects of moderately oxidised dietary oil with or without vitamin E supplementation on performance, nutrient digestibility, some blood traits, lipid peroxidation and antioxidant defence of male broilers. J Sci Food Agric. *91*(7):1277-1282.

Aebi, H. (1984). Catalase in vitro. Methods Enzymol.*105*:121-126.

Albano, E. (2008). Oxidative mechanisms in the pathogenesis of alcoholic liver disease: Review. Mol Aspects Med. *29*:9–16.

Albano, E. (2008). New concepts in the pathogenesis of alcoholic liver disease. Expert Rev Gastroenterol Hepatol. *6*:749-759.

Babcock, J.L., Suber, R.L., Frith, C.H. and Geren C.R. (1981). Systemic effect in mice of venom apparatus extract and toxin from the brown recluse spider (Loxosceles reclusa). Toxicon. *19*:463–471.

Bharrhan, S., Koul, A., Chopra, K. and Rishi, P. (2011). Catechin suppresses an array of signalling molecules and modulates alcohol-induced endotoxin mediated liver injury in a rat model. PLoS One. *6*(6):e20635.

Bird, G.L. and Williams, R. (1998). Factors determining cirrhosis in alcoholic liver disease. Mol Aspects Med. *10*(2):97–105.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. USA Anal Biochem. *72*:248-254.

Brandon-Warner, E., Schrum, L.W., Schmidt, C.M. and McKillop, I.H. (2012). Rodent models of alcoholic liver disease: of mice and men. Alcohol. *46*(8):715-725.

Byun, J.S. and Jeong, W.I. (2010). Involvement of hepatic innate immunity in alcoholic liver disease. Immune Netw. *10*:6:181-187.

Caballero, V.J., Mendieta, J.R., Giudici, A.M., Crupkin, A.C., Barbeito, C.G., Ronchi, V.P., Chisari, A.N. and Conde, R.D. (2011). Alternation between dietary protein depletion and normal feeding cause liver damage in mouse. J Physiol Biochem. *67*(1), 43-52.

Chacko, B.K., Srivastava, A., Johnson, M.S., Benavides, G.A., Chang, M.J., Ye, Y., Jhala, N, Murphy, M.P., Kalyanaraman, B. and Darley-Usmar, V.M. (2011). Mitochondria-targeted ubiquinone (MitoQ) decreases ethanol-dependent micro and macro hepatosteatosis. Hepatology. *54*:153-163.

Comporti, M., Signorini, C., Leoncini, S., Gardi, C., Ciccoli, L., Giardini, A., Vecchio, D. and Arezzini, B. (2010). Ethanol-induced oxidative stress: basic knowledge. Genes Nutr. *5*:101–109.

Curry-McCoy, T.V., Osna, N.A., Nanji, A.A. and Donohue, T.M.Jr. (2010). Chronic ethanol consumption results in atypical liver injury in copper⁄zinc superoxide dismutase deficient mice. Alcohol Clin Exp Res. *34*:251-261.

Das, S.K. and Vasudevan, D.M. (2007). Alcohol-induced oxidative stress. Life Sci. *81*:177–187.

De Oliveira Christoff, A., de Oliveira, A., Chaim, O.M., Lugarini, D., Bastos Pereira, A.L., Paludo, K.S., Queiroz Telles, J.E., Bracht, A., Veiga, S.S. and Acco, A. (2008). Effects of the venom and the dermonecrotic toxin LiRecDT1 of Loxosceles intermedia in the rat liver. Toxicon. *52*:695–704.

Demori, I., Voci, A., Fugassa, E. and Burlando, B. (2006). Combined effects of high-fat diet and ethanol induce oxidative stress in rat liver. Alcohol. *40*:185-191.

Dey, A. and Cederbaum, A.I. (2006). Alcohol and oxidative liver injury. Hepatology. *43*(2 Suppl 1):S63-74.

Di Pascoli, L., Lion, A., Milazzo, D. and Caregaro, L. (2004). Acute liver damage in anorexia nervosa. Int J Eat Disord. *36*(1):114-117.

