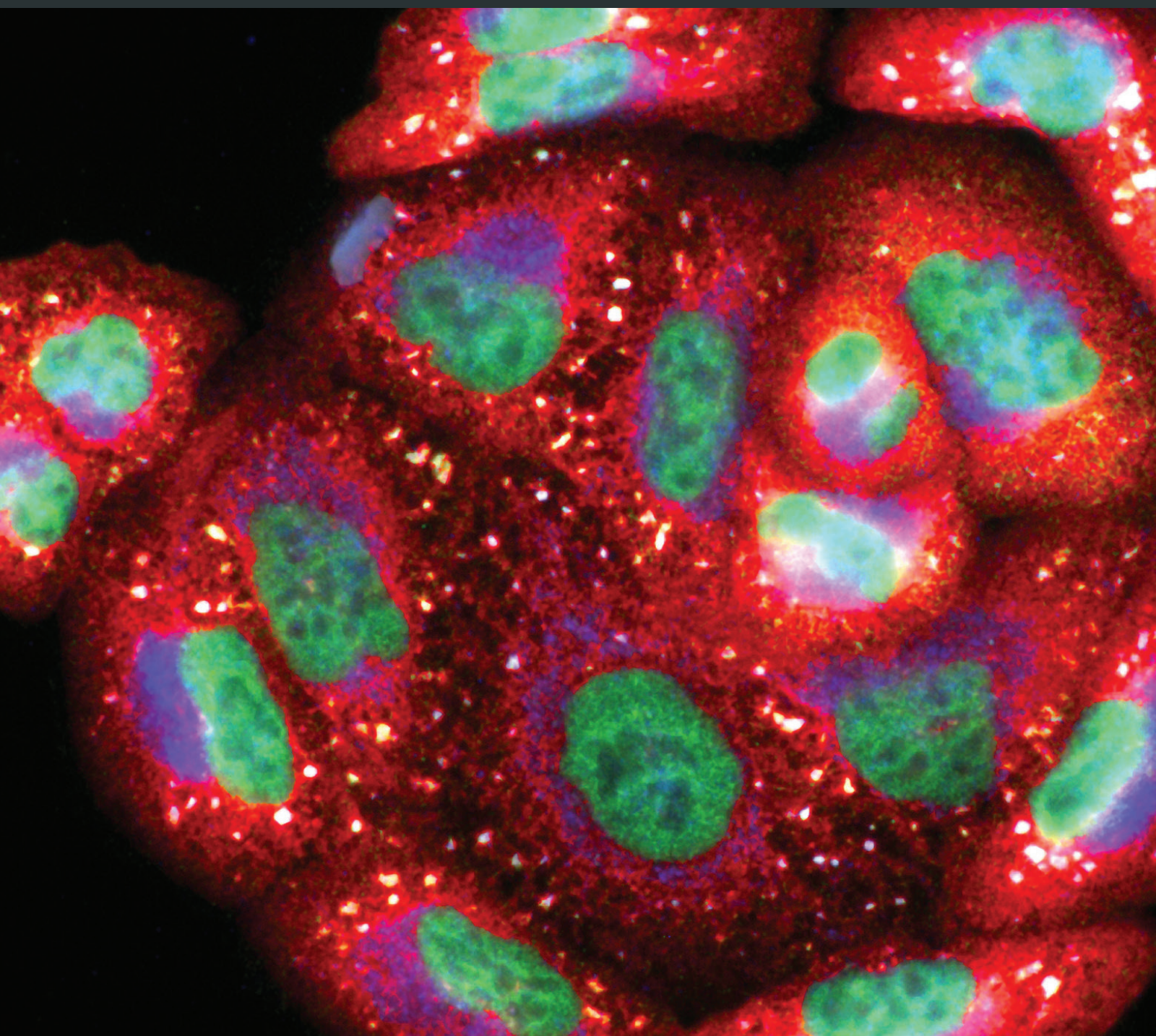
Oxidative Medicine and Cellular Longevity

Role of Oxidative Stress in Liver Health and Disease

Guest Editors: Pablo Muriel and Karina R. Gordillo



Role of Oxidative Stress in Liver Health and Disease

Oxidative Medicine and Cellular Longevity

Role of Oxidative Stress in Liver Health and Disease

Guest Editors: Pablo Muriel and Karina R. Gordillo

Copyright © 2016 Hindawi Publishing Corporation. All rights reserved.

This is a special issue published in "Oxidative Medicine and Cellular Longevity." All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Editorial Board

Mohammad Abdollahi, Iran
Antonio Ayala, Spain
Neelam Azad, USA
Peter Backx, Canada
Damian Bailey, UK
Consuelo Borrás, Spain
Vittorio Calabrese, Italy
Angel Catalá, Argentina
Shao-Yu Chen, USA
Zhao Zhong Chong, USA
Giuseppe Cirillo, Italy
Massimo Collino, Italy
Mark J. Crabtree, UK
Manuela Curcio, Italy
Andreas Daiber, Germany
Felipe Dal Pizzol, Brazil
Francesca Danesi, Italy
Domenico D'Arca, Italy
Yolanda de Pablo, Sweden
James Duce, UK
Grégory Durand, France
Javier Egea, Spain
Amina El Jamali, USA
Ersin Fadillioglu, Turkey
Qingping Feng, Canada
Giuseppe Filomeni, Italy
Swaran J. S. Flora, India
Rodrigo Franco, USA
José Luís García-Giménez, Spain
Janusz Gebicki, Australia
Husam Ghanim, USA
Laura Giamperi, Italy
Daniela Giustarini, Italy
Saeid Golbidi, Canada
Tilman Grune, Germany
Hunjoo Ha, Republic of Korea
Guido Haenen, Netherlands
Nikolas Hodges, UK
Tim Hofer, Norway
Silvana Hrelia, Italy
Maria G. Isaguliants, Sweden
Vladimir Jakovljevic, Serbia
Peeter Karihtala, Finland
Raouf A. Khalil, USA
Kum Kum Khanna, Australia
Neelam Khaper, Canada
Thomas Kietzmann, Finland
Mike Kingsley, UK
Ron Kohen, Israel
W. J.H. Koopman, Netherlands
Jean-Claude Lavoie, Canada
Christopher Horst Lillig, Germany
Paloma B. Liton, USA
Nageswara Madamanchi, USA
Kenneth Maiese, USA
Tullia Maraldi, Italy
Reiko Matsui, USA
Steven McAnulty, USA
Bruno Meloni, Australia
Trevor A. Mori, Australia
Ryuichi Morishita, Japan
Ange Mouithys-Mickalad, Belgium
Hassan Obied, Australia
Pál Pacher, USA
Valentina Pallottini, Italy
David Pattison, Australia
Serafina Perrone, Italy
Tiziana Persichini, Italy
Vincent Pialoux, France
Chiara Poggi, Italy
Aurel Popa-Wagner, Germany
Ada Popolo, Italy
José L. Quiles, Spain
Walid Rachidi, France
Kota V. Ramana, USA
Pranela Rameshwar, USA
Sidhartha D. Ray, USA
Alessandra Ricelli, Italy
Francisco J. Romero, Spain
Vasantha Rupasinghe, Canada
Gabriele Saretzki, UK
Honglian Shi, USA
Cinzia Signorini, Italy
Dinender K. Singla, USA
Richard Siow, UK
Shane Thomas, Australia
Rosa Tundis, Italy
Giuseppe Valacchi, Italy
Jeannette Vasquez-Vivar, USA
Victor M. Victor, Spain
Michal Wozniak, Poland
Sho-ichi Yamagishi, Japan
Liang-Jun Yan, USA
Guillermo Zalba, Spain
Jacek Zielonka, USA

Contents

Role of Oxidative Stress in Liver Health and Disease
Pablo Muriel and Karina R. Gordillo
Volume 2016, Article ID 9037051, 2 pages

Ion Channels and Oxidative Stress as a Potential Link for the Diagnosis or Treatment of Liver Diseases
Ana Ramírez, Alma Yolanda Vázquez-Sánchez, Natalia Carrión-Robalino, and Javier Camacho
Volume 2016, Article ID 3928714, 17 pages

Protective Role of Dietary Curcumin in the Prevention of the Oxidative Stress Induced by Chronic Alcohol with respect to Hepatic Injury and Antiatherogenic Markers
Ravi Varatharajalu, Mamatha Garige, Leslie C. Leckey, Karina Reyes-Gordillo, Ruchi Shah, and M. Raj Lakshman
Volume 2016, Article ID 5017460, 10 pages

Spironolactone Effect in Hepatic Ischemia/Reperfusion Injury in Wistar Rats
Julio César Jiménez Pérez, Araní Casillas Ramírez, Liliana Torres González, Linda Elsa Muñoz Espinosa, Marlene Marisol Perales Quintana, Gabriela Alarcón Galván, Homero Zapata Chavira, Francisco Javier Guzmán de la Garza, Carlos Rodrigo Cámara Lemarroy, Nancy Esthela Fernández Garza, Edelmiro Pérez Rodríguez, and Paula Cordero Pérez
Volume 2016, Article ID 3196431, 9 pages

Sympathetic Nervous System Control of Carbon Tetrachloride-Induced Oxidative Stress in Liver through α-Adrenergic Signaling
Jung-Chun Lin, Yi-Jen Peng, Shih-Yu Wang, Mei-Ju Lai, Ton-Ho Young, Donald M. Salter, and Herng-Sheng Lee
Volume 2016, Article ID 3190617, 11 pages

Oligonol Ameliorates CCl_4-Induced Liver Injury in Rats via the NF-Kappa B and MAPK Signaling Pathways
Jeonghyeon Bak, Nam Kyung Je, Hae Young Chung, Takako Yokozawa, Sik Yoon, and Jeon-Ok Moon
Volume 2016, Article ID 3935841, 12 pages

Is Liver Enzyme Release Really Associated with Cell Necrosis Induced by Oxidant Stress?
Martha Lucinda Contreras-Zentella and Rolando Hernández-Muñoz
Volume 2016, Article ID 3529149, 12 pages

Oxidative Stress in the Healthy and Wounded Hepatocyte: A Cellular Organelles Perspective
Tommaso Mello, Francesca Zanieri, Elisabetta Ceni, and Andrea Galli
Volume 2016, Article ID 8327410, 15 pages

Cholesterol Enhances the Toxic Effect of Ethanol and Acetaldehyde in Primary Mouse Hepatocytes
Anayelly López-Islas, Victoria Chagoya-Hazas, Benjamin Pérez-Aguilar, Mayrel Palestino-Domínguez, Verónica Souza, Roxana U. Miranda, Leticia Bucio, Luis Enrique Gómez-Quiroz, and María-Concepción Gutiérrez-Ruiz
Volume 2016, Article ID 9209825, 9 pages

The Protective Effects of Trypsin Inhibitor on Hepatic Ischemia-Reperfusion Injury and Liver Graft Survival
Lianyue Guan, Hongyu Liu, Peiyao Fu, Zhuonan Li, Peidong Li, Lijuan Xie, Mingang Xin, Zhanpeng Wang, and Wei Li
Volume 2016, Article ID 1429835, 9 pages

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9037051, 2 pages
http://dx.doi.org/10.1155/2016/9037051

Editorial
Role of Oxidative Stress in Liver Health and Disease

Pablo Muriel[1] and Karina R. Gordillo[2,3]

[1]*Laboratory of Experimental Hepatology, Department of Pharmacology, CINVESTAV-IPN, Avenida Instituto Politécnico Nacional 2508, Colonia San Pedro Zacatenco 07360, Apartado Postal 14-740, 07000 México, D.F., Mexico*
[2]*Lipid Research Laboratory, VA Medical Center, 50 Irving Street NW, Washington, DC 20422, USA*
[3]*Department of Biochemistry and Molecular Medicine, The George Washington University, 2300 I Street NW, Suite 530, Washington, DC 20037, USA*

Correspondence should be addressed to Pablo Muriel; pmuriel@cinvestav.mx

Received 24 November 2015; Accepted 24 November 2015

Copyright © 2016 P. Muriel and K. R. Gordillo. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Liver diseases are a worldwide medical problem because the liver is the principal detoxifying organ and maintains metabolic homeostasis. The liver metabolizes various compounds that produce reactive oxygen radicals (ROS). Prooxidants are ROS which can cause tissue liver damage and whose levels may be increased by certain drugs, infection, external exposures, tissue injury, and so forth. Oxidative stress can result from an increase in prooxidant formation or a decrease or deficiency in antioxidants. Molecular redox switches and oxygen sensing by the thiol redox proteome and by NAD/NADP and phosphorylation/dephosphorylation systems are bias involved in signaling, control, and balance redox of a the liver system.

Because of their reactivity, ROS readily interact with all cellular macromolecules. ROS cleave the phosphodiester bonds holding bases in RNA and DNA together, breaking the chain structure of RNA and DNA. Polyunsaturated fatty acids are also a major target for oxidation by ROS, in a process called lipid peroxidation that disrupts normal membrane structure leading to necrosis. In addition, ROS, especially the hydroxyl radical, oxidize the SH group of cysteine residues of proteins to the disulfides or to the sulfoxide or the sulfonic acid; since enzymatic activity depends on cysteine, enzymes are inactivated by ROS. Also oxidative stress contributes to fibrogenesis by increasing harmful cytokines such as transforming grown factor-β (TGF-β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α).

However, ROS are not always the bad guy; important transcription factors such as Nrf2, NF-kB, and AP-1 are activated by H_2O_2-dependent oxidation of thiol residues. These transcription factors subsequently activate many genes, some of which code for cellular antioxidants. Thus, low levels of ROS can cover up for high levels of ROS. Endogenous (e.g., glutathione) or exogenous antioxidants (mainly from diet) inhibit either formation of ROS or remove/scavenge the generated radicals. Due to the central role of oxidative stress on liver disease, this special issue was devoted to its implication in hepatic health and disease.

From Mexico, Dr. J. Camacho et al. (in "Ion Channels and Oxidative Stress as a Potential Link for the Diagnosis or Treatment of Liver Diseases") reviewed the link of ion channels and oxidative stress in hepatic injury of various etiologies; the main conclusion is that such association may be useful to develop new treatments for the principal liver diseases. The group of Dr. H.-S. Lee et al. from Taiwan (in "Sympathetic Nervous System Control of Carbon Tetrachloride-Induced Oxidative Stress in Liver through α-Adrenergic Signaling") found that the sympathetic nervous system allows oxidative stress to damage the liver, thus suggesting that targeting the hepatic α-adrenergic signaling may provide a therapeutic approach to fight liver disease. Obesity associated with excessive alcohol consumption produces fatty liver; in this regard. Professor M.-C. Gutiérrez-Ruiz et al. from Mexico (in "Cholesterol Enhances the Toxic Effect of Ethanol and Acetaldehyde in Primary Mouse Hepatocytes") found that the combination of ethanol and cholesterol in vitro produced a potent damage in steatotic hepatocyte. From Italy, Professor A. Galli et al. (in "Oxidative Stress in the Healthy and

Wounded Hepatocyte: A cellular Organelles Perspective") provided us with a very original review about the evolving concept of oxidative stress in the cellular hepatocyte compartments, highlighting the essential mechanisms of damage caused by free radicals.

The protective effect of dietary curcumin against alcohol induced liver disease and atherosclerosis was reported by the group of Professor M. R. Lakshman et al. from Washington (in "Protective Role of Dietary Curcumin in the Prevention of the Oxidative Stress Induced by Chronic Alcohol with respect to Hepatic Injury and Anti-atherogenic Markers"). Since ischemia/reperfusion (IR) injury is still an unsolved problem in the clinical practice, efforts are being made to prevent it; in this regard, Dr. P. C. Pérez et al. from Mexico demonstrated that spironolactone reduced liver damage produced by IR by increasing IL-6 production and catalase activity ("Spironolactone Effect in Hepatic Ischemia/Reperfusion Injury in Wistar Rats"). Oligonol, a low molecular weight polyphenol derived from lychee fruit, was reported by Dr. J.-O. Moon et al., from Korea, to ameliorate CCl_4-induced liver injury by antioxidant effects decreasing NF-κB activation via blockade of the MAPKs and Akt kinases ("Oligonol Ameliorates CCl_4-Induced Liver Injury in Rats via the NF-Kappa B and MAPK Signaling Pathways"). In the review made by Professor R. Hernández-Muñoz et al. from Mexico ("Is Liver Enzyme Release Really Associated with Cell Necrosis Induced by Oxidant Stress?"), the utility of liver enzymes as markers of oxidative stress is challenged. Dr. Li et al., from Taiwan ("The Protective Effects of Trypsin Inhibitor on Hepatic Ischemia-Reperfusion Injury and Liver Graft Survival"), studied the effect of ulinastatin on liver IRI and graft survival in mice and found that this compound affords significant protection to donor livers from cold IRI, probably by inhibition of proinflammatory cytokine release and modulating apoptosis.

These authors highlight both the importance of free radicals in the development, establishment and perpetuation of liver disease, and the potential therapeutic effect of compounds that directly or indirectly interfere with the prooxidant process.

Hopefully, this publication will provide a benchmark for future investigations evaluating a far greater body of basic and clinical evidence regarding the role of oxidative stress in liver health and disease as well the beneficial effect of antioxidant therapy to prevent or reverse hepatic injury.

Pablo Muriel
Karina R. Gordillo

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3928714, 17 pages
http://dx.doi.org/10.1155/2016/3928714

Review Article

Ion Channels and Oxidative Stress as a Potential Link for the Diagnosis or Treatment of Liver Diseases

Ana Ramírez,[1] Alma Yolanda Vázquez-Sánchez,[1] Natalia Carrión-Robalino,[1,2] and Javier Camacho[1]

[1]*Department of Pharmacology, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Avenida Instituto Politécnico Nacional 2508, 07360 Mexico City, DF, Mexico*
[2]*Departamento de Ciencias de la Vida, Universidad de las Fuerzas Armadas, ESPE, Avenida General Rumiñahui, 171-5-231-B Sangolquí, Ecuador*

Correspondence should be addressed to Javier Camacho; fcamacho@cinvestav.mx

Received 23 July 2015; Revised 22 October 2015; Accepted 27 October 2015

Academic Editor: Karina R. Gordillo

Copyright © 2016 Ana Ramírez et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Oxidative stress results from a disturbed balance between oxidation and antioxidant systems. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be either harmful or beneficial to the cells. Ion channels are transmembrane proteins that participate in a large variety of cellular functions and have been implicated in the development of a variety of diseases. A significant amount of the available drugs in the market targets ion channels. These proteins have sulfhydryl groups of cysteine and methionine residues in their structure that can be targeted by ROS and RNS altering channel function including gating and conducting properties, as well as the corresponding signaling pathways associated. The regulation of ion channels by ROS has been suggested to be associated with some pathological conditions including liver diseases. This review focuses on understanding the role and the potential association of ion channels and oxidative stress in liver diseases including fibrosis, alcoholic liver disease, and cancer. The potential association between ion channels and oxidative stress conditions could be used to develop new treatments for major liver diseases.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced during mitochondrial electron transport or by other enzyme systems comprising several oxidoreductases (such as NADPH oxidase which is critical for the bactericidal action of phagocytes) in all cells types, including hepatocytes [1, 2].

ROS play a dual role, because they can be either harmful or beneficial to the cells. The normal physiological ROS-mediated processes include cellular growth, cell proliferation and regeneration, apoptosis, and microbial killing by phagocytes [3]. The most relevant ROS in the cell physiology are superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) while the more common RNS are nitric oxide (NO) and peroxynitrite ($ONOO^{-\bullet}$).

ROS generation is essential to maintain cellular functions and ensure cell survival [4]; this is achieved through the activation of transcription factors, such as NF-kappa-B and hypoxia-inducible-factor-1α (HIF-1α). ROS also participate in vascular processes [5] and regulate the activation of the immune system by acting as intermediates for cytokines like tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) [6, 7]. However, overproduction of ROS and/or RNS, associated with a failure in the antioxidant system (superoxide dismutase, catalase, and glutathione peroxidase activities [8]), causes cellular oxidative stress that promotes DNA, protein, and lipid damage, triggering the development of several diseases [9–11].

Many oxidative stress-related diseases have been reported since the first introduction of this term in 1985 by Sies [12]. Some of the most studied conditions are neurodegenerative

diseases (Alzheimer, Parkinson, and others) [13–16], cellular aging [17], cardiovascular diseases (hypercholesterolaemia, heart failure, hypertension, myocardial infarction, ischemia/reperfusion injury, and atherosclerosis) [18–20], and pathologies involving chronic inflammation [21]. However, recent studies have also associated oxidative stress with diabetes mellitus [22], obesity [23], infectious diseases (HCV [24], malaria [25]), epilepsy [26], chronic pain [27, 28], and even preeclampsia [29].

2. Oxidative Stress and Ion Channels

Ion channels are multimeric proteins located in the plasma membrane and inner cell compartments forming ion-selective pores that open or close in response to specific stimuli such as membrane potential, ligand-binding, temperature, and mechanical stimuli [30]. ROS and RNS can directly induce posttranslational modification of ion channels leading to oxidation, nitrosylation, and/or nitration of specific amino acid residues (sulfhydryl groups or disulfide linkages involving cysteine residues) or indirectly modulate channel function by affecting the signaling pathways that control gene transcription, trafficking, and turnover [31, 32]. The association of ion channels in oxidative stress-related diseases is reported mostly in cardiovascular [33, 34] and neurodegenerative illnesses [35–38]. Multiple studies have reported the involvement of calcium [39–41], potassium [27, 30, 36, 42, 43], sodium [34], and chloride channels [44, 45] in the development of pathologies where oxidative stress plays a major role (Table 1). Ion channels have been also suggested to be associated with oxidative stress in the liver [32, 46] (Table 2). Next, we will describe some of the most studied plasma membrane and mitochondrial ion channels associated with oxidative stress damage, focusing primarily on their potential participation in liver pathological conditions.

2.1. Participation of Mitochondrial Ion Channels in Different Pathologies. Generation of high energy molecules is carried out by the mitochondria electron chain transport; hence a great amount of ROS is generated during this metabolism [47, 48]. Because mitochondria are involved in the defense against ROS and its effects on intracellular redox equilibrium, the study of mitochondria in pathological conditions associated with oxidative stress is fundamental [49, 50].

There is a huge diversity of ion channels in both the outer and inner mitochondrial membrane (OMM, IMM). Some of the ion channels located on the outer and inner membrane include voltage dependent anion channel (VDAC), Ca^{2+} uniporter, permeability transition pore (PTP), calcium-activated potassium channels ($K_{Ca^{2+}}$), ATP-sensitive potassium channels (K_{ATP}), and inner membrane anion channel (IMAC) [51]. The field of mitochondrial ion channels has recently seen some progress through an integrative approach using genetics, physiology, pharmacology, and cell biology tools, which have helped to elucidate the possible functions of these channels [52]. Some of these ion channels participate in several processes such as apoptosis [53, 54], necrosis, thermogenesis, and volume regulation.

The relevance of mitochondrial ion channel research is observed in numerous studies that have demonstrated its participation in the development of pathological conditions like neurodegenerative [35, 55] and cardiac diseases [51, 56–60]. Even though mitochondrial dysfunction is associated with different liver pathologies, such as alcoholic liver diseases (ALD) [49, 50, 61, 62], metabolic syndromes (insulin resistance) [63], and nonalcoholic fatty liver disease (NAFLD) [64], the participation of mitochondrial ion channels in the onset or development of oxidative stress-related liver diseases has not been fully explored.

Nakagawa et al. [65] reported the presence of mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channels in rat primary hepatocytes. This study demonstrated that diazoxide (a selective opener of mitoK_{ATP} channels) enhanced liver regeneration by keeping a higher ATP content in the liver tissue. They concluded that diazoxide sustains the hepatocytes mitochondrial energetics promoting liver regeneration after partial hepatectomy, which is characterized by the presence of oxidative stress. Indeed, potassium channel openers acting on mitochondria have proved to reduce cell damage observed in ischemia. For instance, the effect of cromakalim and diazoxide on cardioprotection against ischemia-reperfusion injury has been shown [66]. Another study carried out by Shimizu et al. [67] assessed the effect of diazoxide against brain damage after middle cerebral artery occlusion (MCAO) in male Wistar rats. The neurological score was improved in animals treated with diazoxide in comparison with the control group; the effects of diazoxide were prominent in the cerebral cortex. Accordingly, the protective effect was reversed with pretreatment of 5-hydroxydecanoate, a selective blocker of mitoK_{ATP}, proving that selective opening of mitoK_{ATP} channel has neuroprotective effects against ischemia-reperfusion injury in the rat brain.

Conductances reported for mitochondrial K_{ATP} channels of different cell types are lower than those reported for plasma membrane K_{ATP} variants, under the same ionic conditions. Moreover, Szabo and Zoratti [52] mention that small-conductance Ca^{2+}-activated K^+ channels have conductances compatible with the lower values reported for mitoK_{ATP} channels. Pharmacological approaches have their own complications due to the nonspecific targeting exhibited by some mitoK_{ATP} inhibitors (5-hydroxydecanoate) and openers (such as diazoxide and cromakalim) which can also act on plasma membrane K_{ATP} channels, according to some studies. Activators of K_{ATP} and SK_{Ca}/IK_{Ca} (small-conductance, SK_{Ca}; and intermediate-conductance (IK_{Ca}) calcium-activated potassium channels) have structural similarities, suggesting the possibility of a pharmacological crossover [52].

Interestingly, mitochondria associated-endoplasmic reticulum membranes (MAMs), which are important for Ca^{2+}, lipid, and metabolite exchange, were investigated by Arruda et al. [63] in the liver. They reported that the reorganization of MAMs in a background of obesity resulted in mitochondrial Ca^{2+} overload, which compromised mitochondrial oxidative capacity and increased oxidative stress. Even though this study did not address Ca^{2+} flux in mitochondria, its results indicate that obesity drives an abnormal increase in MAMs formation, along with an alteration in Ca^{2+} flux from the ER

Table 1: Ion channels involved in oxidative stress-related diseases.

Type	Channel	Type of dysregulation	Oxidative stress-related disease	Model	Alteration/pathophysiological effect	Ref.
Na^+ voltage-gated sodium channels (VGSCs)	$Na_v1.1$	Missense mutation	Idiopathic epilepsy	Patients	Increase in sodium influx. Patients show variable seizure types, including absence, myoclonic, tonic-clonic, and partial seizures.	[26, 37, 193–196]
	$Na_v1.2$	Missense mutation				
	$Na_v1.5$	Punctual mutation (SNP)	Coronary microvascular dysfunction and ischemic heart disease (IHD)	Patients/population study	Polymorphism rs1805124_GG associated with a higher risk to develop IDH.	[34, 193, 195]
	$Na_v1.6$	Large persistent sodium current	Neurodegenerative diseases	Mouse model	The large persistent current produced by $Na_v1.6$ may play a role in a damaging injury cascade when coexpressed with Na+/Ca+ exchanger in demyelinated axons.	[193, 195, 197]
	$Na_v1.7$	Gain-of-function mutation	Neuropathic pain	Patients	Hyperexcitability of neurons; acute or chronic pain.	[28, 195, 198, 199]
		Loss-of-function mutation	Congenital insensitivity to pain	Patients	Indifference to pain.	[28, 195, 198–200]
	$Na_v1.8$	Gain-of-function mutation	Neuropathic pain	Patients	Mutations contribute to painful peripheral neuropathy by enhancement of the channel's response to depolarization and produce hyperexcitability in DRG neurons.	[193, 195, 201]
	$Na_v1.9$	Gain-of-function mutation	Neuropathic pain	Patients	Gain-of-function mutations in this channel are suggested to contribute to pain, autonomic dysfunction, and axonal degeneration in patients with peripheral neuropathy.	[193, 195, 202]
Potassium channels	$K_{ir}6.1$	Punctual mutation (SNP)	Coronary microvascular dysfunction and ischemic heart disease (IHD)	Patients	The polymorphism rs5219_AA of $K_{ir}6.2$ is associated with a protective effect in the development of IHD.	[34, 193]
	$K_{Ca}1.1$	Overactivation	Alzheimer disease (AD)	Mouse model	Increased availability of ROS in mouse models of AD, so BK channels are extensively oxidized.	[203]
	$K_{Ca}3.1$	Overexpression	Diabetic nephropathy	Mouse model and human tissue	Knockout of $K_{Ca}3.1$ reduces renal fibrosis in a mouse model of diabetic nephropathy.	[204]
Voltage-gated chloride channels (VGClCs)	CLIC1	Single nucleotide polymorphisms	Idiopathic epilepsy	Patients	Possible contribution of the "skeletal" chloride channel ClC-1 to the regulation of brain excitability.	[205]
Acid-sensing ion channels	ASIC1a	Overexpressed	HCC	Liver tumor tissues and SMMC-7721 cells	Suppression of ASIC1α expression by RNAi attenuates the malignant phenotype of HCC cells.	[206]

Table 2: Ion channels involved in oxidative stress in the liver.

Ion channel	Pathology	Model	Oxidative stress effect	Reference
$K_v2.1$	Hepatoma	Huh-7 cell line	HCV inhibits $K_v2.1$, suppressing apoptosis in response to oxidative stress.	[24]
$K_{ir}6.2$	Acute liver injury	LPS-induced mouse model of liver injury	$K_{ir}6.2$ knockout exacerbates LPS-induced endoplasmic reticulum stress in the liver.	[207]
TRPM2	Acetaminophen-induced liver damage	TRPM2 KO mice	$H_2O_2^-$ and acetaminophen-activated Ca^{2+} entry is attenuated in TRPM2 KO mouse hepatocytes.	[208]
TRPM7	Liver fibrosis	Rat hepatic stellate cells	Blockage of TRPM7 causes HSC death induced by ER stress-mediated apoptosis.	[138]
TRPV4	Liver fibrosis	Human liver fibrotic tissues HSC-T6 cells Rat liver fibrosis model, CCl_4	TRPV4 expression correlates with HSC activation and in HSC-T6 induction of α-SMA and Col1α1.	[144]
P2Y	Liver fibrosis	Rat liver fibrosis model, CCl_4	Blockage of P2Y receptors inhibited CCl_4-induced liver fibrosis in rats.	[131]
P2X7	Liver fibrosis NASH	Mouse liver fibrosis model, CCL_4 Mouse diet-induced obesity (DIO)	P2X7 blockage attenuates mouse liver fibrosis and P2X7 gene-deleted mice decreased α-SMA, Col1α1, and TGF-β1 in DIO treated mice.	[117, 133]
CLIC1	Hepatocarcinoma	Mouse hepatocarcinoma ascites cell line (Hca-F)	Overexpression of CLIC1 contributes to cell proliferation, apoptosis, migration, and invasion.	[170]
ASIC1a	Liver fibrosis	Rat liver fibrosis model, CCl_4	ASIC1a increases in HSC and inhibition of ASIC1a suppresses PDGF-induced profibrogenic effects of activated HSC.	[160]
VSOR	Hepatoma	Rat hepatoma (HTC) cells	Activated by H_2O_2 regulating cell volume and cell proliferation.	[170]
VDAC	Acute ethanol intoxication	Rat primary hepatocytes	Bax interacts with the PTP component protein VDAC and likely causes PTP opening, cytochrome c release, caspase activation, and apoptosis.	[209]

to mitochondria. Transient increase in Ca^{2+} level activates mitochondrial matrix enzymes and stimulates oxidative phosphorylation; thus, a high Ca^{2+} flux to the mitochondria is detrimental, promoting oxidative metabolism and consequently ROS production [68].

Mitochondrial ion channels are also considered oncological targets due to the fact that cancer transformation involves reprogramming of mitochondrial metabolic and apoptotic functions, which are necessary to ensure proliferation of neoplastic cells and promote metastasis [53, 69–73].

3. Alcoholic Liver Disease

Alcohol is a psychoactive substance with dependence-producing properties and is considered a causal risk factor for a broad spectrum of diseases and injury conditions including alcohol dependence, liver cirrhosis, and cancer [74]. Emerging evidence suggests that alcohol consumption is also related to the incidence of infectious diseases such as tuberculosis and HIV/AIDS [74]. Chronic alcohol (ethanol) consumption is a well-reviewed risk factor of liver diseases [75–78]. In fact, at least 15% of alcoholic cirrhosis cases end up in hepatocellular carcinoma (HCC), the most common type of liver cancer [79], which has one of the highest mortality rates worldwide [80].

Many investigations strongly suggest that liver damage produced by alcohol is mediated through oxidative stress [76, 81–83]. The multistage process observed in ethanol-induced liver diseases (also called alcoholic induced liver diseases (ALD)) covers a broad spectrum of morphological changes from hepatic steatosis (fatty liver), alcoholic hepatitis, chronic hepatitis, and fibrosis ultimately to cirrhosis, which leads to hepatocarcinoma [79]. Ethanol abuse is directly involved in the generation of ROS and RNS that affect the intracellular redox balance within hepatocytes and other cell types in the liver, such as immune cells (neutrophils), sinusoidal endothelial cells (SECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs). Actually, ethanol exposure impairs the structure and function of mitochondria; thus when ROS production is uncontrolled, several responses that promote immune cell activation are triggered [84].

3.1. Ethanol-Induced Oxidative Stress. The liver oxidizes and completely metabolizes alcohol, for which cytosolic and mitochondrial enzymes are required. Ethanol metabolism is carried out through three main pathways involving the following enzymes: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), microsomal ethanol oxidation system (MEOS) via catalysis by cytochrome P450 isoenzymes (2E1, 1A2, and 3A4 isoforms) [85], and catalase. Any type of

ethanol metabolism will lead to free radical generation that affects the antioxidant defensive system of the cells [86].

Some of the mechanisms by which ethanol impairs the oxidative balance within hepatic cells are acetaldehyde production by ADH, ethanol-induced hypoxia, mitochondria damage, effects on the immune system [87], induction of CYP2E1, mobilization of iron, and alteration of antioxidant enzymes and chemicals [88]. Ethanol metabolism causes depletion of reduced glutathione (GSH) levels and elevates malondialdehyde (MDA), hydroxyethyl radical (HER), and hydroxynonenal (HNE) protein adducts [86], leading to structural and functional abnormalities in the liver. Moreover, alcohol metabolism *via* CYP2E1 activates stress proteins, promotes endoplasmic reticulum stress, and impairs lysosomal function and autophagy [82]. Additionally, some of the mitochondrial alterations caused by ethanol-induced oxidative stress are DNA damage, ribosomal defects, and inhibition of protein synthesis, which in turn affects the integrity of the electron transport chain (complexes I and II) and the oxidative phosphorylation system that is carried by this organelle [50, 79, 89].

3.2. Ion Channels in ALD. The association of ion channels in the mechanism of ethanol-induced oxidative stress to the progression of ALD remains elusive and represents a very interesting field of research. The mitochondrial alterations observed under these conditions include the mitochondrial membrane potential and permeability transition (PT) and changes promoting apoptosis [90]. Alteration of mitochondrial membrane potential has been examined in rat hepatocytes exposed to ethanol using rhodamine 123 (Rh123), an indicator of mitochondrial membrane potential. Acute ethanol administration decreased mitochondrial membrane potential in hepatocytes within 30 min, which indicates that mitochondrial alteration is an early event of ethanol-induced hepatocyte injury. Additionally, the increase in PT is induced by opening of the mitochondrial megachannel also known as permeability transition pore (PTP). PTP is regulated by mitochondrial matrix conditions: electrical membrane potential, thiols, oxidants, pH, and calcium concentration; these are factors disturbed as a consequence of ethanol metabolism [91].

Furthermore, Yan et al. [92] evaluated the effect of ethanol on PTP, mitochondrial membrane potential, and intracellular calcium concentration in cultured hepatocytes. Male Wistar rats were administrated intragastrically with alcohol plus olive oil diet; the control group was given an equal amount of normal saline. Ultramicrostructural changes in mitochondria, PTP opening, mitochondrial membrane potential, mitochondrial mass, and intracellular calcium concentration of isolated hepatocytes were measured. The results showed that the mitochondria of the model group had different shapes and that the PTP was disturbed, causing mitochondria swelling. Moreover, mitochondria transmembrane potential was decreased in comparison with the control group. Intracellular calcium concentration was also increased in the liver cells of the group treated with alcohol. These results indicate that ethanol-induced chondriosome injury could be an important early step in ALD pathogenesis.

The molecular nature of PTP is not completely solved. In the last decade findings made by Bernardi and collaborators [93–95] suggested that reconstituted dimers of the F_0F_1 ATP synthase (or complex V) form a channel that exhibits identical properties to those attributed to the mitochondrial megachannel. Indeed, dimers of the ATP synthase treated with Ca^{2+} generate currents indistinguishable from MMC, while monomers lack any channel activity, strongly suggesting that PTP forms from a specific Ca^{2+} dependent conformation of the dimers. Moreover, inducers (thiol oxidants, benzodiazepine (Bz-423)) and inhibitors (Mg^{2+}, adenine nucleotides) of PTP channel opening have the same effect on ATP synthase. PTP modulators such as membrane potential and matrix pH also constitute key regulators of the ATP synthase. Open questions remain, and further studies are needed to clarify the effect and mechanism of action of other PTP regulators (e.g., rotenone and quinones) and additional issues concerning the dimer hypothesis. According to these findings, it seems that complex V plays a dual function: ATP synthesis and PTP formation.

PTP participates also in mitochondrial calcium release [96]. Pioneer work established its role regulating mitochondrial Ca^{2+} homeostasis in hepatocytes. Cyclosporin A (CsA) is a potent inhibitor of prooxidant-induced release of Ca^{2+} from isolated mitochondria. Pretreatment of hepatocytes with CsA before exposure to prooxidants (*tert*-butyl hydroperoxide, cumene hydroperoxide, or 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine) protected hepatocytes from prooxidant injury. This prevented excessive Ca^{2+} cycling (maintaining mitochondrial Ca^{2+} pool) that leads to alterations of the transmembrane potential and ATP synthesis and consequently compromises mitochondrial functioning and cell survival [97].

Participation of HSCs in ALD has been proposed in various studies [98–101]. HSCs are located in the space of Disse (the liver space between a hepatocyte and a sinusoid) and these cells participate in the process of ECM remodeling (collagen secretion, etc.) after liver injury. L-type voltage-operated Ca^{2+} channels (VOCC) regulate calcium entry into the cytoplasm and subsequently cell contraction, which has been well studied in cardiac and smooth muscle cells. HSCs activated by transforming growth factor-β1 (TGF-β1) express VOCC [102], suggesting that voltage-operated Ca^{2+} channels could also regulate hepatic microcirculation via cell contraction. Itatsu and collaborators [103] evaluated the effect of ethanol on VOCC in HSCs activation. HSCs are known to proliferate in response to liver injury, changing from a "quiescent phenotype" to the "activated phenotype." This study showed that VOCC expression in activated HSCs is significantly increased after 14 days of ethanol exposure in comparison with untreated cells. Ethanol increases the secretion of TGF-β1 that also induces ROS production and downregulates antioxidant enzymes, participating in fibrogenesis and tumorigenesis [104]; however the precise link between TGF-β1, oxidative stress, and VOCC remains elusive.

4. Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease reported in western nations. NAFLD prevalence is 30–45% in Americans. Hispanics have the highest prevalence, followed by Caucasians and African Americans [105]. NAFLD is the accumulation of fat in hepatocytes corresponding to more than 5% of the liver weight in patients and that is not associated with a significant alcohol consumption (conventionally defined as an ethanol intake >20 g/day). The histopathological spectrum includes steatosis (fatty liver) and nonalcoholic steatohepatitis (NASH) which can progress to cirrhosis and finally to hepatocellular carcinoma. NAFLD is associated with the presence of metabolic syndrome. Steatosis is usually associated with a benign prognosis and does not affect overall survival of patients. NASH represents 2–5% of NAFLD cases and is related to increased mortality [106, 107]. The pathophysiology of NASH is complex, involving free fatty acid accumulation (fatty liver), hepatic inflammation (cytokine production), and oxidative stress and lipid peroxidation, as well as hepatocellular damage with or without the presence of fibrosis [108]. Oxidative stress triggers necroinflammation in the liver and contributes to the pathogenesis of NASH. ROS generated during fatty acid metabolism in microsomes, peroxisomes, and mitochondria are the main source of oxidative stress [109].

Mitochondrial dysfunction and oxidative stress play an important role in the pathogenesis of NAFLD, observed in rodent models as well as in patients [110, 111]. Increased serum oxidative markers such as thioredoxin, oxidized LDL, and malondialdehyde have been reported in NASH patients. Lipid accumulation within hepatocytes impairs oxidative capacity of the mitochondria, stimulating peroxisomal, and microsomal pathways of fat oxidation, which results in lipotoxicity. Oxidative stress then promotes apoptosis of hepatic cells via ATP, NAD, and glutathione depletion, as well as by DNA, lipid, and protein damage [112].

A few studies on the participation of ion channels in the pathogenesis of NAFLD or NASH have been reported. Recent investigations reported that cation channels may generate calcium signals during endolysosomal fusion and vesicle trafficking. Two-pore channels (TPCs) are cation-selective intracellular ion channels, expressed mostly in the endosomal system (TPC1) and on late endosomes and lysosomes (TPC2). Activation of TPCs mediates calcium release from lysosomal stores. TPC2 leads to trafficking defects, promoting hepatic cholesterol accumulation, hyperlipoproteinaemia, and finally NASH. Grimm et al. [113] explored the role of TPC2 *in vitro* and *in vivo*. Embryonic mouse hepatocytes lacking TPC2 displayed a significant impairment of LDL-cholesterol and EGF/EGF-receptor trafficking which can be attributed to a dysfunction of the endolysosomal degradation pathway. TPC2-deficient mice also presented cholesterol overload and liver damage consistent with NAFLD. These results suggest that TPC2 plays an important role in trafficking in the endolysosomal degradation pathway of lipids and is potentially involved in the homeostatic control of various molecules.

NAFLD and obesity are frequently associated between them and are characterized by the formation of protein inclusions and ubiquitinated proteins. Moreover, it has been suggested that autophagy deregulation during obesity contributes to protein inclusion and progression to fatty liver pathologies, such as steatohepatitis and HCC. To elucidate how lipotoxicity and obesity can affect autophagy, an *in vitro* system in HepG2 cells was established. HepG2 cells cultured with saturated fatty acid exhibited the accumulation of ubiquitinated proteins in soluble inclusion bodies. Increased cytosolic calcium levels in the hepatocytes were observed in an obesity mouse model. The calcium channel blocker verapamil was proven to restore autophagic flux and suppress protein inclusions in both models. Verapamil also reduced hepatic lipid droplet accumulation, insulin resistance, inflammation, and steatohepatitis, which suggest that calcium channel blockers can be used in NAFLD pathologies [114, 115].