Eder, K. and Kirchgessner, M. (1999). The effect of a moderately thermoxidised dietary fat on the vitamin E status, the fatty acid composition of tissue lipids, and the susceptibility of low-density lipoproteins to lipid peroxidation in rats. Lipids. *101*:178–184.

Eder, K. (1999). The effect of an oxidised dietary oil on plasma cholesterol and thyroid hormone concentrations in miniature pigs fed on a hyperlipidaemic diet. J Anim Physiol Anim Nutr. *82*:271–281.

Eder, K. (1999). The effects of a dietary oxidised oil on lipid metabolism in rats. Lipids. *34*:717–725.

Eder, K. and Stangl, G.I. (2000). Plasma thyroxine and cholesterol concentrations of miniature pigs are influenced by thermally oxidised dietary lipids. J Nutr. *130*:116-121.

Eder, K., Keller, U., Hirche, F. and Brandsch, C. (2003). Thermally oxidised dietary fats increase the susceptibility of rat LDL to lipid peroxidation but not their uptake by macrophages. J Nutr.*133*:2830–2837.

Fromenty, B., Berson, A. and Pessayre, D. (1997). Microvesicular steatosis and steatohepatitis: role of mitochondrial dysfunction and lipid peroxidation. J Hepatol. *26*(Suppl 1):13-22.

Gao, R. et al. (1998). Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. Bioelectrochem Bioenerg. *45*(1):41-45.

Habig, W.H., Papst, M.J. and Jakoby W.B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem. *249*(22):7130-7139.

Hochgraf, E., Mokady, S. and Cogan, U. (1997). Dietary oxidised linoleic acid modifies lipid composition of rat liver microsomes and increases their fluidity. J Nutr. *127*:681–686.

Jabbar, M.A., Ahmad, S. and Riffat, S. (2008). Effect of replacing cotton seed cake with sunflower meal in the rations of lactating crossbred cows. J Vet Anim Sci. *1*:11–13.

Jiang, Z.Y., Woollard, A.C. and Wolff, S.P. (1991). Lipid hydroperoxide measurement by oxidation of Fe_2^+ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids. *26*(10):853-856.

Korourian, S., Hakkak, R., Ronis, M.J., Shelnutt, S.R., Waldron, J., Ingelman-Sundberg, M. and Badger, T.M. (1999). Diet and risk of ethanol-induced hepatotoxicity: carbohydrate-fat relationships in rats. Toxicol Sci. *47*(1):110-117.

Liber, C. S. (2004). Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol. *34*:9–19.

Lívero, F.A.R. (2012). Esteatose hepática alcoólica: correlações com receptor nuclear FXR e estresse oxidativo (FXR agonist 6ECDCA reduces hepatic steatosis and oxidative stress induced by ethanol in mice). (2012) Master Thesis. Federal University of Paraná, Curitiba, Brazil, http://hdl.handle.net/1884/27139.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement whit the Folin phenol reagent. J Biol Chem. *193*:265-275.

Mason, T.L. *et al*. (1973) Cytocrome c oxidase from bakers yeast, isolation and properties. J Biol Chem. *248*:1346–1354.

Miranda-Mendez, A., Lugo-Baruqui, A. and Armendariz-Borunda, J. (2010). Molecular basis and current treatment for alcoholic liver disease. Int J Environ Res Public Health. *7*:1872-1888.

Oller do Nascimento, C.M. and Williamson, D.H. (1986). Evidence for conservation of dietary lipid in the rat during lactation and the immediate period after removal of the litter. Decreased oxidation of oral [1-14C] triolein. Biochem J. *239*:233–236.

Pessayre, D. and Fromenty, B. (2005). NASH a mitochondrial disease. J Hepatol. *42*:928–940.

Pessayre, D., Fromenty, B., Berson, A., Robin, M.A., Lettéron, P., Moreau, R. and Mansouri, A. (2012). Central role of mitochondria in drug-induced liver injury. Drug Metab Rev. *44*(1):34-87.

Powel, E.E., Jonsson, J.R. and Clouston, A.D. (2005). Steatosis: co-factor in other liver diseases. Hepatology. *42*:5–13.

Rautou, P.E., Cazals-Hatem, D., Moreau, R., Francoz, C., Feldmann, G., Lebrec, D., Ogier-Denis, E., Bedossa, P., Valla, D. and Durand, F. (2008). Acute liver cell damage in patients with anorexia nervosa: a possible role of starvation-induced hepatocyte autophagy. Gastroenterology. *135*(3):840-848, 848.e1-3.

Ronis, M.J., Korourian, S., Blackburn, M.L., Badeaux, J. and Badger, T.M. (2010). The role of ethanol metabolism in development of alcoholic steatohepatitis in the rat. Alcohol. *44*(2):157-169.

Sathaye, S., Bagul, Y., Gupta, S., Kaur, H. and Redkar, R. (2011). Hepatoprotective effects of aqueous leaf extract and crude isolates of Murraya koenigii against in vitro ethanol-induced hepatotoxicity model. Exp Toxicol Pathol. *63*:587–591.

Sedlak, J. and Lindsay, R.H. (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. *25* (1):192-205.

Singer, T.P. and Gutman, M. (1971). The DPNH dehydrogenase of the mitochondrial respiratory chain. Adv Enzymol Relat Areas Mol Biol. *34:*79-153.

Somlo, M. (1965). Induction des lactico-cytocrome c reductases (D-ET L-) de la levure aerobie par les lactates (D-ET-L). Biochim Biophys Acta *97*:183–201.

Sozio, M.S., Liangpunsakul, S. and Crabb, D. (2010). The role of lipid metabolism in the pathogenesis of alcoholic and nonalcoholic hepatic steatosis. Semin Liver Dis. *30*(4):378-390.

Tilg, H., Moschen, A.R. and Kaneider, N.C. (2011). Pathways of liver injury in alcoholic liver disease. J Hepatol. *55*:1159–1161.

Voss, D.O., Campelo, A.P. and Bacila, M. (1961). The respiratory chain and the oxidative phosphorylation of rat brain mitochondria. Biochem Biophys Res Commun. *4*:48-51.

3.8 Supplementary Material

3.8.1 Chemical analysis of the chow used in the experiments

UNIVERSIDADE FEDERAL PARANÁ
DEPARTAMENTO DE ZOOTECNIA
LABORATÓRIO DE NUTRIÇÃO ANIMAL
www.labzoo.ufpr.br
labzoo@ufpr.br
TEL.: (0xx41) 3350-5627
FAX. : (0xx41) 3252-4149

Curitiba, 01 de Agosto de 2012.

LAUDO DE ANÁLISE QUÍMICA

AMOSTRA NÚMERO: 12073001

INGRESSO DA AMOSTRA: 30 / 07 / 2012

MATERIAL: RAÇÃO

RESPONSÁVEL: Dra. ALEXANDRA ACCO

EMPRESA/CIDADE: UFPR

Umidade 105°C	(%):	12,30	Cálcio	(%):	1,32
Proteína Bruta	(%):	21,96	Fósforo	(%):	0,82
Extrato Etéreo	(%):	4,61	Sódio	(%):	-
Resíduo Mineral	(%):	8,36	Potássio	(%):	-
Fibra Bruta	(%):	4,04	Peróxido	(meq/1000g):	-
FDA	(%):	-	Acidez	(mg NaOH/g):	-
FDN	(%):	-			
ENN	(%):	48,73	Energia bruta	(kcal/kg):	3913,0
NDT	(%):	-			

- **Este laudo corresponde à amostra enviada ao Laboratório de Nutrição Animal UFPR**

LAUDOS ENVIADO POR E-MAIL

Prof. Dr. Alex Maiorka CRMV – Z 00667 - PR
Coordenador do Laboratório

3.8.2 Chemical analysis of the sunflower seeds (peeled) used in the experiments

UNIVERSIDADE FEDERAL PARANÁ
DEPARTAMENTO DE ZOOTECNIA
LABORATÓRIO DE NUTRIÇÃO ANIMAL
www.labzoo.ufpr.br
labzoo@ufpr.br
TEL.: (0xx41) 3350-5627
FAX. : (0xx41) 3252-4149

Curitiba, 01 de Agosto de 2012.