5. Fibrosis

Hepatic fibrosis is characterized by the excessive generation and accumulation of extracellular matrix (ECM) constituents, particularly collagen (types I and III) and fibronectin. Liver fibrosis is a progressive pathology resulting in cirrhosis [116] and ultimately contributes to the development of hepatocellular carcinoma, a malignancy of global importance with very poor prognosis [117]. A fundamental cell event in this process is the activation of HSCs into fibrogenic myofibroblast-like cells which is characterized by the expression of alpha-smooth muscle actin (α-SMA) [118]. Following hepatic injury, HSCs become activated by cytokines and ROS released from KCs, causing HSCs to proliferate, synthesize, and secrete ECM components (Figure 1) [119–121]. Liver fibrogenesis is strongly associated with oxidative stress and it may be the liver disease most frequently related with changes in ion channels. Several studies of ion channels and liver fibrosis are next described.

5.1. Purinergic Receptors. Purinergic receptors have binding sites for nucleotides like ATP and are divided into ligand-gated (P2X) and G protein-coupled (P2Y) receptors [122, 123]. Diverse roles of the purinergic signals in the liver have been described in recent years. For example, purinergic receptors are involved in the proliferation [124] and glucose secretion [125] of hepatocytes. They are also related to the secretion [126, 127], proliferation [128], and mechanosensation [129] in cholangiocytes. The first evidence relating purinergic receptor and fibrosis showed the presence of functional P2Y2, P2Y4, and P2Y6 receptors in both quiescent and activated HSCs [130]. Dranoff et al. [131] demonstrated that the administration of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), a synthetic inhibitor of P2Y receptors, markedly inhibited carbon tetrachloride- (CCl_4-) induced liver fibrosis in rats [131].

One of the most studied channels described in liver fibrosis is P2X7 [132]. Increased mRNA and protein expression of P2X7 was observed in CCl_4-induced liver fibrosis in mice compared with vehicle-treated mice. The competitive P2X7 receptor antagonist A438970 significantly attenuated the

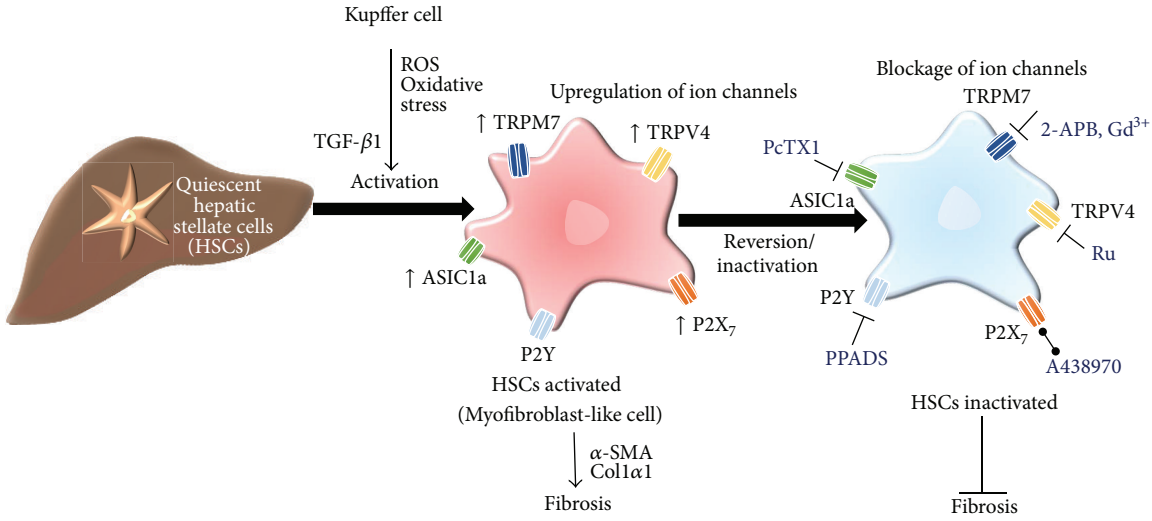


Figure 1: Participation of ion channels in HSCs activation during fibrogenesis. Ion channel upregulation including TRPM7, TRPV4, $P2X_7$, and ASIC1a has been reported during the activation of HSCs, which is a major event during fibrogenesis. Blocking these channels with pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), 2-aminoethoxydiphenyl borate (2-APB), and Gd^{3+}; ruthenium red (Ru); PcTX1 or A438970 reduces proliferation of HSCs and production of profibrotic markers (α-SMA, Col1α1), preventing the progression of fibrosis.

CCl_4-induced necrosis, inflammatory infiltration, and cell injury. The antagonist also reduced collagen accumulation and the production of the proinflammatory mediators TNF-α and IL-1β; in addition it inhibited the activity of NF-κB during inflammation as well as protein expression of profibrotic factors including α-SMA and TGF-β1 [117]. Das et al. [133] demonstrated that mRNA expressions of α-SMA, collagen type 1 alpha 1 (Col1α1), and TGF-β1 were significantly decreased in P2X7 gene-deleted mice compared with wild type mice, suggesting that P2X7 gene-deleted mice are protected from fibrosis. mRNA expression of P2X receptors in human LX-2, hTERT, and FH11 hepatic stellate cell lines has also been demonstrated [134].

5.2. TRPM7 Channels. Transient receptor potential (TRP) channels are a superfamily of cation channels that play critical roles in detecting environmental changes and stimuli. The TRP melastatin-like 7 (TRPM7) channel is selective mainly for Ca^{2+} and is involved in sustaining intracellular Ca^{2+} homeostasis. TRPM7 transcripts are detected in the liver of zebrafish larvae [135] and rat liver [136]. The rat embryonic hepatocyte line RLC-18 expresses a TRPM7-like current suggested to be associated with the proliferation and differentiation of hepatocytes [137]. Likewise, TRPM7 is expressed in HSCs and liver cells from a rat hepatic fibrosis model [138–141]. It is also expressed in rat hepatocytes, the rat hepatoma cells WIF-B cells, and a polarized cell line derived from rat hepatoma-human skin fibroblast cross [139], as well as in H4-IIE cells (rat hepatoma cell line) [140]. Particularly, TRPM7 protein was elevated in fibrotic human liver tissues compared with normal liver tissue and the upregulation of the channel strongly correlated with the increasing levels of α-SMA and Col1α1 proteins. Additionally, cultured rat HSC-T6 cells treated with TGF-β1 showed increased expression of mRNA and protein levels in a time-dependent manner by mechanisms related to the activation of the TGF-β1/Smad3 pathway. The elevated channel levels were correlated with the increasing levels of α-SMA and Col1α1 proteins [141]. Other studies demonstrated that rat primary HSCs displayed increased TRPM7 expression with different stimuli including TGF-β1 or platelet-derived growth factor (PDGF-BB), which are some of the main growth factors stimulating the proliferation of cultured-activated HSCs. This cell proliferation was decreased by the treatment with different TRPM7 nonspecific inhibitors such as 2-aminoethoxydiphenyl borate (2-APB) and Gd^{3+}. Both inhibitors were able to decrease cell viability in a dose-dependent manner via the activation of the apoptotic pathway [138, 142, 143] and decreased the expression of α-SMA and collagen I. Indeed, the treatment with 2-APB and Gd^{3+} inhibited TRPM7 protein expression [143]. It has been also suggested that silencing TRPM7 in the activated HSCs may promote collagen degradation by increasing the levels of hepatic matrix metalloproteinases (MMPs) such as MMP-13 and decreasing the levels of tissue inhibitors of metalloproteinases (TIMPs) like TIMP-1 and TIMP-2 expression [141].

5.3. TRPV4. Increased mRNA and protein levels of TRP vanilloid 4 (TRPV4) have been detected in CCl_4-treated rat livers and in cultured rat HSC-T6 cells. Additionally, the expression of α-SMAD and Col1α1 were elevated according to the progression of HSC-T6 cell activation and correlated with the levels of TRPV4. The blockage of the channel with ruthenium red (a nonspecific TRPV4 channel blocker) or TRPV4 silencing inhibited the proliferation of TGF-β1-treated HSC-T6 cells and decreased profibrotic marker expression. The TRPV4 expression was directly regulated by miR-203 in TGF-β1-induced HSCs. Interestingly, increased TRPV4 protein

expression was found in the liver tissues from liver fibrosis patients compared to normal liver [144].

5.4. Large Conductance Ca^{2+} and Voltage-Activated K^+ Channels. The large conductance Ca^{2+} and voltage-activated K^+ ($K_{Ca}1.1$, BK) channels are activated by membrane depolarization and/or elevations in intracellular Ca^{2+} concentration. They are expressed in almost every tissue in the body participating in numerous cellular functions including the regulation of neurotransmitter release and neuronal excitability, relaxation in smooth muscle cells, hormone release in endocrine and exocrine cells, and blood pressure control [145, 146]. Recently attention has been drawn to BK channels as critical targets of oxidative stress which modifies the gating properties of the channel and is associated with numerous diseases [147], mainly those associated with vascular impair, vascular relaxation, and restricted blood flow [148]. Contraction of smooth vascular cells is given by high elevations of intracellular Ca^{2+} which makes the plasma membrane permeable to K^+ ions by activating BK channels which hyperpolarizes the membrane and causes relaxation [149]. The participation of BK channels has been studied in the modulation of the intrahepatic vascular tone in normal and cirrhotic livers using male Wistar rats exposed to CCL_4. Cirrhotic livers displayed increased activity of BK channels and blockage of the channel increased the baseline portal perfusion pressure in cirrhotic livers [150]. They also used a vasoconstrictor compound (methoxamine) in combination with a channel opener (NS1619) observing a decrease in the baseline portal perfusion pressure in cirrhotic livers, which indicates the participation of BK channels in the modulation of the intrahepatic vascular tone in cirrhosis. Also, in normal human livers, incubating HSCs with the vasodilator NO (nitric oxide) increases the open probability of BK [151]. ROS have been considered to inhibit vascular BK channels. Tang et al. [152] have found that H_2O_2 greatly inhibits BK channels by oxidizing a single cysteine residue (Cys911) near the intracellular Ca^{2+} binding site (Ca^{2+} bowl) in the BK α subunit, disrupting the Ca^{2+}-dependent activation of the channel. These channels have big amounts of cysteines and methionines in their α and β subunits [32, 147]; oxidative molecules could cause alterations in these amino acids and therefore impair the channel activity causing important pathophysiological alterations, as it has been seen in vascular relaxation and blood pressure in different diseases.

5.5. Chloride Channels. Chloride channels are ubiquitously expressed and are localized in the plasma membrane and intracellular organelles. These channels participate in cell volume regulation, maintain intracellular pH, and are involved in transepithelial transport, cell cycle, and electrical excitability [153]. Nonspecific chloride channel blockers including 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and indanyloxyacetic acid (IAA-94) prevented the increase of intracellular levels of $O_2^{-\bullet}$ and inhibited the activation of the HSC human cell line LX-2, induced by the free radical. These findings suggest that the $O_2^{-\bullet}$ radical may enter through chloride channels producing the HSC activation which is critical to fibrosis development [154]. Other authors, Hawkins et al. [155], have demonstrated that $O_2^{-\bullet}$ flux across the endothelial cell plasma membrane of immortalized human pulmonary microvascular endothelial cells (HPMVEC) occurs through ClC-3 channels and induces intracellular Ca^{2+} release, which activates mitochondrial $O_2^{-\bullet}$ production. Also, studies in erythrocytes [156] and amniotic cells [157] have provided evidence for $O_2^{-\bullet}$ transport through anion channels, which could be effectively blocked by DIDS.

5.6. Acid-Sensing Ion Channels. The acid-sensing ion channels (ASICs) are members of the degenerin/epithelial Na^+ channel superfamily, which are activated by extracellular protons and induce an amiloride-sensitive cation current ($Na^+ > Ca^{2+} > K^+$) [158]. The ASICs family is composed of mammals by four different genes encoding seven isoforms: ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3, and ASIC4 [159]. ASIC1a, which is also permeable to Ca^{2+}, may play a role in liver fibrosis because the channel mediates the activation of HSCs. ASIC1a is normally expressed in rat liver tissue including primary HSCs, while protein levels were significantly increased in liver fibrosis induced by CCl_4 where the channel was mainly expressed in activated HSCs. Furthermore, the protein levels of ASIC1a increased in a dose- and time-dependent manner in PDGF-activated HSCs while the blockade of ASIC1a by Psalmotoxin (PcTX1) or the downregulation of the channel by si-RNA reduced the activation of HSCs through the mitogen-activated protein kinases (MAPK) signaling pathway; also ASIC1a silencing inhibited extracellular-signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinases (JNK) activation [160].

6. Cancer

HCC represents 80% of primary liver cancers; the causes leading to HCC include hepatitis B and hepatitis C virus infection (HBV and HCV, resp.) alcoholism, NASH, and aflatoxin B_1 dietary exposure. These factors produce chronic inflammation with severe oxidative stress leading to fibrosis and then cirrhotic livers; this is a common initial mechanism for HCC [161, 162]. Several ion channels have been studied in HCC. Below we summarize recent studies that associate oxidative stress and ion channels in liver carcinogenesis.

6.1. K^+ Channels. Potassium channels play an important role in various biological processes including cell proliferation, apoptosis, cell volume regulation, and migration and angiogenesis of a variety of carcinoma cells [43]. Different K^+ channels have been reported to be involved in the pathogenesis of HCC. The intermediate-conductance Ca^{2+} activated K^+ channel 3.1 (IKCa1, $K_{Ca}3.1$) is overexpressed in HCC tissue [163]. Channel blockade with TRAM-34 inhibited HCC cell proliferation in a time- and dose-dependent manner [163, 164]. TRAM-34 inhibited the activation of NF-κB [164] and activated the MAPK signaling pathway in the SK-Hep1, an invasive liver cell line. The activation of this pathway is linked with the progression of malignant carcinomas

[165]. K^+ channels control the membrane potential; thus overexpression in malignant cells is correlated with the progression of the cell cycle from G1 into the S phase, since a transient hyperpolarization is required in this step of the cell cycle. $K_{Ca}3.1$ could maintain the hyperpolarized membrane potential in cancer liver cells, which promotes Ca^{2+} influx and facilitation of mitogenic activation [166]. Astemizole, a nonspecific inhibitor of $K_v10.1$ and $K_v11.1$ potassium channels, significantly decreased cell proliferation and increased apoptosis *in vitro* in the liver cancer cell lines HepG2 and HuH7 [167]. In the same study, the authors observed that astemizole clearly prevented HCC development *in vivo*. Another recent finding [168] reported that several potassium channels were overexpressed in the liver only in the presence of the chemical carcinogen diethylnitrosamine; when the carcinogen treatment finished, the channel mRNA levels returned almost to normal values. The authors suggested that some potassium channels may serve as carcinogen exposure indicators and that gene expression of the Abcc3 transporter may serve as a liver tumor marker.

6.2. Chloride Channels. In response to oxidative stress in hepatocarcinoma, two types of chloride channels have been studied, the volume-sensitive outwardly rectifying (VSOR) Cl^- channel and chloride intracellular channel 1 (CLIC1). VSOR Cl^- channels are ubiquitously expressed in various cell types and are involved in cell volume regulation after osmotic swelling (regulatory volume decrease, RVD); but they also participate in cell proliferation and apoptosis [169]. In rat hepatoma cells (HTC), H_2O_2 enhances Src mediated PLCγ1 phosphorylation, which subsequently increases intracellular Ca^{2+} levels that activate Ca^{2+}-sensitive pathways causing VSOR Cl^- channel activation [170]; these channels are also activated by H_2O_2 in HeLa cells and in both cell lines participate in cell volume regulation and cell proliferation [169]. Thus, activation of VSORs in HTC may provide advantages in the progression of cancer, but further studies are needed to elucidate the potential mechanisms involved. CLIC1 protein has been found to be overexpressed in liver cancer tissues compared to noncancerous liver tissue and significantly correlated with tumor size, metastasis, and poor prognosis [45]. CLIC1 is overexpressed and promotes cell proliferation, migration, and invasion in the mouse hepatocarcinoma ascites cell line Hca-F that has the potential to produce lymphatic metastasis. In accordance, silencing CLIC1 gene expression with shRNA inhibited cell proliferation, induced apoptosis, and decreased migration and invasion [171]. One of the possible mechanisms by which CLIC1 mediates invasion is by regulating maspin (tumor suppressor), matrix metalloproteinases [172], annexin A7, and gelsolin [173].

6.3. T-Type Ca^{2+} Channels. Oxidative stress induces Ca^{2+} cytoplasmic increase via calcium influx through plasma membrane channels or calcium release from the endoplasmic reticulum, increasing calcium influx into the mitochondria and nuclei, where different signaling pathways take place in the presence of Ca^{2+} [174]. T-type calcium channels play a role in cell cycle progression in different types of cancer [70, 175, 176]. The expression of the three T-type calcium channel subunits was observed in six HCC cell lines (HuH-1, PLC/PRF5, SMMC7721, SNU182, SNU449, and SNU475) and in the cell line SNU449 T-type channel blockage with mibefradil decreased cell proliferation [177].

6.4. P2Y Receptor. Extracellular nucleotides, such as ATP, are released from cells in response to various stimuli, such as shear stress, stretching, hypoxia, inflammation, osmotic swelling, and cell death. In HCC cell lines, the levels of P2Y2 receptor are enhanced compared with human normal hepatocytes; these receptors are involved in ATP-induced $[Ca^{2+}]_i$ increase. Silencing P2Y2R expression inhibited ATP-induced human HCC cell proliferation and migration, and, in nude mice that were implanted with human HCC cells, blocking P2Y2R inhibited cell growth [178].

6.5. Hepatitis C Virus. Hepatitis C virus (HCV) infection is a major health problem worldwide; an estimated 130–170 million people of the world's population are infected with HCV [179]. Most of these individuals (around 80%) will develop chronic liver disease predisposing them to fibrosis and serious clinical outcomes such as cirrhosis and hepatocellular carcinoma [180]. HCV is an enveloped positive-single stranded RNA virus whose genome encodes a single polyprotein that produces mature proteins in the host cell: mature viral structural proteins (Core, E1, E2, and possibly p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [181]. Chronic HCV infection is associated with elevated levels of ROS and RNS leading to an overall increase of oxidative stress within the liver of patients; this redox perturbation has been recognized as a key player in HCV-associated liver diseases. Ion channels have been involved in the development of the redox state in hepatocytes [182, 183], but their role in hepatitis C virus infection remains largely unstudied. However, some studies have reported changes in ion channels in the presence of HCV.

6.6. K^+ Channels. Voltage-gated potassium channels are essential for several cellular processes participating in proliferation, migration, survival, and apoptosis [184, 185]. In HCV-infected cells, the NS5A viral protein inhibits apoptosis in response to oxidative stress by disrupting the function of the $K_v2.1$ channel [24]. In response to oxidative stress, mixed lineage kinase 3 (MLK3) is activated leading to activation of p38-MAPK that phosphorylates $K_v2.1$ at a serine residue in the cytoplasmic C terminus (S800), which is then trafficked to the plasma membrane resulting in an outward K^+ current, which is involved in causing apoptosis. NS5A inhibits MLK3 activation then preventing $K_v2.1$ phosphorylation and channel activity in the plasma membrane and avoiding apoptosis [186]. This is advantageous to the infected cells that are able to avoid apoptosis induced by oxidative stress.

6.7. Chloride Channels. Activation of chloride channels is required for the life cycle of HCV; blocking chloride channels inhibits HCV genome replication and reduces NS5A expression [187]. This might occur because Cl^- channels are responsible for endosome and lysosomal acidification [153],

which is necessary to initiate entry fusion of HCV envelope proteins to the membrane and viral genome release [188]. HCV induces ROS production in liver infected cells and activation of chloride channels may take place to maintain the oxidative state in the infected cells. It has been proposed that when ROS are produced by the NAPDH oxidase, the transfer electrons from the intracellular donor NADPH to extracellular oxygen gives rise to an outward flow of negative charges that depolarizes the plasma membrane in leukocytes. To counteract the more negative potential, the activation of chloride channels may take place modifying the membrane potential and allowing an optimal ROS production [44, 189]. Thus, HCV-infected cells may balance the loss of negative charges in the cell by activating chloride channels.

6.8. P2X Receptors. P2X receptors regulate proliferation and glucose release in hepatocytes [125, 190]. Transcripts of P2X1, P2X2, P2X3, P2X4, and P2X7 have been observed in rat liver cells and rat hepatocytes [125]. Huh-7 cells transfected with the HCV proteins E1E2 showed increased P2X4 gene expression in comparison with control Huh-7 cells [191]. P2X4 is one of the most responsive subtypes of P2X receptors and may participate in mediating glucose-release via glycogenolysis [192].

7. Conclusions

Ion channels play an important role in cellular processes associated with oxidative stress in health and disease. These proteins are very important pharmacological targets for several human pathologies. Therefore, more research on the participation of ion channels in oxidative stress-associated liver diseases should help to find new early-detection tools as well as novel therapeutic strategies.

Abbreviations

ASICs: Acid-sensing ion channels
ANT: Adenine nucleotide translocator
ADH: Alcohol dehydrogenase
ALD: Alcoholic liver diseases
ALDH: Aldehyde dehydrogenase
α-SMA: Alpha-smooth muscle actin
2-APB: 2-Aminoethoxydiphenyl borate
Bz-423: 1,4-Benzodiazepine
K_{ATP}: ATP-sensitive potassium channels
$K_{Ca^{2+}}$: Calcium-activated potassium channels
CCl_4: Carbon tetrachloride
CLIC1: Chloride intracellular channel 1
Col1α1: Collagen type 1 alpha 1
CsA: Cyclosporin A
DIDS: Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate
ER: Endoplasmic reticulum
EGF: Epidermal growth factor
ECM: Extracellular matrix
ERK1/2: Extracellular-signal-regulated kinases 1 and 2
P2Y: G protein-coupled purinergic receptors 2Y
HSCs: Hepatic stellate cells
HBV: Hepatitis B virus
HCV: Hepatitis C virus
HCC: Hepatocellular carcinoma
HTC: Hepatoma cells
HIV/AIDS: Human immunodeficiency virus infection and acquired immune deficiency syndrome
HER: Hydroxyethyl radical
H_2O_2: Hydrogen peroxide
$^{\bullet}OH$: Hydroxyl radical
HNE: Hydroxynonenal
HIF-1α: Hypoxia-inducible-factor-1α
IAA-94: Indanyloxyacetic acid
IMAC: Inner membrane anion channel
IMM: Inner mitochondrial membrane
IL-1β: Interleukin-1β
IK_{Ca}: Intermediate-conductance Ca^{2+} activated K^+ channel
JNK: c-Jun N-terminal kinases
KCs: Kupffer cells
K_{ATP}: ATP-sensitive K^+
P2X: Ligand-gated purinergic receptor
VOCC: L-type voltage-operated Ca^{2+} channels
MAPK: Mitogen-activated protein kinases
MDA: Malondialdehyde
MMPs: Matrix metalloproteinases
MCAO: Middle cerebral artery occlusion
MAMs: Mitochondria associated-endoplasmic reticulum membranes
mitoK_{ATP}: Mitochondrial ATP-sensitive K^+
MEOS: Microsomal ethanol oxidation system
PT: Permeability transition
MLK3: Mixed lineage kinase 3
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
NADH: Nicotinamide adenine dinucleotide (reduced)
NO: Nitric oxide
NPPB: 5-Nitro-2-(3-phenylpropyl-amino)benzoic acid
NF-kappa-B: Nuclear factor kappa-light-chain-enhancer of activated B cells
OMM: Outer mitochondrial membrane
PTP: Permeability transition pore
$ONOO^{-\bullet}$: Peroxynitrite
PLCγ1: Phospholipase C gamma 1
PDGF-BB: Platelet-derived growth factor
PcTX1: Psalmotoxin
PPADS: Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate
ROS: Reactive oxygen species
RNS: Reactive nitrogen species
GSH: Reduced glutathione
RVD: Regulatory volume decrease
Rh123: Rhodamine 123
SECs: Sinusoidal endothelial cells
SK_{Ca}: Small-conductance Ca^{2+} activated K^+ channel

$O_2^{-\bullet}$: Superoxide anion
TIMPs: Tissue inhibitors of metalloproteinases
TGF-β1: Transforming growth factor-beta-1
TRP: Transient receptor potential
TRPM7: Transient receptor potential melastatin-like 7
TRPV4: Transient receptor potential vanilloid 4
TNF-α: Tumor necrosis factor alpha
TPCs: Two-pore channels
VDAC: Voltage dependent anion channel
VSOR: Volume-sensitive outwardly rectifying.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The liver disease research related to this topic in the laboratory of Dr. Camacho has been partially funded by the Conacyt Grant 168102 to Javier Camacho.

References

[1] M. Marí, A. Colell, A. Morales, C. Von Montfort, C. Garcia-Ruiz, and J. C. Fernández-Checa, "Redox control of liver function in health and disease," *Antioxidants and Redox Signaling*, vol. 12, no. 11, pp. 1295–1331, 2010.

[2] H. J. Forman and M. Torres, "Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling," *American Journal of Respiratory and Critical Care Medicine*, vol. 166, no. 12, part 2, pp. S4–S8, 2002.

[3] C. Esquivel-Chirino, J. Esquivel-Soto, J. A. Morales-González et al., "Inflammatory environmental, oxidative stress in tumoral progression," in *Oxidative Stress and Chronic Degenerative Diseases—A Role for Antioxidants*, chapter 8, InTech, Rijeka, Croatia, 2013.

[4] C. Gorrini, I. S. Harris, and T. W. Mak, "Modulation of oxidative stress as an anticancer strategy," *Nature Reviews Drug Discovery*, vol. 12, no. 12, pp. 931–947, 2013.

[5] M. Y. Lee and K. K. Griendling, "Redox signaling, vascular function, and hypertension," *Antioxidants and Redox Signaling*, vol. 10, no. 6, pp. 1045–1059, 2008.

[6] B. Poljsak, "Strategies for reducing or preventing the generation of oxidative stress," *Oxidative Medicine and Cellular Longevity*, vol. 2011, Article ID 194586, 15 pages, 2011.

[7] Z. Durackova, "Some current insights into oxidative stress," *Physiological Research*, vol. 59, no. 4, pp. 459–469, 2010.

[8] F. Farinati, M. Piciocchi, E. Lavezzo, M. Bortolami, and R. Cardin, "Oxidative stress and inducible nitric oxide synthase induction in carcinogenesis," *Digestive Diseases*, vol. 28, no. 4-5, pp. 579–584, 2010.

[9] A. A. Alfadda and R. M. Sallam, "Reactive oxygen species in health and disease," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 936486, 14 pages, 2012.

[10] S. Noori, "An overview of oxidative stress and antioxidant defensive system," *Journal of Clinical & Cellular Immunology*, vol. 1, no. 8, 2012.

[11] A. Rahal, A. Kumar, V. Singh et al., "Oxidative stress, prooxidants, and antioxidants: the interplay," *BioMed Research International*, vol. 2014, Article ID 761264, 19 pages, 2014.

[12] H. Sies, "Oxidative stress: introductory remarks," in *Oxidative Stress*, pp. 1–8, Academic Press, London, UK, 1985.

[13] B. Uttara, A. V. Singh, P. Zamboni, and R. T. Mahajan, "Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options," *Current Neuropharmacology*, vol. 7, no. 1, pp. 65–74, 2009.

[14] R. Thanan, S. Oikawa, Y. Hiraku et al., "Oxidative stress and its significant roles in neurodegenerative diseases and cancer," *International Journal of Molecular Sciences*, vol. 16, no. 1, pp. 193–217, 2014.

[15] Y. T. Chang, W. Chang, N. Tsai et al., "The roles of biomarkers of oxidative stress and antioxidant in Alzheimer's disease: a systematic review," *BioMed Research International*, vol. 2014, Article ID 182303, 14 pages, 2014.

[16] C. Henchcliffe and M. F. Beal, "Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis," *Nature Clinical Practice Neurology*, vol. 4, no. 11, pp. 600–609, 2008.

[17] W. Dröge and H. M. Schipper, "Oxidative stress and aberrant signaling in aging and cognitive decline," *Aging Cell*, vol. 6, no. 3, pp. 361–370, 2007.

[18] C. Ceconi, A. Boraso, A. Cargnoni, and R. Ferrari, "Oxidative stress in cardiovascular disease: myth or fact?" *Archives of Biochemistry and Biophysics*, vol. 420, no. 2, pp. 217–221, 2003.

[19] M. Hristova and M. Penev, "Oxidative stress and cardiovascular diseases," *Trakia Journal of Science*, vol. 12, no. 3, pp. 296–303, 2014.

[20] R. Hutcheson and P. Rocic, "The metabolic syndrome, oxidative stress, environment, and cardiovascular disease: the great exploration," *Experimental Diabetes Research*, vol. 2012, Article ID 271028, 13 pages, 2012.

[21] S. A. Noeman, H. E. Hamooda, and A. A. Baalash, "Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats," *Diabetology and Metabolic Syndrome*, vol. 3, no. 1, article 17, 2011.

[22] A. C. Maritim, R. A. Sanders, and J. B. Watkins III, "Diabetes, oxidative stress, and antioxidants: a review," *Journal of Biochemical and Molecular Toxicology*, vol. 17, no. 1, pp. 24–38, 2003.

[23] L. Marseglia, S. Manti, G. D'Angelo et al., "Oxidative stress in obesity: a critical component in human diseases," *International Journal of Molecular Sciences*, vol. 16, no. 1, pp. 378–400, 2014.

[24] J. Mankouri, M. L. Dallas, M. E. Hughes et al., "Suppression of a pro-apoptotic K^+ channel as a mechanism for hepatitis C virus persistence," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 37, pp. 15903–15908, 2009.

[25] S. Percário, D. Moreira, B. Gomes et al., "Oxidative stress in malaria," *International Journal of Molecular Sciences*, vol. 13, no. 12, pp. 16346–16372, 2012.

[26] S. Waldbaum and M. Patel, "Mitochondria, oxidative stress, and temporal lobe epilepsy," *Epilepsy Research*, vol. 88, no. 1, pp. 23–45, 2010.

[27] E. A. Afify, M. M. Khedr, A. G. Omar, and S. A. Nasser, "The involvement of K(ATP) channels in morphine-induced antinociception and hepatic oxidative stress in acute and inflammatory pain in rats," *Fundamental and Clinical Pharmacology*, vol. 27, no. 6, pp. 623–631, 2013.

[28] S. D. Dib-Hajj, T. R. Cummins, J. A. Black, and S. G. Waxman, "From genes to pain: $Na_v1.7$ and human pain disorders," *Trends in Neurosciences*, vol. 30, no. 11, pp. 555–563, 2007.

[29] M. Can, B. Guven, S. Bektas, and I. Arikan, "Oxidative stress and apoptosis in preeclampsia," *Tissue and Cell*, vol. 46, no. 6, pp. 477–481, 2014.

[30] H. Akbarali, "Oxidative stress and ion channels," in *Systems Biology of Free Radicals and Antioxidants*, I. Laher, Ed., pp. 355–373, Springer, Berlin, Germany, 2014.

[31] L. Annunziato, A. Pannaccione, M. Cataldi et al., "Modulation of ion channels by reactive oxygen and nitrogen species: a pathophysiological role in brain aging?" *Neurobiology of Aging*, vol. 23, no. 5, pp. 819–834, 2002.

[32] Y. Liu and D. D. Gutterman, "Oxidative stress and potassium channel function," *Clinical and Experimental Pharmacology and Physiology*, vol. 29, no. 4, pp. 305–311, 2002.

[33] K. Takahashi, Y. Kakimoto, K. Toda, and K. Naruse, "Mechanobiology in cardiac physiology and diseases," *Journal of Cellular and Molecular Medicine*, vol. 17, no. 2, pp. 225–232, 2013.

[34] F. Fedele, M. Mancone, W. M. Chilian et al., "Role of genetic polymorphisms of ion channels in the pathophysiology of coronary microvascular dysfunction and ischemic heart disease," *Basic Research in Cardiology*, vol. 108, no. 6, article 387, 2013.

[35] C. Chinopoulos and V. Adam-Vizi, "Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme," *The FEBS Journal*, vol. 273, no. 3, pp. 433–450, 2006.

[36] F. Sesti, S. Liu, and S.-Q. Cai, "Oxidation of potassium channels by ROS: a general mechanism of aging and neurodegeneration?" *Trends in Cell Biology*, vol. 20, no. 1, pp. 45–51, 2010.

[37] M. Mantegazza, G. Curia, G. Biagini, D. S. Ragsdale, and M. Avoli, "Voltage-gated sodium channels as therapeutic targets in epilepsy and other neurological disorders," *The Lancet Neurology*, vol. 9, no. 4, pp. 413–424, 2010.

[38] G. Zündorf and G. Reiser, "Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection," *Antioxidants and Redox Signaling*, vol. 14, no. 7, pp. 1275–1288, 2011.

[39] M. Naziroğlu, D. Merve Dikici, and Ş. Dursun, "Role of oxidative stress and Ca^{2+} signaling on molecular pathways of neuropathic pain in diabetes: focus on TRP channels," *Neurochemical Research*, vol. 37, no. 10, pp. 2065–2075, 2012.

[40] J. Striessnig, A. Pinggera, G. Kaur, G. Bock, and P. Tuluc, "L-type Ca^{2+} channels in heart and brain," *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling*, vol. 3, no. 2, pp. 15–38, 2014.

[41] B. Brawek and O. Garaschuk, "Network-wide dysregulation of calcium homeostasis in Alzheimer's disease," *Cell and Tissue Research*, vol. 357, no. 2, pp. 427–438, 2014.

[42] J. I. Vandenberg, "Oxidative stress fine-tunes the dance of hERG K^+ channels," *Journal of Physiology*, vol. 588, no. 16, pp. 2975–2975, 2010.

[43] L. A. Pardo and W. Stühmer, "The roles of K^+ channels in cancer," *Nature Reviews Cancer*, vol. 14, no. 1, pp. 39–48, 2014.

[44] S. Averaimo, R. H. Milton, M. R. Duchen, and M. Mazzanti, "Chloride intracellular channel 1 (CLIC1): sensor and effector during oxidative stress," *FEBS Letters*, vol. 584, no. 10, pp. 2076–2084, 2010.

[45] S. Zhang, X.-M. Wang, Z.-Y. Yin et al., "Chloride intracellular channel 1 is overexpression in hepatic tumor and correlates with a poor prognosis," *APMIS*, vol. 121, no. 11, pp. 1047–1053, 2013.

[46] F. Simon, D. Varela, and C. Cabello-Verrugio, "Oxidative stress-modulated TRPM ion channels in cell dysfunction and pathological conditions in humans," *Cellular Signalling*, vol. 25, no. 7, pp. 1614–1624, 2013.

[47] A. Boveris and B. Chance, "The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen," *Biochemical Journal*, vol. 134, no. 3, pp. 707–716, 1973.

[48] H. Sies, "Role of metabolic H_2O_2 generation: redox signaling and oxidative stress," *Journal of Biological Chemistry*, vol. 289, no. 13, pp. 8735–8741, 2014.

[49] J. B. Hoek, A. Cahill, and J. G. Pastorino, "Alcohol and mitochondria: a dysfunctional relationship," *Gastroenterology*, vol. 122, no. 7, pp. 2049–2063, 2002.

[50] S. M. Bailey, "A review of the role of reactive oxygen and nitrogen species in alcohol-induced mitochondrial dysfunction," *Free Radical Research*, vol. 37, no. 6, pp. 585–596, 2003.

[51] B. O'Rourke, "Pathophysiological and protective roles of mitochondrial ion channels," *Journal of Physiology*, vol. 529, no. 1, pp. 23–36, 2000.

[52] I. Szabo and M. Zoratti, "Mitochondrial channels: ion fluxes and more," *Physiological Reviews*, vol. 94, no. 2, pp. 519–608, 2014.

[53] B. O'Rourke, S. Cortassa, and M. A. Aon, "Mitochondrial ion channels: gatekeepers of life and death," *Physiology*, vol. 20, no. 5, pp. 303–315, 2005.

[54] S.-Y. Ryu, P. M. Peixoto, O. Teijido, L. M. Dejean, and K. W. Kinnally, "Role of mitochondrial ion channels in cell death," *BioFactors*, vol. 36, no. 4, pp. 255–263, 2010.

[55] A. A. Starkov, C. Chinopoulos, and G. Fiskum, "Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury," *Cell Calcium*, vol. 36, no. 3-4, pp. 257–264, 2004.

[56] G. J. Grover and K. D. Garlid, "ATP-sensitive potassium channels: a review of their cardioprotective pharmacology," *Journal of Molecular and Cellular Cardiology*, vol. 32, no. 4, pp. 677–695, 2000.

[57] B. O'Rourke, S. Cortassa, F. Akar, and M. Aon, "Mitochondrial ion channels in cardiac function and dysfunction," *Novartis Foundation Symposia*, vol. 287, pp. 140–156, 2007.

[58] G. Plank, L. Zhou, J. L. Greenstein et al., "From mitochondrial ion channels to arrhythmias in the heart: computational techniques to bridge the spatio-temporal scales," *Philosophical Transactions of the Royal Society of London. Series A. Mathematical, Physical and Engineering Sciences*, vol. 366, no. 1879, pp. 3381–3409, 2008.

[59] P. M. Peixoto, S.-Y. Ryu, and K. W. Kinnally, "Mitochondrial ion channels as therapeutic targets," *FEBS Letters*, vol. 584, no. 10, pp. 2142–2152, 2010.

[60] H. Nishida, A. Matsumoto, N. Tomono, T. Hanakai, S. Harada, and H. Nakaya, "Biochemistry and physiology of mitochondrial ion channels involved in cardioprotection," *FEBS Letters*, vol. 584, no. 10, pp. 2161–2166, 2010.

[61] F. Nassir and J. A. Ibdah, "Role of mitochondria in alcoholic liver disease," *World Journal of Gastroenterology*, vol. 20, no. 9, pp. 2136–2142, 2014.

[62] S. M. Bailey and C. C. Cunningham, "Contribution of mitochondria to oxidative stress associated with alcoholic liver disease," *Free Radical Biology and Medicine*, vol. 32, no. 1, pp. 11–16, 2002.

[63] A. P. Arruda, B. M. Pers, G. Parlakgül, E. Güney, K. Inouye, and G. S. Hotamisligil, "Chronic enrichment of hepatic endoplasmic reticulum—mitochondria contact leads to mitochondrial dysfunction in obesity," *Nature Medicine*, vol. 20, no. 12, pp. 1427–1435, 2014.

[64] F. Nassir and J. A. Ibdah, "Role of mitochondria in nonalcoholic fatty liver disease," *International Journal of Molecular Sciences*, vol. 15, no. 5, pp. 8713–8742, 2014.

[65] Y. Nakagawa, M. Yoshioka, Y. Abe et al., "Enhancement of liver regeneration by adenosine triphosphate-sensitive K+ channel opener (diazoxide) after partial hepatectomy," *Transplantation*, vol. 93, no. 11, pp. 1094–1100, 2012.

[66] K. D. Garlid, P. Paucek, V. Yarov-Yarovoy et al., "Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: possible mechanism of cardioprotection," *Circulation Research*, vol. 81, no. 6, pp. 1072–1082, 1997.

[67] K. Shimizu, Z. Lacza, N. Rajapakse, T. Horiguchi, J. Snipes, and D. W. Busija, "$Mito_{KATP}$ opener, diazoxide, reduces neuronal damage after middle cerebral artery occlusion in the rat," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 283, no. 3, pp. H1005–H1011, 2002.

[68] R. Rizzuto, D. De Stefani, A. Raffaello, and C. Mammucari, "Mitochondria as sensors and regulators of calcium signalling," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 9, pp. 566–578, 2012.

[69] L. Leanza, L. Biasutto, A. Managò, E. Gulbins, M. Zoratti, and I. Szabò, "Intracellular ion channels and cancer," *Frontiers in Physiology*, vol. 4, article 227, 2013.

[70] A. Das, C. Pushparaj, J. Herreros et al., "T-type calcium channel blockers inhibit autophagy and promote apoptosis of malignant melanoma cells," *Pigment Cell and Melanoma Research*, vol. 26, no. 6, pp. 874–885, 2013.

[71] L. Leanza, E. Venturini, S. Kadow, A. Carpinteiro, E. Gulbins, and K. A. Becker, "Targeting a mitochondrial potassium channel to fight cancer," *Cell Calcium*, vol. 58, no. 1, pp. 131–138, 2015.

[72] L. Leanza, M. Zoratti, E. Gulbins, and I. Szabo, "Mitochondrial ion channels as oncological targets," *Oncogene*, vol. 33, no. 49, pp. 5569–5581, 2014.

[73] S. M. Madamba, K. N. Damri, L. M. Dejean, and P. M. Peixoto, "Mitochondrial ion channels in cancer transformation," *Frontiers in Oncology*, vol. 5, article 120, 2015.

[74] WHO, *Global Status Report on Alcohol and Health 2014*, WHO, Geneva, Switzerland, 2014.

[75] R. Bruha, K. Dvorak, and J. Petrtyl, "Alcoholic liver disease," *World Journal of Hepatology*, vol. 4, no. 3, pp. 81–90, 2012.

[76] E. Ceni, T. Mello, and A. Galli, "Pathogenesis of alcoholic liver disease: role of oxidative metabolism," *World Journal of Gastroenterology*, vol. 20, no. 47, pp. 17756–17772, 2014.

[77] B. Gao and R. Bataller, "Alcoholic liver disease: pathogenesis and new therapeutic targets," *Gastroenterology*, vol. 141, no. 5, pp. 1572–1585, 2011.

[78] A. Gramenzi, F. Caputo, M. Biselli et al., "Review article: Alcoholic liver disease—pathophysiological aspects and risk factors," *Alimentary Pharmacology and Therapeutics*, vol. 24, no. 8, pp. 1151–1161, 2006.

[79] B. Sid, J. Verrax, and P. B. Calderon, "Role of oxidative stress in the pathogenesis of alcohol-induced liver disease," *Free Radical Research*, vol. 47, no. 11, pp. 894–904, 2013.

[80] J. Ferlay, I. Soerjomataram, M. Ervik et al., *GLOBOCAN 2012 V1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11*, International Agency for Research on Cancer, Lyon, France, 2013.

[81] E. Albano, "Oxidative mechanisms in the pathogenesis of alcoholic liver disease," *Molecular Aspects of Medicine*, vol. 29, no. 1-2, pp. 9–16, 2008.

[82] A. Ambade and P. Mandrekar, "Oxidative stress and inflammation: essential partners in alcoholic liver disease," *International Journal of Hepatology*, vol. 2012, Article ID 853175, 9 pages, 2012.

[83] J. I. Beier and C. J. McClain, "Mechanisms and cell signaling in alcoholic liver disease," *Biological Chemistry*, vol. 391, no. 11, pp. 1249–1264, 2010.