LAUDO DE ANÁLISE QUÍMICA

AMOSTRA NÚMERO: 12073002

INGRESSO DA AMOSTRA: 30 / 07 / 2012

MATERIAL: GIRASSOL SEM CASCA

RESPONSÁVEL: Dra. ALEXANDRA ACCO

EMPRESA/CIDADE: UFPR

Umidade 105°C	(%):	2,93	Cálcio	(%):	0,22
Proteína Bruta	(%):	28,19	Fósforo	(%):	0,89
Extrato Etéreo	(%):	58,62	Sódio	(%):	-
Resíduo Mineral	(%):	3,97	Potássio	(%):	-
Fibra Bruta	(%):	0,36	Peróxido	(meq/1000g):	-
FDA	(%):	-	Acidez	(mg NaOH/g):	-
FDN	(%):	-			
ENN	(%):	2,93	Energia bruta	(kcal/kg):	6888,0
NDT	(%):	-			

- Este laudo corresponde à amostra enviada ao Laboratório de Nutrição Animal UFPR

LAUDOS ENVIADO POR E-MAIL

Prof. Dr. Alex Maiorka CRMV – Z 00667 - PR
Coordenador do Laboratório

4. CONSIDERAÇÕES FINAIS

Com os resultados obtidos neste trabalho, podemos concluir que:

- No modelo proposto, a função hepática foi modificada e as alterações foram encontradas tanto nas análises morfológicas, pela presença de tumefação e acúmulo de lipídeos intracelulares, quanto nos biomarcadores de estresse oxidativo SOD, GST, GSH e LPO, e nos parâmetros plasmáticos ALT e colesterol. Igualmente, a atividade mitocondrial hepática foi inibida nos complexos I e II pela dieta com etanol e semente de girassol.

- A combinação de etanol 10% associado à semente de girassol produz um modelo de estudo alternativo para a esteatose hepática alcoólica em ratos, levando à evolução da enfermidade com fidedignidade à doença em humanos. Deste modo, o modelo servirá como uma ferramenta para futuros estudos mecanísticos e farmacológicos da EHA.

5. REFERÊNCIAS BIBLIOGRÁFICAS ADICIONAIS

ARTEEL, G. *et al*. Advances in alcoholic liver disease. **Best Practice & Research Clinical Gastroenterology**, v. 17, n. 4, p. 625-647, 2003.

ARTEEL, G. Animal models of alcoholic liver disease. **Digestive diseases**, v. 28, n. 6, p. 729-736, 2010.

BAILEY, S. M. *et al*. Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1 system and protein oxidation status in rat liver. **Alcoholism, Clinical and Experimental Research.** v. 25, n. 5, p. 726-733, 2001.

BATRA, S. C. Gastric metabolism of ethanol in Syrian golden hamster. **Digestive Disease Science**, v. 40, n. 12, p. 2712-2716, 1995.

BERR, C. *et al.* Alcohol: Health effects. **Expertise Collective**. France. 2001

BRASIL. Ministério da Saúde, A Política do Ministério da Saúde para a Atenção Integral a Usuários de Álcool e outras Drogas. Série B. Textos Básicos de Saúde Brasília – DF, 2003. Acesso no site http://bvsms.saude.gov.br/bvs/publicacoes/pns_alcool_drogas.pdf em 16/08/2011 às 12:45hr.

ANDERSON, C. L. *et al*. Protein-calorie malnutrition associated with alcoholic hepatitis. Veterans Administration Cooperative Study Group on Alcoholic Hepatitis. **American Journal of Medicine**, v. 76, n. 2, p. 211–222, 1984.

CARLINI, E. A. *et al.* II Levantamento domiciliar sobre o uso de drogas psicotrópicas no Brasil: estudo envolvendo as 108 maiores cidades do país – 2005. Supervisão E.A.Carlini; Coordenação J.C.F Galduróz; Brasília: **Secretaria Nacional Antidrogas**, 2007.

CHRISTENSEN, E.; GLUUD, C. Glucocorticoids are ineffective in alcoholic hepatitis: a meta-analysis adjusting for confounding variables. ***Gut* - BMJ Journals**, v. 37, n. 1, p. 113–118, 1995.

COLELL, A. *et al*. Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. **Gastroenterology**, v. 115, n. 6, p. 1541-1551, 1998.

COMPORTI, M. *et al.* Ethanol-induced oxidative stress: basic knowledge. Review. **Genes Nutrition**. v. 5, n. 2, p. 101–109, 2010.

CORTEZ-PINTO, H. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. **JAMA**, v. 282, n. 17, p. 1659-1664, 1999.

CRABB, D.W. *et al*. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. **Proceeding of the Nutrition Society**, v. 63, n. 1, p. 49-63, 2004.

CURRY-MCCOY, T. V. *et al.* Chronic Ethanol Consumption Results in AtypicalLiver Injury in Copper⁄Zinc Superoxide Dismutase Deficient Mice. **Alcoholism: Clinical and Experimental Research**, v. 34, n. 2, p. 251–261, 2010.

DAS, S. K.; VASUDEVAN, D. M. Alcohol-induced oxidative stress. **Life Sciences**, v. 81, n. 3, p. 177–187, 2007.

DeNUCCI, S. M. *et al*. Rat strain differences in susceptibility to alcohol-induced chronic liver injury and hepatic insulin resistance. **Gastroenterology Research and Practice**, Article ID 312790 , 2010.

DEY, A.; CEDERBAUM, A. I. Alcohol and Oxidative Liver Injury. **Hepatology**, v. 43, n. 2, Suppl. 1, 2006.

FELDSTEIN, A. Hepatocytes apoptosis in patients with non-alcoholic steatohepatitis. Quantification and clinical relevance. **Gastroenterology**, v. 124, p. 703, 2003.

FERNANDEZ-CHECA, J. C.; OOKHTENS, M.; KAPLOWITZ, N. Effects of chronic ethanol feeding on rat hepatocytic glutathione: relationship of cytosolic glutathione to efflux and mitochondrial sequestration. **The Journal of Clinical Investigation**, v. 83, n. 4, p. 1247-1252, 1989.

FERNANDEZ-CHECA, J. C.; OOKHTENS, M.; KAPLOWITZ, N. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol- fed rats: tracer kinetic studies in vitro and in vivo and susceptibility to oxidant stress. **The Journal of Clinical Investigation**, v. 87, n. 2, p. 397-405, 1991.

FERNANDEZ-CHECA, J. C. Alcohol-induced liver disease: when fat and oxidative stress meet. **Annals of Hepatology**, v. 2, n. 2, p. 69–75, 2003.

FERNANDEZ-CHECA, J. C.; KAPLOWITZ, N. Hepatic mitochondrial glutathione: transport and role in disease and toxicity. **Toxicology and Applied Pharmacology**, v. 204, n. 3, p. 263–273, 2005.

FERNANDEZ-CHECA, J. C.; OOKHTENS, M.; KAPLOWITZ, N. Effects of chronic ethanol feeding on rat hepatocyte glutathione: compartmentation, efflux, and response to incubation with ethanol. **The Journal of Clinical Investigation**, v. 80, n. 1, p. 57–62, 1987.

FRENCH, S. W. *et al*. Effect of ethanol on cytochrome P450 2E1 (CYP2E1), lipid peroxida- tion, and serum protein adduct formation in relation to liver pathology pathogenesis. **Experimental and Molecular Pathology**, v. 58, p. 61-75, 1993.

GABBITA, S. P. *et al*. Redox regulatory mechanisms of cellular signal transduction. **Archives of Biochemistry and Biophysics**. v. 376, n. 1, p. 1-13, 2000.

GARCIA-RUIZ, C. *et al*. Evidence that the rat hepatic mitochondrial carrier is distinct from the sinusoidal and canalicular transporters for reduced glutathione: expression studies in Xenopus laevis oocytes. **Journal of Biological Chemistry**, v. 270, p. 15946-15949, 1995.