[84] A. M. Miller, N. Horiguchi, W.-I. Jeong, S. Radaeva, and B. Gao, "Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines," *Alcoholism: Clinical and Experimental Research*, vol. 35, no. 5, pp. 787–793, 2011.

[85] C. S. Lieber, "Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968–1998)—a review," *Alcoholism: Clinical and Experimental Research*, vol. 23, no. 6, pp. 991–1007, 1999.

[86] S. K. Das and D. M. Vasudevan, "Alcohol-induced oxidative stress," *Life Sciences*, vol. 81, no. 3, pp. 177–187, 2007.

[87] M. Vidali, S. F. Stewart, and E. Albano, "Interplay between oxidative stress and immunity in the progression of alcohol-mediated liver injury," *Trends in Molecular Medicine*, vol. 14, no. 2, pp. 63–71, 2008.

[88] A. Dey and A. I. Cederbaum, "Alcohol and oxidative liver injury," *Hepatology*, vol. 43, no. 2, supplement 1, pp. S63–S74, 2006.

[89] S. M. Bailey, E. C. Pietsch, and C. C. Cunningham, "Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III," *Free Radical Biology and Medicine*, vol. 27, no. 7-8, pp. 891–900, 1999.

[90] M. Adachi and H. Ishii, "Role of mitochondria in alcoholic liver injury," *Free Radical Biology and Medicine*, vol. 32, no. 6, pp. 487–491, 2002.

[91] I. Kurose, H. Higuchi, S. Kato et al., "Oxidative stress on mitochondria and cell membrane of cultured rat hepatocytes and perfused liver exposed to ethanol," *Gastroenterology*, vol. 112, no. 4, pp. 1331–1343, 1997.

[92] M. Yan, P. Zhu, H.-M. Liu, H.-T. Zhang, and L. Liu, "Ethanol induced mitochondria injury and permeability transition pore opening: role of mitochondria in alcoholic liver disease," *World Journal of Gastroenterology*, vol. 13, no. 16, pp. 2352–2356, 2007.

[93] P. Bernardi, "The mitochondrial permeability transition pore: a mystery solved?" *Frontiers in Physiology*, vol. 4, article 95, 2013.

[94] P. Bernardi, A. Rasola, M. Forte, and G. Lippe, "The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology," *Physiological Reviews*, vol. 95, no. 4, pp. 1111–1155, 2015.

[95] P. Bernardi, F. Di Lisa, F. Fogolari, and G. Lippe, "From ATP to PTP and back: a dual function for the mitochondrial ATP synthase," *Circulation Research*, vol. 116, no. 11, pp. 1850–1862, 2015.

[96] C. Brenner and M. Moulin, "Physiological roles of the permeability transition pore," *Circulation Research*, vol. 111, no. 9, pp. 1237–1247, 2012.

[97] G. E. N. Kass, M. J. Juedes, and S. Orrenius, "Cyclosporin a protects hepatocytes against prooxidant-induced cell killing. A study on the role of mitochondrial Ca^{2+} cycling in cytotoxicity," *Biochemical Pharmacology*, vol. 44, no. 10, pp. 1995–2003, 1992.

[98] M. L. Hautekeete, I. Dodeman, V. Azais-Braesco, K. D. Van Berg, C. Seynaeve, and A. Geerts, "Hepatic stellate cells and liver retinoid content in alcoholic liver disease in humans," *Alcoholism: Clinical and Experimental Research*, vol. 22, no. 2, pp. 494–500, 1998.

[99] N. I. Han, K. W. Chung, B. M. Ahn et al., "Ultrastructural changes of hepatic stellate cells in the space of Disse in alcoholic fatty liver," *Korean Journal of Internal Medicine*, vol. 16, no. 3, pp. 160–166, 2001.

[100] M. Vera and N. Nieto, "Hepatic stellate cells and alcoholic liver disease," *Revista Espanola de Enfermedades Digestivas*, vol. 98, no. 9, pp. 674–684, 2006.

[101] J.-H. Wang, R. G. Batey, and J. George, "Role of ethanol in the regulation of hepatic stellate cell function," *World Journal of Gastroenterology*, vol. 12, no. 43, pp. 6926–6932, 2006.

[102] H. Oide and R. G. Thurman, "Hepatic Ito cells contain calcium channels: increases with transforming growth factor-β1," *Hepatology*, vol. 20, no. 4, part 1, pp. 1009–1014, 1994.

[103] T. Itatsu, H. Oide, S. Watanabe, M. Tateyama, R. Ochi, and N. Sato, "Alcohol stimulates the expression of L-type voltage-operated Ca^{2+} channels in hepatic stellate cells," *Biochemical and Biophysical Research Communications*, vol. 251, no. 2, pp. 533–537, 1998.

[104] J. Krstić, D. Trivanović, S. Mojsilović, and J. F. Santibanez, "Transforming growth factor-beta and oxidative stress interplay: implications in tumorigenesis and cancer progression," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 654594, 15 pages, 2015.

[105] C. D. Williams, J. Stengel, M. I. Asike et al., "Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study," *Gastroenterology*, vol. 140, no. 1, pp. 124–131, 2011.

[106] C. M. Fielding and P. Angulo, "Hepatic steatosis and steatohepatitis: are they really two distinct entities?" *Current Hepatitis Reports*, vol. 13, no. 2, pp. 151–158, 2014.

[107] S. Bellentani, F. Scaglioni, M. Marino, and G. Bedogni, "Epidemiology of non-alcoholic fatty liver disease," *Digestive Diseases*, vol. 28, no. 1, pp. 155–161, 2010.

[108] M. Varela-Rey, N. Embade, U. Ariz, S. C. Lu, J. M. Mato, and M. L. Martínez-Chantar, "Non-alcoholic steatohepatitis and animal models: understanding the human disease," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 5, pp. 969–976, 2009.

[109] A. Takaki, D. Kawai, and K. Yamamoto, "Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH)," *International Journal of Molecular Sciences*, vol. 14, no. 10, pp. 20704–20728, 2013.

[110] C. P. M. S. Oliveira, A. M. M. Coelho, H. V. Barbeiro et al., "Liver mitochondrial dysfunction and oxidative stress in the pathogenesis of experimental nonalcoholic fatty liver disease," *Brazilian Journal of Medical and Biological Research*, vol. 39, no. 2, pp. 189–194, 2006.

[111] S. K. Das and D. M. Vasudevan, "Monitoring oxidative stress in patients with non-alcoholic and alcoholic liver diseases," *Indian Journal of Clinical Biochemistry*, vol. 20, no. 2, pp. 24–28, 2005.

[112] A. P. Rolo, J. S. Teodoro, and C. M. Palmeira, "Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis," *Free Radical Biology and Medicine*, vol. 52, no. 1, pp. 59–69, 2012.

[113] C. Grimm, L. M. Holdt, C.-C. Chen et al., "High susceptibility to fatty liver disease in two-pore channel 2-deficient mice," *Nature Communications*, vol. 5, article 4699, 2014.

[114] H. W. Park, I. A. Semple, I. Jang et al., "Pharmacological correction of obesity-induced autophagy arrest using calcium channel blockers," *Nature Communications*, vol. 5, article 4834, 2014.

[115] H.-W. Park and J. H. Lee, "Calcium channel blockers as potential therapeutics for obesity-associated autophagy defects and fatty liver pathologies," *Autophagy*, vol. 10, no. 12, pp. 2385–2386, 2015.

[116] G. Poli, "Pathogenesis of liver fibrosis: role of oxidative stress," *Molecular Aspects of Medicine*, vol. 21, no. 3, pp. 49–98, 2000.

[117] C. Huang, W. Yu, H. Cui et al., "P2X7 blockade attenuates mouse liver fibrosis," *Molecular Medicine Reports*, vol. 9, no. 1, pp. 57–62, 2014.

[118] R. K. Moreira, "Hepatic stellate cells and liver fibrosis," *Archives of Pathology and Laboratory Medicine*, vol. 131, no. 11, pp. 1728–1734, 2007.

[119] J. Jiao, S. L. Friedman, and C. Aloman, "Hepatic fibrosis," *Current Opinion in Gastroenterology*, vol. 25, no. 3, pp. 223–229, 2009.

[120] A. Casini, E. Ceni, R. Salzano et al., "Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide," *Hepatology*, vol. 25, no. 2, pp. 361–367, 1997.

[121] P. P. Simeonova, R. M. Gallucci, T. Hulderman et al., "The role of tumor necrosis factor-α in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride," *Toxicology and Applied Pharmacology*, vol. 177, no. 2, pp. 112–120, 2001.

[122] V. Ralevic and G. Burnstock, "Receptors for purines and pyrimidines," *Pharmacological Reviews*, vol. 50, no. 3, pp. 413–492, 1998.

[123] D. Lu and P. A. Insel, "Cellular mechanisms of tissue fibrosis. 6. Purinergic signaling and response in fibroblasts and tissue fibrosis," *The American Journal of Physiology—Cell Physiology*, vol. 306, no. 9, pp. C779–C788, 2014.

[124] G. Beldi, Y. Wu, X. Sun et al., "Regulated catalysis of extracellular nucleotides by vascular CD39/ENTPD1 is required for liver regeneration," *Gastroenterology*, vol. 135, no. 5, pp. 1751–1760, 2008.

[125] D. S. Emmett, A. Feranchak, G. Kilic et al., "Characterization of ionotrophic purinergic receptors in hepatocytes," *Hepatology*, vol. 47, no. 2, pp. 698–705, 2008.

[126] J. A. Dranoff, A. I. Masyuk, E. A. Kruglov, N. F. LaRusso, and M. H. Nathanson, "Polarized expression and function of P2Y ATP receptors in rat bile duct epithelia," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 281, no. 4, pp. G1059–G1067, 2001.

[127] N. Minagawa, J. Nagata, K. Shibao et al., "Cyclic AMP regulates bicarbonate secretion in cholangiocytes through release of ATP into bile," *Gastroenterology*, vol. 133, no. 5, pp. 1592–1602, 2007.

[128] M. N. Jhandier, E. A. Kruglov, É. G. Lavoie, J. Sévigny, and J. A. Dranoff, "Portal fibroblasts regulate the proliferation of bile duct epithelia via expression of NTPDase2," *The Journal of Biological Chemistry*, vol. 280, no. 24, pp. 22986–22992, 2005.

[129] A. I. Masyuk, S. A. Gradilone, J. M. Banales et al., "Cholangiocyte primary cilia are chemosensory organelles that detect biliary nucleotides via $P2Y_{12}$ purinergic receptors," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 295, no. 4, pp. G725–G734, 2008.

[130] J. A. Dranoff, M. Ogawa, E. A. Kruglov et al., "Expression of P2Y nucleotide receptors and ectonucleotidases in quiescent and activated rat hepatic stellate cells," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 287, no. 2, pp. G417–G424, 2004.

[131] J. A. Dranoff, E. A. Kruglov, O. Abreu-Lanfranco, T. Nguyen, G. Aurora, and D. Jain, "Prevention of liver fibrosis by the purinoceptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPDAS)," *In Vivo*, vol. 21, no. 6, pp. 957–966, 2007.

[132] A. Surprenant, F. Rassendren, E. Kawashima, R. A. North, and G. Buell, "The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7)," *Science*, vol. 272, no. 5262, pp. 735–738, 1996.

[133] S. Das, R. K. Seth, A. Kumar et al., "Purinergic receptor X7 is a key modulator of metabolic oxidative stress-mediated autophagy and inflammation in experimental nonalcoholic steatohepatitis," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 305, no. 12, pp. G950–G963, 2013.

[134] J. R. Goree, E. G. Lavoie, M. Fausther, and J. A. Dranoff, "Expression of mediators of purinergic signaling in human liver cell lines," *Purinergic Signalling*, vol. 10, no. 4, pp. 631–638, 2014.

[135] M. R. Elizondo, B. L. Arduini, J. Paulsen et al., "Defective skeletogenesis with kidney stone formation in dwarf zebrafish mutant for trpm7," *Current Biology*, vol. 15, no. 7, pp. 667–671, 2005.

[136] L. W. Runnels, L. Yue, and D. E. Clapham, "TRP-PLIK, a bifunctional protein with kinase and ion channel activities," *Science*, vol. 291, no. 5506, pp. 1043–1047, 2001.

[137] D. H. Lam, C. E. Grant, and C. E. Hill, "Differential expression of TRPM7 in rat hepatoma and embryonic and adult hepatocytes," *Canadian Journal of Physiology and Pharmacology*, vol. 90, no. 4, pp. 435–444, 2012.

[138] Y. Zhu, R. Men, M. Wen, X. Hu, X. Liu, and L. Yang, "Blockage of TRPM7 channel induces hepatic stellate cell death through endoplasmic reticulum stress-mediated apoptosis," *Life Sciences*, vol. 94, no. 1, pp. 37–44, 2014.

[139] R. Mishra, V. Rao, R. Ta, N. Shobeiri, and C. E. Hill, "Mg^{2+}- and MgATP-inhibited and Ca^{2+}/calmodulin-sensitive TRPM7-like current in hepatoma and hepatocytes," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 297, no. 4, pp. G687–G694, 2009.

[140] G. J. Barritt, J. Chen, and G. Y. Rychkov, "Ca^{2+}-permeable channels in the hepatocyte plasma membrane and their roles in hepatocyte physiology," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1783, no. 5, pp. 651–672, 2008.

[141] L. Fang, C. Huang, X. Meng et al., "TGF-β1-elevated TRPM7 channel regulates collagen expression in hepatic stellate cells via TGF-β1/Smad pathway," *Toxicology and Applied Pharmacology*, vol. 280, no. 2, pp. 335–344, 2014.

[142] L. Fang, S. Zhan, C. Huang et al., "TRPM7 channel regulates PDGF-BB-induced proliferation of hepatic stellate cells via PI3K and ERK pathways," *Toxicology and Applied Pharmacology*, vol. 272, no. 3, pp. 713–725, 2013.

[143] H. Liu, J. Li, Y. Huang, and C. Huang, "Inhibition of transient receptor potential melastain 7 channel increases HSCs apoptosis induced by TRAIL," *Life Sciences*, vol. 90, no. 15-16, pp. 612–618, 2012.

[144] Y. Songa, L. Zhan, M. Yu et al., "TRPV4 channel inhibits TGF-β1-induced proliferation of hepatic stellate cells," *PLoS ONE*, vol. 9, no. 7, Article ID e101179, 2014.

[145] B. H. Bentzen, S.-P. Olesen, L. C. B. Rønn, and M. Grunnet, "BK channel activators and their therapeutic perspectives," *Frontiers in Physiology*, vol. 5, article 389, 2014.

[146] T. Hoshi, A. Pantazis, and R. Olcese, "Transduction of voltage and Ca^{2+} signals by Slo1 BK channels," *Physiology*, vol. 28, no. 3, pp. 172–189, 2013.

[147] A. Hermann, G. Sitdikova, and T. Weiger, "Oxidative stress and maxi calcium-activated potassium (BK) channels," *Biomolecules*, vol. 5, no. 3, pp. 1870–1911, 2015.

[148] R. Köhler, B. P. Kaistha, and H. Wulff, "Vascular KCa-channels as therapeutic targets in hypertension and restenosis disease," *Expert Opinion on Therapeutic Targets*, vol. 14, no. 2, pp. 143–155, 2010.

[149] E. A. Ko, J. Han, I. D. Jung, and W. S. Park, "Physiological roles of K^+ channels in vascular smooth muscle cells," *Journal of Smooth Muscle Research*, vol. 44, no. 2, pp. 65–81, 2008.

[150] A. Rodríguez-Vilarrupla, M. Graupera, V. Matei et al., "Large-conductance calcium-activated potassium channels modulate vascular tone in experimental cirrhosis," *Liver International*, vol. 28, no. 4, pp. 566–573, 2008.

[151] X. Gasull, R. Bataller, P. Ginès et al., "Human myofibroblastic hepatic stellate cells express Ca^{2+}-activated K^+ channels that modulate the effects of endothelin-1 and nitric oxide," *Journal of Hepatology*, vol. 35, no. 6, pp. 739–748, 2001.

[152] X. D. Tang, M. L. Garcia, S. H. Heinemann, and T. Hoshi, "Reactive oxygen species impair Slo1 BK channel function by altering cysteine-mediated calcium sensing," *Nature Structural and Molecular Biology*, vol. 11, no. 2, pp. 171–178, 2004.

[153] T. Stauber, T. Weinert, and T. J. Jentsch, "Cell biology and physiology of CLC chloride channels and transporters," *Comprehensive Physiology*, vol. 2, no. 3, pp. 1701–1744, 2012.

[154] G. J. M. den Hartog, S. Qi, J. H. O. Van Tilburg, G. H. Koek, and A. Bast, "Superoxide anion radicals activate hepatic stellate cells after entry through chloride channels: a new target in liver fibrosis," *European Journal of Pharmacology*, vol. 724, no. 1, pp. 140–144, 2014.

[155] B. J. Hawkins, M. Madesh, C. J. Kirkpatrick, and A. B. Fisher, "Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling," *Molecular Biology of the Cell*, vol. 18, no. 6, pp. 2002–2012, 2007.

[156] R. E. Lynch and I. Fridovich, "Permeation of the erythrocyte stroma by superoxide radical," *The Journal of Biological Chemistry*, vol. 253, no. 13, pp. 4697–4699, 1978.

[157] Y. Ikebuchi, N. Masumoto, K. Tasaka et al., "Superoxide anion increases intracellular pH, intracellular free calcium, and arachidonate release in human amnion cells," *Journal of Biological Chemistry*, vol. 266, no. 20, pp. 13233–13237, 1991.

[158] R. Waldmann, G. Champigny, F. Bassilana, C. Heurteaux, and M. Lazdunski, "A proton-gated cation channel involved in acid-sensing," *Nature*, vol. 386, no. 6621, pp. 173–177, 1997.

[159] Z.-G. Xiong, X.-P. Chu, and R. P. Simon, "Ca^{2+}-permeable acid-sensing ion channels and ischemic brain injury," *The Journal of Membrane Biology*, vol. 209, no. 1, pp. 59–68, 2006.

[160] F.-R. Wu, C.-X. Pan, C. Rong et al., "Inhibition of acid-sensing ion channel 1a in hepatic stellate cells attenuates PDGF-induced activation of HSCs through MAPK pathway," *Molecular and Cellular Biochemistry*, vol. 395, no. 1-2, pp. 199–209, 2014.

[161] A. Arzumanyan, H. M. G. P. V. Reis, and M. A. Feitelson, "Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma," *Nature Reviews Cancer*, vol. 13, no. 2, pp. 123–135, 2013.

[162] B. Sun and M. Karin, "Inflammation and liver tumorigenesis," *Frontiers of Medicine*, vol. 7, no. 2, pp. 242–254, 2013.

[163] X.-W. Yang, J.-W. Liu, R.-C. Zhang, Q. Yin, W.-Z. Shen, and J.-L. Yi, "Inhibitory effects of blockage of intermediate conductance Ca^{2+}-activated K^+ channels on proliferation of hepatocellular carcinoma cells," *Journal of Huazhong University of Science and Technology [Medical Sciences]*, vol. 33, no. 1, pp. 86–89, 2013.

[164] C. Freise, M. Ruehl, D. Seehofer, J. Hoyer, and R. Somasundaram, "The inhibitor of Ca^{2+}-dependent K^+ channels TRAM-34 blocks growth of hepatocellular carcinoma cells via down-regulation of estrogen receptor alpha mRNA and nuclear factor-κB," *Investigational New Drugs*, vol. 31, no. 2, pp. 452–457, 2013.

[165] B. Minguez, V. Tovar, D. Chiang, A. Villanueva, and J. M. Llovet, "Pathogenesis of hepatocellular carcinoma and molecular therapies," *Current Opinion in Gastroenterology*, vol. 25, no. 3, pp. 186–194, 2009.

[166] W. Lai, S. Chen, H. Wu et al., "PRL-3 promotes the proliferation of LoVo cells via the upregulation of KCNN4 channels," *Oncology Reports*, vol. 26, no. 4, pp. 909–917, 2011.

[167] M. de Guadalupe Chávez-López, J. I. Pérez-Carreón, V. Zuñiga-García et al., "Astemizole-based anticancer therapy for hepatocellular carcinoma (HCC), and Eag1 channels as potential early-stage markers of HCC," *Tumor Biology*, vol. 36, no. 8, pp. 6149–6158, 2015.

[168] V. Zúñiga-García, M. de Guadalupe Chávez-López, V. Quintanar-Jurado et al., "Differential expression of ion channels and transporters during hepatocellular carcinoma development," *Digestive Diseases and Sciences*, vol. 60, no. 8, pp. 2373–2383, 2015.

[169] D. Varela, F. Simon, A. Riveros, F. Jørgensen, and A. Stutzin, "NAD(P)H oxidase-derived H_2O_2 signals chloride channel activation in cell volume regulation and cell proliferation," *The Journal of Biological Chemistry*, vol. 279, no. 14, pp. 13301–13304, 2004.

[170] D. Varela, F. Simon, P. Olivero et al., "Activation of H_2O_2-induced VSOR Cl^- currents in HTC cells require phospholipase Cγ1 phosphorylation and Ca^{2+} mobilisation," *Cellular Physiology and Biochemistry*, vol. 20, no. 6, pp. 773–780, 2007.

[171] R.-K. Li, J. Zhang, Y.-H. Zhang, M.-L. Li, M. Wang, and J.-W. Tang, "Chloride intracellular channel 1 is an important factor in the lymphatic metastasis of hepatocarcinoma," *Biomedicine and Pharmacotherapy*, vol. 66, no. 3, pp. 167–172, 2012.

[172] X. Wei, J. Li, H. Xie et al., "Chloride intracellular channel 1 participates in migration and invasion of hepatocellular carcinoma by targeting maspin," *Journal of Gastroenterology and Hepatology*, vol. 30, no. 1, pp. 208–216, 2015.

[173] J. Zhang, M. Li, M. Song et al., "Clic1 plays a role in mouse hepatocarcinoma via modulating Annexin A7 and Gelsolin in vitro and in vivo," *Biomedicine and Pharmacotherapy*, vol. 69, pp. 416–419, 2015.

[174] G. Ermak and K. J. A. Davies, "Calcium and oxidative stress: from cell signaling to cell death," *Molecular Immunology*, vol. 38, no. 10, pp. 713–721, 2002.

[175] W. Huang, C. Lu, Y. Wu, S. Ouyang, and Y. Chen, "T-type calcium channel antagonists, mibefradil and NNC-55-0396 inhibit cell proliferation and induce cell apoptosis in leukemia cell lines," *Journal of Experimental & Clinical Cancer Research*, vol. 34, no. 1, p. 54, 2015.

[176] B. Dziegielewska, L. S. Gray, and J. Dziegielewski, "T-type calcium channels blockers as new tools in cancer therapies," *Pflugers Archiv European Journal of Physiology*, vol. 466, no. 4, pp. 801–810, 2014.

[177] Y. Li, S. Liu, F. Lu et al., "A role of functional T-type Ca2+ channel in hepatocellular carcinoma cell proliferation," *Oncology Reports*, vol. 22, no. 5, pp. 1229–1235, 2009.

[178] R. Xie, J. Xu, G. Wen et al., "The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP," *The Journal of Biological Chemistry*, vol. 289, no. 27, pp. 19137–19149, 2014.

[179] D. Lavanchy, "The global burden of hepatitis C," *Liver International*, vol. 29, supplement 1, pp. 74–81, 2009.

[180] S. L. Chen and T. R. Morgan, "The natural history of hepatitis C virus (HCV) infection," *International Journal of Medical Sciences*, vol. 3, no. 2, pp. 47–52, 2006.

[181] F. V. Chisari, "Unscrambling hepatitis C virus-host interactions," *Nature*, vol. 436, no. 7053, pp. 930–932, 2005.

[182] X. Li and S. A. Weinman, "Chloride channels and hepatocellular function: prospects for molecular identification," *Annual Review of Physiology*, vol. 64, pp. 609–633, 2002.

[183] J. Graf and D. Häussinger, "Ion transport in hepatocytes: mechanisms and correlations to cell volume, hormone actions and metabolism," *Journal of Hepatology*, vol. 24, supplement 1, pp. 53–77, 1996.

[184] A. Girault and E. Brochiero, "Evidence of K^+ channel function in epithelial cell migration, proliferation, and repair," *American Journal of Physiology: Cell Physiology*, vol. 306, no. 4, pp. C307–C319, 2014.

[185] H. A. Kolb, "Potassium channels in excitable and non-excitable cells," *Reviews of Physiology Biochemistry and Pharmacology*, vol. 115, pp. 51–91, 1990.

[186] Y. Amako, Z. Igloi, J. Mankouri et al., "Hepatitis C virus NS5A inhibits mixed lineage kinase 3 to block apoptosis," *The Journal of Biological Chemistry*, vol. 288, no. 34, pp. 24753–24763, 2013.

[187] Z. Igloi, B. Mohl, J. D. Lippiat, M. Harris, J. Mankouri, and M. S. Diamond, "Requirement for chloride channel function during the hepatitis C virus life cycle," *Journal of Virology*, vol. 89, no. 7, pp. 4023–4029, 2015.

[188] M. B. Zeisel, H. Barth, C. Schuster, and T. F. Baumert, "Hepatitis C virus entry: molecular mechanisms and targets for antiviral therapy," *Frontiers in Bioscience*, vol. 14, no. 9, pp. 3274–3285, 2009.

[189] J. G. Moreland, A. P. Davis, G. Bailey, W. M. Nauseef, and F. S. Lamb, "Anion channels, including ClC-3, are required for normal neutrophil oxidative function, phagocytosis, and transendothelial migration," *Journal of Biological Chemistry*, vol. 281, no. 18, pp. 12277–12288, 2006.

[190] S. Thevananther, H. Sun, D. Li et al., "Extracellular ATP activates c-jun N-terminal kinase signaling and cell cycle progression in hepatocytes," *Hepatology*, vol. 39, no. 2, pp. 393–402, 2004.

[191] S. Manzoor, M. Idrees, J. Ashraf et al., "Identification of ionotrophic purinergic receptors in Huh-7 cells and their response towards structural proteins of HCV genotype 3a," *Virology Journal*, vol. 8, article 431, 2011.

[192] V. C. Minguetti-Câmara, J. Constantin, F. Suzuki-Kemmelmeier, E. L. Ishii-Iwamoto, and A. Bracht, "Hepatic heterogeneity in the response to ATP studied in the bivascularly perfused rat liver," *Molecular and Cellular Biochemistry*, vol. 179, no. 1-2, pp. 35–48, 1998.

[193] A. M. Waszkielewicz, A. Gunia, N. Szkaradek, K. Sloczynska, S. Krupinska, and H. Marona, "Ion channels as drug targets in central nervous system disorders," *Current Medicinal Chemistry*, vol. 20, no. 10, pp. 1241–1285, 2013.

[194] R. Köhling, "Voltage-gated sodium channels in epilepsy," *Epilepsia*, vol. 43, no. 11, pp. 1278–1295, 2002.

[195] W. A. Catterall, A. L. Goldin, and S. G. Waxman, "International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels," *Pharmacological Reviews*, vol. 57, no. 4, pp. 397–409, 2005.

[196] K. Kamiya, M. Kaneda, T. Sugawara et al., "A nonsense mutation of the sodium channel gene *SCN2A* in a patient with intractable epilepsy and mental decline," *Journal of Neuroscience*, vol. 24, no. 11, pp. 2690–2698, 2004.

[197] A. M. Rush, S. D. Dib-Hajj, and S. G. Waxman, "Electrophysiological properties of two axonal sodium channels, Nav1.2 and Nav1.6, expressed in mouse spinal sensory neurones," *Journal of Physiology*, vol. 564, part 3, pp. 803–815, 2005.

[198] J. D. England, L. T. Happel, D. G. Kline et al., "Sodium channel accumulation in humans with painful neuromas," *Neurology*, vol. 47, no. 1, pp. 272–276, 1996.

[199] J. G. J. Hoeijmakers, C. G. Faber, I. S. J. Merkies, and S. G. Waxman, "Painful peripheral neuropathy and sodium channel mutations," *Neuroscience Letters*, vol. 596, pp. 51–59, 2015.

[200] E. M. Nagasako, A. L. Oaklander, and R. H. Dworkin, "Congenital insensitivity to pain: an update," *Pain*, vol. 101, no. 3, pp. 213–219, 2003.

[201] S. D. Dib-Hajj, A. M. Binshtok, T. R. Cummins, M. F. Jarvis, T. Samad, and K. Zimmermann, "Voltage-gated sodium channels in pain states: role in pathophysiology and targets for treatment," *Brain Research Reviews*, vol. 60, no. 1, pp. 65–83, 2009.

[202] J. Huang, C. Han, M. Estacion et al., "Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy," *Brain*, vol. 137, part 6, pp. 1627–1642, 2014.

[203] H. Ye, S. Jalini, S. Mylvaganam, and P. Carlen, "Activation of large-conductance Ca^{2+}-activated K^+ channels depresses basal synaptic transmission in the hippocampal CA1 area in APP (swe/ind) TgCRND8 mice," *Neurobiology of Aging*, vol. 31, no. 4, pp. 591–604, 2010.

[204] C. Huang, S. Shen, Q. Ma et al., "Blockade of KCa3.1 ameliorates renal fibrosis through the tgf-β1/smad pathway in diabetic mice," *Diabetes*, vol. 62, no. 8, pp. 2923–2934, 2013.

[205] T. T. Chen, T. L. Klassen, A. M. Goldman, C. Marini, R. Guerrini, and J. L. Noebels, "Novel brain expression of ClC-1 chloride channels and enrichment of CLCN1 variants in epilepsy," *Neurology*, vol. 80, no. 12, pp. 1078–1085, 2013.

[206] C. Jin, Q.-H. Ye, F.-L. Yuan et al., "Involvement of acid-sensing ion channel 1α in hepatic carcinoma cell migration and invasion," *Tumor Biology*, vol. 36, no. 6, pp. 4309–4317, 2015.

[207] R.-H. Du, J. Tan, N. Yan et al., "Kir6.2 knockout aggravates lipopolysaccharide-induced mouse liver injury via enhancing NLRP3 inflammasome activation," *Journal of Gastroenterology*, vol. 49, no. 4, pp. 727–736, 2014.

[208] E. Kheradpezhouh, L. Ma, A. Morphett, G. J. Barritt, and G. Y. Rychkov, "TRPM2 channels mediate acetaminophen-induced liver damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 8, pp. 3176–3181, 2014.

[209] M. Adachi, H. Higuchi, S. Miura et al., "Bax interacts with the voltage-dependent anion channel and mediates ethanol-induced apoptosis in rat hepatocytes," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 287, no. 3, pp. G695–G705, 2004.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5017460, 10 pages
http://dx.doi.org/10.1155/2016/5017460

Research Article

Protective Role of Dietary Curcumin in the Prevention of the Oxidative Stress Induced by Chronic Alcohol with respect to Hepatic Injury and Antiatherogenic Markers

Ravi Varatharajalu, Mamatha Garige, Leslie C. Leckey, Karina Reyes-Gordillo, Ruchi Shah, and M. Raj Lakshman

Lipid Research Laboratory, VA Medical Center and Department of Biochemistry and Molecular Medicine, The George Washington University, Washington, DC 20422, USA

Correspondence should be addressed to M. Raj Lakshman; raj.lakshman@va.gov

Received 18 June 2015; Revised 10 November 2015; Accepted 12 November 2015

Academic Editor: Silvana Hrelia

Copyright © 2016 Ravi Varatharajalu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Curcumin, an antioxidant compound found in Asian spices, was evaluated for its protective effects against ethanol-induced hepatosteatosis, liver injury, antiatherogenic markers, and antioxidant status in rats fed with Lieber-deCarli low menhaden (2.7% of total calories from ω-3 polyunsaturated fatty acids (PUFA)) and Lieber-deCarli high menhaden (13.8% of total calories from ω-3 PUFA) alcohol-liquid (5%) diets supplemented with or without curcumin (150 mg/kg/day) for 8 weeks. Treatment with curcumin protected against high ω-3 PUFA and ethanol-induced hepatosteatosis and increase in liver injury markers, alanine aminotransferase, and aspartate aminotransferase. Curcumin upregulated paraoxonase 1 (PON1) mRNA and caused significant increase in serum PON1 and homocysteine thiolactonase activities as compared to high ω-3 PUFA and ethanol group. Moreover, treatment with curcumin protected against ethanol-induced oxidative stress by increasing the antioxidant glutathione and decreasing the lipid peroxidation adduct 4-hydroxynonenal. These results strongly suggest that chronic ethanol in combination with high ω-3 PUFA exacerbated hepatosteatosis and liver injury and adversely decreases antiatherogenic markers due to increased oxidative stress and depletion of glutathione. Curcumin supplementation significantly prevented these deleterious actions of chronic ethanol and high ω-3 PUFA. Therefore, we conclude that curcumin may have therapeutic potential to protect against chronic alcohol-induced liver injury and atherosclerosis.

1. Introduction

Chronic alcohol consumption leads to alcoholic hepatosteatosis and consequently to inflammation, necrosis, fibrosis, and finally cirrhosis that afflicts millions worldwide and is one of the major causes of mortality in developed countries [1]. Liver alcohol dehydrogenase is primarily responsible for the oxidation of ethanol to acetaldehyde as well as hydroxyl radicals leading to the generation of reactive oxygen species (ROS). In addition, chronic ethanol-induced cytochrome P4502E1 (CYP2E1) mediated oxidation of ethanol also produces a state of oxidative stress by generating ROS within the cells including the α,β-unsaturated aldehyde, 4-hydroxy-2-nonenal (4-HNE) that may be more harmful than ROS because it has a longer half-life and can easily diffuse into cellular membranes [2]. The 4-HNE, in turn, is likely to play a major role for the progression of alcoholic fatty liver and liver disease. Whereas high ω-3 PUFA fish oil diet (~14% of dietary calories from ω-3 PUFA) causes severe hepatosteatosis liver injury [3], we showed that ω-3 PUFA fish oil diet (~2.7% of dietary calories from ω-3 PUFA) had protective effects [4].

Excess of ROS in alcoholics causes increased low density lipoproteins (LDL) oxidation that is avidly scavenged by macrophages leading to their transformation to foam cells that are atherogenic. Further, oxidized LDL is capable of stimulating endothelial cells to express adhesion molecules, which attract excess of foamy macrophages under the subendothelial layer, thereby promoting the seeding process of plaque formation. Incidence of atherosclerosis is high in chronic alcoholics due to higher level of ROS [5], homocysteine,

and homocysteine thiolactone (HTL) [6]. Antiatherogenic enzyme, paraoxonase 1 (PON1), is predominantly synthesized in the liver and secreted into circulation, where it avidly binds and becomes an integral part of high density lipoproteins (HDL) [7]. Antiatherosclerotic role of PON1 is to inhibit the oxidation of LDL as well as to inactivate oxidized lipid molecules by hydrolyzing cholesteryl linoleate hydroxide (CL-OH) and cholesteryl linoleate hydroperoxide (CL-OOH) groups by its esterase activity, thus facilitating reverse cholesterol transport (RCT) from macrophages to the liver for degradation [8, 9]. Other crucial function of PON1 is to hydrolyze highly toxic homocysteine thiolactone ultimately to methionine by methionine synthase pathway [10, 11].

A number of etiological agents of liver disease such as smoking, alcohol, high fat and high cholesterol, high polyunsaturated fatty acids (PUFA), lipopolysaccharide (LPS), iron, homocysteine, and HTL, ROS, and PON1 gene polymorphism adversely affect PON1 activity leading to cardiovascular disease (CVD) [11–18]. Selective antioxidants such as quercetin, pomegranate juice, vitamin C and folic acid, vitamins C and E, resveratrol, and betaine have been shown to protect against CVD by restoring PON activity [11, 19–24].

Curcumin (diferuloylmethane), a derivative of the spice turmeric (*Curcuma longa*), has been used in traditional medicine for thousands of years [25]. It is known for its use in wound healing [26] and also as an antibacterial, antiviral, and antifungal agent [27]. Curcumin also has antioxidant and anti-inflammatory properties and prevents alcohol- or carbon tetrachloride-induced liver injury by inhibiting the activation of NFκB signaling cascade, and the subsequent induction of proinflammatory cytokines, as well as chemokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in Kupffer cells [28, 29]. A number of studies have shown that curcumin can reduce lipid peroxidation and liver and serum cholesterol in several liver injury models [30–32].

Therefore, in this present study, we have addressed for the first time the concerted protective role played by dietary curcumin on chronic ethanol and PUFA mediated adverse effects on hepatic liver injury due to oxidative stress as evidenced by alterations in liver (i) ROS, (ii) endogenous antioxidant, and reduced glutathione (GSH), as well as (iii) hepatic lipid score. In addition, we also evaluated the possible protective action of curcumin on the antiatherogenic gene, PON1/HTLase, as well as on plasma and liver lipids.

2. Materials and Methods

2.1. Animals. Female Wistar-Furth rats weighing between 130 and 150 g were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Female animals were chosen because they are known to show more severe alcohol-induced liver injury than males [33–35]. They were housed in pairs per cage in plastic cages (40 × 24 × 18 cm), in a temperature-controlled room at 25°C with 12-hour light : dark cycle. All rats were fed a pelleted commercial diet (Purina Rodent Chow, #500, TMI Nutrition, St. Louis, MO) during the first week of acclimation period after arrival. They were then divided randomly into the following low ω-3 PUFA (LFO) and high ω-3 PUFA (HFO) groups as depicted in Table 1. Each of the LFO ($n = 12$) and HFO ($n = 12$) groups were further subdivided separately into three groups of 4 each: LFO or HFO (controls), LFO or HFO plus ethanol (LFOE or HFOE, 35% of dietary calories derived from ethanol), and LFOE or HFOE supplemented with curcumin 150 mg/day/kg [36] body weight (LFOEC or HFOEC). All LFO and HFO diets were made isoenergetic by substituting ethanol calories with dextrin-maltose and all these groups were pair-fed for 8 weeks. The composition of each diet (Dyets Inc., Bethlehem, PA) according to individual ingredients is described in Table 2.

Table 1: Composition of various experimental diets, total fat, ω-3 PUFA, and ethanol.

Dietary groups ($n = 4$)	As percent of total calories			
	Total Fat	Ethanol	Protein	Carbohydrate
Low ω-3 PUFA control (LFO)	35*	0	18	47
Low ω-3 PUFA + ethanol (LFOE)	35*	36	18	11
Low ω-3 PUFA + ethanol + curcumin (LFOEC)	35*	36	18	11
High ω-3 PUFA control (HFO)	35†	0	18	47
High ω-3 PUFA + ethanol (HFOE)	35†	36	18	11
High ω-3 PUFA + ethanol + curcumin (HFOEC)	35†	36	18	11

*2.7% of total calories came from ω-3 PUFA; †13.8% of total calories came from ω-3 PUFA.

Table 2: Composition of various diets according to individual ingredients.

Diet ingredient	Composition, grams/L			
	LFO	LFOE	HFO	HFOE
Casein	41.4	41.4	41.4	41.4
L-Cysteine	0.5	0.5	0.5	0.5
DL-Methionine	0.3	0.3	0.3	0.3
Menhaden oil	8.5	8.5	39.6	39.6
Olive oil	28.4	28.4	0	0
Safflower oil	2.7	2.7	0	0
Maltose dextrin	115.2	25.6	115.2	25.6
Cellulose	10	10	10	10
Mineral mix	8.75	8.75	8.75	8.75
Vitamin mix	2.5	2.5	2.5	2.5
Choline bitartrate	0.53	0.53	0.53	0.53
Xanthan gum	3	3	3	3

2.2. Lipid Analysis. Blood samples were collected and centrifuged at 3100 rpm (Beckman J6M) for 10 min at 4°C. Separated serum, plasma, and liver samples were frozen at −80°C until assayed. Liver lipids were extracted as previously

described [37]. Both plasma and liver lipids were determined using enzymatic reagents as described by the manufacturer (Teco Diagnostics, Anaheim, CA).

2.3. Quantification of Hepatosteatosis by Oil Red O. Livers from various experimental groups were cut into small pieces and washed immediately with ice cold PBS and mounted on optimum cutting temperature (OCT) embedding compound in peel-a-way embedding molds (Electron Microscope Sciences, Hatfield, PA). Liver tissues were cryosectioned and stained with oil red O to measure accumulation of lipid using an automated histometric system (Image-Pro Plus 6.1, Media Cybernetics, Bethesda, MD) as described previously [11]. The data are expressed as average oil red O percentage area of lipid staining. Values are means ± SEM.

2.4. 4-Hydroxynonenal (4-HNE) Staining of Liver Sections. Liver tissue sections were dewaxed in xylene and rehydrated and antigen was retrieved in heat incubated citrate buffer for 3 minutes. After washing in Tris buffered saline (TBS/T) containing Tween 20 (0.1%), sections were incubated overnight at 4°C in primary antibody raised in goat against 4-HNE (Millipore Incorporation, MA, USA) that was diluted to 1 : 100 in CAS-Block (Life Technologies Grand Island, NY) reagent. After an hour of secondary antibody incubation, a DAB Plus Substrate kit (Life Technologies) was used as the chromogen, and then slides were counterstained with hematoxylin. Intensity of 4-HNE-positive brown colored area was detected under optical microscopy (Observer Z1, Carl Zeiss Microimaging, Inc., Thornwood, NY) and % of brown pixels were calculated using AxioVision Rel.4.8.2 software in 4 randomly selected microscopic fields (magnification 40x) per section of all groups ($n = 4$).

2.5. Serum Biomarkers of Liver Injury. Leakage of liver transaminases into the blood compartment was assessed by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in each animal from the various experimental groups using commercial kits according to manufacturer's instructions (Teco Diagnostics, Anaheim, CA). Mean serum ALT and AST activity in each group are expressed as international units per liter (IU/L) ± SEM.

2.6. RNA Isolation and Real-Time PCR. The total RNA was isolated from each liver using the Tri-Reagent (Molecular Research Center, Cincinnati, OH) as per manufacturer's instructions. Isolated total RNA was reverse transcribed by *in vitro* transcription as described by the manufacturer (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using a Bio-Rad iCycler using the SYBR green PCR mix (Bio-Rad, Hercules, CA). To amplify the coding region of PON1 and β-actin, we used gene-specific primers for rat PON1 forward primer 5′-TGCTGGCTCACAAGATTCAC-3′ and reverse primer 5′-TTCCTTTGTACACAGCAGCG-3′ and β-actin (forward: 5′-GTCAGGTCACTATCGGC-3′; reverse: 5′-CATGGATGCCACAGGATTCC-3′). Data were normalized to β-actin and then quantified.