GARCIA-RUIZ, C. *et al*. Feeding S-adenosyl-L-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. **Hepatology**, v. 21, p.207-214, 1995.

GARCIA-RUIZ, C. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-kb: studies with isolated mitochondria and rat hepatocytes. **Molecular Pharmacology**, v. 48, 1995.

GILPIN, N. W. Vapor inhalation of alcohol in rats. **Current Protocols in Neuroscience**, v. 9, 2008

GRIFFITH C. M.; SCHENKER, S. The Role of Nutritional Therapy in Alcoholic Liver **Disease. Alcohol Research & Health** . v. 29, n. 4, p. 296-306, 2006

GYAMFI, M. A.; WAN, Y. J. Y. Pathogenesis of alcoholic liver disease: the role of nuclear receptors. Minireview. **Experimental Biology and Medicine**, v. 235, n. 4, p. 547–560, 2010.

HENZEL, K. *et al.* Toxicity of ethanol and acetaldehyde in hepatocytes treated with ursodeoxycholic or tauroursodeoxycholic acid. **Biochimica et Biophysica Acta**, v. 1644, p. 37– 45, 2004.

HIRANO, T. Hepatic mitochondrial glutathione depletion and progression of experimental alcoholic liver disease in rats. **Hepatology**, v. 16, p. 1423-1427, 1992.

HIRSCH, S.; DE LA MAZA, M.P.; GATTAS, V.; ET AL. Nutritional support in alcoholic cirrhotic patients improves host defenses. **Journal of the American College of Nutrition**, v. 18, n. 5, p. 434–441, 1999.

HOBBS, W. R.; RALL, T. W.; VERDOORN, T. A. Hypnotics and sedatives; ethanol. In: Hardman JG & Limbird LE (Ed). Goodman & Gilman's The Pharmacological Basis of Therapeutics. 9th ed**. McGraw Hill**,. 1905 p.p. 361-396, 1996.

HOYUMPA, A. M. Jr. *et al*. Fatt liver: biochemical and clinical considerations. **American Journal of Digestive Diseases**, v. 20, p. 1142-1170, 1975.

LIMURO, Y. *et al.* The glutathione precursor L-2-oxothiazolidine-4-carboxylic acid protects against liver injury due to chronic enteral ethanol exposure in the rat. **Hepatology**, v. 31, p. 391-398, 2000.

LIMURO, Y. *et al.* Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. **Hepatology**, v. 26, n. 6, p. 1530–1537, 1997.

KLAUS, S. Increasing the Protein: Carbohydrate Ratio in a high-fat diet delays the development of adiposity and improves glucose homeostasis in Mice1. **Journal of Nutrition**, v. 135, p. 1854-8, 2005.

KNECHT, K. T. *et al*. Free radical adducts in the bile of rats treated chronically with intragastric alcohol: inhibition by destruction of Kupffer cells. **Molecular Pharmacology**, v. 47, p. 1028-1034, 1995.

KNECHT, K. T.; THURMAN, R. G.; MASON, R. P. Role of superoxide and trace transition metals in the production of alpha-hydroxyethyl radical from ethanol by microsomes from alcohol dehydrogenase-deficient deermice. **Archives of Biochemistry and Biophysics**, v. 303, n. 2, p. 339–348, 1993.

KONISHI, T. The ADH3_2 and CYP2E1 c2 alleles increase the risk of alcoholism in Mexican American men. **Experimental and Molecular Pathology**. v. 74, n. 2, p. 183-189, 2003.

KONISHI, T. ADH1B_1, ADH1C_2, DRD2 (–141C Ins), and 5-HTTLPR are associated with alcoholism in Mexican American men living in Los Angeles. **Alcoholism: Clinical and Experimental Research**, v. 28, n. 8, p. 1145–1152, 2004.

LADERO, J. M. Polymorphisms of the glutathione S-transferases mu-1 (GSTM1) and theta-1 (GSTT1) and the risk of advanced alcoholic liver disease. **Scandinavian Journal** of **Gastroenterology**, v. 40, n. 3, p. 348–53, 2005.