2.7. Serum PON1 and HTLase Activity Measurement. Serum PON1 enzyme activity was determined with paraoxon (Sigma-Aldrich Inc., St. Louis, MO) as the substrate. PON1 activity was measured as described by us previously [11, 13]. One unit of international enzyme activity is equal to 1 nmol of paraoxon hydrolyzed per min per mL serum. PON1 activity in each experimental group was expressed as percent of the activity in the corresponding control group.

Serum HTLase activity was measured using 10 mM γ-thiobutyrolactone (Sigma Inc.) as the substrate [11, 38] and Ellman's procedure to monitor the accumulation of free sulfhydryl groups via coupling with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid). The assay conditions were the same as for PON1 assay above and the rate of γ-thiobutyrolactone hydrolysis was expressed as nmoles per mL per min.

2.8. Liver Reduced Glutathione (GSH) Measurement. 1 g of tissue was weighed and washed with ice cold saline and homogenized in 10 mL of ice cold homogenizing buffer (10 mM Tris-HCl, pH 7.2; 250 mM Sucrose; 1 mM EDTA) containing 2x protease inhibitor cocktail. Reduced GSH was quantified according to manufacturer's instructions (Sigma Inc.).

2.9. Statistical Analysis. All data are presented as means ± standard error of the mean (SEM). The data were analyzed as one-way ANOVA. This design was selected rather than the 3-way factorial design in order to save unnecessary animals in the curcumin treatment control groups. Pairwise comparisons and linear contrasts were not made where the overall ANOVA p value exceeded 0.05. In order to protect against inflation of the type 1 error rate, a Bonferroni adjustment (SAS Institute Inc., Cary, NC, USA) was made to the critical alpha value that was selected by dividing the 0.05 alpha by the number of preplanned comparisons. A p value that was ≤ 0.05 was considered significant.

3. Results

3.1. Influence of Chronic Ethanol, Low ω-3 PUFA, High ω-3 PUFA, and Curcumin on Growth Pattern and Hepatosomatic Index in Various Experimental Groups. As shown in Table 3, animals from all experimental groups grew normally with significant gain in body weights, although the gains in the mean body weight in the ethanol fed groups were not as significant as in the corresponding control groups even when supplemented with curcumin. The mean liver weight relative to the mean body weight (hepatosomatic index) increased significantly by 23% ($p < 0.0001$), in the high HFOE group but not in the LFOE group.

3.2. Influence of Chronic Ethanol, Low ω-3 PUFA, High ω-3 PUFA, and Curcumin on the Status of Plasma and Liver Lipids. Table 4 shows that plasma triglycerides, VLDL-C, LDL-C, HDL-C, and total cholesterol were insignificantly altered in rats fed low PUFA alcohol group compared to control low PUFA group. LFOEC group showed significantly lowered plasma triglycerides compared to the LFOE group

Table 3: Effect of ethanol and curcumin in low and high ω-3 PUFA on liver weight to body weight ratio.

Dietary groups ($n = 4$)	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Final liver weight (g)	Hepatosomatic index
LFO	130.1 ± 3.9	244.5 ± 5.9	114.4 ± 6.2	7.8 ± 0.4	3.2 ± 0.16
LFOE	160 ± 4.8	233.4 ± 4.8	73.4 ± 4.8*	7.5 ± 0.3	3.2 ± 0.20
LFOEC	156.5 ± 3.4	209.0 ± 4.4	53 ± 4.1†	6.6 ± 0.7†	3.0 ± 0.24
HFO	159.4 ± 2.9	268.2 ± 7.3	108.8 ± 4.3§	8.4 ± 0.2	3.1 ± 0.18
HFOE	178.3 ± 4.2	228.3 ± 6.7	50.3 ± 4.6*	8.8 ± 0.3*	3.8 ± 0.21*
HFOEC	157.4 ± 3.3	216.5 ± 0.6	59 ± 2.8‡	7.72 ± 0.1†	3.6 ± 0.26*

Values are means ± SEM. Means in a column with different superscripts differ significantly ($p < 0.05$) as calculated by t-test with Bonferroni correction. $^{*}p < 0.0001$, compared to corresponding LFO or HFO groups; $^{\dagger}p < 0.0001$, compared to the corresponding LFOE or HFOE groups; $^{\ddagger}p < 0.0001$ compared to HFO; $^{\S}p < 0.02$ compared to LFO. Hepatosomatic index = (Liver weight × 100)/body weight.

Table 4: Effect of Chronic ethanol and curcumin on blood and livers lipids of rats fed low and high ω-3 PUFA diets.

Groups $n = 4$	Plasma (mg/dL)					Liver (mg/100 g)	
	Triglycerides	VLDL-C	LDL-C	HDL-C	Total-C	Triglycerides	Total-C
LFO	83 ± 5.5	17 ± 1.8	24 ± 2	26 ± 3	66 ± 8	132 ± 16	56 ± 6
LFOE	75 ± 4.5*	15 ± 1.7	20 ± 4	25 ± 6	60 ± 6	146 ± 14	165 ± 8*
LFOEC	64 ± 5†	13 ± 0.9	15 ± 3	20 ± 5	48 ± 7$^{\dagger *}$	117 ± 12	117 ± 12$^{\dagger *}$
HFO	104 ± 8*	21 ± 1.3*	6 ± 0.6*	14 ± 2$^{\dagger *}$	40 ± 7*	549 ± 42*	201 ± 8.0$^{\dagger *}$
HFOE	74 ± 6.3‡	15 ± 1.5‡	11 ± 0.8‡	21 ± 4‡	47 ± 9	734 ± 49‡	393 ± 53‡
HFOEC	86 ± 7‡,	17 ± 2.0‡	10 ± 0.7‡	14 ± 2	40 ± 7	633 ± 55†‡	217 ± 5.0†‡

Values are means ± SEM. Means in a column with different superscripts differ significantly ($p < 0.05$) as calculated by t-test with Bonferroni correction. $^{*}p < 0.05$, compared to LFO; $^{\dagger}p < 0.05$, compared to the corresponding LFOE; $^{\ddagger}p < 0.05$ compared to the corresponding HFO.

($p < 0.002$); however plasma triglycerides significantly increased in HFOEC group compared to HFOE group ($p < 0.04$). HFOE group had significantly lower VLDL-C compared to HFO group ($p < 0.0001$). There was a tendency for plasma LDL-C, HDL-C, and total cholesterol to increase in HFOE group compared to HFOEC group, although the results were not statistically significant. Compared to respective control groups, both LFOE ($p < 0.0001$) and HFOE ($p < 0.0005$) groups exhibited significant increase in both liver triglycerides and cholesterol. Both liver triglycerides and cholesterol were significantly lower in LFOEC and HFOEC groups compared to the corresponding LFOE ($p < 0.0001$, $p < 0.0001$) and HFOE ($p < 0.0001$, $p < 0.0001$) groups, respectively.

3.3. Influence of Chronic Ethanol, Low ω-3 PUFA, High ω-3 PUFA, and Curcumin on Hepatosteatosis. As shown in Figures 1(a) and 1(b), histochemistry of oil red O stained liver sections of low ω-3 PUFA ethanol fed rats showed no significant changes of fat deposition compared to control. Ethanol significantly increased hepatosteatosis in rats fed high ω-3 PUFA compared to other groups ($p < 0.0001$). Curcumin significantly reduced hepatosteatosis in HFOEC group compared to HFOE group ($p < 0.002$).

3.4. Influence of Chronic Ethanol, Low ω-3 PUFA, High ω-3 PUFA, and Curcumin on Liver Injury. As shown in Figures 2(a) and 2(b), serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) did not alter significantly between LFO and HFO groups. However, while serum AST and ALT increased significantly by 28% ($p < 0.008$) and 40% ($p < 0.008$), respectively, in LFOE group compared to LFO group, these markers were significantly restored to the level of LFO group by curcumin supplementation ($p < 0.0002$). Furthermore, serum ALT and AST increased dramatically by 60% ($p < 0.0001$) and by 50% ($p < 0.0001$), respectively, in HFOE compared to the HFO group. Curcumin feeding significantly blocked these dramatic increases in serum ALT and AST as compared to HFOE group ($p < 0.0001$).

3.5. Influence of Chronic Ethanol, Low ω-3 PUFA, High ω-3 PUFA, and Curcumin on Liver PON1 mRNA Expression. As shown in Figure 3, liver PON1 mRNA expression was decreased by 23% ($p < 0.01$) in the high ω-3 PUFA-fed group compared to the low ω-3 PUFA group. Chronic ethanol feeding decreased liver PON1 mRNA expression by 25% ($p < 0.001$) and 30% ($p < 0.01$) in both low and high ω-3 PUFA groups, respectively, compared to low ω-3 PUFA group. Curcumin significantly increased liver PON1 mRNA expression in high PUFA alcohol group ($p < 0.04$) but insignificantly increased PON1 mRNA in low PUFA alcohol groups.

3.6. Influence of Chronic Alcohol, Low ω-3 PUFA, High ω-3 PUFA, and Curcumin on Serum PON1 and HTLase Activity. As shown in Figure 4, high ω-3 PUFA significantly decreased serum PON1 activity (Figure 4(a)) by 20% ($p < 0.003$) and HTLase activity (Figure 4(b)) by 28% ($p < 0.0001$,

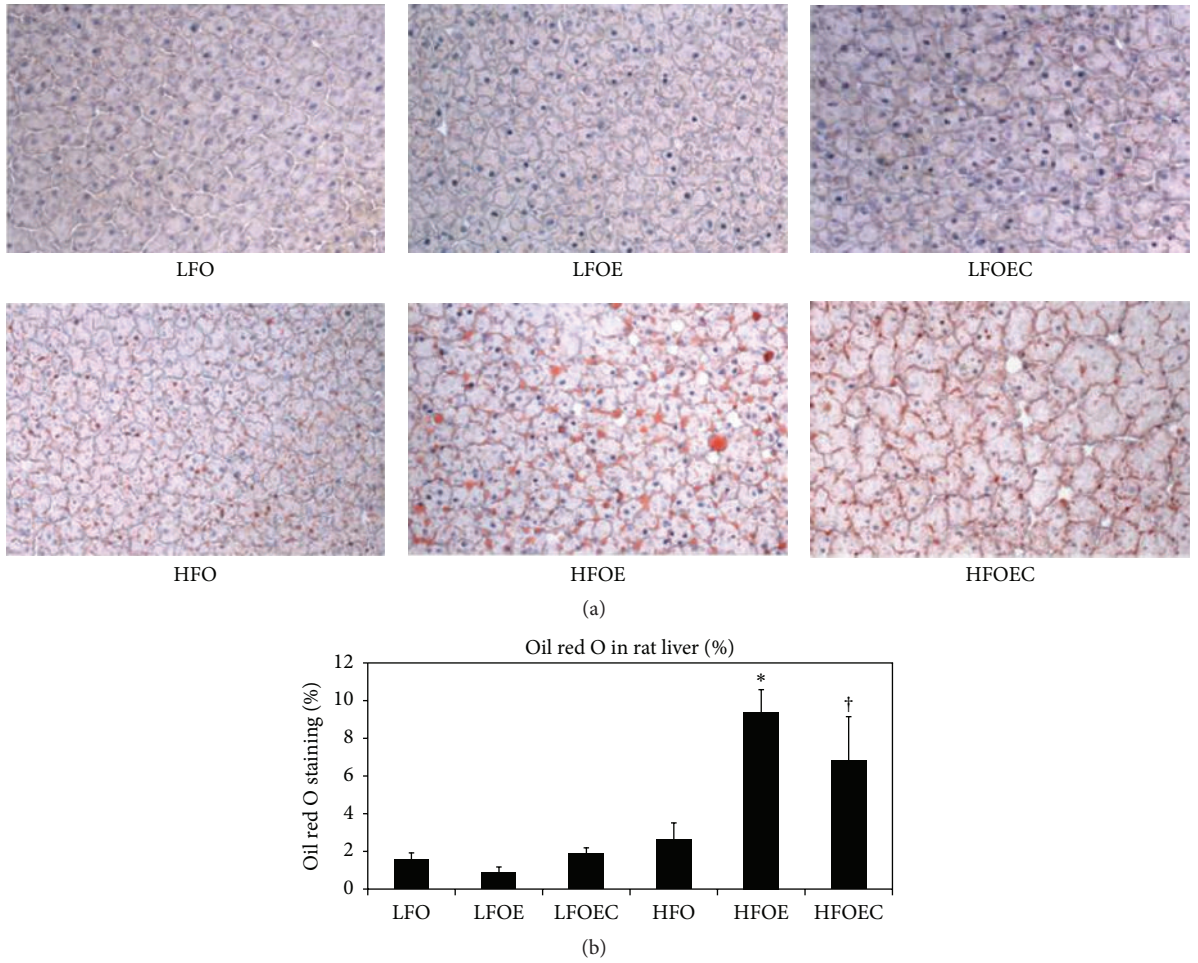


Figure 1: Histopathology of rat liver specimens and lipid deposits in the study groups. Cryosections of tissues were prepared as described in Section 2. Sections were stained with oil red O and counterstained with hematoxylin. (a) Representative 20x photomicrograph from the various groups. (b) Quantification of oil droplets in oil red O stained sections as determined by the Image-Pro Plus version 6.1 method. The data are expressed as average oil red O percentage area of lipid staining. Values are means ± SEM. Statistical significance of variance was calculated using t-test with Bonferroni correction; $^{*}p < 0.0001$ compared to all groups except HFOEC group; $^{\dagger}p < 0.002$ compared to HFOE group.

Figure 4(b)) compared to low ω-3 PUFA. Correspondingly, serum PON1 activity (Figure 4(a)) decreased by 23% ($p < 0.05$) in LFOE group and 58% ($p < 0.0001$) in HFOE group while serum HTLase activity (Figure 4(b)) decreased by 25% ($p < 0.0001$) and 59% ($p < 0.0001$) in the low and high ω-3 PUFA alcohol groups, respectively. Curcumin moderately stimulated serum PON1 activity by 15% ($p < 0.05$) (Figure 4(a)) in the high ω-3 PUFA alcohol group compared to HFOE group while it had no effect as compared to the LFOE group. Curcumin caused similar changes in serum HTLase activity only in the high ω-3 PUFA ethanol group.

3.7. Influence of Chronic Ethanol, PUFA, and Curcumin on Liver GSH. As shown in Figure 5, there was no significant difference in the liver GSH levels in low or high ω-3 PUFA fed groups. However, chronic ethanol significantly decreased liver GSH levels by 27% ($p < 0.0001$) in low ω-3 PUFA group and by 38% ($p < 0.005$) in high ω-3 PUFA group. Curcumin restored liver GSH levels in both low and high ω-3 PUFA alcohol groups nearly to the control level ($p < 0.05$).

3.8. Influence of Chronic Ethanol, PUFA, and Curcumin on Lipid Peroxidation. To determine if the treatment with curcumin may have protected livers from the onset of alcohol-induced hepatitis, we determined levels of 4-HNE protein adduct which is an indicator of lipid peroxidation. Figure 6 shows the staining of 4-HNE protein adducts was found to be significantly elevated in livers of LFOE (Figure 6(b)) and HFO (Figure 6(d)) and HFOE (Figure 6(e)) as compared to LFO (Figure 6(a)) group ($p < 0.0001$). Curcumin feeding significantly lowered levels of 4-HNE protein adducts in both LFOEC (Figure 6(c)) and HFOEC (Figure 6(f)) as compared to LFOE and HFOE groups ($p < 0.001$), respectively.

4. Discussion

This study clearly demonstrates the ability of dietary curcumin to significantly attenuate chronic ethanol and ω-3 PUFA

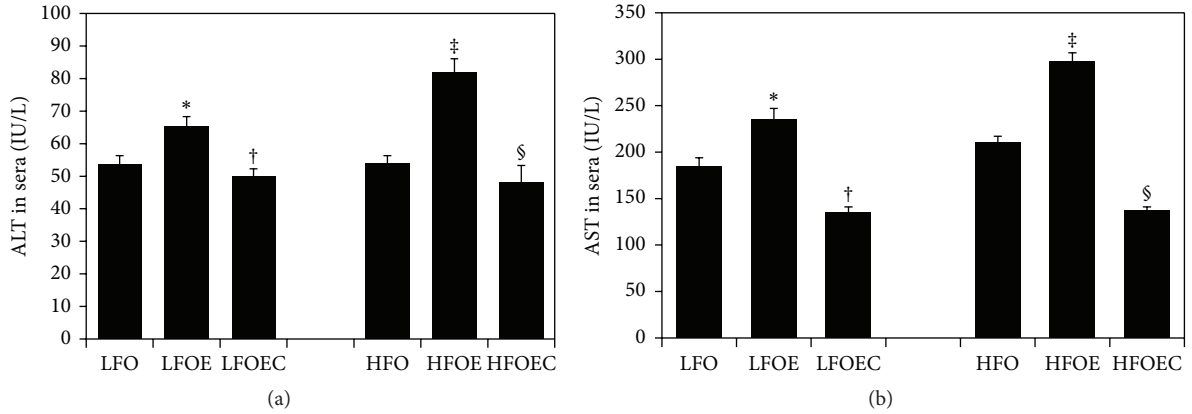


Figure 2: Influence of chronic ethanol, ω-3 PUFA, and curcumin on serum ALT and AST in rats fed low and high ω-3 PUFA diets. The animals in the indicated groups ($n = 4$) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of curcumin for 8 weeks after which the animals were killed and each serum sample was analyzed. (a) ALT and (b) AST levels of these liver markers were measured as described in Section 2. The relative ALT and AST activities in the various experimental groups are expressed as international units per liter. The data are Means ± SEM. Statistical significance of variance was calculated using t-test with Bonferroni correction; $^{*}p < 0.008$ compared to LFO group; $^{\dagger}p < 0.0002$ compared to the corresponding LFOE group; $^{\ddagger}p < 0.0001$ compared to HFO group; $^{\S}p < 0.0001$ compared to HFOE group.

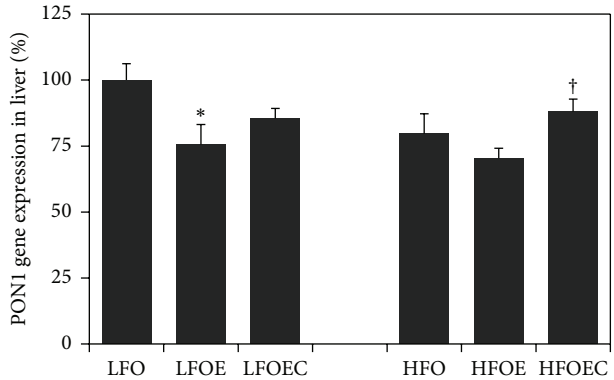


Figure 3: Effect of chronic ethanol and curcumin onPON1 mRNA expression in livers of rats fed low and high ω-3 PUFA diets. The animals in the indicated groups ($n = 4$) were pair-fed their respective Lieber-DeCarli control or alcohol containing liquid diets for 8 weeks after which the animals were killed and each liver was analyzed for PON1 mRNA as described in Section 2. The relative expression of PON1 mRNA in the HFO group is expressed as percent of the corresponding values in the LFO group. The data are Means ± SEM. Statistical significance of variance was calculated using t-test with Bonferroni correction; $^{*}p < 0.0001$ compared to LFO group; $^{\dagger}p < 0.04$ compared to HFOE group.

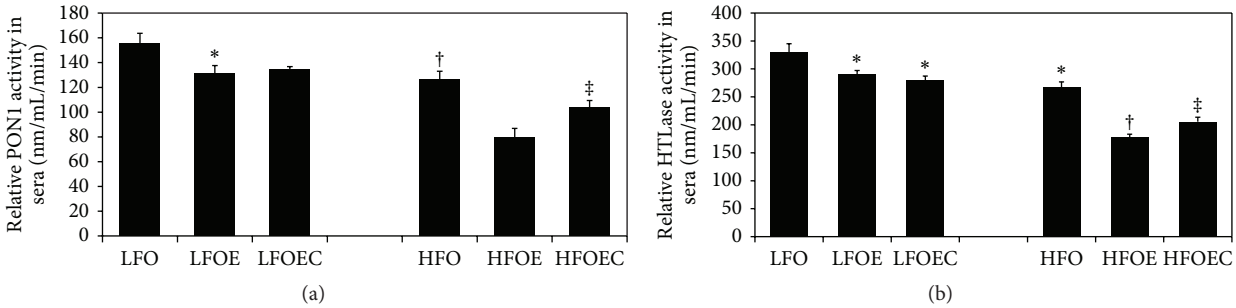


Figure 4: Influence of chronic ethanol and curcumin on serum PON1 and homocysteine thiolactonase activities were measured in rats fed low and high ω-3 PUFA diets. The animals in the indicated groups ($n = 4$) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of the curcumin for 8 weeks after which the animals were killed and serum sample was analyzed. (a) Serum PON1 activity measured using paraoxon as substrate. (b) Serum homocysteine thiolactonase activity was measured using γ-thiobutyrolactone as substrate. Enzyme activities in the various experimental groups are expressed as percent of the corresponding values in the LFO group. The data are Means ± SEM. Statistical significance of variance was calculated using t-test with Bonferroni correction; $^{*}p < 0.0001$ compared to LFO group; $^{\dagger}p < 0.0001$ compared to LFO group; $^{\ddagger}p < 0.05$ compared HFOE group.

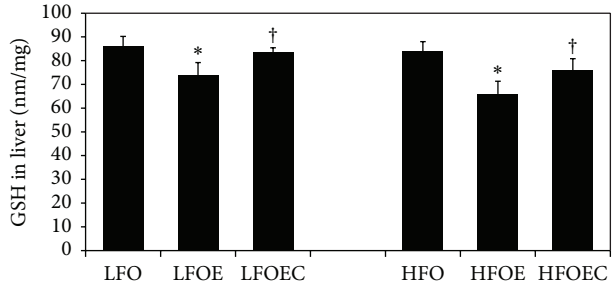


Figure 5: Influence of chronic ethanol, ω-3 PUFA, and curcumin on liver GSH level in rats fed low and high ω-3 PUFA diets. The animals in the indicated groups ($n = 4$) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of curcumin for 8 weeks after which the animals were killed and each liver was analyzed for reduced GSH level in liver quantified as described in Section 2. The data are Means ± SEM. Statistical significance of variance was calculated using t-test with Bonferroni correction; $^{*}p < 0.0001$ compared to corresponding LFO or HFO group; $^{\dagger}p < 0.005$ compared to the corresponding LFOE or HFOE group.

mediated hepatomegaly (Table 3), hyperlipidemia (Table 4), hepatosteatosis (Figure 1), steatohepatitis (Figure 2), and antiatherogenic markers. Furthermore, the protective properties of curcumin were associated with its antioxidant actions. This was assessed by biochemical and histological methods.

Consistent with numerous alcohol feeding studies, the present study confirms that alcohol-fed animals do not seem to gain as much weight as the control animals in spite of pair-feeding leading to increased hepatosomatic index (Table 3). Strikingly, curcumin feeding seems to further reduce the gain in body weight resulting in greater increase in the hepatosomatic index in both a low and a high PUFA alcohol diet, showing its ameliorative effects on hepatomegaly. These results may be related in part to the ability of curcumin to suppress adipogenesis.

The significant decreases in plasma triglycerides in LFOEC group compared to LFOE group (Table 4) could be explained due to the potential inhibitory action of curcumin on hepatic triglyceride and VLDL synthesis. This is reflected in significant decreases in plasma VLDL-C and total cholesterol. The present work clearly shows that feeding a high PUFA alcohol diet increased hepatosteatosis (measured with oil red O staining) and liver triglycerides and total cholesterol (Table 4), whereas curcumin feeding significantly prevented these effects on the hepatosteatotic action of chronic ethanol. These results are in accord with the effects of curcumin in ob/ob male mice fed high fat diet [39].

Levels of ALT and AST were increased in animals fed with high PUFA alcohol (Figure 2), indicating hepatocellular injury and dysfunction. It must be pointed out that the gross liver appearance and liver histopathology as well as ALT and AST data show that the liver injury caused by chronic ethanol is markedly alleviated by curcumin feeding in high ω-3 fatty acid fed animals. Supporting our observations, curcumin has been shown to decrease hepatocellular injury and dysfunction in other hepatic injury models such as carbon tetrachloride [27, 36, 40], LPS/D-glucosamine [41], and methionine/choline deficiency [42].

Our present study clearly shows that feeding both low and high ω-3 PUFA significantly downregulates the gene expression and activity of the multifunctional antioxidant enzyme PON1 in both total liver and serum (Figure 3). These results are consistent with our previous study [11] and with those of Kudchodkar et al. [43]. Since chronic ethanol-induced cytochrome P4502E1 (Cyp2E1) exacerbates the accelerated generation of ROS in the presence of high ω-3 PUFA, it is reasonable that this increased ROS may be responsible for these deleterious effects on PON1 status that is vulnerable to oxidative stress. The ability of curcumin to partially restore chronic ethanol and ω-3 PUFA-mediated downregulation of serum PON1 activity and also PON1 mRNA in the liver as well as liver HTLase activity supports its antiatherogenic role of this antioxidant enzyme. Beneficial effects of other antioxidants such as pomegranate [44], vitamins C and E [22], and catechin [45] on serum PON1 activity are known. However, to our knowledge, this is the first time the protective effect of curcumin on chronic ethanol in combination with high ω-3 PUFA mediated decrease in serum PON1 activity has been demonstrated. Since serum PON1 is an integral component of serum HDL-C there is a direct positive correlation between serum HDL-C and serum PON1 level. Therefore, the inability of curcumin to completely restore serum PON1 and HTLase activities in HFOEC group to their corresponding levels in HFO group may be due to the fact that curcumin also independently lowers plasma HDL-C (Table 4). It is well known that, apart from plasma HDL, PON1 is the only other most important antiatherogenic marker because of its ability to not only prevent LDL oxidation, but also destroy the other atherogenic molecule, homocysteine [6–9, 13–20].

The above antihepatosteatotic and antiatherogenic effects of curcumin are accompanied by reciprocal restoration of severely depleted liver GSH level and a reduction of 4-HNE lipid peroxidation product in chronic ethanol-fed group. These results are consistent with previous reports [27, 40] indicating that curcumin acts as an antioxidant. Our data are also consistent with previous studies, which demonstrated that curcumin induced *de novo* synthesis of GSH [46, 47]. These results strongly suggest the antioxidant capacity of curcumin in nullifying ethanol-induced ROS generation.

5. Conclusion

In conclusion, we have demonstrated that dietary curcumin not only effectively protects against the deleterious effects

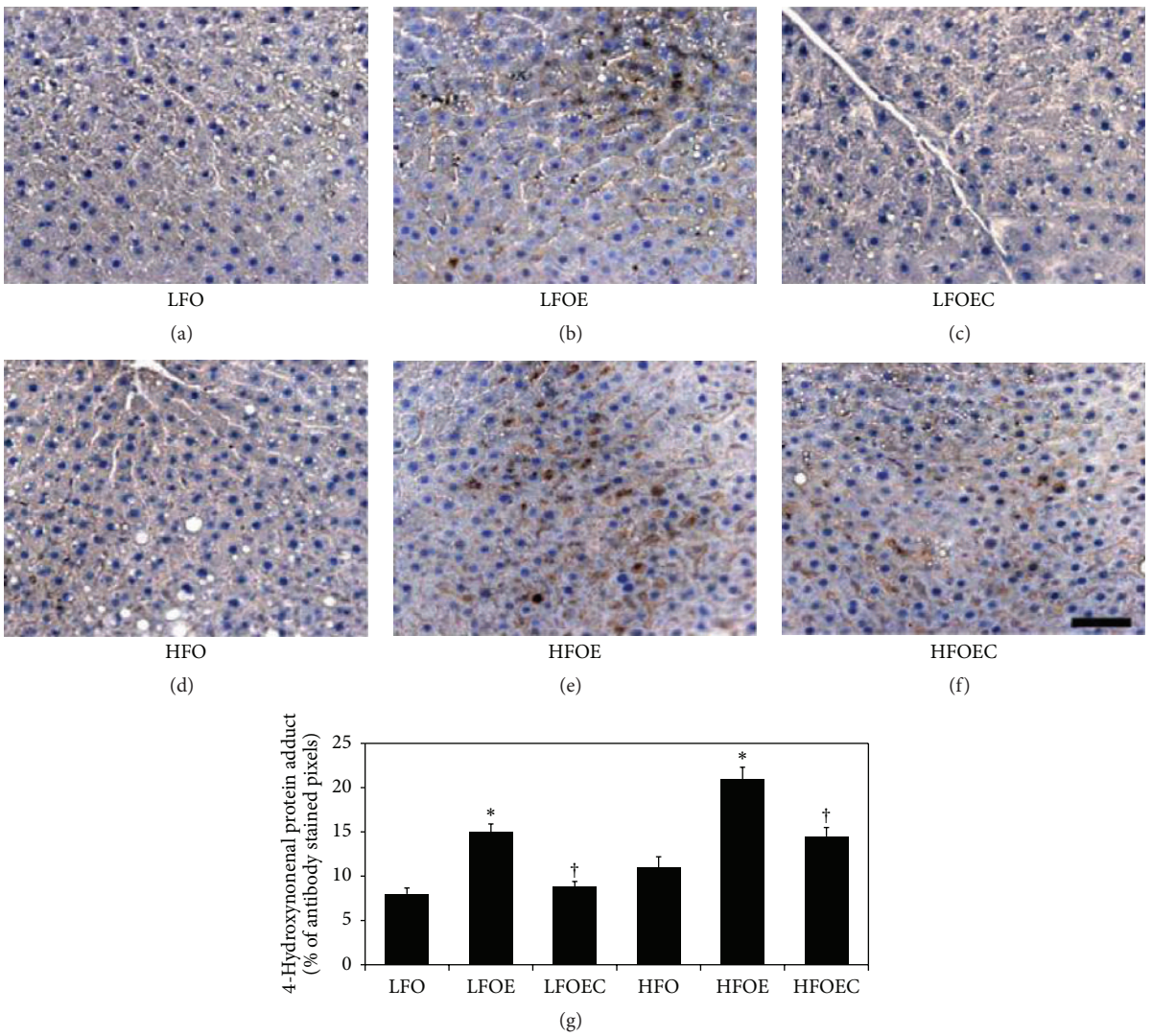


Figure 6: Effect of chronic alcohol, ω-3 PUFA, and curcumin on 4-HNE protein adducts levels (×200) in liver samples of various groups. The animals in the indicated groups ($n = 4$) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of curcumin for 8 weeks after which the animals were killed and each rat liver sample was paraffin sectioned and stained with 4-HNE specific antibody. (1) Representative photomicrographs of immunohistochemically stained 4-HNE protein adducts (scale bars: 200 μm) in liver sections of following groups shown in (a) LFO; (b) LFOE; (c) LFOEC; (d) HFO; (e) HFOE; and (f) HFOEC and (g). Bar graph shows the % of pixel values of brown color representing 4-HNE specific antibody reactivity from four randomly selected regions per section of each group ($n = 4$) measured using AxioVision Rel.4.8.2 software. The data are Means ± SEM. Statistical significance of variance was calculated using t-test with Bonferroni correction; $^{*}p < 0.0001$ compared to all groups except HFOEC group; $^{\dagger}p < 0.001$ compared to HFOE group.

of chronic ethanol in combination with high PUFA on liver injury, but also favorably improves the antiatherogenic markers such as PON1/HTLase and GSH. These findings raise the possibilities that consuming traditional natural antioxidants compounds like curcumin may benefit health by modulating the lipid metabolism in alleviating hepatosteatosis and suppressing atherogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported partly by a Merit Grant from Department of Veterans Affairs and partly by NIH Grant R01AA0207020.

References

[1] P. E. Molina, C. McClain, D. Valla et al., "Molecular pathology and clinical aspects of alcohol-induced tissue injury," *Alcoholism: Clinical and Experimental Research*, vol. 26, no. 1, pp. 120–128, 2002.

[2] H. Esterbauer, R. J. Schaur, and H. Zollner, "Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes," *Free Radical Biology and Medicine*, vol. 11, no. 1, pp. 81–128, 1991.

[3] A. A. Nanji, S. Zhao, S. M. H. Sadrzadeh, A. J. Dannenberg, S. R. Tahan, and D. J. Waxman, "Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-ethanol-fed rats," *Alcoholism: Clinical and Experimental Research*, vol. 18, no. 5, pp. 1280–1285, 1994.

[4] M. R. Lakshman, S. J. Chirtel, and L. L. Chambers, "Roles of omega 3 fatty acids and chronic ethanol in the regulation of plasma and liver lipids and plasma apoproteins A1 and E in rats," *Journal of Nutrition*, vol. 118, no. 11, pp. 1299–1303, 1988.

[5] W. Wu and A. I. Cederbaum, *Alcohol, Oxidative Stress, and Free Radical Damage*, NIAAA Publication, 2004.

[6] M. L. Cravo, L. M. Gloria, J. Selhub et al., "Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status," *The American Journal of Clinical Nutrition*, vol. 63, no. 2, pp. 220–224, 1996.

[7] S. Deakin, I. Leviev, M. Gomaraschi, L. Calabresi, G. Franceschini, and R. W. James, "Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism," *Journal of Biological Chemistry*, vol. 277, no. 6, pp. 4301–4308, 2002.

[8] M. Aviram, M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo-Parmo, and B. N. La Du, "Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase," *The Journal of Clinical Investigation*, vol. 101, no. 8, pp. 1581–1590, 1998.

[9] O. Rozenberg, D. M. Shih, and M. Aviram, "Human serum paraoxonase 1 decreases macrophage cholesterol biosynthesis: possible role for its phospholipase-A_2-like activity and lysophosphatidylcholine formation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 3, pp. 461–467, 2003.

[10] H. Jakubowski, "Calcium-dependent human serum homocysteine thiolactone hydrolase. A protective mechanism against protein N-homocysteinylation," *The Journal of Biological Chemistry*, vol. 275, no. 6, pp. 3957–3962, 2000.

[11] R. Varatharajalu, M. Garige, L. C. Leckey, M. Gong, and M. R. Lakshman, "Betaine protects chronic alcohol and ω-3 PUFA-mediated down-regulations of PON1 gene, serum PON1 and homocysteine thiolactonase activities with restoration of liver GSH," *Alcoholism: Clinical and Experimental Research*, vol. 34, no. 3, pp. 424–431, 2010.

[12] E. Nishio and Y. Watanabe, "Cigarette smoke extract inhibits plasma paraoxonase activity by modification of the enzyme's free thiols," *Biochemical and Biophysical Research Communications*, vol. 236, no. 2, pp. 289–293, 1997.

[13] M. N. Rao, P. Marmillot, M. Gong et al., "Light, but not heavy alcohol drinking, stimulates paraoxonase by up regulating liver mRNA in rats and humans," *Metabolism*, vol. 52, no. 10, pp. 1287–1294, 2003.

[14] I. Durak, H. Özbek, E. Devrim, N. Karagenç, and I. B. Ergüder, "Effects of cholesterol supplementation on antioxidant enzyme activities in rat hepatic tissues: possible implications of hepatic paraoxonase in atherogenesis," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 14, no. 4, pp. 211–214, 2004.

[15] L. G. Costa and C. E. Furlong, Eds., *Paraoxonase (PON1) in Health and Disease: Basic and Clinical Aspects*, Kluwer Academic Publishers, Norwell, Mass, USA, 2002.

[16] K. Trudel, D. Sinnett, R. W. James et al., "Iron-ascorbic acid-induced oxidant stress and its quenching by paraoxonase 1 in HDL and the liver: comparison between humans and rats," *Journal of Cellular Biochemistry*, vol. 96, no. 2, pp. 404–411, 2005.

[17] G. Ferretti, T. Bacchetti, E. Marotti, and G. Curatola, "Effect of homocysteinylation on human high-density lipoproteins: a correlation with paraoxonase activity," *Metabolism*, vol. 52, no. 2, pp. 146–151, 2003.

[18] M.-C. Blatter Garin, R. W. James, P. Dussoix et al., "Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes," *Journal of Clinical Investigation*, vol. 99, no. 1, pp. 62–66, 1997.

[19] M. Gong, M. Garige, R. Varatharajalu et al., "Quercetin up-regulates paraoxonase 1 gene expression with concomitant protection against LDL oxidation," *Biochemical and Biophysical Research Communications*, vol. 379, no. 4, pp. 1001–1004, 2009.

[20] M. Aviram, M. Rosenblat, D. Gaitini et al., "Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation," *Clinical Nutrition*, vol. 23, no. 3, pp. 423–433, 2004.

[21] M. F. Gursu, M. Onderci, F. Gulcu, and K. Sahin, "Effects of vitamin C and folic acid supplementation on serum paraoxonase activity and metabolites induced by heat stress in vivo," *Nutrition Research*, vol. 24, no. 2, pp. 157–164, 2004.

[22] G. P. Jarvik, N. T. Tsai, L. A. McKinstry et al., "Vitamin C and E intake is associated with increased paraoxonase activity," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 8, pp. 1329–1333, 2002.

[23] B. F. Curtin, K. I. Seetharam, P. Dhoieam, R. K. Gordon, B. P. Doctor, and M. P. Nambiar, "Resveratrol induces catalytic bioscavenger paraoxonase 1 expression and protects against chemical warfare nerve agent toxicity in human cell lines," *Journal of Cellular Biochemistry*, vol. 103, no. 5, pp. 1524–1535, 2008.

[24] M. Garige, M. Gong, R. Varatharajalu, and M. R. Lakshman, "Quercetin up-regulates paraoxonase 1 gene expression via sterol regulatory element binding protein 2 that translocates from the endoplasmic reticulum to the nucleus where it specifically interacts with sterol responsive element-like sequence in paraoxonase 1 promoter in HuH7 liver cells," *Metabolism*, vol. 59, no. 9, pp. 1372–1378, 2010.

[25] S. Prasad and B. B. Aggarwal, "Turmeric, the golden spice: from traditional medicine to modern medicine," in *Herbal Medicine: Biomolecular and Clinical Aspects*, I. F. F. Benzie and S. Wachtel-Galor, Eds., chapter 13, CRC Press, Boca Raton, Fla, USA, 2nd edition, 2011.

[26] D. Akbik, M. Ghadiri, W. Chrzanowski, and R. Rohanizadeh, "Curcumin as a wound healing agent," *Life Sciences*, vol. 116, no. 1, pp. 1–7, 2014.

[27] S. Z. Moghadamtousi, H. Abdul Kadir, P. Hassandarvish, H. Tajik, S. Abubakar, and K. Zandi, "A review on antibacterial, antiviral, and antifungal activity of curcumin," *BioMed Research International*, vol. 2014, Article ID 186864, 12 pages, 2014.

[28] K. Reyes-Gordillo, J. Segovia, M. Shibayama, P. Vergara, M. G. Moreno, and P. Muriel, "Curcumin protects against acute liver damage in the rat by inhibiting NF-κB, proinflammatory cytokines production and oxidative stress," *Biochimica et Biophysica Acta*, vol. 1770, no. 6, pp. 989–996, 2007.

[29] A. A. Nanji, K. Jokelainen, G. L. Tipoe, A. Rahemtulla, P. Thomas, and A. J. Dannenberg, "Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-κB-dependent genes," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 284, no. 2, pp. G321–G327, 2003.

[30] H.-I. Lee, R. A. McGregor, M.-S. Choi et al., "Low doses of curcumin protect alcohol-induced liver damage by modulation of the alcohol metabolic pathway, CYP2E1 and AMPK," *Life Sciences*, vol. 93, no. 18-19, pp. 693–699, 2013.

[31] M. Y. Um, K. H. Hwang, J. Ahn, and T. Y. Ha, "Curcumin attenuates diet-induced hepatic steatosis by activating AMP-activated protein kinase," *Basic and Clinical Pharmacology and Toxicology*, vol. 113, no. 3, pp. 152–157, 2013.

[32] H. M. M. Arafa, "Curcumin attenuates diet-induced hypercholesterolemia in rats," *Medical Science Monitor*, vol. 11, no. 7, pp. BR228–BR234, 2005.

[33] H. Kono, M. D. Wheeler, I. Rusyn et al., "Gender differences in early alcohol-induced liver injury: role of CD14, NFκB and TNFα," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 278, pp. G652–G661, 2000.

[34] A. A. Nanji, K. Jokelainen, M. Fotouhinia et al., "Increased severity of alcoholic liver injury in female rats: role of oxidative stress, endotoxin, and chemokines," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 281, no. 6, pp. G1348–G1356, 2002.

[35] S. D. Tadic, M. S. Elm, H.-S. Li et al., "Sex differences in hepatic gene expression in rat model of ethanol-induced liver injury," *Journal of Applied Physiology*, vol. 93, no. 3, pp. 1057–1068, 2002.

[36] E.-J. Park, C. H. Jeon, G. Ko, J. Kim, and D. H. Sohn, "Protective effect of curcumin in rat liver injury induced by carbon tetrachloride," *Journal of Pharmacy and Pharmacology*, vol. 52, no. 4, pp. 437–440, 2000.

[37] R. B. Shireman and J. Durieux, "Microplate methods for determination of serum cholesterol, high density lipoprotein cholesterol, triglyceride and apolipoproteins," *Lipids*, vol. 28, no. 2, pp. 151–155, 1993.

[38] T. Kosaka, M. Yamaguchi, T. Motomura, and K. Mizuno, "Investigation of the relationship between atherosclerosis and paraoxonase or homocysteine thiolactonase activity in patients with type 2 diabetes mellitus using commercially available assay," *Clinica Chimica Acta*, vol. 359, no. 1-2, pp. 156–162, 2005.

[39] S. P. Weisberg, R. Leibel, and D. V. Tortoriello, "Dietary curcumin significantly improves obesity-associated inflammation and diabetes in mouse models of diabesity," *Endocrinology*, vol. 149, no. 7, pp. 3549–3558, 2008.

[40] K. Reyes-Gordillo, J. Segoviab, M. Shibayama et al., "Curcumin prevents and reverses cirrhosis induced by bile duct obstruction or CCl_4 in rats: role of TGF-β modulation and oxidative stress," *Fundamental and Clinical Pharmacology*, vol. 22, no. 4, pp. 417–427, 2008.

[41] S. S. Yun, S. P. Kim, M. Y. Kang, and S. H. Nam, "Inhibitory effect of curcumin on liver injury in a murine model of endotoxemic shock," *Biotechnology Letters*, vol. 32, no. 2, pp. 209–214, 2010.

[42] F. Vizzutti, A. Provenzano, S. Galastri et al., "Curcumin limits the fibrogenic evolution of experimental steatohepatitis," *Laboratory Investigation*, vol. 90, no. 1, pp. 104–115, 2010.

[43] B. J. Kudchodkar, A. G. Lacko, L. Dory, and T. V. Fungwe, "Dietary fat modulates serum paraoxonase 1 activity in rats," *Journal of Nutrition*, vol. 130, no. 10, pp. 2427–2433, 2000.