LI Y. M. Effect of acute alcoholism on hepatic enzymes and oxidation ⁄ antioxidant in rats. **Hepatobiliary & Pancreatic Diseases International**. v. 3, p. 241–244, 2004.

Li, T. K. Quantifying the risk for alcohol-use and alcohol-attributable health disorders: present findings and future research needs. **Journal of Gastroenterology and Hepatology**, v. 23, Suppl. 1, p. S2-S8, 2008

LIEBER C. S. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. **Alcohol**, v. 34, n. 1, p. 9–19, 2004.

LIEBER, C. S. Choline fails to prevent liver fibrosis in ethanol-fed baboons but causes toxicity. **Heptology**, v. 5, n. 4, p. 561-572, 1985.

MANDAYAM, S.; JAMAL, M. M.; MORGAN, T. R. Epidemiology of alcoholic liver disease. **Seminars in Liver Disease**, v. 24, n. 3, p. 217-232, 2004.

MANN, R. E.; SMART, R. G.; GOVONI, R. The epidemiology of alcoholic liver disease. **Alcohol Research and Health**, v. 27, p. 209-219, 2003.

MCCULLOUGH, A. J.; O'CONNOR, J. F. Alcoholic liver disease: Proposed recommendations for the American College of Gastroenterology. **American Journal of Gastroenterology**, v. 93, n. 11, p. 2022– 2036, 1998.

MENDENHALL, C. *et al*. Relationship of protein calorie malnutrition to alcoholic liver disease: A reexamination of data from two Veterans Administration Cooperative Studies. **Alcoholism: Clinical and Experimental Research**, v. 19, n. 3, p. 635–641, 1995.

MENDEZ, E. *et al*. Molecular Basis and Current Treatment for Alcoholic Liver Disease. Review. **International Journal of Environmental Research and Public Health**, v. 7, n. 7, p. 1872–1888, 2010.

MEZEY, E. Dietary fat and alcoholic liver disease. **Hepatology**, v. 28, n. 4, p. 901-905, 1998.

MEZEY, E. Interaction between alcohol and nutrition in the pathogenesis of alcoholic liver disease. **Seminars in Liver Disease**, v. 11, n. 4, p. 340–348, 1991.

MILLER, A. M. *et al*. Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines. **Alcoholism: Clinical & Experimental Research**, v. 35, n. 5, p. 787-793, 2012.

MITSUYOSHI, H. *et al.* Ursodeoxycholic acid protects hepatocytes against oxidative injury via induction of antioxidants. **Biochemical and Biophysical Research Communications**, v. 263, p. 537–542, 1999.

MURRAY, C. J. L.; LOPEZ, A. D. The global burden of disease: a comprehensive assessment of mortality and disability, form diseases, injuries and risk factors in 1990 and projected to 2020. Cambridge, Massachusetts Harvard School of Public Health to World Health Organization and World Bank. **Global Burden of Disease and Injury Series**, v I, 1996.

NANJI, A. A. *et al*. Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-ethanol-fed rats. **Alcoholism: Clinical & Experimental Research**, v. 18, p. 1280-1285, 1994.

NATH, B.; SZABO, G. Alcohol-induced modulation of signaling pathways in liver parenchymal and nonparenchymal cells: implications for immunity. **Seminars in Liver Disease**, v. 29, n. 2, p. 166-177, 2009.

OH, S. I. *et al*. Chronic ethanol consumption affects glutathione status in rat liver. **The Journal of Nutrition**, v. 128, n. 4, p. 758-763, 1998.

OZARAS, R. N-acetylcysteine attenuates alcohol-induced oxidative stress in the rat. **The World Journal of Gastroenterology**, v. 9, n. 1, p. 125–128, 2003

PAQUETTE, A. Effects of ovariectomy on PPARalpha, SREBP-1c and SCD-1 gene expression in the rat liver. **Menopause**, v. 15, n. 6, p. 1169-1175, 2008.

PESSAYRE, D.; FROMENTY, B. NASH a mitochondrial disease. **Journal of Hepatology**, v. 42, n. 6, p. 928-940, 2005.