[44] B. Fuhrman, N. Volkova, and M. Aviram, "Pomegranate juice polyphenols increase recombinant paraoxonase-1 binding to high-density lipoprotein: Studies in vitro and in diabetic patients," *Nutrition*, vol. 26, no. 4, pp. 359–366, 2010.

[45] J. Hamelet, K. Demuth, J. Dairou et al., "Effects of catechin on homocysteine metabolism in hyperhomocysteinemic mice," *Biochemical and Biophysical Research Communications*, vol. 355, no. 1, pp. 221–227, 2007.

[46] R. A. Sharma, C. R. Ireson, R. D. Verschoyle et al., "Effects of dietary curcumin on glutathione S-transferase and malondialdehyde-DNA adducts in rat liver and colon mucosa: relationship with drug levels," *Clinical Cancer Research*, vol. 7, no. 5, pp. 1452–1458, 2001.

[47] S. V. Singh, X. Hu, S. K. Srivastava et al., "Mechanism of inhibition of benzo[a]pyrene-induced forestomach cancer in mice by dietary curcumin," *Carcinogenesis*, vol. 19, no. 8, pp. 1357–1360, 1998.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3196431, 9 pages
http://dx.doi.org/10.1155/2016/3196431

Research Article

Spironolactone Effect in Hepatic Ischemia/Reperfusion Injury in Wistar Rats

Julio César Jiménez Pérez,[1] Araní Casillas Ramírez,[2] Liliana Torres González,[1] Linda Elsa Muñoz Espinosa,[1] Marlene Marisol Perales Quintana,[3] Gabriela Alarcón Galván,[4] Homero Zapata Chavira,[3] Francisco Javier Guzmán de la Garza,[5] Carlos Rodrigo Cámara Lemarroy,[5] Nancy Esthela Fernández Garza,[5] Edelmiro Pérez Rodríguez,[3] and Paula Cordero Pérez[1]

[1]*Liver Unit, Department of Internal Medicine, University Hospital "Dr. José E. González", The Autonomous University of Nuevo León, 64460 Monterrey, NL, Mexico*
[2]*Hospital Regional de Alta Especialidad de Ciudad Victoria "Bicentenario 2010", 87087 Cuidad Victoria, TAMPS, Mexico*
[3]*Transplant Service, University Hospital "Dr. José E. González", The Autonomous University of Nuevo León, 64460 Monterrey, NL, Mexico*
[4]*Department of Pathology, University Hospital "Dr. José E. González", The Autonomous University of Nuevo León, 64460 Monterrey, NL, Mexico*
[5]*Department of Physiology, School of Medicine, The Autonomous University of Nuevo León, 64460 Monterrey, NL, Mexico*

Correspondence should be addressed to Paula Cordero Pérez; paucordero@yahoo.com.mx

Received 24 July 2015; Revised 7 October 2015; Accepted 11 October 2015

Academic Editor: Karina R. Gordillo

Copyright © 2016 Julio César Jiménez Pérez et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction. Ischemia/reperfusion (IR) injury, often associated with liver surgery, is an unresolved problem in the clinical practice. Spironolactone is an antagonist of aldosterone that has shown benefits over IR injury in several tissues, but its effects in hepatic IR are unknown. *Objective*. To evaluate the effect of spironolactone on IR-induced damage in liver. *Materials and Methods*. Total hepatic ischemia was induced in rats for 20 min followed by 60 min of reperfusion. Spironolactone was administered and hepatic injury, cytokine production, and oxidative stress were assessed. *Results*. After IR, increased transaminases levels and widespread acute inflammatory infiltrate, disorganization of hepatic hemorrhage trabeculae, and presence of apoptotic bodies were observed. Administration of SPI reduced biochemical and histological parameters of liver injury. SPI treatment increased IL-6 levels when compared with IR group but did not modify either IL-1β or TNF-α with respect to IR group. Regarding oxidative stress, increased levels of catalase activity were recorded in IR + SPI group in comparison with group without treatment, whereas MDA levels were similar in IR + SPI and IR groups. *Conclusions*. Spironolactone reduced the liver damage induced by IR, and this was associated with an increase in IL-6 production and catalase activity.

1. Introduction

Clamping of the hepatic pedicle during resection of liver tumors or liver transplantation is often unavoidable, and during these conditions hepatic ischemia/reperfusion (IR) injury may occur. IR injury is the main cause of primary graft dysfunction or nonfunction after liver transplantation. In addition, the liver suffers from warm IR injury during tissue resections (Pringle Maneuver), hemorrhagic or endotoxin shock, and thermal injury [1]. Hepatic IR involves a complex series of processes that comprises microcirculatory failure, followed by necrosis and cell death [2]. The destructive effects of IR are in part triggered by the acute generation of reactive oxygen species following reoxygenation, which causes direct tissue injury and initiates a chain of deleterious cellular responses leading to inflammation and cell death, which

eventually culminate in target organ failure [3]. Current strategies for the treatment of liver IR injury are either preventive [4] or pharmacological [5]. Pharmacological modulation may have a more universal application; however, several therapeutic formulations have been studied and none has been fully successful in preventing mortality associated with liver IR [6, 7]. Thus, the development of new strategies for prevention and treatment of liver damage due to IR is critical to improving outcomes for patients under such conditions.

Recent studies in humans and experimental models have shown that aldosterone plays a pivotal role in the pathophysiology of cardiovascular and renal injury. In this regard, clinical trials have evidenced that mineralocorticoid receptor (MR) blockade improves the survival of patients with chronic heart disease and chronic renal failure [8–11]. The protective effect of MR blockade is associated with decreased fibrosis and vascular inflammation, suggesting that aldosterone is a profibrotic hormone [12, 13]. Spironolactone (SPI) is a synthetic 17-lactone steroid, which is a competitive aldosterone antagonist in a class of pharmaceuticals called potassium-sparing diuretics. SPI is considered fourth line therapy for hypertension in patients already treated with multiple medications [14, 15]. Antagonists of aldosterone have shown beneficial effects in IR experimental models in retina [16], intestine [17], heart [18], kidney [19], and brain [20], but nothing has been reported yet in the setting of hepatic IR injury.

In this study, we sought to evaluate the effect of SPI in livers undergoing normothermic IR injury and to investigate if the protective effects of SPI could be associated with a reduction in oxidative stress and the inflammatory response.

2. Materials and Methods

2.1. Animals. Animal procedures were performed in accordance with the proper use and care of laboratory animals, approved by the ethics committee of our institution. Experiments were performed using 15 male Wistar rats weighing 200–250 g. Animals were maintained under standard conditions such as stable room temperature (24 ± 3°C) and a 12-hour light-dark cycle and were allowed access to commercial rat pellets and water ad libitum.

2.2. Experimental Model. Briefly, after 24 hours of fasting, the animals were anesthetized with pentobarbital sodium anesthesia (60 mg/kg i.p.) and were placed below a heating lamp to maintain constant temperature (37°C), and an identical midline abdominal incision was performed.

2.2.1. Total Hepatic Ischemia. The hepatic hilum was identified and complete warm hepatic ischemia was induced by Pringle Maneuver [21, 22] with microvascular bulldog clamps; ischemia was noticed by color changes in the liver and intestinal tissue. Hepatic ischemia was maintained for 20 minutes, and then clamps were removed to allow 60-minute reperfusion, after which blood and liver samples were collected, and rats were humanely sacrificed.

2.2.2. Partial Hepatic Ischemia. A model of 70% hepatic ischemia was also used, following procedures described in the literature [23]. Briefly, after midline laparotomy, the liver was freed from its ligaments and subsequently all structures of the portal triad of the left and median hepatic lobes were occluded for 60 minutes with a microvascular clamp (Aesculap, San Francisco, CA). In this model, mesenteric congestion is prevented by allowing intestinal blood flow through the right and caudate lobes. The clamps were then removed to allow 6-hour reperfusion, after which blood and liver samples were collected, and rats were humanely sacrificed.

2.3. Experimental Protocol

Protocol 1 (dose-response study to assess the effect of SPI on hepatic injury). To determine the most effective dose of SPI in reducing hepatic injury in conditions of complete warm ischemia, a dose-response study was carried out to evaluate the effect of several doses of SPI as follows:

(1.A) IR group (n = 5 rats): animals were subject to total hepatic ischemia as described above and received vehicle only (saline).

(1.B) IR + SPI group (n = 5 rats): it is as in group B, but animals received SPI at dose of 1000 μg/kg orally 20 hours before induction of IR.

(1.C) IR + SPI group (n = 5 rats): it is as in group B, but animals received SPI at dose of 2600 μg/kg orally 20 hours before induction of IR.

(1.D) IR + SPI group (n = 5 rats): it is as in group B, but animals received SPI at dose of 5000 μg/kg orally 20 hours before induction of IR.

(1.E) IR + SPI group (n = 5 rats): it is as in group B, but animals received SPI at dose of 10,000 μg/kg orally 20 hours before induction of IR.

(1.F) IR + SPI group (n = 5 rats): it is as in group B, but animals received SPI at dose of 20,000 μg/kg orally 20 hours before induction of IR.

Protocol 2 (effect of SPI on hepatic injury associated with normothermic IR). To evaluate whether spironolactone treatment at the most effective dose could reduce hepatic injury in conditions of either total or partial normothermic IR, the following experimental groups were performed:

(2.A) Sham group (n = 5 rats): animals received only Sham surgery, where laparotomy was performed but liver was only manipulated and warm hepatic ischemia was not induced.

(2.B) IR group (n = 5 rats): animals were subject to total hepatic ischemia as described above and received vehicle only (saline).

(2.C) IR + SPI group (n = 5 rats): it is as in group B, but animals received SPI (2.6 mg/kg) orally 20 hours before induction of IR.

(2.D) PIR group (n = 5 rats): animals were subject to partial hepatic ischemia as described above and received vehicle only (saline).

(2.E) PIR + SPI group (n = 5 rats): it is as in group D, but animals received SPI (2.6 mg/kg) orally 20 hours before induction of IR.

2.4. Histological Examination. Immediately after obtaining the liver, the sample was fixed in 10% neutral buffered formalin. Samples were then embedded in paraffin, and 4 μm thick sections were stained with hematoxylin and eosin and examined under light microscope by a blinded pathologist. The hepatic histological damage and hepatocellular necrosis were evaluated according to the Shen [24] and Chen [25] scales, respectively.

The hepatic histological damage scale consists in 4 degrees (G0–G3): grade 0 indicates minimal or no evidence of injury; grade 1 indicates mild injury with cytoplasm vacuolization and focal nuclear pyknosis; grade 2 indicates moderate-to-severe injury with extensive nuclear pyknosis, loss of intercellular borders, and mild-to-moderate neutrophil infiltration; grade 3 indicates severe injury with disintegration of hepatic cords, hemorrhage, and severe PMN infiltration. The hepatocellular necrosis scale consists in 4 degrees (G0–G3): none is grade 0, single cell is grade 1, −30% is grade 2, and >30% is grade 3.

2.5. Biochemical Analysis

2.5.1. Measurements of Transaminases. Blood samples were used to determine serum levels of ALT and AST by standard commercial biochemical assay kits, using DT6011 analyzer (Vitros DTII Systems Chemistry, module DTSCII; Johnson & Johnson Ortho-Clinical Diagnostics, New Brunswick, NJ, USA).

2.5.2. Cytokine Determination. Serum levels of tumour necrosis factor-alpha (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6) were determined using a rat TNF-alpha, IL-1, and IL-6 enzyme, linked immunosorbent assay (ELISA) kit (Peprotech, México).

2.5.3. Oxidative Stress Parameters. In serum samples, total antioxidant capacity was determined using an Antioxidant Assay Kit, which assesses the combination of both small molecule and protein antioxidants (Cayman Chemical Company, Michigan, USA); catalase activity using a Catalase Assay Kit (Cayman Chemical Company, Michigan, USA); and malondialdehyde (MDA) using a MDA Assay Kit (Cayman Chemical Company, Michigan, USA).

2.6. Statistical Analysis. The SPSS 22.0 statistical software package (SPSS Inc. Software, Chicago, Illinois, USA) was used to analyze data using one-way analysis of variance (ANOVA) and Tukey's post hoc test to determine comparison between groups and differences between groups, respectively. All values are expressed as mean ± standard deviation (SD) and $P < 0.05$ was considered statistically significant. Pearson's chi-square test was applied for histological examination; P value < 0.05 was considered statistically significant. The dose-response study results were analyzed using Prism version 6 (GraphPad Software Inc., San Diego, CA). Data were evaluated by one-way analysis of variance and Bonferroni's post-test.

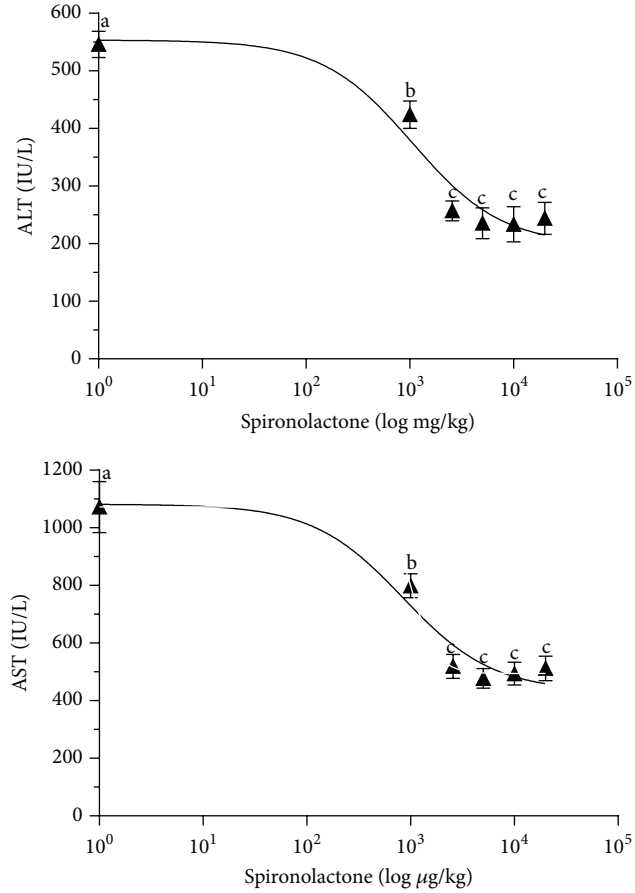


Figure 1: Dose-response study of spironolactone on hepatic injury in total normothermic IR. The effects of SPI treatment on ALT and AST levels were assessed. Rats were treated with SPI (0, 1,000, 2,600, 5,000, 10,000, and 20,000 μg/kg). Transaminase levels were measured after 1 h of reperfusion. Means without a common letter are different; $P < 0.05$.

3. Results

3.1. Dose-Response Effect of SPI on Hepatic Injury in Total Warm Ischemia. We administered SPI at doses of 1,000, 2,600, 5,000, 10,000, and 20,000 μg/kg in rats 20 hours before the surgical procedure, and the effects on hepatic injury were determined 1 h after reperfusion. Our results indicated that SPI protected livers against damage in a dose-dependent manner. The ED50 values for ALT and AST were 1,056 μg/kg and 1,030 μg/kg, respectively. The most effective dose of SPI in reducing the parameters of hepatic injury in liver undergoing warm ischemia was 2,600 μg/kg (2.6 mg/kg). This dose was then used in the rest of the experimental procedures. Higher doses were not associated with lower hepatic damage (Figure 1).

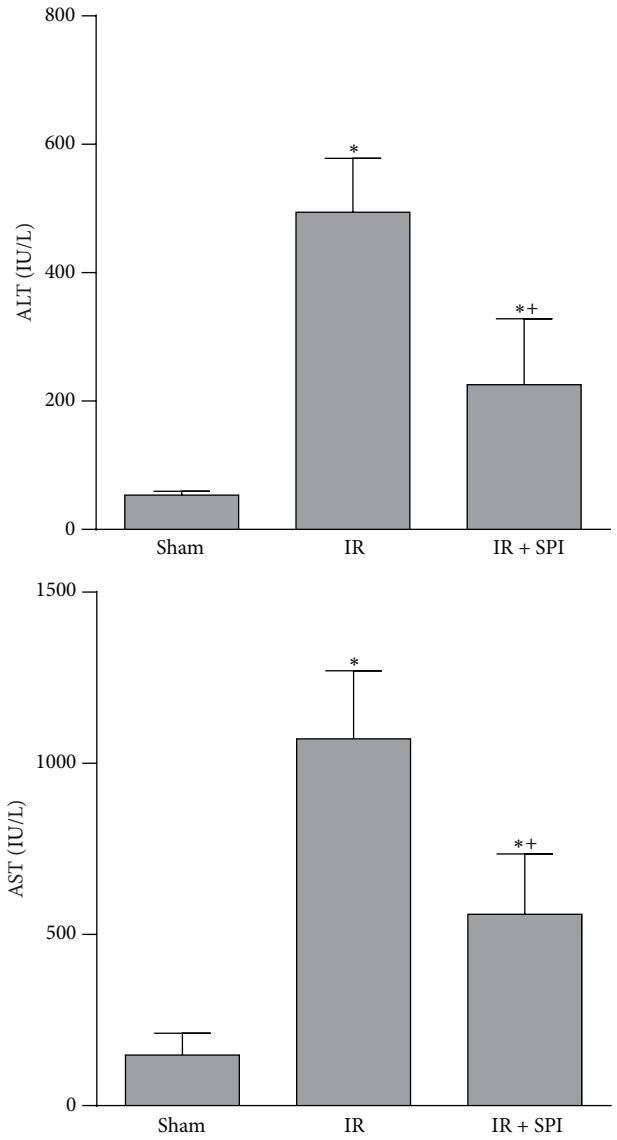


Figure 2: Effect of spironolactone on biochemical parameters of liver injury in total normothermic IR. ALT and AST levels were measured in plasma. $^{*}P < 0.05$ versus Sham; $^{+}P < 0.05$ versus IR.

3.2. Spironolactone as Pharmaceutical Strategy to Reduce Hepatic IR Injury. In the total liver normothermic IR model, the administration of spironolactone at the selected dose of 2.6 mg/kg (IR + SPI group) reduced ALT and AST levels compared with the results obtained in IR group (ALT values: 494 ± 83.9 and 226 ± 103 IU/L for the IR and IR + SPI, resp.; AST values: 1072 ± 198 and 559 ± 176 IU/L, for the IR and IR + SPI, resp.) (Figure 2). Biochemical parameters of hepatic injury were consistent with histological study of the liver. The IR group showed extensive inflammatory infiltrate with presence of apoptotic bodies. The IR + SPI group showed conserved cellular architecture, isolated pockets of acute inflammation, and apoptotic bodies (Figure 3). Significantly lower histological damage and hepatocellular necrosis scores were found in the IR + SPI group when compared with IR group at the end of reperfusion (Table 1).

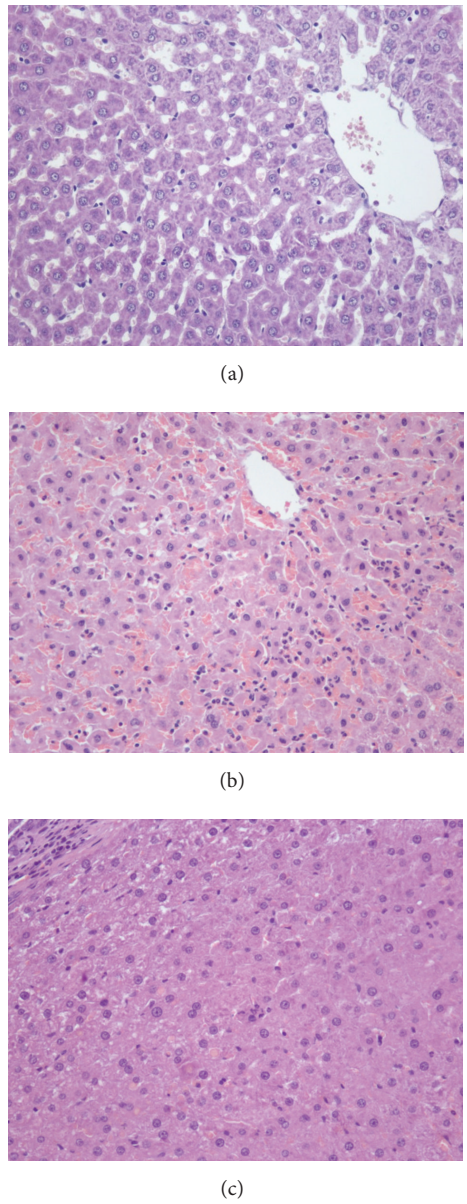


Figure 3: Hematoxylin and eosin staining of hepatic tissue. The Sham group (a) showed conserved cellular architecture. IR group (b) showed numerous inflammatory cell groups predominantly perivenular and presence of apoptotic bodies isolated surrounded by inflammation. The IR + SPI group (c) showed conserved cellular architecture, isolated pockets of acute inflammation, and apoptotic bodies.

Table 1: Evaluation of hepatic tissue according to histological damage.

	Hepatic histological damage scale	Hepatocellular necrosis scale
Sham	1	0
IR	2*	3*
IR + SPI	1#	1#

∗ indicates $P < 0.05$ versus Sham.
# indicates $P < 0.05$ versus IR.

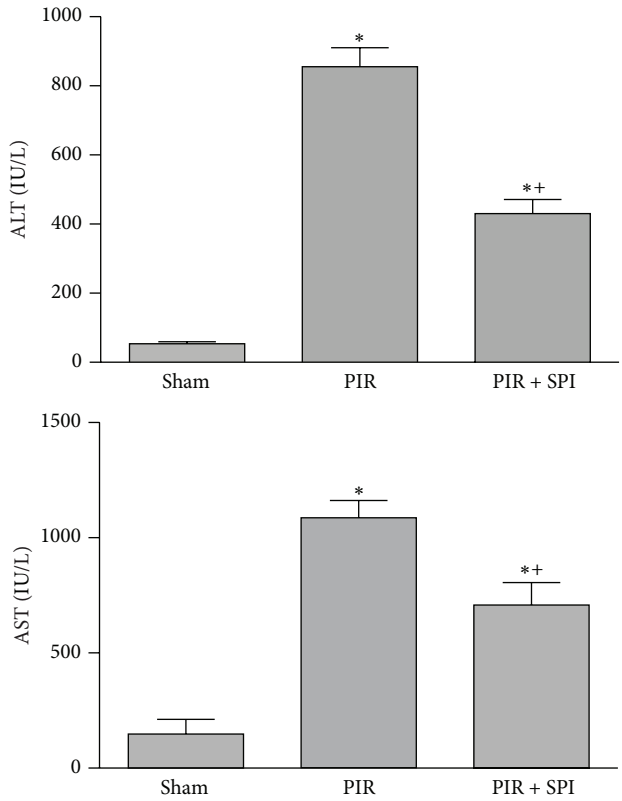


Figure 4: Effect of spironolactone on biochemical parameters of liver injury in normothermic PIR. ALT and AST levels were measured in plasma. $^{*}P < 0.05$ versus Sham; $^{+}P < 0.05$ versus PIR.

Similar results were obtained in the partial liver IR model, where the administration of spironolactone at the selected dose of 2.6 mg/kg (PIR + SPI group) reduced ALT and AST levels compared with the results obtained in PIR group (ALT values: 855 ± 55 and 431 ± 41 IU/L for the PIR and PIR + SPI, resp.; AST values: 1085 ± 75 and 708 ± 80 IU/L, for the PIR and PIR + SPI, resp.) (Figure 4).

3.3. Effect of SPI on Cytokine Production and Oxidative Stress in Hepatic Normothermic IR Injury. As shown in Figure 5, IL-1β, TNF-α, and IL-6 levels of the IR group were of the same order as those of the Sham group (IL-1β values: 1.38 ± 0.25 and 1.32 ± 0.28 ng/mL, in IR and Sham groups, resp.; TNF-α values: 1.01 ± 0.61 ng/mL and 1.06 ± 0.46 ng/mL, in IR and Sham groups,; IL-6 values: 0.48 ± 0.23 and 0.32 ± 0.29 ng/mL, in IR and Sham groups, resp.). Treatment with spironolactone did not result in changes in plasma IL-1β and TNF-α levels with regard to those found in the IR group (IL-1β values: 1.55 ± 0.24 and 1.38 ± 0.25 ng/mL, in IR + SPI and IR groups, resp.; TNF-α values: 1.42 ± 0.47 and 1.01 ± 0.61 ng/mL, in IR + SPI and IR groups, resp.). However, IR + SPI group showed increased IL-6 levels when compared with the IR group (IL-6 values: 2.15 ± 0.53 and 0.48 ± 0.23 ng/mL, in IR + SPI and IR groups, resp.) (Figure 5).

Regarding oxidative stress parameters, total antioxidant capacity of the IR and Sham groups was similar (total antioxidant capacity values: 3.07 ± 0.52 and 2.96 ± 0.52 mM, in IR and Sham groups, resp.). IR increased catalase activity and MDA levels when compared with the Sham group (catalase activity values: 214.22 ± 61.1 and 18.9 ± 8.50 nmol/min/mL, in IR and Sham groups, resp.; MDA values: 18.0 ± 2.75 and 11.1 ± 0.96 μM, in IR and Sham groups, resp.). Spironolactone did not have a significant effect over total antioxidant capacity, since this parameter in the IR + SPI group was similar to that recorded in IR group (total antioxidant capacity values: 3.07 ± 0.52 and 2.89 ± 0.41 mM, in IR + SPI and IR groups, resp.). Results showed an increase in catalase activity in the IR + SPI group in comparison with the IR group (catalase activity values: 651.55 ± 57 and 214 ± 61.1 nmol/min/mL, in IR + SPI and IR groups, resp.). Treatment with spironolactone did not modify MDA levels with respect to the IR group (MDA values: 19.1 ± 3.61 and 18.0 ± 2.75 μM, in IR + SPI and IR groups, resp.) (Figure 6).

4. Discussion

We found that SPI was able to reduce liver IR injury in total liver IR models, as evidenced by attenuation of the histopathological alterations associated with IR injury as well as by reduction of serum levels of AST and ALT. Spironolactone is widely used in clinical practice [25–27]. In fact, several studies have evaluated the usefulness of spironolactone in the treatment of ischemia reperfusion in organs such as kidney and heart in clinical studies, and the results obtained have shown beneficial effects of this drug [28–30]. Our results reveal that pretreatment with spironolactone could open new pathways for protecting liver against IR injury, a strategy that could turn out to be clinically relevant.

As the experimental model of total hepatic IR involves the fact that both gut and liver are subjected to ischemic conditions, it is possible to consider that the observed effects on the liver might thus represent gut-originating responses to ischemia and their modification by SPI. To assess this possibility, an experimental model of partial hepatic IR was carried out to evaluate the effect of SPI on hepatic injury, since in this model intestinal congestion is prevented. Our results indicated that the same dose of SPI was able to reduce biochemical parameters of hepatic injury, thus indicating a liver-specific protective effect for SPI in normothermic hepatic IR.

It is well known that, during hepatic IR, cytokines are released through the induction of adhesion molecules (ICAM and vascular cell adhesion molecule [VCAM]) and CXC chemokine which leads to neutrophil activation and accumulation. These neutrophils then extravasate, causing parenchymal injury by ROS production [31]. Several experimental studies in IR models in tissues different to liver have demonstrated that SPI has anti-inflammatory activity, which may rely on its ability to modulate the production of cytokines including IL-1β, TNF-α, and IL-6 [17, 32, 33]. By analyzing these parameters in our study, the benefits of spironolactone could be associated only with increased IL-6 production. IL-6 treatment has been found to have protective effects against warm IR injury in rodents [34]. One study showed worse IR injury in livers of IL-6 knockout mice than wild type mice, which was restored to the wild type injury patterns

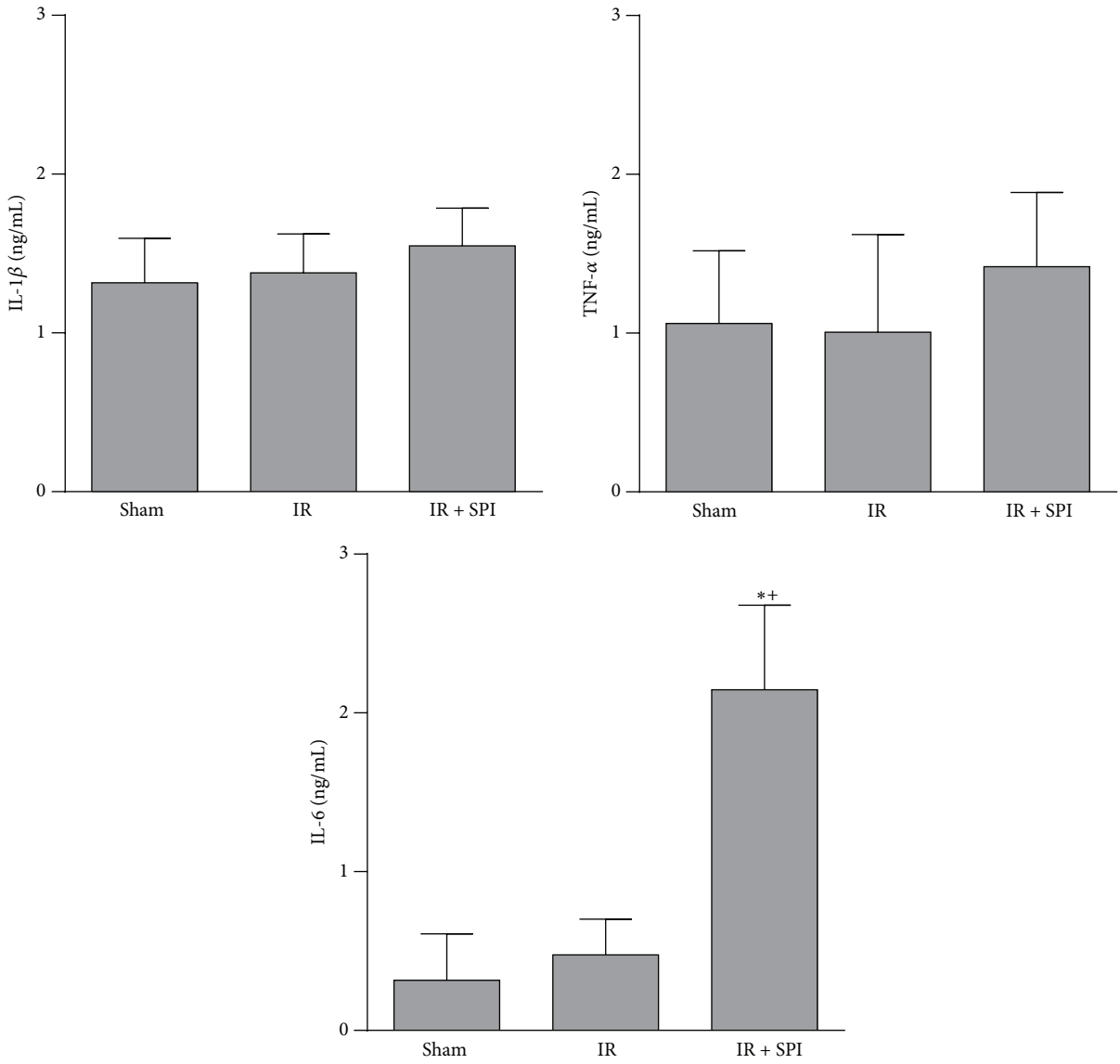


Figure 5: Effect of spironolactone on cytokine production in normothermic IR injury. IL-1β, IL-6, and TNF-α levels were measured in plasma. $^{*}P < 0.05$ versus Sham; $^{+}P < 0.05$ versus IR.

by administration of recombinant IL-6 to the knockout mice before ischemia [35]. These results suggest that SPI-induced protection against IR injury could be partly explained by modulation of IL-6 levels but not by any effects over IL-1β or TNF-α.

Spironolactone had a protective effect in several models of IR injury model through amelioration of oxidative stress. [16, 17]. In this study, three oxidative stress mediators were analyzed: total antioxidants, catalase activity, and MDA. Unlike other studies that have reported reduction in oxidative stress parameters after SPI administration [17, 19, 28], we found no relevant changes in MDA. This could be due to differences in the experimental conditions in the models used. Our results suggest that, in the conditions evaluated herein, SPI did not reduce markers of oxidative stress. We evaluated total antioxidant capacity but results indicated that SPI treatment did not induce any change in this parameter. Then, we decided to assess catalase activity, since this enzyme is one of the most important antioxidants in the context of hepatic IR. [25, 36]. The effect of SPI on catalase was evaluated, and we found that SPI increased catalase activity at the systemic level. Thus, in addition to inducing IL-6 production, SPI increased antioxidant enzymes, resulting in the preservation of hepatic structure and reduction of liver injury, as was shown by the light microscopic findings and the biochemical liver injury markers. This may indicate that SPI is inducing endogenous protective mechanisms in hepatic tissue as a way to counteract the injurious effects of normothermic IR.

Studies on myocardial infarction models have also shown that mineralocorticoid receptor blockers can modulate macrophage function, thus diminishing the cellular inflammatory response [37]. The proteolytic enzyme cathepsin was also shown to be modulated by MR blockade in an

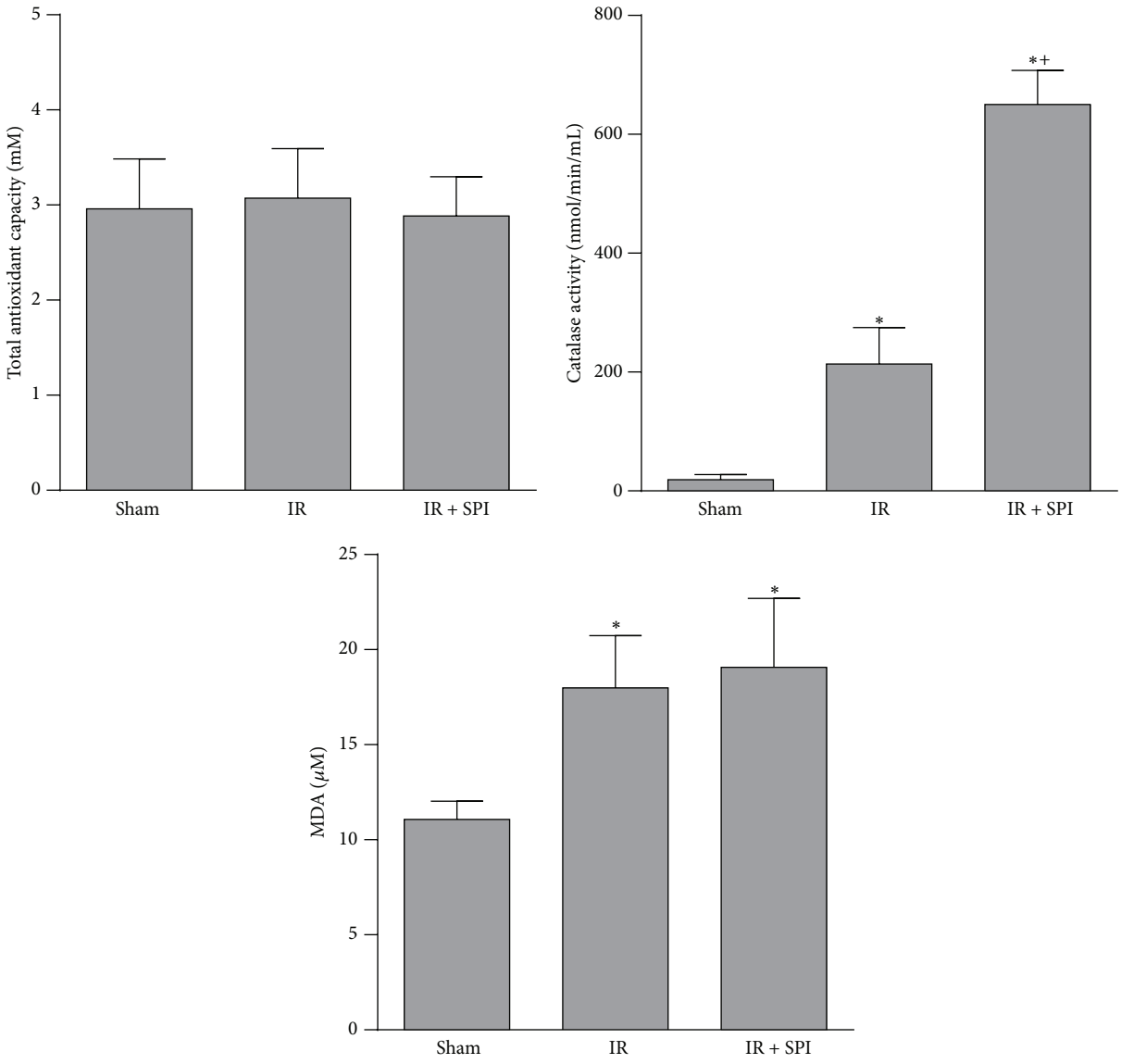


Figure 6: Effect of spironolactone on oxidative stress in normothermic IR injury. Total antioxidant capacity, catalase activity, and MDA levels were measured in plasma. $^{*}P < 0.05$ versus Sham; $^{+}P < 0.05$ versus IR.

intestinal IR injury model [38]. Whether these mechanisms are implicated in SPI-induced hepatoprotection in liver IR injury remains unknown and deserves further studies.

There are some limitations in this study. The sample size is small, and molecular mediators were assessed on serum and not on tissue. This could reflect more a systemic response to IR, irrespective of the beneficial effects we observed over histopathological scores. Finally, further studies would be necessary to conclude that these results are clinically relevant.

5. Conclusions

In conclusion, the present study demonstrated for the first time that SPI has hepatoprotective properties in IR liver injury model. This effect was associated with the induction of protective mechanisms in hepatic tissue such as IL-6 production and increased catalase activity.

Abbreviations

IR: Ischemia reperfusion
MDA: Malondialdehyde
ROS: Reactive oxygen species
SD: Standard deviation
SPI: Spironolactone.

Conflict of Interests

No conflict of interests, financial or otherwise, is declared by the authors.

References

[1] H. Jaeschke, "Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning," *Journal of Investigative Surgery*, vol. 16, no. 3, pp. 127–140, 2003.

[2] J. W. Kupiec-Weglinski and R. W. Busuttil, "Ischemia and reperfusion injury in liver transplantation," *Transplantation Proceedings*, vol. 37, no. 4, pp. 1653–1656, 2005.

[3] P. Pacher and G. Haskó, "Endocannabinoids and cannabinoid receptors in ischaemia-reperfusion injury and preconditioning," *British Journal of Pharmacology*, vol. 153, no. 2, pp. 252–262, 2008.

[4] N. R. Banga, S. Homer-Vanniasinkam, A. Graham, A. Al-Mukhtar, S. A. White, and K. R. Prasad, "Ischaemic preconditioning in transplantation and major resection of the liver," *The British Journal of Surgery*, vol. 92, no. 5, pp. 528–538, 2005.

[5] G. J. Carrizo, R. Wu, X. Cui, A. J. Dwivedi, H. H. Simms, and P. Wang, "Adrenomedullin and adrenomedullin-binding protein-1 downregulate inflammatory cytokines and attenuate tissue injury after gut ischemia-reperfusion," *Surgery*, vol. 141, no. 2, pp. 245–253, 2007.

[6] C. Fondevila, R. W. Busuttil, and J. W. Kupiec-Weglinski, "Hepatic ischemia/reperfusion injury—a fresh look," *Experimental and Molecular Pathology*, vol. 74, no. 2, pp. 86–93, 2003.

[7] H. Jaeschke, "Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 284, no. 1, pp. G15–G26, 2003.

[8] B. Pitt, F. Zannad, W. J. Remme et al., "The effect of spironolactone on morbidity and mortality in patients with severe heart failure," *The New England Journal of Medicine*, vol. 341, no. 10, pp. 709–717, 1999.

[9] A. Nishiyama and Y. Abe, "Molecular mechanisms and therapeutic strategies of chronic renal injury: renoprotective effects of aldosterone blockade," *Journal of Pharmacological Sciences*, vol. 100, no. 1, pp. 9–16, 2006.

[10] B. Pitt, W. Remme, F. Zannad et al., "Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction," *The New England Journal of Medicine*, vol. 348, no. 14, pp. 1309–1321, 2003.

[11] N. J. Brown, "Aldosterone and end-organ damage," *Current Opinion in Nephrology and Hypertension*, vol. 14, no. 3, pp. 235–241, 2005.

[12] T. H. Hostetter and H. N. Ibrahim, "Aldosterone in chronic kidney and cardiac disease," *Journal of the American Society of Nephrology*, vol. 14, no. 9, pp. 2395–2401, 2003.

[13] H. V. Joffe and G. K. Adler, "Effect of aldosterone and mineralocorticoid receptor blockade on vascular inflammation," *Heart Failure Reviews*, vol. 10, no. 1, pp. 31–37, 2005.

[14] J. Batterink, S. N. Stabler, A. M. Tejani, and C. T. Fowkes, "Spironolactone for hypertension," *The Cochrane Database of Systematic Reviews*, vol. 8, Article ID CD008169, 2010.

[15] F. de Souza, E. Muxfeldt, R. Fiszman, and G. Salles, "Efficacy of spironolactone therapy in patients with true resistant hypertension," *Hypertension*, vol. 55, no. 1, pp. 147–152, 2010.

[16] Y. Liu, K. Hirooka, A. Nishiyama et al., "Activation of the aldosterone/mineralocorticoid receptor system and protective effects of mineralocorticoid receptor antagonism in retinal ischemia-reperfusion injury," *Experimental Eye Research*, vol. 96, no. 1, pp. 116–123, 2012.

[17] H. S. Ozacmak, V. H. Ozacmak, F. Barut, M. Arasli, and B. H. Ucan, "Pretreatment with mineralocorticoid receptor blocker reduces intestinal injury induced by ischemia and reperfusion: involvement of inhibition of inflammatory response, oxidative stress, nuclear factor κB, and inducible nitric oxide synthase," *The Journal of Surgical Research*, vol. 191, no. 2, pp. 350–361, 2014.

[18] W. Chai, I. M. Garrelds, R. de Vries, and A. H. J. Danser, "Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone?" *Hypertension*, vol. 47, no. 4, pp. 665–670, 2006.

[19] J. M. Mejía-Vilet, V. Ramírez, C. Cruz, N. Uribe, G. Gamba, and N. A. Bobadilla, "Renal ischemia-reperfusion injury is prevented by the mineralocorticoid receptor blocker spironolactone," *The American Journal of Physiology—Renal Physiology*, vol. 293, no. 1, pp. F78–F86, 2007.

[20] A. M. Dorrance, H. L. Osborn, R. Grekin, and R. C. Webb, "Spironolactone reduces cerebral infarct size and EGF-receptor mRNA in stroke-prone rats," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 281, no. 3, pp. R944–R950, 2001.