PICCHI, M. G. *et al*. A high-fat diet as a model of fatty liver disease in rats. **Acta Cirúrgica Brasileira**, v. 26, n. Suppl. 2, 2011.

PLEVRIS, J. N.; HAYES, P. C.; BOUCHIER, I. A. D. Ursodeoxycholic acid in the treatment of alcoholic liver disease. **Eur J Gastroenterol Hepatology**, v. 3, p. 653-656, 1991.

POLAVARAPU, R. *et al*. Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil. **Hepatology**, v. 27, n. 5, p. 1317-1323, 1998.

ROGERS, C. Q.; AJMO, J. M.; YOU, M. Adiponectin and Alcoholic fatty liver disease. **IUBMB Life**, v. 60, n. 12, p. 790-797, 2008.

ROUACH, H. *et al*. Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology. **Hepatology**, v. 25, n. 2, p. 351-355, 1997.

SANAL, M. G. The blind men ‘see’ the elephant – the many faces of fatty liver disease. **World Journal of Gastroenterology**, v. 14, n. 6, p. 831-844, 2008.

SCHENKER, S.; HALFF G. A. Nutritional therapy in alcoholic liver disease. **Seminars in Liver Disease**, v. 13, n. 2, p. 196–209, 1993.

SCHRAUWEN, P.; WESTERTERP, K. R. The role of high-fat diets and physical activity in the regulation of body Weight. **British Journal of Nutrition**. v. 84, n. 4, p. 417-27. 2000.

SHUKLA, S. D. *et al*. Binge Ethanol and Liver: New Molecular Developments. **Alcoholism: Clinical & Experimental Research**, p. 1–8, 2013.

SILVEIRA, D. X.; MOREIRA, F. G. Panorama atual de drogas e dependências. **Atheneu**, 2006.

SOZIO, M.; CRABB, D. W. Alcohol and lipid metabolism. **American Journal of Physiology**, v. 295, p. E10-E16, 2008.

SZABO, G.; BALA, S. Alcoholic liver disease and the gut-liver axis. **World Journal of Gastroenterology**, v. 16, n. 11, p. 1321-1329, 2010.

TSUCHIYA, M. *et al*. Interstrain differences in liver injury and one-carbon metabolism in alcohol-fed mice. **Hepatology**, v. 56, n. 1, p. 130-139, 2012.

TSUKAMOTO, H. *et al*. “Second hit” models of alcoholic liver disease. **Seminars in Liver Disease**, v. 29, n. 2, p. 178-187, 2009.

VALKO, M. *et al.* Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chemico-Biological Interactions**, v. 160, n. 1, p. 1–40, 2006.

VENKATRAMAN, A. *et al*.. Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. **Journal of Biological Chemistry**, v. 279, n. 21, p. 22092-22101, 2004.

WILFRED DE ALWIS, N. M.; DAY, C. P. Genetics of alcoholic liver disease and nonalcoholic fatty liver disease. **Seminars in Liver Disease**, v. 27, n. 1, p. 44–54, 2007.

WILLETT, W. C. Is dietary fat a major determinant of body fat? **American Journal of Clinical Nutrition**. v. 67, Suppl 3, p. 556S-562S, 1998.

WU, D.; CEDERBAUM, A. I. Oxidative stress and alcoholic liver disease. **Seminars in Liver Disease**, v. 29, n. 2, p. 141-54, 2009.

YANG S. *et al*. Mitochondrial adaptations to obesity-related oxidant stress. **Archives of Biochemistry and Biophysics**, v. 378, n. 2, p. 259-268, 2000.

YOU, M. *et al*. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). **The Journal of Biological Chemistry**, v. 277, n. 32, p. 29342-29347, 2002.

YOU, M. et al. Role of adiponectin in the protective action of dietary saturated fat against alcoholic fatty liver in mice. **Hepatology**, v. 42, n. 3, p. 568–577, 2005.

ZAKHARI, S.; LI T. K. Determinants of alcohol use and abuse: impact of quantity and frequency patterns on liver disease. **Hepatology**. v. 46, n. 6, p. 2032–9, 2007.