[21] W.-Y. Lau, E. C. H. Lai, and S. H. Y. Lau, "Methods of vascular control technique during liver resection: a comprehensive review," *Hepatobiliary and Pancreatic Diseases International*, vol. 9, no. 5, pp. 473–481, 2010.

[22] E. K. Chouillard, A. A. Gumbs, and D. Cherqui, "Vascular clamping in liver surgery: physiology, indications and techniques," *Annals of Surgical Innovation and Research*, vol. 4, article 2, 2010.

[23] S. S. Yadav, D. Sindram, D. K. Perry, and P.-A. Clavien, "Ischemic preconditioning protects the mouse liver by inhibition of apoptosis through a caspase-dependent pathway," *Hepatology*, vol. 30, no. 5, pp. 1223–1231, 1999.

[24] S.-Q. Shen, Y. Zhang, J.-J. Xiang, and C.-L. Xiong, "Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes," *World Journal of Gastroenterology*, vol. 13, no. 13, pp. 1953–1961, 2007.

[25] Y.-X. Chen, M. Sato, K. Kawachi, and Y. Abe, "Neutrophil-mediated liver injury during hepatic ischemia-reperfusion in rats," *Hepatobiliary & Pancreatic Diseases International*, vol. 5, no. 3, pp. 436–442, 2006.

[26] K. M. Kurrelmeyer, Y. Ashton, J. Xu, S. F. Nagueh, G. Torre-Amione, and A. Deswal, "Effects of spironolactone treatment in elderly women with heart failure and preserved left ventricular ejection fraction," *Journal of Cardiac Failure*, vol. 20, no. 8, pp. 560–568, 2014.

[27] Y. Ito, M. Mizuno, Y. Suzuki et al., "Long-term effects of spironolactone in peritoneal dialysis patients," *Journal of the American Society of Nephrology*, vol. 25, no. 5, pp. 1094–1102, 2014.

[28] M. Ojeda-Cervantes, J. Barrera-Chimal, J. Alberú, R. Pérez-Villalva, L. E. Morales-Buenrostro, and N. A. Bobadilla, "Mineralocorticoid receptor blockade reduced oxidative stress in renal transplant recipients: a double-blind, randomized pilot study," *American Journal of Nephrology*, vol. 37, no. 5, pp. 481–490, 2013.

[29] M. J. Li, C. X. Huang, E. Okello, T. Yanhong, and S. Mohamed, "Treatment with spironolactone for 24 weeks decreases the level of matrix metalloproteinases and improves cardiac function in patients with chronic heart failure of ischemic etiology," *The Canadian Journal of Cardiology*, vol. 25, no. 9, pp. 523–526, 2009.

[30] N. C. Shah, S. D. Pringle, P. T. Donnan, and A. D. Struthers, "Spironolactone has antiarrhythmic activity in ischaemic cardiac patients without cardiac failure," *Journal of Hypertension*, vol. 25, no. 11, pp. 2345–2351, 2007.

[31] A. Casillas-Ramírez, I. B. Mosbah, F. Ramalho, J. Roselló-Catafau, and C. Peralta, "Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation," *Life Sciences*, vol. 79, no. 20, pp. 1881–1894, 2006.

[32] Y.-M. Kang, Z.-H. Zhang, R. F. Johnson et al., "Novel effect of mineralocorticoid receptor antagonism to reduce proinflammatory cytokines and hypothalamic activation in rats with ischemia-induced heart failure," *Circulation Research*, vol. 99, no. 7, pp. 758–766, 2006.

[33] P. R. Hansen, K. Rieneck, and K. Bendtzen, "Spironolactone inhibits production of proinflammatory cytokines by human mononuclear cells," *Immunology Letters*, vol. 91, no. 2-3, pp. 87–91, 2004.

[34] G. Datta, B. J. Fuller, and B. R. Davidson, "Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models," *World Journal of Gastroenterology*, vol. 19, no. 11, pp. 1683–1698, 2013.

[35] C. A. Camargo Jr., J. F. Madden, W. Gao, R. S. Selvan, and P.-A. Clavien, "Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent," *Hepatology*, vol. 26, no. 6, pp. 1513–1520, 1997.

[36] S.-Q. He, Y.-H. Zhang, S. K. Venugopal et al., "Delivery of antioxidative enzyme genes protects against ischemia/reperfusion-induced liver injury in mice," *Liver Transplantation*, vol. 12, no. 12, pp. 1869–1879, 2006.

[37] N. Rafatian, K. V. Westcott, R. A. White, and F. H. H. Leenen, "Cardiac macrophages and apoptosis after myocardial infarction: effects of central MR blockade," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 307, no. 7, pp. R879–R887, 2014.

[38] W.-F. Liu, S.-H. Wen, J.-H. Zhan et al., "Treatment with recombinant *Trichinella spiralis* cathepsin B-like protein ameliorates intestinal ischemia/reperfusion injury in mice by promoting a switch from M1 to M2 macrophages," *The Journal of Immunology*, vol. 195, no. 1, pp. 317–328, 2015.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3190617, 11 pages
http://dx.doi.org/10.1155/2016/3190617

Research Article

Sympathetic Nervous System Control of Carbon Tetrachloride-Induced Oxidative Stress in Liver through α-Adrenergic Signaling

Jung-Chun Lin,[1,2] Yi-Jen Peng,[1,3] Shih-Yu Wang,[3,4] Mei-Ju Lai,[3] Ton-Ho Young,[5] Donald M. Salter,[6] and Herng-Sheng Lee[1,3,4]

[1] *Graduate Institute of Medical Sciences, National Defense Medical Center and Department of Pathology, Tri-Service General Hospital, 325 Section 2, Cheng-gong Road, Neihu, Taipei 114, Taiwan*
[2] *Division of Gastroenterology and Hepatology, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[3] *Department of Pathology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[4] *Department of Pathology and Laboratory Medicine, Kaohsiung Veterans General Hospital, No. 386, Dazhong 1st Road, Zuoying District, Kaohsiung 81362, Taiwan*
[5] *Division of Gastroenterology, Department of Internal Medicine, Cardinal Tien Hospital, Fu Jen Catholic University, Taipei, Taiwan*
[6] *Center for Molecular Medicine, MRC IGMM, University of Edinburgh, Edinburgh, UK*

Correspondence should be addressed to Herng-Sheng Lee; herngsheng131419@gmail.com

Received 6 May 2015; Revised 17 August 2015; Accepted 29 September 2015

Academic Editor: Karina R. Gordillo

Copyright © 2016 Jung-Chun Lin et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In addition to being the primary organ involved in redox cycling, the liver is one of the most highly innervated tissues in mammals. The interaction between hepatocytes and sympathetic, parasympathetic, and peptidergic nerve fibers through a variety of neurotransmitters and signaling pathways is recognized as being important in the regulation of hepatocyte function, liver regeneration, and hepatic fibrosis. However, less is known regarding the role of the sympathetic nervous system (SNS) in modulating the hepatic response to oxidative stress. Our aim was to investigate the role of the SNS in healthy and oxidatively stressed liver parenchyma. Mice treated with 6-hydroxydopamine hydrobromide were used to realize chemical sympathectomy. Carbon tetrachloride (CCl_4) injection was used to induce oxidative liver injury. Sympathectomized animals were protected from CCl_4 induced hepatic lipid peroxidation-mediated cytotoxicity and genotoxicity as assessed by 4-hydroxy-2-nonenal levels, morphological features of cell damage, and DNA oxidative damage. Furthermore, sympathectomy modulated hepatic inflammatory response induced by CCl_4-mediated lipid peroxidation. CCl_4 induced lipid peroxidation and hepatotoxicity were suppressed by administration of an α-adrenergic antagonist. We conclude that the SNS provides a permissive microenvironment for hepatic oxidative stress indicating the possibility that targeting the hepatic α-adrenergic signaling could be a viable strategy for improving outcomes in patients with acute hepatic injury.

1. Introduction

The liver is one of the most highly innervated tissues in mammals. The interaction between hepatocytes and sympathetic, parasympathetic, and peptidergic nerve fibers through a variety of neurotransmitters and signaling pathways is recognized as being important in the regulation of hepatocyte function and hepatic response to injury [1]. Studies of hepatic architecture identify adrenergic nerve fibers extending from perivascular plexus in the portal space into the lobule [2]. Sympathetic nervous system (SNS) transmission to hepatocytes occurs through release of norepinephrine and epinephrine as neurotransmitters from intrahepatic nerve endings and by delivery as hormones from adrenal glands. In addition to being important for a range of functions such as regulating hepatic circulation, metabolism, and bile

formation the SNS is also known to modulate both liver regeneration and fibrosis [3–5].

Potential associations between the SNS and oxidative stress are indicated from previous studies. Hepatic monoamine oxidases catalyze oxidative deamination of catecholamines such as norepinephrine and epinephrine. During this process hydrogen peroxide (H_2O_2) is generated and further converted to water by glutathione peroxidase during which glutathione is utilized [6]. Thus, oxidation of catecholamines is a source of reactive oxygen species (ROS) [7]. Long-term elevation of epinephrine can deplete hepatic glutathione as epinephrine both decreases the rate of glutathione synthesis in the liver and increases the rate of glutathione release from the liver whilst decreasing the rate of recycling of oxidized glutathione [8–10]. Similarly, epinephrine stimulates H_2O_2 production via cyclic 3′-5′-adenosine monophosphates in macrophages [11]. Intense physical work is known to increase sympathetic activity and ROS production in the rodent heart [12]. The knowledge that β-adrenergic stimulation is the main driver of ROS generation in mitochondria [13] has indicated the use of β-adrenergic receptor blockers to reduce oxidative stress in cardiac failure [14, 15].

Although these studies clearly suggest a role for catecholamines in the modulation of oxidative stress, whether the SNS affects oxidative stress in the liver has yet to be established. CCl_4 is a classic model compound for inducing free radical damage in the liver [16, 17], being metabolized to form trichloromethyl and trichloromethyl peroxy radicals which covalently bind to proteins, lipids, and nucleic acids to initiate lipid peroxidation, generate 4-hydroxy-2-nonenal (4-HNE), and thus induce liver damage [17]. This model is also useful for characterization of xenobiotic-induced hepatotoxicity, screening of hepatoprotective effects of drugs, and studying mechanisms of human liver injury [18, 19]. An ablation of the SNS exerts a protective effect against CCl_4 induced acute liver injury in mice [20]. In the current study peripheral injection of the neurotoxin 6-hydroxydopamine hydrobromide (6-OHDA) was used to induce chemical sympathectomy and the effects on acute CCl_4 induced hepatic lipid peroxidation were assessed. We also tested the hypothesis that adrenergic signaling is required for control of the oxidative stress in hepatocytes after acute CCl_4 exposure. The observation that chemical sympathectomy or treatment with the α-adrenoreceptor antagonist has profound inhibitory effects on CCl_4 induced hepatic oxidative injury has important implications for understanding of how the response to liver injury may be controlled and, on a clinically applicable basis, indicate potential novel strategies for management of acute or, indeed, chronic liver injury.

2. Materials and Methods

2.1. Animal Care. Male C57Bl/6JNarl mice aged 8 weeks were obtained from the National Laboratory Animal Center in Taipei, Taiwan. They were maintained under controlled conditions (22 ± 1°C and 12 h day/night rhythm) and fed standard laboratory food.

2.2. Chemical Sympathectomy and Antagonists. Mice were intraperitoneally injected with 100 mg kg^{-1} 6-OHDA (Sigma, St. Louis, MO, USA) in 0.1% ascorbic acid (Sigma) in phosphate buffered saline (PBS, Gibco, Gaithersburg, MD) daily over 5 consecutive days. Peripheral administration of 6-OHDA results in a "chemical sympathectomy" by depleting sympathetic fibers [20] and has previously been found to induce an 85% decrease in norepinephrine levels in the liver [21]. Control mice received injections of 0.1% ascorbic acid in PBS. One day following the final treatment with 6-OHDA, mice were subjected to the acute oxidative liver injury protocol described below. Phentolamine (10 mg kg^{-1}) and nadolol (20 mg kg^{-1}) were injected in 0.9% saline daily intraperitoneally, commencing 5 days before CCl_4 administration, while control mice were injected with 0.9% saline vehicle.

2.3. Acute Oxidative Liver Injury Induced by CCl_4. Mice were injected intraperitoneally with a single dose of CCl_4 (Sigma) (12.5% in olive oil (Sigma), 2 mL kg^{-1}). Control groups were treated with vehicle (2 mL kg^{-1} of olive oil). At 24 h after CCl_4 or vehicle treatment, mice were weighed and then euthanized by carbon dioxide asphyxiation. Blood was collected from the heart for analysis of serum AST, ALT, LDH, and ALP by standard enzymatic methods. The liver was then removed, weighed, and processed for further analysis as described.

2.4. Immunostaining and Assessment of Hepatocyte Nuclear Morphology. Samples of liver from mice with or without chemical sympathectomy were embedded in optimum cutting temperature (OCT) (Tissue-Tek, Sakura, CA, USA) compound and quickly frozen. Five μm thick sections were cut and fixed with methanol/acetone. Immunohistochemistry for identification of sympathetic nerve fibers was performed with a primary antibody against tyrosine hydroxylase (the key enzyme for norepinephrine production in sympathetic nerve endings, Millipore, Billerica, MA, USA) and an Alexa 488 conjugated secondary antibody (against rabbit IgG, Invitrogen Corporation, Carlsbad, CA, USA). Following a final incubation for 1 h with propidium iodide (50 μg/mL) (Invitrogen) sections were viewed with a Zeiss LSM 510 Meta inverted confocal microscope using a LD-Achroplan 20x lens.

For assessment of nuclear morphology sections were washed twice in TBST (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) before 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) diluted 1 : 10,000 in PBS for 5 min at room temperature. Slides were mounted with glycerin, coverslipped, and examined using a fluorescence microscope (Olympus BX-51).

2.5. Histological Analysis of the Liver. The liver left lateral lobes were removed and fixed in 10% buffered formalin before standard tissue processing, embedding in paraffin wax, and cutting into 5 μm sections which were stained with hematoxylin and eosin. Histological examination was performed in a blinded fashion by three experienced pathologists. The severity of hepatic injury was scored as described by Camargo et al. [22] according to the following criteria: 0, minimal

or no evidence of injury; 1, mild injury with cytoplasmic vacuolization and focal nuclear pyknosis; 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. Areas of hepatocyte necrosis were expressed as the percentage of damaged liver architecture, measured in 10 randomly selected high-power fields per section using ImageJ software (version 1.48u, US National Institutes of Health).

2.6. Estimation of Hepatic Lipid Peroxidation Assay. Samples of liver were homogenized on ice in PBS containing butylhydroxytoluene and centrifuged at 10,000 g (4°C, 5 min). 4-HNE adducts were measured in supernatants with the OxiSelect HNE-His adduct enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs, Inc., San Diego, CA) in accordance with the manufacturers' instructions. The concentration of 4-HNE adducts was normalized per microgram of total protein, the concentration of the latter being determined by the Lowry method.

2.7. Transmission Electron Microscopy. Liver tissues were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in PBS, pH 7.4, and prepared routinely for transmission electron microscopy with final embedding in LR white resin. Ultrathin sections (70–80 nm) were cut, placed on a nickel grid, and then examined under a Hitachi H-600 transmission electron microscope.

2.8. 8-Hydroxy-2-deoxyguanosine (8-OHdG) Assay. Total DNA from liver was extracted using the Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). A NanoDrop 1000 spectrophotometer (Thermo Fisher, Pittsburgh, PA, USA) was utilized to measure DNA purity. Briefly, pH of DNA (25 μg) was adjusted to 5.2 with 3 M sodium acetate. The DNA reaction mixture was subjected to 1 μL of nuclease P1 (1 U/μL) digestion for 2 h at 37°C. After 2 h of incubation, 15 μL of 1 M Tris-HCl (pH 8.0) was used to bring the pH back to 7.4, followed by treatment with 5 μL of alkaline phosphatase (1 U/μL stock) for 1 h. The reaction mixture was centrifuged for 5 min at 6,000 g, and the supernatant was collected for the 8-OHdG assay using the OxiSelect oxidative DNA damage ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions. Known standards were also included in the assay to allow accurate quantitation. Six livers were used for each experimental group.

2.9. Cytokines/Chemokines Antibody Array. Livers were homogenized in lysis buffer (kit component) and then centrifuged to collect the supernatant to detect 40 cytokines/chemokines on the RayBio Mouse Inflammation Antibody Array 1 membrane (RayBiotech, Inc., Norcross, GA, USA), according to the manufacturer's protocol. We pooled six samples per group to obtain 300 μg total proteins per membrane (one membrane for each group). The membranes were blocked in 2 mL of blocking buffer for 30 min and then incubated with pooled supernatant at room temperature for 2 h. The samples were then decanted from each container, and the membranes were washed three times with 2 mL of wash buffer I, followed by two washes with 2 mL of 1x wash buffer II at room temperature with shaking. The membranes were incubated in 1 : 250-diluted biotin-conjugated primary antibodies at room temperature for 2 h and washed as above before incubation in 1 : 1000-diluted horseradish peroxidase-conjugated streptavidin. After incubation in horseradish peroxidase-conjugated streptavidin for 1 h, the membranes were washed thoroughly and exposed to a peroxidase substrate for 5 min in the dark before imaging. The membranes were scanned and analyzed by using TotalLab Quant software (TotalLab Ltd., Newcastle upon Tyne, UK). Proteins with >2-fold differences in their expression levels between the saline + CCl_4 group and the saline + olive oil group were considered as differentially expressed.

2.10. Statistical Analysis. The results were expressed as mean ± standard deviation (SD). Results were analyzed using Student's t-test for unpaired data. The statistical significance level was a p value of < 0.05.

3. Results

3.1. Alleviation Effect of Chemical Sympathectomy on Lipid Peroxidation in CCl_4 Induced Hepatic Injury. Tyrosine hydroxylase-expressing fibers were readily identified in the border of periportal areas of saline-treated mice but were essentially absent in 6-OHDA-treated animals (Supplementary Data, Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3190617) confirming successful hepatic sympathectomy. There was no hepatic necrosis in saline- or 6-OHDA-treated mice treated with olive oil alone. The extent of CCl_4 induced hepatic necrosis was significantly greater in saline-treated mice than in 6-OHDA-treated mice (32.1 ± 8.6% versus 9.2 ± 4.4%, $p = 0.0007$) (Supplementary Data, Figures S2(a) and S2(b)). Similarly the severity score of hepatic injury was significantly greater (Supplementary Data, Figure S2(c), $p < 0.0001$) in the saline + CCl_4 group (2.7 ± 0.5) when compared to the 6-OHDA + CCl_4 group (1.5 ± 0.5). Biochemical markers of liver injury, including aspartate aminotransferase (AST; 4,115 ± 1,755 versus 17,830 ± 3,078 IU/L, $p < 0.0001$), alanine aminotransferase (ALT; 7,809 ± 2,527 versus 15,519 ± 4,678, $p = 0.0052$), alkaline phosphatase (ALP; 56 ± 26 versus 128 ± 11, $p = 0.0013$), and lactate dehydrogenase (LDH; 11,139 ± 5,496 versus 28,764 ± 8,063, $p = 0.0001$) levels 24 h after CCl_4 treatment were lower in serum from 6-OHDA-treated mice compared to the saline-treated group (Supplementary Data, Table S1).

As secondary products during lipid peroxidation, malondialdehyde appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic and considered as "one of major generators of oxidative stress" and a "major lipid peroxidation product" [23–25]. Thus, we investigated ROS-induced lipid damage as measured by changes in 4-HNE levels in the livers of saline- and 6-OHDA-treated mice 24 h after CCl_4 administration (Table 1). As

Table 1: Effect of sympathectomy on CCl_4 induced lipid peroxidation in liver.

Parameter	Saline + olive oil ($n = 6$)	6-OHDA + olive oil ($n = 6$)	Saline + CCl_4 ($n = 6$)	6-OHDA + CCl_4 ($n = 6$)
4-Hydroxy-2-nonenal (4-HNE, μg/μg protein)	0.23 ± 0.02	0.18 ± 0.02**	0.33 ± 0.07*	0.23 ± 0.03##,††

The results are presented as mean ± SD.
* denotes significant differences compared with the saline + olive oil group ($p < 0.05$).
** denotes significant differences compared with the saline + olive oil group ($p < 0.01$).
denotes significant differences compared with the saline + CCl_4 group ($p < 0.01$).
†† denotes significant differences compared with the olive oil + 6-OHDA group ($p < 0.001$).

expected, 4-HNE was elevated in the livers of saline-treated mice 24 h after CCl_4 treatment ($p = 0.0122$). This increase in lipid peroxidation was blocked when mice were treated with 6-OHDA prior to CCl_4 ($p = 0.0053$).

3.2. Protective Effect of Chemical Sympathectomy on the Ultrastructure of Hepatocyte Injury. Elevation in lipid peroxidation levels has been reported to correlate with ultrastructural changes in hepatocytes following CCl_4 exposure since 4-HNE can promote organelle damage [25–27]. In the current study the ultrastructure of hepatocytes was normal in the olive oil-treated groups (Figures 1(a), 1(b), 1(e), and 1(f)). Twenty-four hours after CCl_4 administration, ultrastructural changes including expansion of the perinuclear space with a pyknotic condensed nucleus, increased number and size of lipid globules, glycogen loss, and mitochondrial swelling with loss of cristae in addition to dilation of the cisternae of rough endoplasmic reticulum were evident (Figures 1(c) and 1(g)). In comparison to the saline + CCl_4 mice, the number and size of lipid globules were decreased and glycogen deposits were readily identifiable within hepatocytes in the 6-OHDA + CCl_4 group. Similarly the organelle and cytoplasmic structures appeared preserved from the deleterious effects of CCl_4 (Figures 1(d) and 1(h)).

3.3. Attenuation Effect of Chemical Sympathectomy on Hepatic Nuclear Damage. Lipid peroxidation products, in particular, 4-HNE, are known to promote hepatocyte nuclear loss or injury [24, 27, 28] which can be visualized by DAPI staining [29]. The nuclei in hepatocytes of olive oil alone-treated mice were round and emitted even blue fluorescence (Figure 2). In the necrotic pericentral region livers of saline-treated mice 24 h after CCl_4 administration nuclei were absent, condensed, or fragmented. 6-OHDA effectively preserved the integrity and morphology of hepatic nuclei in CCl_4-treated mice (Figure 2).

3.4. Inhibition Action of Chemical Sympathectomy on Oxidative DNA Damage in Liver. Since 4-HNE also has high capability of reaction with DNA to cause DNA damage [25, 30], we examined whether sympathectomy attenuates liver oxidative DNA damage by analyzing 8-OHdG content in hepatocyte. The level of 8-OHdG in CCl_4-treated livers without sympathectomy was increased by 94% when compared with that in olive oil-treated livers without sympathectomy (Figure 3, $p = 0.0092$). Pretreatment with 6-OHDA, on the other hand, abrogated this increase ($p = 0.0113$). There was no significant difference in levels of 8-OHdG between the 6-OHDA + olive oil group and saline + olive oil group (Figure 3).

3.5. Analysis of Cytokine and Chemokine Protein Profiling in Livers. It is known that 4-HNE plays a role as mediator of inflammatory processes with interactions with the cytokine networks [31–33]. In the current study we investigated the inflammatory response using a multiplex of 40 cytokines and chemokines. The results are shown in Figure 4. Following administration of CCl_4 there was increased expression of a number of cytokines including IL-1α (2.28-fold, $p = 0.0192$), IL-10 (3.64-fold, $p = 0.0070$), leptin (9.27-fold, $p = 0.0496$), tissue inhibitor of metalloproteinase-2 (TIMP-2, 2.90-fold, $p = 0.0087$), soluble tumor necrosis factor receptor I (sTNFR I, 3.62-fold, $p = 0.0228$), and granulocyte-macrophage colony-stimulating factor (GM-CSF, 5.58-fold, $p = 0.0019$). There was also an increase in expression of 5 inflammation-associated chemokines. These were CCL3 (3.80-fold, $p = 0.0243$), CCL5 (2.42-fold, $p = 0.0153$), CCL9 (3.57-fold, $p = 0.0064$), CCL11 (3.79-fold, $p = 0.0344$), and CXCL11 (6.71-fold, $p = 0.0057$). With chemical sympathectomy, in the absence of CCl_4, there was reduction of protein levels of CCL5 (0.66-fold, $p = 0.0022$) and CCL9 (0.54-fold, $p = 0.0083$) when compared with the saline + olive oil group but no significant change in the levels of other cytokines and chemokines was evident. Pretreatment with 6-OHDA inhibited the increase in hepatic cytokine and chemokine levels induced by CCl_4.

3.6. Hepatoprotective and Antioxidant Effects of α-Adrenergic Blockade against CCl_4 Induced Oxidative Damage. Our findings strongly implicate an important role for the SNS in modulating CCl_4 induced oxidative stress in liver, based strictly on 6-OHDA ablation of the peripheral SNS. However, it is unclear whether 6-OHDA could affect other cell types, for example, hepatocytes, in mice and through an alternative route directly or indirectly affect oxidative stress or the hepatic antioxidant status. To address this concern, we treated mice with drugs that selectively block α- or β-adrenergic receptors. Phentolamine, an α-blocker, and nadolol, a β-blocker, were delivered intraperitoneally over 5 consecutive days before injection with CCl_4. Nadolol had no significant effect on hepatic injury and antioxidant status (Table 2), indicating that β-adrenergic stimulation may not enhance or suppress hepatic oxidative stress responses to CCl_4. Strikingly, phentolamine enhanced antioxidant status

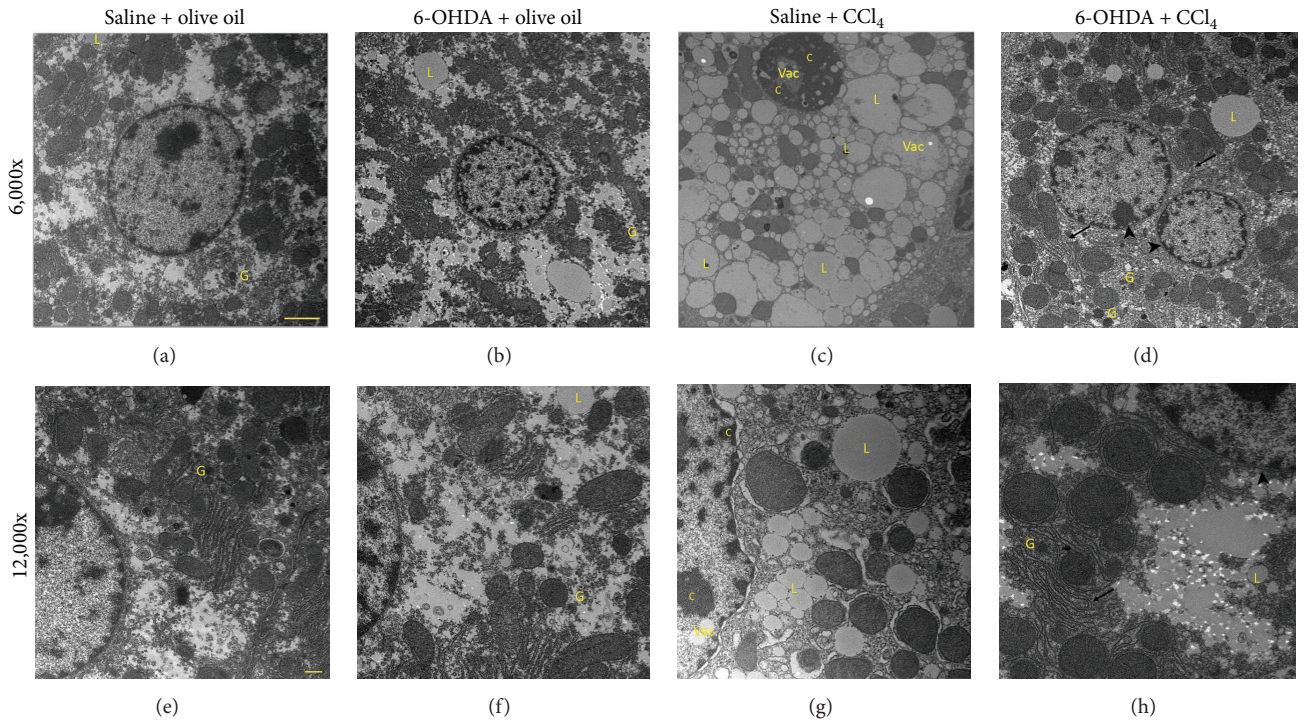


Figure 1: Effect of 6-OHDA on CCl_4 induced changes on hepatocyte morphology. Representative micrographs of transmission electron microscopy (magnification 6,000x or 12,000x) in the liver tissues. (a and e) The saline + olive oil group; (b and f) the 6-OHDA + olive oil group; (c and g) the saline + CCl_4 group; and (d and h) the 6-OHDA + CCl_4 group. Arrows denote rough endoplasmic reticulum. Arrowheads denote perinuclear space. C, chromatin; G, glycogen deposits; L, lipid drops; Vac, vacuolization. Scale bar denotes 100 nm in (a)–(d) and 500 nm in (e)–(h).

Table 2: Hepatic lipid peroxidation and serum biochemical markers in mice after 24 hours of CCl_4 treatment with or without pretreatment of α-adrenergic blocker or β-adrenergic blocker.

Parameter	Saline + CCl_4 ($n = 4$)	Phentolamine + CCl_4 ($n = 4$)	Nadolol + CCl_4 ($n = 4$)
4-Hydroxy-2-nonenal (4-HNE, μg/μg protein)	0.34 ± 0.04	0.25 ± 0.01*	0.28 ± 0.02
Aspartate transaminase (AST, IU/L)	16,695 ± 5,203	6,038 ± 3,320*	13,571 ± 5,758
Alanine transaminase (ALT, IU/L)	14,559 ± 3,268	7,318 ± 3,799*	10,830 ± 4,381
Alkaline phosphatase (ALP, IU/L)	131 ± 14	106 ± 21*	140 ± 31
Lactate dehydrogenase (LDH, IU/L)	30,656 ± 9,387	8,994 ± 5,986*	17,483 ± 10,584

The results are presented as mean ± SD.
* denotes significant differences compared with the saline + CCl_4 group ($p < 0.05$).

in liver (Table 2, $p = 0.0238$) and suppressed both the extent of hepatic necrosis (11.62 ± 2.76% versus 27.55 ± 12.60%, $p = 0.0009$) (Figure 5) and serum levels of AST ($p = 0.0478$), ALT ($p = 0.0387$), ALP ($p = 0.0346$), and LDH ($p = 0.0247$) (Table 2) following CCl_4 administration. Pretreatment with phentolamine abrogated the increased level of 8-OHdG in CCl_4-treated livers ($p = 0.0193$). There was no significant difference in levels of 8-OHdG between the nadolol + CCl_4 group and saline + CCl_4 group (Figure 5(c)). The magnitude of the effect was similar to that induced by 6-OHDA treatment, pointing to an important role in SNS activation of α-adrenergic receptors in regulating antioxidant status in liver.

4. Discussion

In the current study we show that the SNS has major roles in regulating lipid peroxidation, oxidative DNA damage, and proinflammatory cytokine production in the liver associated with CCl_4 toxicity with chemical sympathectomy essentially preventing the major hepatotoxic effects seen within 24 h of CCl_4 administration. We also present that α-adrenergic signaling is required for control of the oxidative stress in hepatocytes after acute CCl_4 exposure.

The changes of hepatocyte ultrastructure identified in the current study following CCl_4 administration are in line with previous observations [26] and are likely to be the result of

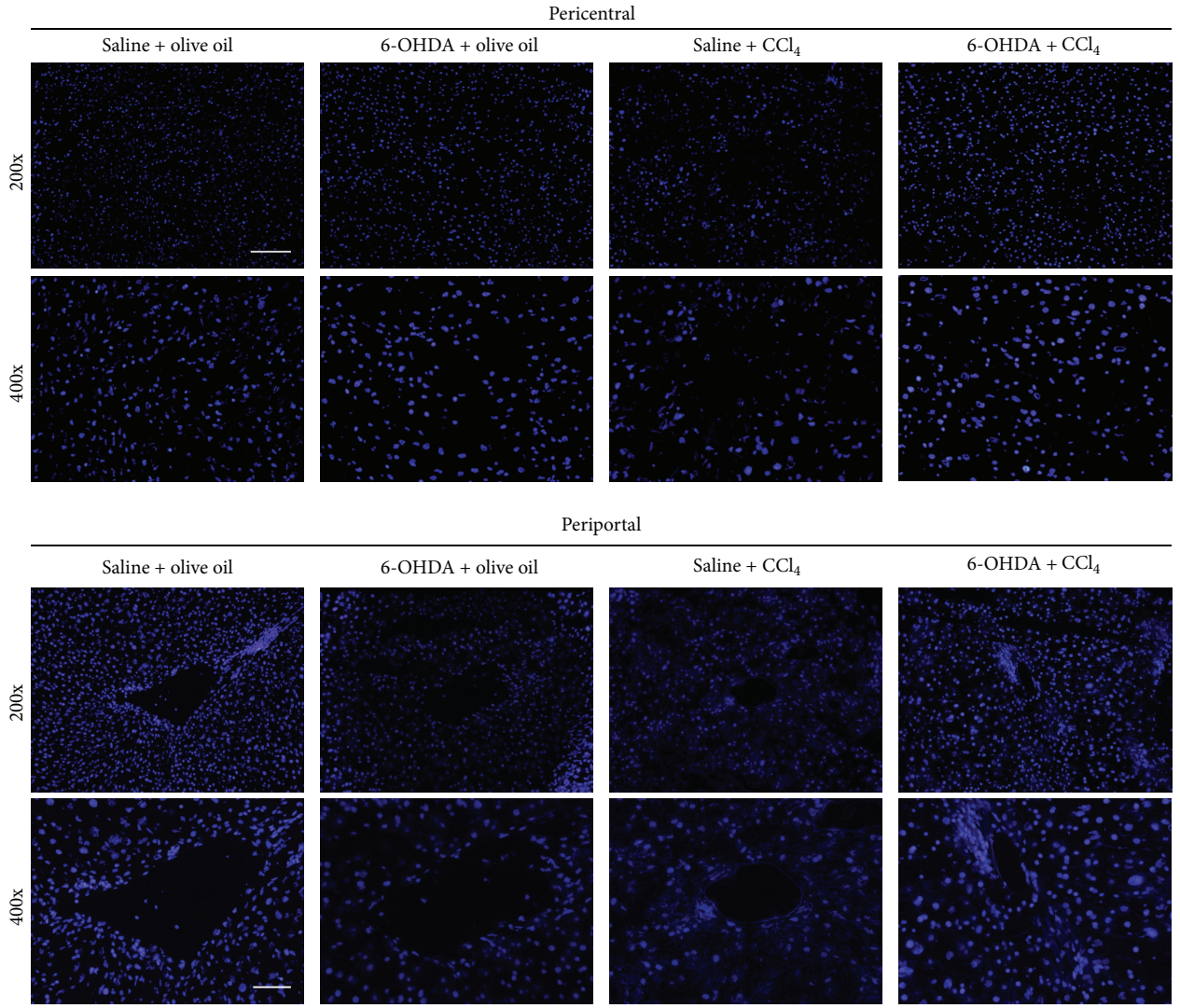


Figure 2: Effect of 6-OHDA on CCl_4 induced changes in hepatocyte nuclei. Representative micrographs of DAPI stained nuclei (magnification 200x or 400x) in the liver tissues. Typical images were selected from each experimental group (original magnification 200x or 400x). Scale bar = 100 μm in 200x and 50 μm in 400x.

damage to cellular and organelle membrane structure caused by lipid peroxidation [25, 27]. As chemical sympathectomy prevents these changes it is probable that sympathectomy supports the antioxidant defense system, maintaining membrane integrity. Sympathectomy also attenuated CCl_4 toxicity characterized by the appearance of dysfunctional hepatic nuclei which arise as a result of enhanced lipid peroxidation [24, 27, 28] further supporting the idea that the mode of action of the beneficial effect of sympathectomy is through reducing oxidative stress. Similarly, the observation that 6-OHDA treatment inhibited oxidative stress-mediated DNA damage in CCl_4-treated mice suggests that sympathectomy, at least in part, may attenuate oxidative stress-induced DNA damage through the inhibition of lipid peroxidation and the reduction of 4-HNE generation because 4-HNE is biologically reactive and known to cause DNA damage [25, 30].

The cytokine/chemokine array data also demonstrate an inhibitory effect of chemical sympathectomy on CCl_4 induced hepatic proinflammatory responses. CCl_4 induced production of inflammation-associated cytokines (IL-1α, IL-10, leptin, TIMP-2, and sTNFR I) in the liver, in part a consequence of combined lipid peroxidation [31–33] and hepatocyte necrosis [34], results in an immunostimulatory environment [16, 35, 36]. Presumably the inhibitory effects of sympathectomy on CCl_4 induced liver cell damage were sufficient to prevent activation of inflammatory cascades. It may also be possible that the SNS has a direct regulatory effect on resident Kupffer [37] and stellate cells [38] within the liver. We also identified an effect of sympathectomy on elevation of hepatic leptin following CCl_4 induced injury. There is increasing evidence that leptin augments inflammatory and profibrogenic responses to hepatic injury [39, 40] whilst downregulation of leptin decreases liver fibrosis [40, 41]. Sympathectomy appears therefore to potentially have both protective anti-inflammatory and potentially antifibrogenic effects.

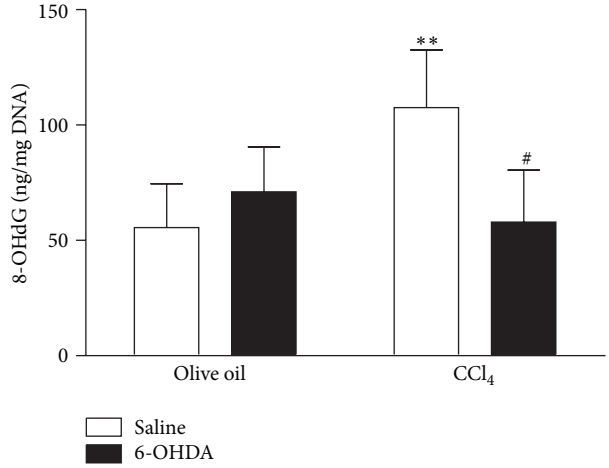


Figure 3: Effect of 6-OHDA on CCl_4 induced oxidative DNA damage. Oxidized DNA in liver was measured 24 hours after exposure to olive oil or CCl_4 following pretreatment with saline of 6-OHDA. Data plotted are mean and SD ($n = 6$ animals in each group). ** denotes significant differences ($p < 0.01$) compared with the saline + olive oil group. # denotes significant differences ($p < 0.05$) compared with the saline + CCl_4 group.

CCl_4 also induced or upregulated the protein expression levels of 5 inflammation-associated chemokines (CCL3, CCL5, CCL9, CCL11, and CXCL11), a response inhibited by pretreatment with 6-OHDA. Increased expression of CCL3 and CCL5 following CCl_4 treatment is consistent with previous studies [42, 43]. To the best of our knowledge, this is the first demonstration of expression of CCL9, a mouse CC chemokine and strong chemoattractant for bone marrow cells [44], in liver. CCL11 is a known potent inducer of eosinophil chemotaxis and regulates the recruitment to the liver after CCl_4 induced hepatic injury to facilitate liver regeneration [45]. CXCL11 release has previously been shown to be induced by oxidative stress exposure [46] and liver ischemia/reperfusion injury [47] supporting the idea that the antioxidant effect of sympathectomy is possibly attributable to the downregulation of CXCL11 and possibly the other cytokines and chemokines studied including GM-CSF [48].

Studies describing an interaction with pharmacological sympathetic blockade have been reported for CCl_4. Pretreatment with either prazosin, an α_1-selective adrenoreceptor antagonist, or yohimbine, an α_2-selective adrenoreceptor antagonist, abolishes methamphetamine potentiation of CCl_4 hepatotoxicity. However, neither prazosin nor yohimbine has any effects on toxicity produced by CCl_4 alone [5, 49]. The ability of phenoxybenzamine, a nonselective, irreversible α-adrenergic receptor antagonist, to counteract the hepatotoxic effect of CCl_4 by preventing the action of catecholamines [50], has been reported. A similar observation was noticed in the recent study of the interaction between dopaminergic agonist piribedil and CCl_4. Administration of piribedil results in amelioration of CCl_4 induced liver damage probably due to its α_2-adrenoceptor antagonist properties to reduce sympathetic outflow and then decrease the extent of lipid peroxidation [51]. Since both phenoxybenzamine and piribedil cross the blood-brain barrier, they would be expected to antagonize pan α- or α_2-adrenoreceptors, respectively, within the central nervous system (CNS). Our findings that phentolamine, a pan α-adrenergic antagonist which has no access to the CNS, suppresses hepatic injury through oxidative stress suggest that suppression of antioxidant status by SNS is associated with the peripheral release of catecholamines. Moreover, we show that it is unlikely that the β-adrenergic signaling modulates CCl_4 induced oxidative stress by pretreatment with nadolol. This observation appears to be consistent with a role for isoproterenol, an agonist at both β_1- and β_2-adrenoreceptors, found to have no effect on potentiation of CCl_4 induced hepatotoxicity [52] and may be due to the concept that β-receptor activation decreases H_2O_2 synthesis in hepatocyte plasma membrane [53, 54]. Although we favor the idea that the α-blocker acts directly on hepatocytes we cannot eliminate the potential contribution of catecholamines on blood flow to liver. Activation of the SNS results in production of both "classical" neurotransmitters norepinephrine and epinephrine and cotransmitters such as adenosine triphosphate and adenosine. Hepatocytes express various adrenergic and purinergic receptors that are sensitive to these molecules, and the production of cytokines/chemokines is probably modulated by activation of these receptors [1, 55] and not necessarily by adrenergic activity alone.

5. Conclusions

In conclusion, we found that sympathectomy or α-adrenergic blockade decreased hepatic lipid peroxidation in CCl_4 induced liver injury. Our results also suggest that the SNS may regulate inflammatory cytokine and chemokine production following hepatic injury through regulation of lipid peroxidation. This study demonstrates that modulation of the SNS can potentially influence the outcome of acute liver damage and provides a basis to explore the effects of adrenergic modifiers on treatment of drug-induced liver injury in humans.

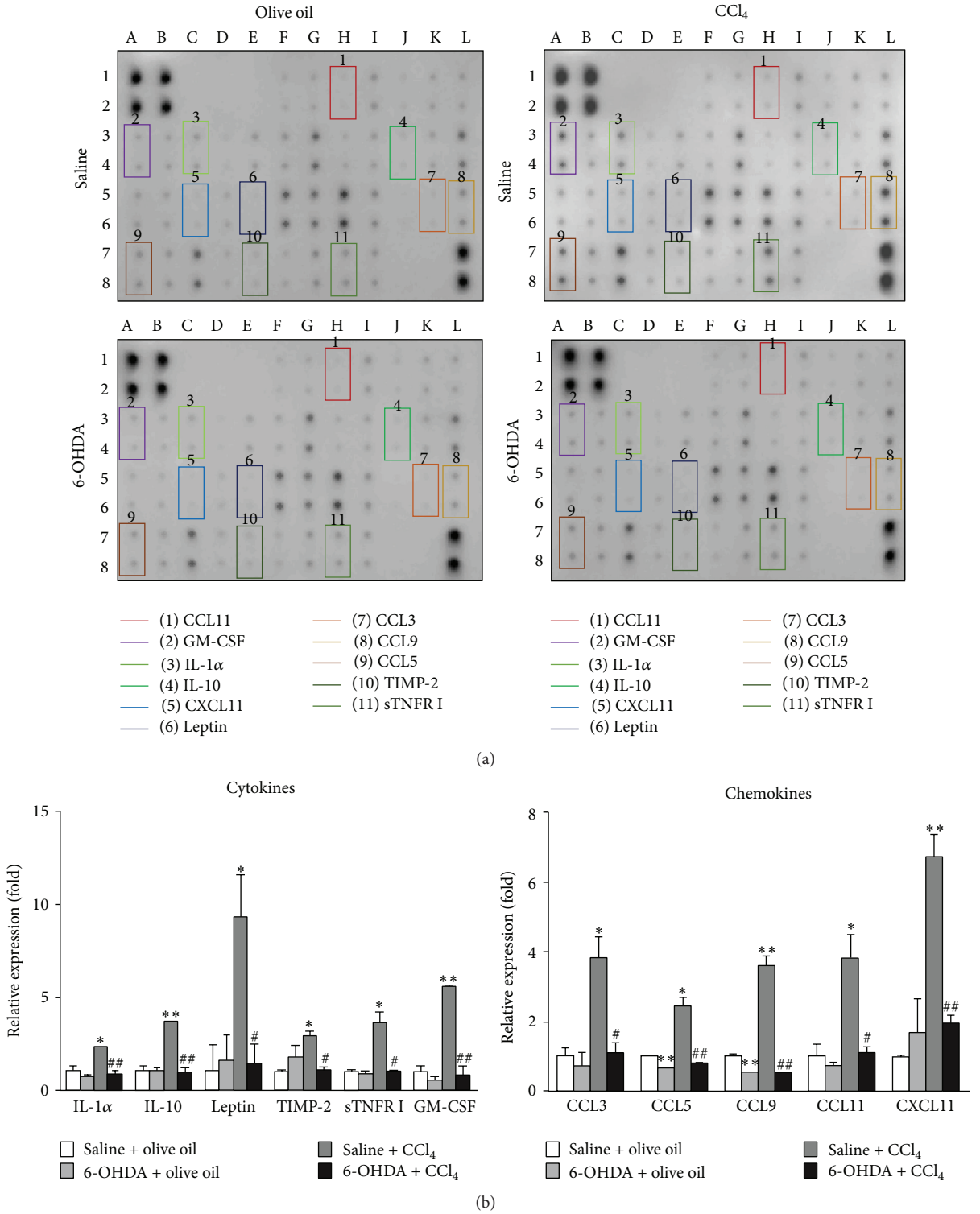


FIGURE 4: Effect of 6-OHDA on levels of hepatic cytokines and chemokines in the absence and following CCl_4 treatment. (a) A cytokine array assay in the liver of the saline + olive oil group, the 6-OHDA + olive oil group, the saline + CCl_4 group, and the 6-OHDA + CCl_4 group was measured. Altered cytokines (twofold or more), including IL-1α, IL-10, leptin, TIMP-2, sTNFR I, GM-CSF, CCL3, CCL5, CCL9, CCL11, and CXCL11, are indicated by boxes. (b) The relative density of cytokines and chemokines was normalized with the internal control and expressed as a ratio of the expression level of cytokines and chemokines in each group divided by the expression level in the saline + olive oil group. Each value represents the average of two replicated spots on the membrane. In all figures, $*$ denotes significant differences compared with the saline + olive oil group ($p < 0.05$). $**$ denotes significant differences compared with the saline + olive oil group ($p < 0.01$). # denotes significant differences compared with the saline + CCl_4 group ($p < 0.05$). ## denotes significant differences compared with the saline + CCl_4 group ($p < 0.01$).

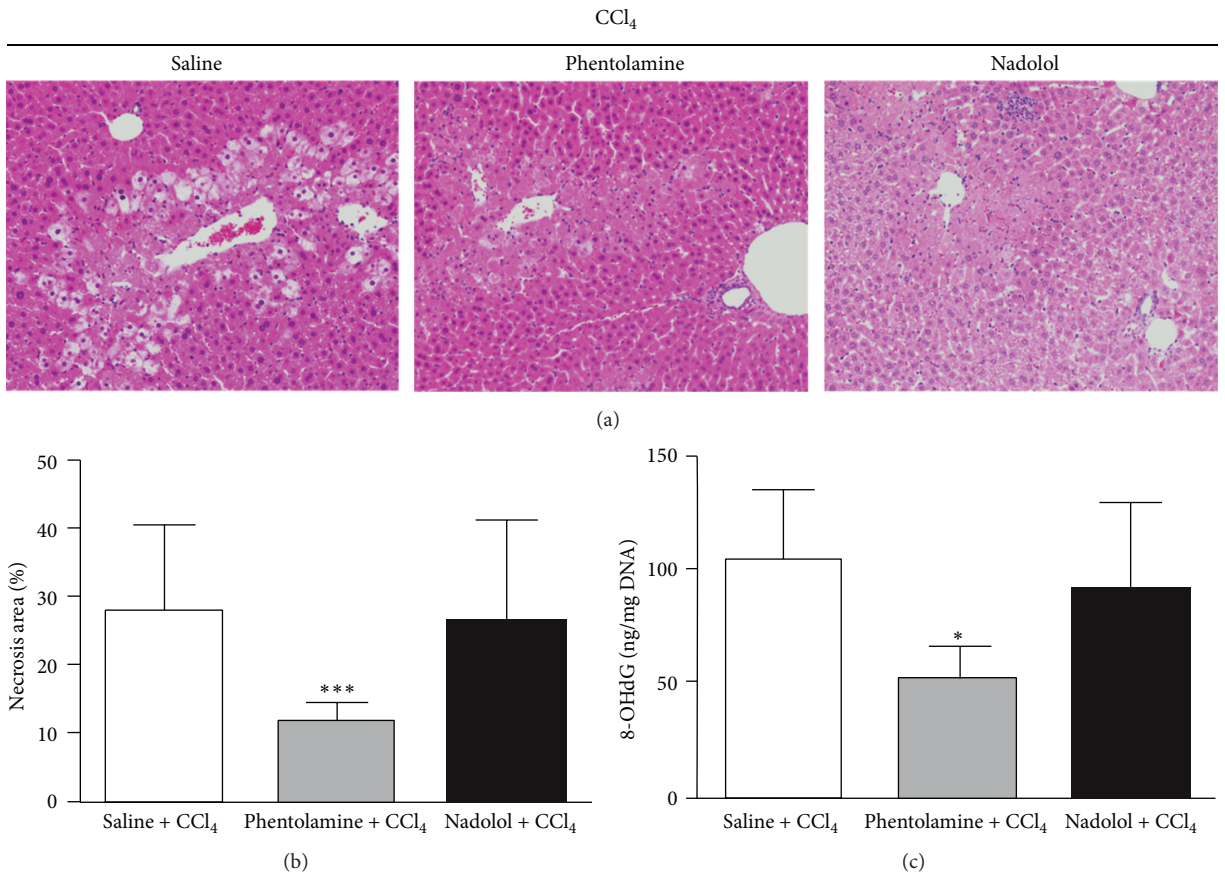


Figure 5: Effect of α- or β-adrenergic blocker on CCl_4 induced hepatic injury and oxidative DNA damage. Histological features (a) and area of necrosis (b) of liver sections stained with hematoxylin and eosin 24 h after CCl_4 treatment. Typical images were selected from each experimental group (original magnification 200x). The saline + CCl_4 group showing hepatocellular necrosis; the phentolamine + CCl_4 group showing mild hepatocellular necrosis. (c) Oxidized DNA in liver was measured 24 hours after exposure to CCl_4 following pretreatment with saline, phentolamine, or nadolol. Data plotted are mean and SD ($n = 4$ animals in each group). $*$ denotes significant differences ($p < 0.05$) compared with the saline + CCl_4 group. $***$ denotes significant differences ($p < 0.001$) compared with the saline + CCl_4 group.

Ethical Approval

All experimental procedures and animal care were performed in accordance with the guidelines approved by the Committee on Institutional Animal Care and Use (IACUC-14-123 and IACUC-15-033) of National Defense Medical Center (Taipei, Taiwan).

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Jung-Chun Lin and Herng-Sheng Lee conceived and designed the experiments. Jung-Chun Lin, Yi-Jen Peng, and Shih-Yu Wang performed the experiments. Jung-Chun Lin, Yi-Jen Peng, Shih-Yu Wang, Mei-Ju Lai, Ton-Ho Young, and Herng-Sheng Lee analyzed the data. Jung-Chun Lin wrote the paper. Donald M. Salter and Herng-Sheng Lee provided advice in designing experiments and writing the paper. All authors have reviewed the paper.

Acknowledgments

The authors acknowledge the skilled technical assistance of Fang-Show Liou, Yu-Ju Lin, Chih-Shan Chang, Sz-Chi Chen, Ya-Ting Ku, and Chih-Min Kuo. This study was supported in part by grant from Ministry of Science and Technology, Taiwan (MOST 103-2320-B-075B-003), Department of Medical Research of Cardinal Tien Hospital, Taiwan (CTH-103-1-2C04), Tri-Service General Hospital, National Defense Medical Center, Taiwan (TSGH-C102-069, TSGH-C103-078, TSGH-C103-080, TSGH-C104-175, TSGH-C104-183, TSGH-C105-061, TSGH-C105-074, and TSGH-C105-189), and Medical Affairs Bureau, Ministry of National Defense, Taiwan (MAB-103-066 and MAB-104-089). They would also like to thank Dr. Tung-Hung Chueh for statistical analysis and acknowledge the service of Instrument Center of National Defense Medical Center for the Zeiss LSM 510 Meta inverted

confocal microscope, especially Po-Li Chen for her technical support.

References

[1] L. A. Streba, C. C. Vere, A. G. Ionescu, C. T. Streba, and I. Rogoveanu, "Role of intrahepatic innervation in regulating the activity of liver cells," *World Journal of Hepatology*, vol. 6, no. 3, pp. 137–143, 2014.

[2] A. Geerts, "Hepatic nervous system: foreword," *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology*, vol. 280, no. 1, pp. 805–806, 2004.

[3] H. Kato and T. Shimazu, "Effect of autonomic denervation on DNA synthesis during liver regeneration after partial hepatectomy," *European Journal of Biochemistry*, vol. 134, no. 3, pp. 473–478, 1983.

[4] C.-T. Hsu, "The role of the autonomic nervous system in chemically-induced liver damage and repair—using the essential hypertensive animal model (SHR)," *Journal of the Autonomic Nervous System*, vol. 51, no. 2, pp. 135–142, 1995.

[5] L. Dubuisson, A. Desmoulière, B. Decourt et al., "Inhibition of rat liver fibrogenesis through noradrenergic antagonism," *Hepatology*, vol. 35, no. 2, pp. 325–331, 2002.

[6] B. Pereira, L. F. B. P. Costa-Rosa, E. J. H. Bechara, P. Newsholme, and R. Curi, "Changes in the TBARs content and superoxide dismutase, catalase and glutathione peroxidase activities in the lymphoid organs and skeletal muscles of adrenodemedullated rats," *Brazilian Journal of Medical and Biological Research*, vol. 31, no. 6, pp. 827–833, 1998.

[7] S. L. Jewett, L. J. Eddy, and P. Hochstein, "Is the autoxidation of catecholamines involved in ischemia-reperfusion injury?" *Free Radical Biology and Medicine*, vol. 6, no. 2, pp. 185–188, 1989.

[8] J. M. Estrela, F. Gil, J. M. Vila, and J. Vina, "Alpha-adrenergic modulation of glutathione metabolism in isolated rat hepatocytes," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 255, no. 6, part 1, pp. E801–E805, 1988.

[9] P. M. Toleikis and D. V. Godin, "Alteration of antioxidant status following sympathectomy: differential effects of modified plasma levels of adrenaline and noradrenaline," *Molecular and Cellular Biochemistry*, vol. 152, no. 1, pp. 39–49, 1995.

[10] Z. Song, D. Cawthon, K. Beers, and W. G. Bottje, "Hepatic and extra-hepatic stimulation of glutathione release into plasma by norepinephrine in vivo," *Poultry Science*, vol. 79, no. 11, pp. 1632–1639, 2000.

[11] L. F. Costa Rosa, R. Curi, C. Murphy, and P. Newsholme, "Effect of adrenaline and phorbol myristate acetate or bacterial lipopolysaccharide on stimulation of pathways of macrophage glucose, glutamine and O_2 metabolism. Evidence for cyclic AMP-dependent protein kinase mediated inhibition of glucose-6-phosphate dehydrogenase and activation of NADP+-dependent 'malic' enzyme," *Biochemical Journal*, vol. 310, part 2, pp. 709–714, 1995.

[12] H. Bo, N. Jiang, G. Ma et al., "Regulation of mitochondrial uncoupling respiration during exercise in rat heart: role of reactive oxygen species (ROS) and uncoupling protein 2," *Free Radical Biology and Medicine*, vol. 44, no. 7, pp. 1373–1381, 2008.

[13] M. Ishizawa, K. Mizushige, T. Noma et al., "An antioxidant treatment potentially protects myocardial energy metabolism by regulating uncoupling protein 2 expression in a chronic beta-adrenergic stimulation rat model," *Life Sciences*, vol. 78, no. 25, pp. 2974–2982, 2006.

[14] M. L. Kukin, J. Kalman, R. H. Charney et al., "Prospective, randomized comparison of effect of long-term treatment with metoprolol or carvedilol on symptoms, exercise, ejection fraction, and oxidative stress in heart failure," *Circulation*, vol. 99, no. 20, pp. 2645–2651, 1999.

[15] K. Kawai, F. Qin, J. Shite, W. Mao, S. Fukuoka, and C.-S. Liang, "Importance of antioxidant and antiapoptotic effects of beta-receptor blockers in heart failure therapy," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 287, no. 3, pp. H1003–H1012, 2004.

[16] N. Nakamoto, S. Tada, K. Kameyama et al., "A free radical scavenger, edaravone, attenuates steatosis and cell death via reducing inflammatory cytokine production in rat acute liver injury," *Free Radical Research*, vol. 37, no. 8, pp. 849–859, 2003.

[17] J. D. Morrow, J. A. Awad, T. Kato et al., "Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation," *The Journal of Clinical Investigation*, vol. 90, no. 6, pp. 2502–2507, 1992.

[18] J. Liu, K. C. Wu, Y.-F. Lu, E. Ekuase, and C. D. Klaassen, "NRF2 protection against liver injury produced by various hepatotoxicants," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 305861, 8 pages, 2013.

[19] F. Zhang, X. Wang, X. Qiu et al., "The protective effect of esculentoside a on experimental acute liver injury in mice," *PLoS ONE*, vol. 9, no. 11, Article ID e113107, 2014.

[20] J. C. Lin, Y. J. Peng, S. Y. Wang et al., "Role of the sympathetic nervous system in carbon tetrachloride-induced hepatotoxicity and systemic inflammation," *PLOS ONE*, vol. 10, no. 3, Article ID e0121365, 2015.

[21] H. Terazono, T. Mutoh, S. Yamaguchi et al., "Adrenergic regulation of clock gene expression in mouse liver," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 11, pp. 6795–6800, 2003.

[22] C. A. Camargo Jr., J. F. Madden, W. Gao, R. S. Selvan, and P.-A. Clavien, "Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent," *Hepatology*, vol. 26, no. 6, pp. 1513–1520, 1997.

[23] H. Esterbauer, P. Eckl, and A. Ortner, "Possible mutagens derived from lipids and lipid precursors," *Mutation Research/Reviews in Genetic Toxicology*, vol. 238, no. 3, pp. 223–233, 1990.

[24] P. M. Eckl, "Genotoxicity of HNE," *Molecular Aspects of Medicine*, vol. 24, no. 4-5, pp. 161–165, 2003.

[25] A. Ayala, M. F. Muñoz, and S. Argüelles, "Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 360438, 31 pages, 2014.

[26] L. Knockaert, A. Berson, C. Ribault et al., "Carbon tetrachloride-mediated lipid peroxidation induces early mitochondrial alterations in mouse liver," *Laboratory Investigation*, vol. 92, no. 3, pp. 396–410, 2012.

[27] L. Moldovan and N. I. Moldovan, "Oxygen free radicals and redox biology of organelles," *Histochemistry and Cell Biology*, vol. 122, no. 4, pp. 395–412, 2004.

[28] J. Gewiese-Rabsch, C. Drucker, S. Malchow, J. Scheller, and S. Rose-John, "Role of IL-6 trans-signaling in CCl_4 induced liver damage," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1802, no. 11, pp. 1054–1061, 2010.

[29] N. Atale, S. Gupta, U. C. S. Yadav, and V. Rani, "Cell-death assessment by fluorescent and nonfluorescent cytosolic and

nuclear staining techniques," *Journal of Microscopy*, vol. 255, no. 1, pp. 7–19, 2014.

[30] K. E. McElhanon, C. Bose, R. Sharma, L. Wu, Y. C. Awasthi, and S. P. Singh, "*Gsta4* null mouse embryonic fibroblasts exhibit enhanced sensitivity to oxidants: role of 4-hydroxynonenal in oxidant toxicity," *Open Journal of Apoptosis*, vol. 2, no. 1, pp. 1–11, 2013.

[31] O. Sommerburg, T. Grune, S. Klee, F. R. Ungemach, and W. G. Siems, "Formation of 4-hydroxynonenal and further aldehydic mediators of inflammation during bromotrichlorornethane treatment of rat liver cells," *Mediators of Inflammation*, vol. 2, no. 1, pp. 27–31, 1993.

[32] L. Milkovic, A. Cipak Gasparovic, and N. Zarkovic, "Overview on major lipid peroxidation bioactive factor 4-hydroxynonenal as pluripotent growth-regulating factor," *Free Radical Research*, vol. 49, no. 7, pp. 850–860, 2015.

[33] U. C. S. Yadav and K. V. Ramana, "Regulation of NF-κB-induced inflammatory signaling by lipid peroxidation-derived aldehydes," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 690545, 11 pages, 2013.

[34] T. Sakurai, G. He, A. Matsuzawa et al., "Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis," *Cancer Cell*, vol. 14, no. 2, pp. 156–165, 2008.

[35] Y. Yamada and N. Fausto, "Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor," *The American Journal of Pathology*, vol. 152, no. 6, pp. 1577–1589, 1998.

[36] E. Roeb, E. Purucker, B. Breuer et al., "TIMP expression in toxic and cholestatic liver injury in rat," *Journal of Hepatology*, vol. 27, no. 3, pp. 535–544, 1997.

[37] M. A. Ajakaiye, A. Jacob, R. Wu et al., "Upregulation of Kupffer cell α2A-Adrenoceptors and downregulation of MKP-1 mediate hepatic injury in chronic alcohol exposure," *Biochemical and Biophysical Research Communications*, vol. 409, no. 3, pp. 406–411, 2011.

[38] B. Sigala, C. McKee, J. Soeda et al., "Sympathetic nervous system catecholamines and neuropeptide Y neurotransmitters are upregulated in human NAFLD and modulate the fibrogenic function of hepatic stellate cells," *PLoS ONE*, vol. 8, no. 9, Article ID e72928, 2013.

[39] K. Ikejima, H. Honda, M. Yoshikawa et al., "Leptin augments inflammatory and profibrogenic responses in the murine liver induced by hepatotoxic chemicals," *Hepatology*, vol. 34, no. 2, pp. 288–297, 2001.

[40] J. A. Handy, P. P. Fu, P. Kumar et al., "Adiponectin inhibits leptin signalling via multiple mechanisms to exert protective effects against hepatic fibrosis," *Biochemical Journal*, vol. 440, no. 3, pp. 385–395, 2011.

[41] B. Lu, L. Yu, S. Li, S. Si, and Y. Zeng, "Alleviation of CCl4-induced cirrhosis in rats by tetramethylpyrazine is associated with downregulation of leptin and TGF-β1 pathway," *Drug and Chemical Toxicology*, vol. 33, no. 3, pp. 310–315, 2010.

[42] A. Nellen, D. Heinrichs, M.-L. Berres et al., "Interference with oligomerization and glycosaminoglycan binding of the chemokine CCL5 improves experimental liver injury," *PLoS ONE*, vol. 7, no. 5, Article ID e36614, 2012.

[43] R. G. Gieling, A. M. Elsharkawy, J. H. Caamã No et al., "The c-Rel subunit of nuclear factor-κb regulates murine liver inflammation, wound-healing, and hepatocyte proliferation," *Hepatology*, vol. 51, no. 3, pp. 922–931, 2010.

[44] M. Yang and P. R. Odgren, "Molecular cloning and characterization of rat CCL9 (MIP-1γ), the ortholog of mouse CCL9," *Cytokine*, vol. 31, no. 2, pp. 94–102, 2005.

[45] Y. P. S. Goh, N. C. Henderson, J. E. Heredia et al., "Eosinophils secrete IL-4 to facilitate liver regeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 24, pp. 9914–9919, 2013.

[46] J. Øvrevik, M. Refsnes, P. Schwarze, and M. Låg, "The ability of oxidative stress to mimic quartz-induced chemokine responses is lung cell line-dependent," *Toxicology Letters*, vol. 181, no. 2, pp. 75–80, 2008.

[47] Y. Zhai, X.-D. Shen, W. W. Hancock et al., "$CXCR3^+CD4^+$ T cells mediate innate immune function in the pathophysiology of liver ischemia/reperfusion injury," *Journal of Immunology*, vol. 176, no. 10, pp. 6313–6322, 2006.

[48] H. Nagashima, K. Nakamura, and T. Goto, "Rubratoxin B induced the secretion of hepatic injury-related colony stimulating factors in human hepatoma cells," *Toxicology Letters*, vol. 145, no. 2, pp. 153–159, 2003.

[49] S. M. Roberts, R. D. Harbison, and R. C. James, "Methamphetamine potentiation of carbon tetrachloride hepatotoxicity in mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 271, no. 2, pp. 1051–1057, 1994.

[50] T. M. Brody, D. N. Calvert, and A. F. Schneider, "Alteration of carbon tetrachloride-induced pathologic changes in the rat by spinal transection, adrenalectomy and adrenergic blocking agents," *Journal of Pharmacology and Experimental Therapeutics*, vol. 131, no. 3, pp. 341–345, 1961.

[51] O. M. E. Abdel-Salam, A. A. Sleem, and N. Shafee, "The dopamine agonist piribedil exerts hepatoprotective effects on carbon tetrachloride-induced hepatic damage," *Comparative Clinical Pathology*, vol. 22, no. 3, pp. 413–419, 2013.

[52] S. M. Roberts, R. D. Harbison, J. E. Seng, and R. C. James, "Potentiation of carbon tetrachloride hepatotoxicity by phenylpropanolamine," *Toxicology and Applied Pharmacology*, vol. 111, no. 2, pp. 175–188, 1991.

[53] A. Díaz-Cruz, R. Guinzberg, R. Guerra et al., "Adrenaline stimulates H_2O_2 generation in liver via NADPH oxidase," *Free Radical Research*, vol. 41, no. 6, pp. 663–672, 2007.

[54] A. Diaz-Cruz, M. M. Vilchis-Landeros, R. Guinzberg, R. Villalobos-Molina, and E. Piña, "NOX2 activated by α_1-adrenoceptors modulates hepatic metabolic routes stimulated by β-adrenoceptors," *Free Radical Research*, vol. 45, no. 11-12, pp. 1366–1378, 2011.

[55] D. S. Emmett, A. Feranchak, G. Kilic et al., "Characterization of ionotrophic purinergic receptors in hepatocytes," *Hepatology*, vol. 47, no. 2, pp. 698–705, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3935841, 12 pages
http://dx.doi.org/10.1155/2016/3935841

Research Article

Oligonol Ameliorates CCl_4-Induced Liver Injury in Rats via the NF-Kappa B and MAPK Signaling Pathways

Jeonghyeon Bak,[1] Nam Kyung Je,[1] Hae Young Chung,[1] Takako Yokozawa,[2] Sik Yoon,[3] and Jeon-Ok Moon[1]

[1]*College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea*
[2]*Graduate School of Science and Engineering for Research, University of Toyama, Toyama 930-0194, Japan*
[3]*Department of Anatomy, College of Medicine, Pusan National University, Yangsan 626-870, Republic of Korea*

Correspondence should be addressed to Jeon-Ok Moon; mjo@pusan.ac.kr

Received 24 July 2015; Accepted 27 September 2015

Academic Editor: Pablo Muriel

Copyright © 2016 Jeonghyeon Bak et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Oxidative stress is thought to be a key risk factor in the development of hepatic diseases. Blocking or retarding the reactions of oxidation and the inflammatory process by antioxidants could be a promising therapeutic intervention for prevention or treatment of liver injuries. Oligonol is a low molecular weight polyphenol containing catechin-type monomers and oligomers derived from lychee fruit. In this study, we investigated the anti-inflammatory effect of oligonol on carbon tetrachloride- (CCl_4-) induced acute hepatic injury in rats. Oral administration of oligonol (10 or 50 mg/kg) reduced CCl_4-induced abnormalities in liver histology and serum AST and serum ALT levels. Oligonol treatment attenuated the CCl_4-induced production of inflammatory mediators, including TNF-α, IL-1β, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) mRNA levels. Western blot analysis showed that oligonol suppressed proinflammatory nuclear factor-kappa B (NF-κB) p65 activation, phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs) as well as Akt. Oligonol exhibited strong antioxidative activity *in vitro* and *in vivo*, and hepatoprotective activity against *t*-butyl hydroperoxide-induced HepG2 cells. Taken together, oligonol showed antioxidative and anti-inflammatory effects in CCl_4-intoxicated rats by inhibiting oxidative stress and NF-κB activation via blockade of the activation of upstream kinases including MAPKs and Akt.

1. Introduction

Liver inflammation is a common response to various types of chronic liver injury. In the initial stages of inflammation, hepatocytes, Kupffer cells, platelets, and leukocytes are activated and produce reactive oxygen species (ROS) and inflammatory mediators such as platelet-derived growth factor, transforming growth factor-β (TGF-β), connective tissue growth factor, and tissue necrosis factor-α (TNF-α). These factors probably act as paracrine mediators to activate quiescent hepatic stellate cells (HSCs) that are localized in the perisinusoidal space, resulting in abnormal quantity and composition of extracellular matrix [1], which in turn leads to hepatitis, liver fibrosis, and cirrhosis. Thus, it is important to suppress hepatic inflammation in the early stages of liver fibrosis.

Oxidative stress, in particular, lipid peroxidation, has been considered one of the major causes of liver damage and has been reported to be associated with HSC activation [2]. Lipid peroxidation may provoke liver damage by compromising the integrity of membranes and by inducing covalent binding of reactive intermediates to important antioxidants such as glutathione [3]. Antioxidants are potent free radical scavengers and have been documented to protect hepatocytes from lipid peroxidation in the carbon tetrachloride- (CCl_4-) or dimethylnitrosamine- (DMN-) induced liver injury models [4, 5]. Therefore, blocking or retarding the reactions of lipid peroxidation and the inflammatory process by antioxidants could be a promising therapeutic intervention for prevention or treatment of liver injuries.

Dietary phytochemicals of fruits, vegetables, whole grains, and other plant foods were shown to have potent

antioxidant activity, and the mixture or combination of phytochemicals was proposed to be responsible for their strong antioxidant activity [6]. In most cases, the poor absorption rate of polyphenolic substances limits their use as dietary supplements in human. Particularly, many polyphenols exist in polymeric forms of high molecular weight that may further decrease their bioavailability [7]. Oligonol is a phenolic product derived from lychee fruit (*Litchi chinensis* Sonn.) extract by a manufacturing process that converts polyphenol polymers into oligomers. Oligonol comprises 16.0% monomers (catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate), 13.9% dimers (procyanidin A1, A2, B1, and B2), and oligomers of proanthocyanidins [8]. Oligonol delivers higher levels of oligomeric proanthocyanidins compared with fruit and plant sources that contain high molecular weight proanthocyanidins [7].

There is a growing evidence that oligonol can elicit some physiological and biochemical alterations *in vitro* and *in vivo*, such as inhibition of adipogenesis in 3T3-L1 adipocytes [9], improvement of memory and cognition under an amyloid beta-induced Alzheimer's mouse model [10], the induction of apoptosis in MCF-7 and MDA-MB-231 human breast cancer cell lines [11], and antioxidant and anti-inflammatory effects in ultraviolet B- (UVB-) irradiated mouse skin [12]. However, the potential protective activity and mechanism of oligonol on acute liver inflammation induced CCl_4 have not yet been conducted.

Here, we report that oral administration of oligonol exerts an antioxidant, anti-inflammatory, and hepatoprotective effect in CCl_4-induced acute liver injury in rats. The possible molecular mechanism of action of oligonol was explored by analyzing the expression of nuclear factor-kappa B (NF-κB), TNF-α, IL-1β, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), Akt, and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38.

2. Materials and Methods

2.1. Chemicals. Bicinchoninic acid (BCA) solution, bovine serum albumin (BSA), *t*-butyl hydroperoxide (*t*-BHP), butylated hydroxytoluene (BHT), CCl_4, 1,1-diphenyl-2-picrylhydrazyl (DPPH), formalin, sodium carboxymethylcellulose (CMC), 2′,7′-dichlorofluorescin diacetate (DCFDA), phosphoric acid, trichloroacetic acid (TCA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromides (MTT), and Trolox were obtained from Sigma-Aldrich Co. 2-Thiobarbituric acid (TBA) was obtained from Tokyo Chemical Industry Co. Malondialdehyde (MDA) tetrabutylammonium salt was obtained from Fluka. Oligonol is commercially available (Amino Up Chemical Co., Ltd., Sapporo, Japan).

2.2. DPPH Assay. The scavenging activity of oligonol was measured using the stable DPPH free radical, according to a published method [13] with slight modifications. The reaction mixture contained 50 mM phosphate buffer at pH 7.4 (80 μL), 100 μM DPPH dissolved in ethanol (100 μL), and the indicated concentrations of oligonol and Trolox (20 μL). Triplicate reaction tubes were wrapped in aluminum foil and placed at room temperature for 30 min in the dark. Spectrophotometric readings were taken at 517 nm using a microplate reader (Apollo-LB913, Berthold Technologies). The percent inhibition of free radical production was calculated from $([A_0 - (A - A_b)]/A_0) \times 100$, where A_0 is the absorbance of the control, A is the absorbance of the sample, and A_b is the absorbance of the blank sample (containing all reagents except DPPH). IC_{50} values were obtained from the inhibition curves.

2.3. Antioxidative Activity against Lipid Peroxidation Induced $FeSO_4/H_2O_2$ in Rat Liver Homogenates. Lipid peroxidation in rat liver homogenates induced by the Fenton reaction, comprising 0.1 mM $FeSO_4$, 3 mM H_2O_2, various concentrations of the tested substances, and liver homogenates (7.5 mg protein/mL), was measured by the method of Buege and Aust [14] with some modifications. The reaction was started by the addition of $FeSO_4$ and H_2O_2 and then incubated at 37°C for 10 min. The reaction was stopped by mixing with 3 mL of a stock solution of 15% (w/v) TCA, 0.375% (w/v) TBA, 0.125 M hydrochloric acid, and 0.6 mM BHT. The combination of reaction mixture and stock solution was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1,250 g for 20 min. The absorbance of the supernatant was determined at 532 nm, and the MDA concentration was calculated using MDA tetrabutylammonium salt as a standard. Protein concentrations were determined by the BCA assay using BSA as the reference standard.

2.4. Protective Effect of Oligonol on Cell Damage Induced by t-BHP. The human hepatocellular carcinoma cell line HepG2 was purchased from the Korean Cell Line Bank. The cells were cultured in a complete medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2 humidified incubated environment. The cells were placed in 96-well plates at a density of 2.0×10^4 cells per well. After 24 h cultivation, the complete medium of the plates was replaced with serum-free medium, and various concentrations of oligonol (0.5, 2, 5, and 10 μg/mL) were added to the cells. Four hours later, the cells were exposed to 300 μM *t*-BHP for 3 h. After incubation, 100 μL of MTT solution (1 mg/mL in phosphate buffered saline) was added to each well and incubated for another 2 h. After the culture medium had been removed, 100 μL of DMSO was added and mixed to dissolve the MTT formazan crystals. The plates were read on a microplate reader (Apollo-LB913, Berthold Technologies) using a wavelength of 540 nm. The survival values, used to examine the protective effects of the compounds against cell damage by *t*-BHP, were expressed as a percentage of the absorbance of the normal cells.

2.5. Cell Lysis. Cells were washed by phosphate buffered saline (PBS), and then 1 mL of ice-cold PBS was added.

Pellets were harvested at 1,000 g at 4°C for 3 min. The pellets were suspended in ProEX CETi Lysis Buffer (TransLab), incubated on ice for 20 min and then centrifuge at 14,000 g at 4°C for 10 min. The supernatants were used as total protein extraction.

2.6. Animals. Male Sprague-Dawley rats were obtained from Samtako (Osan, Korea). Animals were provided standard rat chow with free access to tap water and were maintained at a controlled temperature (23 ± 3°C) and humidity (50 ± 20%) with a 12 h light-dark cycle. With respect to ethical issues and scientific care, the animal protocol used in this study was reviewed and approved by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; Approval number PNU 2008-0541).

2.7. Induction of Acute Hepatic Inflammation with CCl_4. Twenty-four rats weighing 140–160 g and 5-6 weeks in age were assigned to 4 groups ($n = 6$): control, CCl_4, Oli10, and Oli50. Animals in the control group received olive oil (CCl_4 vehicle) by intraperitoneal (i.p.) injection and CMC (oligonol vehicle) by oral gavage; the CCl_4 group received CCl_4 and CMC, while the Oli10 and Oli50 group received CCl_4 and oligonol at 10 and 50 mg/kg/day, respectively. Liver injury was induced by a single i.p. injection of 25% (w/v) CCl_4 (0.6 g/kg body weight) in olive oil. Oligonol was suspended in 0.5% CMC solution to a concentration of 10 and 50 mg/mL and administered by oral gavage twice, once at 16 h and once at 30 min before CCl_4 intoxication. Twenty-four hours after the CCl_4 injection, all rats were euthanized by ether anesthesia, and the livers were excised and weighed. Blood samples for biochemical analyzes were obtained from the inferior vena cava.

2.8. Liver Homogenate Preparation. The remaining liver tissue was rapidly cut into small pieces and homogenized with two volumes (w/v) of ice-cold potassium phosphate buffer (pH 7.4) using an IKA T10 basic Ultra-Tur Rax homogenizer. Debris and nuclei were removed from the homogenate by centrifugation at 700 ×g at 4°C for 10 min and stored at −80°C for further analysis.

2.9. Histology. Liver specimens were fixed by immersion in 10% neutral buffered formaldehyde solution (NBF) for 24 h and then washed overnight. The samples from each group ($n = 6$) were dehydrated in a graded series of ethanol solutions, cleared in xylene, and embedded in paraffin. Eight to ten tissue sections (6 μm thick) were cut and stained with hematoxylin and eosin (H&E) to assess the architectural alterations. The degree of liver damage was evaluated semiquantitatively using the Ishak system under a light microscope [15].

2.10. Biochemical Analysis of Liver Enzymes. Serum aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured using the method described by Reitman and Frankel [16], using AST/ALT kits (Asan Chemical Co.).

2.11. Determination of MDA Content. MDA levels in the liver tissue were measured by a published method with modifications [17]. Standards were prepared via serial dilution of a stock solution of 10 μM MDA tetrabutylammonium salt in distilled water. For the assay, 10 mg of liver tissue was homogenized with 1 mL of solution containing 26 mM TBA, 0.64 mM BHT, 0.93 M TCA, and 11 mM hydrochloric acid. The homogenates were heated for 1 h in a boiling water bath. After cooling, tubes were centrifuged for 15 min at 2,000 ×g. The absorbance of supernatant was determined at 532 nm and the MDA concentration was calculated using MDA tetrabutylammonium salt as a standard.

2.12. Measurement of ROS Level. A fluorometric assay was used to determine levels of ROS, such as $^{\bullet}O_2^{-}$, $^{\bullet}OH$, and H_2O_2. Nonfluorescent DCFDA was oxidized to the highly fluorescent 2′,7′-dichlorofluorescin (DCF) in the presence of esterases and ROS, including lipid peroxides [18]. For the assay, 50 μM DCFDA was added to liver homogenates for 250 μL of final volume. Changes in fluorescence intensity were measured every 5 min for 30 min on a fluorescence plate reader, GENios (Tecan Instrument, Salzburg, Austria), with excitation and emission wavelengths set at 485 and 530 nm, respectively.

2.13. RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from samples of 100 mg of frozen liver by homogenization in Trizol reagent (Invitrogen). RNA purity was assessed by the absorbance ratio at 260 nm and 280 nm. cDNA was prepared from samples of 1 μg of RNA with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the protocol provided by the manufacturer. PCR was performed in 20 μL of reaction solution containing 1 μg of cDNA and the appropriate primers from Bioneer (Daejeon, Korea) (Table 1) using a Promega GoTaq Flexi DNA Polymerase PCR kit. PCR conditions were as follows: denaturation at 95°C for 10 min, 35 cycles of 30 s at 95°C, 90 s at 60°C, 60 s at 72°C, and a final extension at 72°C for 5 min. GAPDH was measured as an internal control for normalization of mRNA levels. The amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light illumination (Gel Doc/ChemiDoc imager, Azure). All reactions were performed in triplicate.

2.14. Western Blotting Analysis. Nuclear extracts, cytosol extracts, or total proteins of liver tissue were prepared as a published method with modification [19]. The protein concentration was measured by the BCA assay. Aliquots of protein (30 μg) were denatured at 95°C for 5 min before electrophoresis on 10% SDS-polyacrylamide gel. After transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore), the blot was blocked with 5% nonfat milk solution for 1 h at room temperature and then incubated with a 1 : 1,000 dilution of primary antibodies selective against either NF-κB p65, total JNK, p-JNK, total ERK, p-ERK, total Akt, p-Akt, total p38, p-p38, histone, or β-actin (Santa Cruz Biotechnology) in Tris-buffered saline Tween-20 (TBST) at 4°C overnight,

Table 1: Oligonucleotide sequences used in RT-PCR analysis.

Group	Direction	Sequence
TNF-α	Forward	TTCTGTCTACTGAACTTGGGGGTGATCGGTCC
	Reverse	GTATGAGATAGCAAATCGGCTGACGGTGTGGG
IL-1β	Forward	ATGGCAACTGTTCCTGAACTCAACT
	Reverse	CAGGACAGGTATAGATTCTTTCCTTT
COX-2	Forward	CCAGAGCAGAGAGATGAAATACCA
	Reverse	GCAGGGCGGGATACAGTTC
iNOS	Forward	GATTCAGTGGTCCAACCTGCA
	Reverse	CGACCTGATGTTGCCACTGTT
GAPDH	Forward	GACAACTTTGGCATCGTGGA
	Reverse	ATGCAGGGATGATGTTCTGG

TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1 beta; COX-2: cyclooxygenase-2; iNOS: inducible nitric oxide synthase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 2: Antioxidative activities of oligonol against DPPH radical.

Substances	Concentration	Inhibition (%)	IC_{50}
Oligonol (μg/mL)	5	10.7 ± 0.002	50.5
	10	28.5 ± 0.001	
	25	38.9 ± 0.001	
	50	49.9 ± 0.001	
	100	61.1 ± 0.006	
Trolox (μM)	5	19.2 ± 0.008	18.6
	10	26.1 ± 0.003	
	25	50.4 ± 0.007	
	50	65.1 ± 0.001	
	100	71.3 ± 0.006	

The reaction mixture consisted of 0.5 mL of 60 μM ethanolic solution of DPPH and 0.5 mL of various concentrations of sample solution. After allowing the mixture to stand at room temperature for 30 min, the absorbance of the remaining DPPH was determined at 517 nm. Trolox was used as a positive control.

Table 3: Antioxidative activities of oligonol against the lipid peroxidation of rat liver homogenates induced by $FeSO_4$ and H_2O_2.

Substances	Concentration	Inhibition (%)	IC_{50}
Oligonol (μg/mL)	10	47.52 ± 0.06	15.16
	50	62.01 ± 0.01	
	100	74.74 ± 0.06	
	500	93.02 ± 0.01	
BHT (μM)	5	16.51 ± 0.04	15.01
	10	43.43 ± 0.03	
	20	59.82 ± 0.01	
	50	89.61 ± 0.05	

Results are expressed as mean ± SE ($n = 3$). The reaction mixture was composed of the rat liver homogenate, 0.1 mM $FeSO_4$, 3 mM H_2O_2, and various concentrations of oligonol or BHT. After incubation at 37°C for 10 min, the amount of MDA formation was measured by the method of Buege and Aust. Inhibition (%) of MDA formation in oligonol or BHT was calculated based on the amount of MDA formation of the $FeSO_4/H_2O_2$-treated control after subtracting the normal.

followed by 1 h at room temperature. The membrane was washed 3 times for 5 min each with TBST solution. The membranes were incubated with 1 : 10,000 dilution of horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1 h. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection system and the band intensities were determined using a Gel Doc/ChemiDoc imager (Azure). The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.15. Statistical Analyses. All results are expressed as the mean ± SE of the indicated number of replicates. Data were analyzed for statistical differences by one-way analysis of variance (ANOVA). A *p value* of 0.05 or less was considered statistically significant.

3. Results

3.1. Antioxidative Activities of Oligonol against the Lipid Peroxidation of Rat Liver Homogenates Induced by $FeSO_4$ and H_2O_2 and against DPPH Radical. The antioxidant activities of oligonol were investigated by the examination of the inhibitory effect against $FeSO_4/H_2O_2$-induced lipid peroxidation in rat liver homogenates (Table 2) and the DPPH radical scavenging effect (Table 3). As positive control for the inhibition of lipid peroxidation, a well-known antioxidant BHT was tested. Under the reaction condition which allows the IC_{50} of BHT to be 15.01 μM, IC_{50} of oligonol was 15.15 μg/mL. Oligonol showed DPPH free radical scavenging activity (IC_{50} = 50.5 μg) and Trolox was tested as a positive control (IC_{50} = 18.6 μM)

3.2. Hepatoprotective Effect of Oligonol against Cell Damage Induced by t-BHP. *t*-BHP is a cytotoxic agent that is metabolized to free radicals including *t*-butoxyl, *t*-butylperoxyl, or methyl radical that interfere with cellular functions. The protective effect of oligonol on cell damage induced by *t*-BHP was examined (Figure 1). By exposing the cells to 300 μM *t*-BHP for 3 h, cell viability decreased to 46%. However, oligonol was found to protect *t*-BHP-induced cell damage dose-dependently, and the EC_{50} was calculated to be 0.25 μg. In addition, we investigated the effect of oligonol

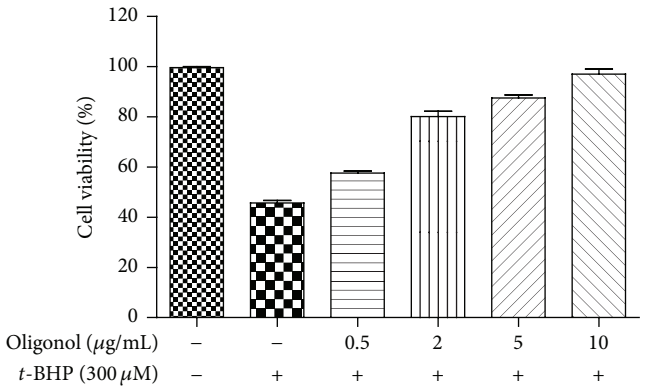


Figure 1: Effect of oligonol on HepG2 cell damage induced by *t*-BHP. Cell viability was assessed using MTT assays. Data shown represent means ± standard deviation of triplicate experiments.

on the induction of proinflammatory mediator COX-2 in *t*-BHP-treated HepG2 cells at the protein level by western blot analysis. As shown in Figure 2, oligonol suppressed the *t*-BHP-induced COX-2 induction.

3.3. Changes in Body and Liver Weight and Serum Parameters in Rats Intoxicated by CCl_4. Treatment with CCl_4 caused a slight increase in the ratio of body weight/liver weight when compared with the control group (Table 4). In contrast, animals injected with CCl_4 and orally administered with oligonol showed significantly reduced ratios of liver weight to body weight, compared to untreated animals injected with CCl_4. These results indicate that oligonol reduces the ratios of body to liver weight induced by CCl_4 intoxication. Biochemical analyses of serum AST and serum ALT activities were performed to determine whether oligonol protected the liver from CCl_4-induced injury (Figures 3(a) and 3(b)). Serum AST and serum ALT levels were significantly higher in rats injected with CCl_4 (475.1 ± 330.3 and 160.0 ± 120.6 U/L, resp.) than in the control rats (35.5 ± 5.2 and 28.4 ± 4.3 U/L, resp.). Serum AST and serum ALT activities were significantly reduced by oral administration of oligonol at both 10 and 50 mg/kg doses; levels of AST and ALT in the Oli10 group were 193.7 ± 61.7 and 72.9 ± 26.0 U/L, respectively, and levels in the Oli50 group were 121.1 ± 29.4 and 44.2 ± 17.6 U/L, respectively.

3.4. Prevention of ROS Production and Lipid Peroxidation by Oligonol. To assess the overall oxidative status, total ROS was measured with DCFDA probe in the liver homogenates. Results show that increased ROS levels with CCl_4 intoxication were suppressed by the administration of oligonol (Figure 4(a)). Induction of lipid peroxidation by CCl_4 was measured by the production of MDA in liver tissues (Figure 4(b)). The MDA content in the livers of CCl_4-treated rats was significantly higher than that in the control animals but significantly reduced in the livers of rats treated with oligonol in a dose-dependent manner, which is consistent with the results of ROS production and the liver function tests.

Table 4: Effects of oligonol on body and liver weights of rats treated with CCl_4.

	Body weight (g)	Liver weight (g)	Ratio (%)[a]
Control	154.02 ± 7.40	5.96 ± 1.25	3.85
CCl_4	158.01 ± 7.29	7.69 ± 0.66**	4.87
Oli10	158.16 ± 4.62	6.98 ± 0.34$^{\#}$	4.42
Oli50	156.75 ± 10.05	6.71 ± 0.29$^{\#}$	4.29

CCl_4: CCl_4 alone treated group; Oli10: oligonol (10 mg/kg) with CCl_4; Oli50: oligonol (50 mg/kg) with CCl_4. [a]Values are expressed as the ratios of liver weight to body weight. Data are the mean ± SE of $n = 6$ rats/group. $^{**}p < 0.01$ compared with the control group and $^{\#}p < 0.05$ compared with the CCl_4 group.

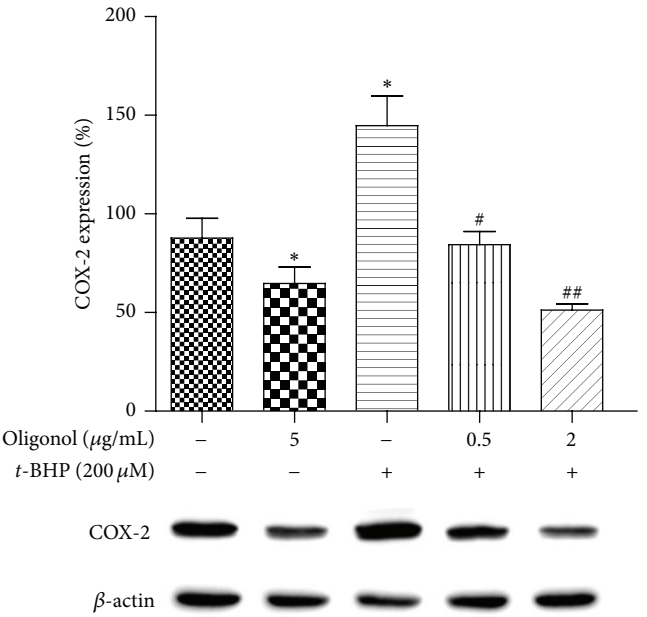


Figure 2: Effect of oligonol on *t*-BHP-induced COX-2 expression on HepG2 cells. HepG2 cells were treated with different concentrations of oligonol (0, 0.5, and 2 μg/mL) for 4 h before being exposed to *t*-BHP (200 μM) for 24 h. Western blotting was performed to detect COX-2 in whole protein (30 μg) from HepG2 cell. One representative blot of each protein is shown from three experiments that yielded similar result, respectively. Values are normalized as percentage of β-actin. Values are mean ± SE of $n = 3$. $^{*}p < 0.05$ compared with the control group, and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the *t*-BHP group.

3.5. Liver Histopathology. The effect of oligonol on CCl_4-induced histopathological changes in the liver was evaluated on H&E stained liver sections (Table 5). Livers of the control group showed normal lobular architecture with central veins and radiating hepatic cords. No histological abnormalities were observed (Figure 5(a)). In contrast, the liver sections from CCl_4-treated animals showed distorted tissue architecture, submassive necrosis, vacuolization, and macrovesicular fatty changes of hepatocytes (Figure 5(b)). Notably, these pathologic changes were markedly reduced dose-dependently in the livers of animals treated with oligonol (Figures 5(c) and 5(d)).

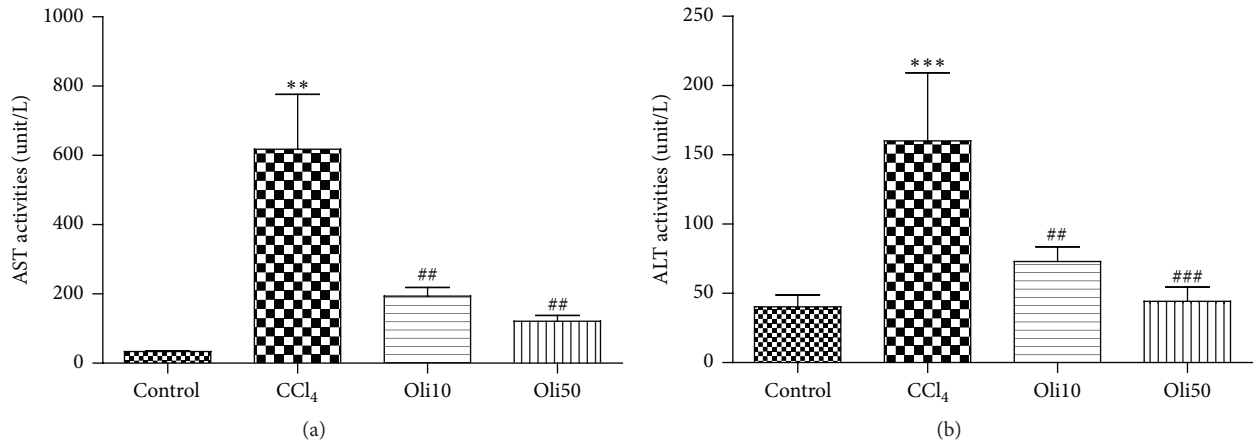


Figure 3: Measurements of serum ALT and serum AST levels. Groups are as described in "Methods." Values are mean ± SE of $n = 6$ rats/group. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the CCl_4 group.

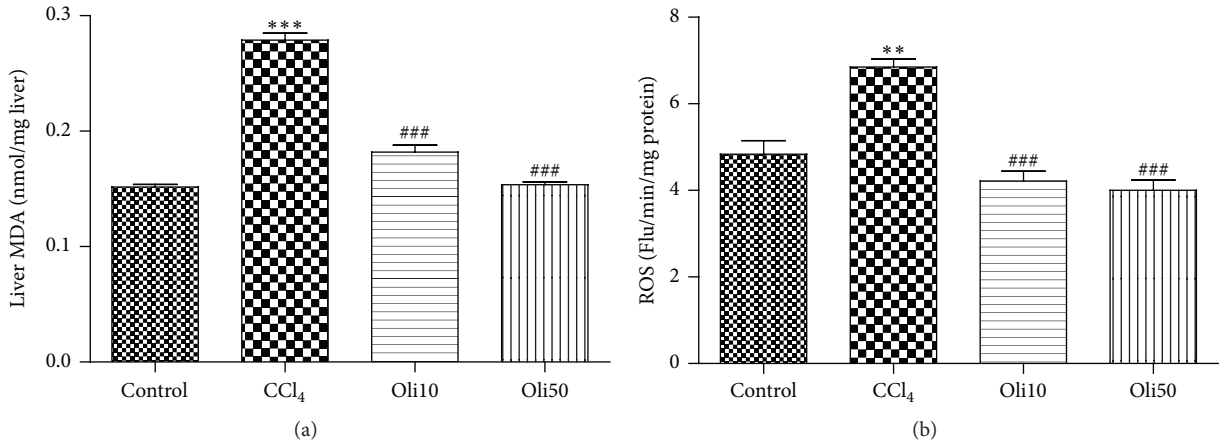


Figure 4: Effect of oligonol on ROS and MDA levels in rat liver intoxicated with CCl_4. ROS generation was measured by DCF formation with a fluorescent probe, DCFDA. Effect of oligonol on CCl_4-induced lipid peroxidation activity from the liver was measured by the method of Buege and Aust. Values are mean ± SE of $n = 6$ rats/group. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group. $^{\#\#\#}p < 0.001$ compared with the CCl_4 group.

Table 5: Effects of oligonol on the histopathological score of liver of CCl_4-treated rats.

Group	Number	Histopathological score of liver				
		0	1	2	3	4
Control	6	6	0	0	0	0
CCl_4	6	0	0	0	1	5
Oli10	6	1	2	1	2	0
Oli50	6	3	1	1	1	0

3.6. Expression of TNF-α, IL-1β, COX-2, and iNOS mRNA. Expression of mRNA of proinflammatory cytokines TNF-α and IL-1β and proinflammatory proteins COX-2 and iNOS in the liver was measured by RT-PCR. Following agarose gel electrophoresis of reaction products, mRNA levels were quantified by normalization against the expression of the housekeeping gene, GAPDH. CCl_4 treatment increased the expression of TNF-α, IL-1β, COX-2, and iNOS mRNA in the liver, but these were dose-dependently and significantly reduced by pretreatment of rats with oligonol (Figures 6 and 7).

3.7. NF-κB Translocation to the Nucleus. The protein level of transcription factor NF-κB was examined. Activation of NF-κB was based on the detection of its translocation into cell nuclei from its initial location in the cytoplasm where it exists in an inactive form. Western blotting of NF-κB p65 protein in nuclear and cytosolic fractions of the liver tissues indicates that CCl_4 treatment exhibited an enhancement of nuclear NF-κB and a reduction of cytosolic NF-κB (Figure 8). Oligonol treatment to CCl_4-intoxicated rat markedly inhibited CCl_4-induced increase of NF-κB in the nuclear fraction of the liver. It also abrogated the reduction of NF-κB in the cytosolic fraction. The relative level of NF-κB p65 in the nuclear and cytosol is compared with β-actin and histon, respectively, and quantified by image analysis.

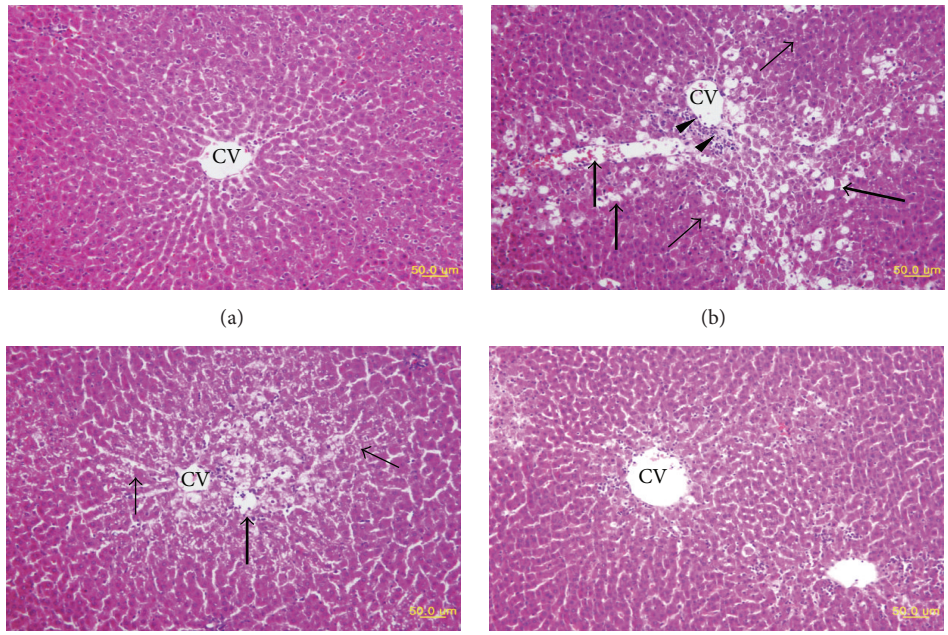


Figure 5: Effects of oligonol on CCl_4-induced histopathological changes in rat livers. Representative H&E stained sections of livers of rats treated as described in methods. (a) Liver section of control group shows normal liver architecture, intact hepatocytes, and radiating hepatic cords from the central vein (CV); (b) CCl_4-induced damage is indicated by distortion of the tissue architecture, submassive necrosis (thick arrows), fatty changes (thin arrows) in hepatocytes, and aggregations of inflammatory cells (arrowheads); (c) oligonol (10 mg/kg) plus CCl_4; and (d) oligonol (50 mg/kg) plus CCl_4; oligonol treatment reduced the pathological alterations induced by CCl_4. All images are original magnification ×400.

It showed that CCl_4-treated rats had significantly increased expression of NF-κB p65 compared with the control animals. Moreover, the expression of NF-κB p65 in the oligonol-treated rats was reduced significantly in a dose-dependent manner. These results demonstrate that oligonol treatment in CCl_4-intoxicated rats strongly inhibited the translocation of NF-κB p65 from the cytosol to the nuclear fraction.

3.8. MAPKs and Akt Signaling Pathways Involved in NF-κB Activation. To investigate the molecular mechanism of NF-κB activation in the CCl_4-intoxicated rat, we measured the expression levels of ERK1/2, JNK, and p38 MAPKs as well as Akt by using western blot analysis. The phosphorylation of MAPKs and Akt was increased in rats treated with CCl_4 alone as compared with the control group. However, treatment with low (10 mg/kg/day) and high (50 mg/kg/day) dose of oligonol in CCl_4-intoxicated rats significantly decreased the expression levels of phosphorylated ERK1/2, JNK, and p38 MAPKs as well as Akt, in a dose-dependent manner (Figure 9).

4. Discussion

Oxidative stress, caused by the increased production of reactive oxygen species (ROS), is thought to be a key risk factor in the development of liver disease [20]. CCl_4 intoxication has been widely used as an experimental model of liver injury. CCl_4 is a substrate for cytochrome P450 2E1 (CYP2E1). It is converted to a CCl_3 radical, which generates $CCl_3OO^{\bullet}$ by reacting with molecular oxygen. Since $CCl_3OO^{\bullet}$ reacts with microsomal membranes and induces lipid peroxidation, membrane damage by free radical chain reaction has been postulated to be the primary cause of hepatocellular injury by this compound [21]. In CCl_4-induced injury, antioxidants are widely known to be able to protect against hepatocyte necrosis because they intercept the CCl_4-induced oxidative stress in hepatocytes by scavenging $^{\bullet}CCl_3$ and lipid peroxy radicals [22].

In this study, a single dose of CCl_4 induced distorted tissue architecture, submassive centrizonal necrosis, fatty changes, and inflammatory cell infiltration. However, these pathologic changes were significantly reduced by pretreatment of 10 and 50 mg/kg of oligonol dose-dependently. Treatment of oligonol effectively improved the CCl_4-induced elevation in serum AST and serum ALT levels, indicating the hepatoprotective effects of oligonol against CCl_4 intoxication. CCl_4 treatment caused high levels of liver oxidative damage, as evidenced by a significant elevation of ROS production and MDA concentration in liver homogenates. However, oligonol markedly inhibits CCl_4-induced oxidative stress in liver of rats in a dose-dependent manner.

Antioxidant activity of oligonol is well established in many studies [23], and the activities were consistent with the results that oligonol exerts the inhibitory effect against $FeSO_4$/ H_2O_2-induced lipid peroxidation in rat liver homogenates and against the DPPH radical scavenging effects. In addition, the protective effect of oligonol via antioxidative

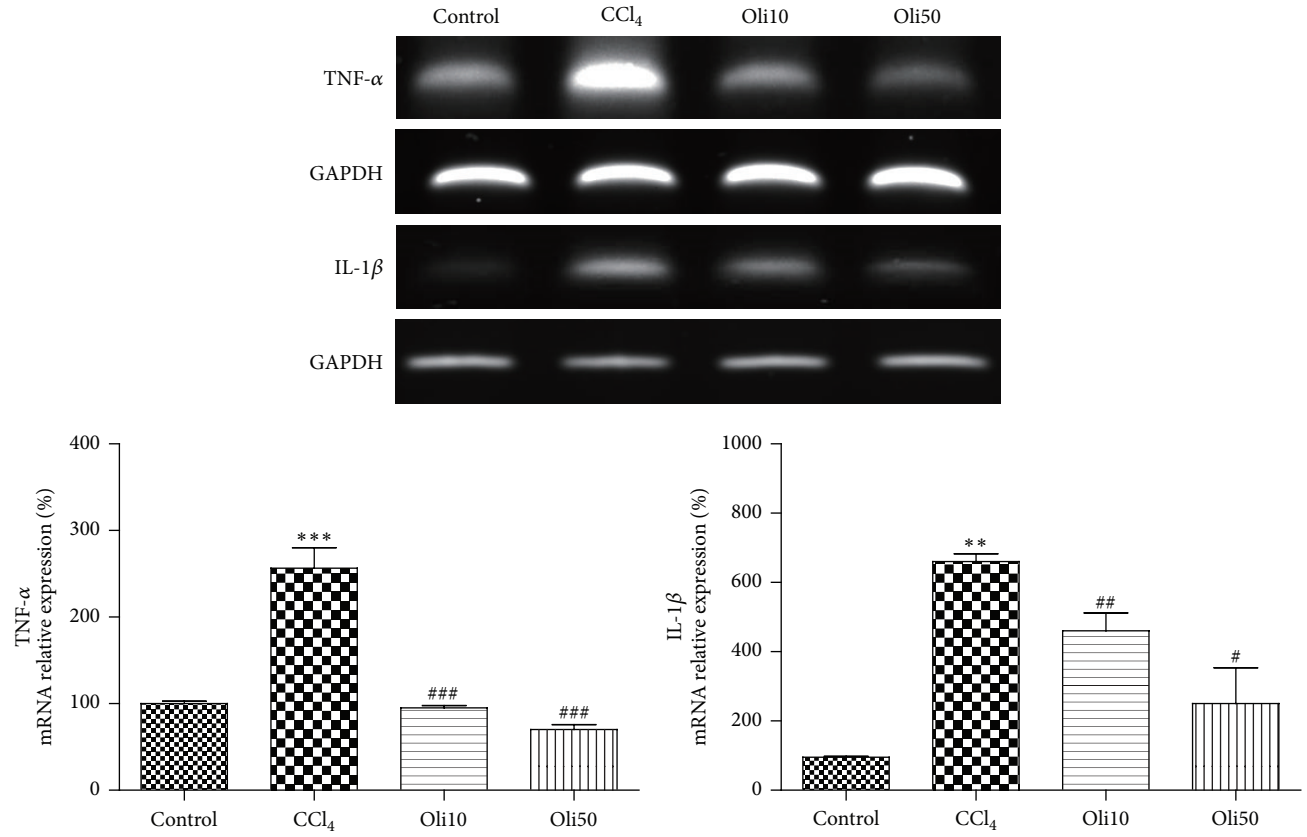


Figure 6: Effects of oligonol on TNF-α and IL-1β mRNA expression after CCl_4 administration. RT-PCR was performed to measure TNF-α and IL-1β mRNA expression in the liver tissues. Values are mean ± SE of $n = 6$. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the CCl_4 group.

activity was assessed in *t*-BHP-induced HepG2 cell damage. HepG2 cells are considered a reasonable model for studying *in vitro* xenobiotic metabolism and liver toxicity since they maintain a majority of specialized functions similar to normal human hepatocytes [24]. The oxidant *t*-BHP is well known to induce oxidative stress [25]. Oligonol showed hepatoprotective effects against *t*-BHP-induced oxidative stress. Taken together, hepatoprotective effects of oligonol in CCl_4-intoxicated rat model and in *t*-BHP-induced HepG2 cells may be due to the potent antioxidant and free radical scavenging activities of oligonol.

Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been the focus of investigations of inflammatory organ injury because the uncontrolled and prolonged action of these proteins is potentially harmful [26]. Considerable evidence suggests that TNF-α and IL-1β contribute to the pathogenesis of liver inflammatory diseases by activating the NF-κB signaling pathway [27], suggesting that it may be important to monitor proinflammatory cytokines when studying liver injury. In our study, we focused on the anti-inflammatory effect of oligonol by analyzing the expression of NF-κB p65, TNF-α, and IL-1β. Our data show that the production of TNF-α and IL-1β were significantly increased by CCl_4-induced hepatotoxicity, which is consistent with the findings of Reyes-Gordillo et al. [28]. We found that the translocation of NF-κB p65 protein into nucleus and TNF-α and IL-1β mRNA expression were inhibited in rat pretreated oligonol, suggesting that oligonol acts, at least in part, by inhibition of NF-κB activity.

Beside proinflammatory cytokines, NF-κB regulates the expression of the inflammatory proteins COX-2 and iNOS. This study showed a significant increase of COX-2 and iNOS mRNA expression levels in the liver treated CCl_4. These increases were attenuated by treatment with oligonol. In the study with HepG2 cells, the increased COX-2 protein levels in *t*-BHP-treated cells were also reduced by pretreatment of oligonol in a dose-dependent manner. These results suggest that oligonol exerts effects in suppressing inflammatory responses caused by CCl_4 or *t*-BHP.

A series of upstream kinases including MAPKs and Akt are involved in a relay in transmitting stimuli-induced signals to the downstream transcription factors like NF-κB by regulating transcriptional activation of a variety of genes encoding COX-2, iNOS, TNF-α, and IL-1β. MAPKs mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, death, and transformation [29]. The mammalian MAPK family consists of ERK, JNK, and p38. The MAPK pathways are activated by diverse extracellular and intracellular stimuli including cytokines and various cellular stressors such as oxidative stress caused ROS and endoplasmic reticulum stress. Activated MAPKs phosphorylate various substrate

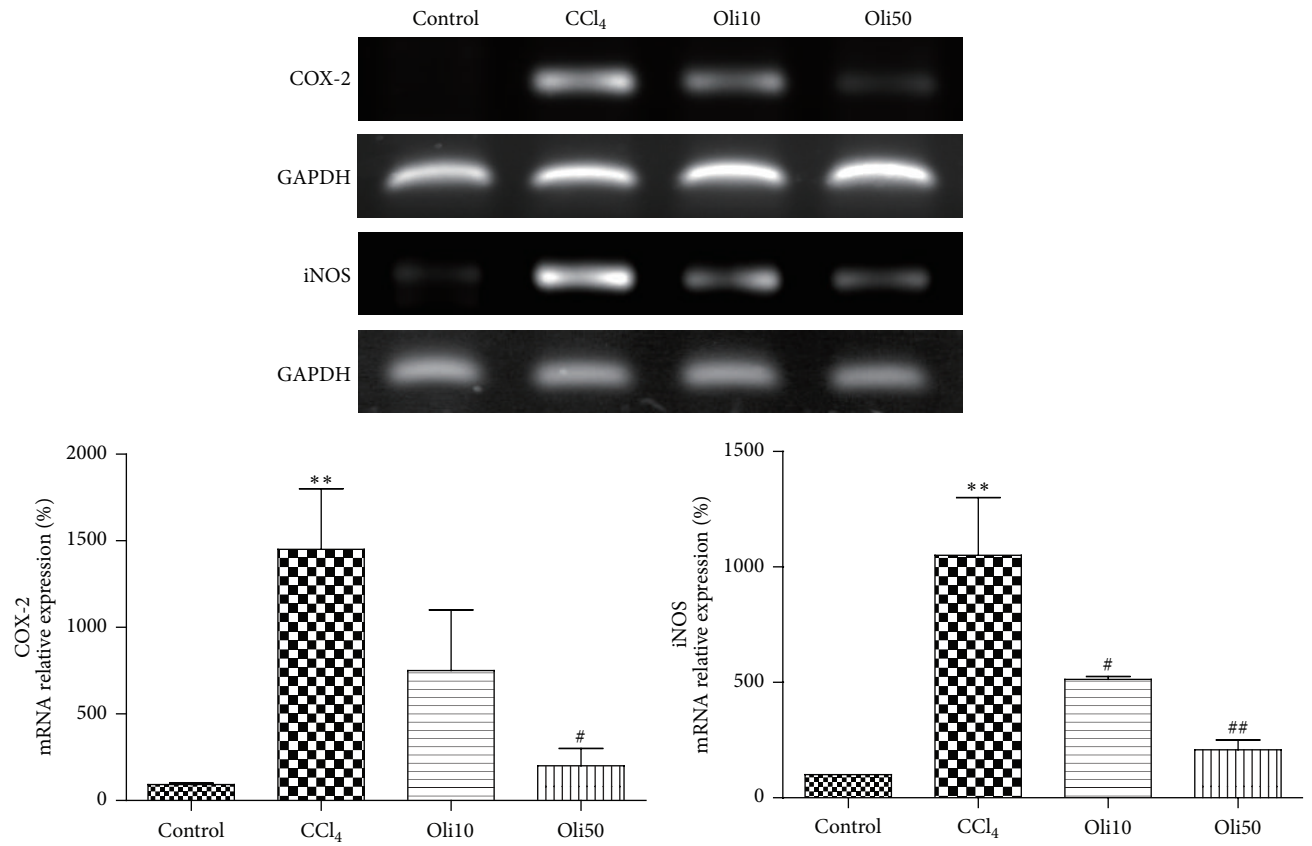


Figure 7: Effects of oligonol on COX-2 and iNOS mRNA expression after CCl_4 administration. RT-PCR was performed to measure COX-2 and iNOS mRNA expression in the liver tissues. Values are mean ± SE of $n = 6$. $^{**}P < 0.01$ compared with the control group and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the CCl_4 group.

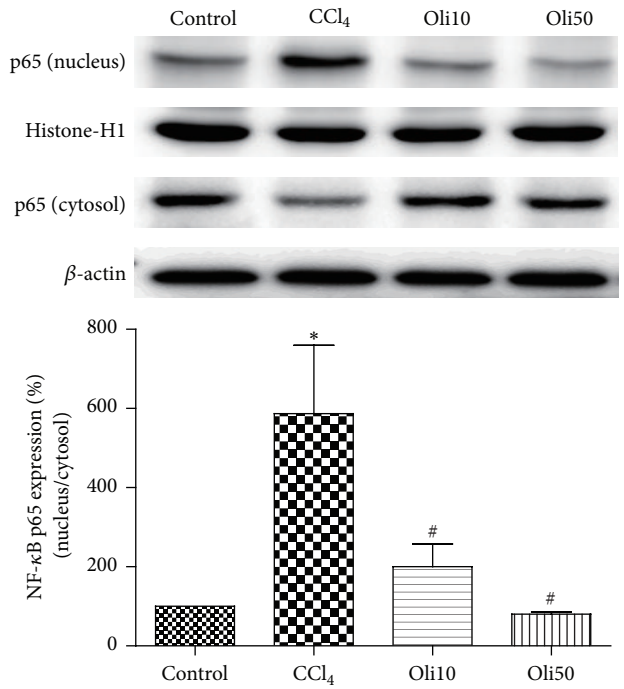


Figure 8: Effects of oligonol on CCl_4-induced NF-κB p65 activation. Western blotting was performed to detect nuclear and cytoplasmic localization of NF-κB p65 in the livers tissues. The relative level of NF-κB p65 in the nuclear and cytosol compared with β-actin and histon, respectively, and quantified by image analysis. Values are mean ± SE of $n = 6$. $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the CCl_4 group.

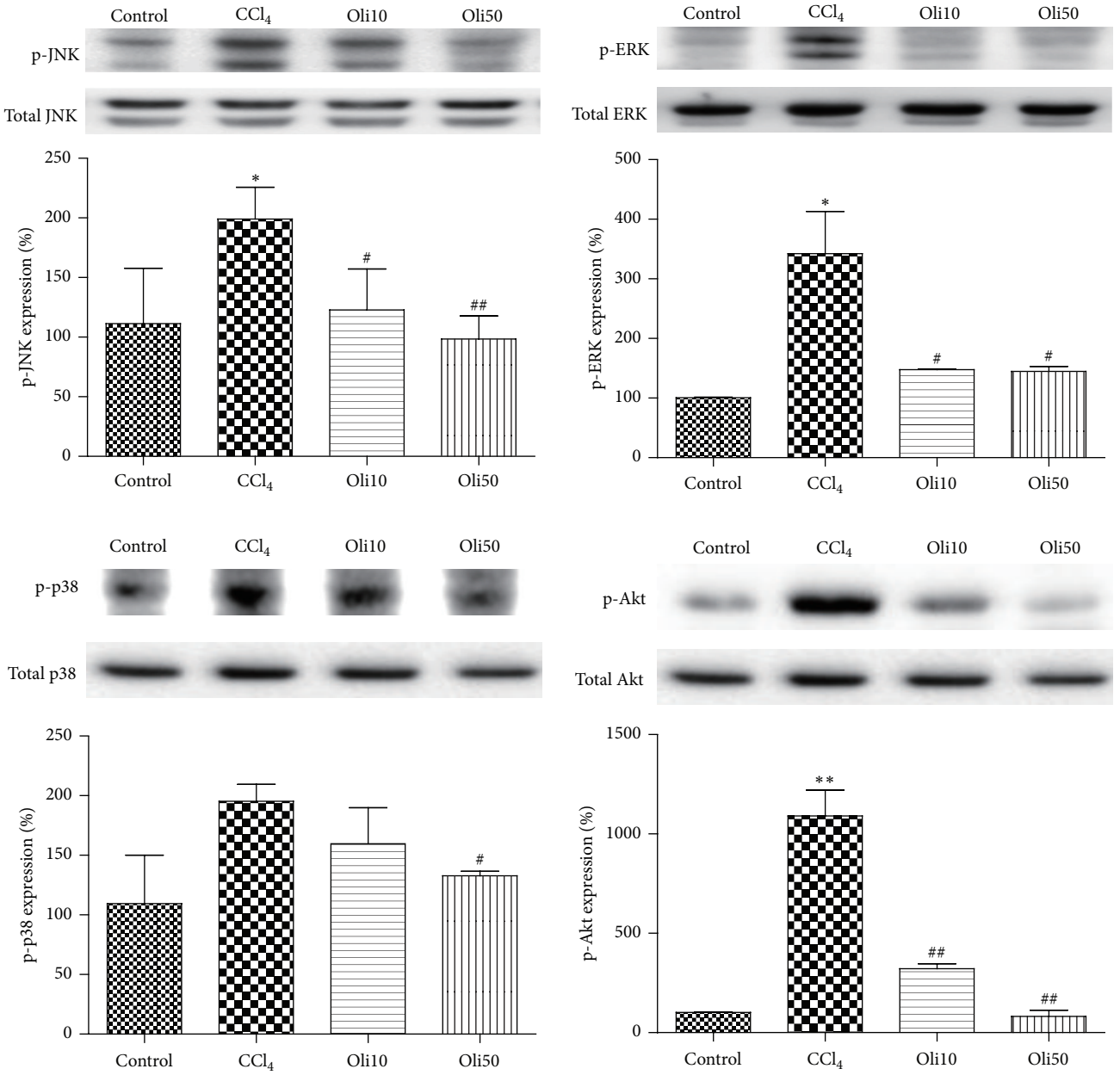


Figure 9: Effects of oligonol on CCl_4-induced MAPKs and Akt activation. Western blotting was performed to detect ERK, JNK, and p38 MAPK, as well as Akt, in liver tissues. Activation of these kinases was detected using their specific phosphorylated antibody. The band intensity of phosphorylated forms of MAPKs and Akt was quantified by densitometry and normalized to total forms of MAPKs and Akt, respectively. Values are mean ± SE of $n = 6$. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the CCl_4 group.

proteins including transcription factors like NF-κB. Our results also showed that CCl_4 exposure induced activation of MAPKs in rat liver, and oligonol inhibited CCl_4-induced JNK, ERK, and p38 phosphorylation. In addition, in order to activate NF-κB in response to specific stimuli, NF-κB needs to first be liberated from its inhibitory IκB partner [30]. On phosphorylation by IκB kinase (IKK), IκB is degraded by the proteasome and NF-κB is set free. IKK can be activated by Akt. In this study, phosphorylation of Akt was increased in CCl_4-intoxicated rats, while oligonol suppressed this Akt activation. This suggests that oligonol is involved with the Akt/NF-κB pathway.

5. Conclusion

The findings of the present study indicate that oligonol is highly effective in preventing CCl_4-induced acute liver damage, which is most likely mediated by its activity to suppress oxidative stress and lipid peroxidation as an antioxidant. It has capacity to inhibit NF-κB p65 activation and the expression of the proinflammatory cytokines, TNF-α and IL-1β, and proinflammatory proteins, such as COX-2 and iNOS. The underlying mechanisms for this NF-κB inactivation may be due to inhibition of the activation of upstream kinases including ERK, JNK, and p38 MAPKs as well as Akt. Our data

suggests that oligonol may be useful as a therapeutic agent for the suppression of hepatic inflammation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (no. 2009-0083538). The authors thank the Aging Tissue Bank (Busan, Korea) for supplying research materials.

References

[1] J. Wu and M. A. Zern, "Hepatic stellate cells: a target for the treatment of liver fibrosis," *Journal of Gastroenterology*, vol. 35, no. 9, pp. 665–672, 2000.

[2] K. S. Lee, S. J. Lee, H. J. Park et al., "Oxidative stress effect on the activation of hepatic stellate cells," *Yonsei Medical Journal*, vol. 42, no. 1, pp. 1–8, 2001.

[3] P. Muriel, "Peroxidation of lipids and liver damage: in Baskin," in *Antioxidants, Oxidants, and Free Radicals*, S. I. Baskin and H. Salem, Eds., Washington, DC, USA, pp. 237–257, Taylor and Francis, 1997.

[4] D.-S. Shin, K. W. Kim, H. Y. Chung, S. Yoon, and J.-O. Moon, "Effect of sinapic acid against dimethylnitrosamine-induced hepatic fibrosis in rats," *Archives of Pharmacal Research*, vol. 36, no. 5, pp. 608–618, 2013.

[5] D.-S. Shin, K. W. Kim, H. Y. Chung, S. Yoon, and J.-O. Moon, "Effect of sinapic acid against carbon tetrachloride-induced acute hepatic injury in rats," *Archives of Pharmacal Research*, vol. 36, no. 5, pp. 626–633, 2013.

[6] R. H. Liu, "Health-promoting components of fruits and vegetables in the diet," *Advances in Nutrition*, vol. 4, no. 3, pp. 384–392, 2013.

[7] M.-H. Li, J.-H. Jang, B. Sun, and Y.-J. Surh, "Protective effects of oligomers of grape seed polyphenols against beta-amyloid-induced oxidative cell death," *Annals of the New York Academy of Sciences*, vol. 1030, pp. 317–329, 2004.

[8] J. S. Noh, H. Y. Kim, C. H. Park, H. Fujii, and T. Yokozawa, "Hypolipidaemic and antioxidative effects of oligonol, a low-molecular-weight polyphenol derived from lychee fruit, on renal damage in type 2 diabetic mice," *British Journal of Nutrition*, vol. 104, no. 8, pp. 1120–1128, 2010.

[9] J. Y. Park, Y. Kim, J. A. Im, S. You, and H. Lee, "Inhibition of adipogenesis by Oligonol through Akt-mTOR inhibition in 3T3-L1 adipocytes," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 895272, 11 pages, 2014.

[10] Y. Y. Choi, T. Maeda, H. Fujii et al., "Oligonol improves memory and cognition under an amyloid β25–35-induced Alzheimer's mouse model," *Nutrition Research*, vol. 34, no. 7, pp. 595–603, 2014.

[11] E.-H. Jo, S.-J. Lee, N.-S. Ahn et al., "Induction of apoptosis in MCF-7 and MDA-MB-231 breast cancer cells by Oligonol is mediated by Bcl-2 family regulation and MEK/ERK signaling," *European Journal of Cancer Prevention*, vol. 16, no. 4, pp. 342–347, 2007.

[12] J. K. Kundu, E.-J. Chang, H. Fujii, B. Sun, and Y.-J. Surh, "Oligonol inhibits UVB-induced COX-2 expression in HR-1 hairless mouse skin—AP-1 and C/EBP as potential upstream targets," *Photochemistry and Photobiology*, vol. 84, no. 2, pp. 399–406, 2008.

[13] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT—Food Science and Technology*, vol. 28, no. 1, pp. 25–30, 1995.

[14] J. A. Buege and S. D. Aust, "Microsomal lipid peroxidation," *Methods in Enzymology*, vol. 52, pp. 302–310, 1978.

[15] K. Ishak, A. Baptista, L. Bianchi et al., "Histological grading and staging of chronic hepatitis," *Journal of Hepatology*, vol. 22, no. 6, pp. 696–699, 1995.

[16] S. Reitman and S. Frankel, "A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases," *American Journal of Clinical Pathology*, vol. 28, no. 1, pp. 56–63, 1957.

[17] R. Agarwal and S. D. Chase, "Rapid fluorimetric-liquid chromatographic determination of malondialdehyde in biological samples," *Journal of Chromatography B: Analytical Technologies in the Biomedical & Life Sciences*, vol. 775, no. 1, pp. 121–126, 2002.

[18] C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress," *Chemical Research in Toxicology*, vol. 5, no. 2, pp. 227–231, 1992.

[19] A. Ejarque-Ortiz, M. G. Medina, J. M. Tusell, A. P. Pérez-González, J. Serratosa, and J. Saura, "Upregulation of CCAAT/enhancer binding protein β in activated astrocytes and microglia," *Glia*, vol. 55, no. 2, pp. 178–188, 2007.

[20] W. Zhu and P. C. W. Fung, "The roles played by crucial free radicals like lipid free radicals, nitric oxide, and enzymes NOS and NADPH in CCl_4-induced acute liver injury of mice," *Free Radical Biology and Medicine*, vol. 29, no. 9, pp. 870–880, 2000.

[21] J. A. Castro and M. I. Diaz Gomez, "Studies on the irreversible binding of 14CCCl4 to microsomal lipids in rats under varying experimental conditions," *Toxicology and Applied Pharmacology*, vol. 23, no. 4, pp. 541–552, 1972.

[22] J. A. Castro, H. A. Sasame, H. Sussman, and J. R. Gillette, "Diverse effects of SKF 525-A and antioxidants on carbon tetrachloride-induced changes in liver microsomal P-450 content and ethylmorphine metabolism," *Life Sciences*, vol. 7, no. 3, pp. 129–136, 1968.

[23] J. H. Ahn, J. W. Choi, J. M. Choi et al., "Protective role of oligonol from oxidative stress-induced inflammation in C6 glial cell," *Nutrition Research and Practice*, vol. 9, no. 2, pp. 123–128, 2015.

[24] R. Krithika, R. Mohankumar, R. J. Verma et al., "Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line," *Chemico-Biological Interactions*, vol. 181, no. 3, pp. 351–358, 2009.

[25] D.-S. Lee, K.-S. Kim, W. Ko et al., "The cytoprotective effect of sulfuretin against tert-butyl hydroperoxide-induced hepatotoxicity through Nrf2/ARE and JNK/ERK MAPK-mediated heme oxygenase-1 expression," *International Journal of Molecular Sciences*, vol. 15, no. 5, pp. 8863–8877, 2014.

[26] P. Muriel, "Cytokines in liver diseases," in *Hepatotoxicity: From Genomics to In Vitro and In Vivo Models*, S. Sahu, Ed., pp. 371–389, John Wiley & Sons, West Sussex, UK, 2007.

[27] P. Muriel, "NF-κB in liver diseases: a target for drug therapy," *Journal of Applied Toxicology*, vol. 29, no. 2, pp. 91–100, 2009.

[28] K. Reyes-Gordillo, J. Segovia, M. Shibayama, P. Vergara, M. G. Moreno, and P. Muriel, "Curcumin protects against acute liver damage in the rat by inhibiting NFkappaB, proinflammatory cytokines production and oxidative stress," *Biochimica et Biophysica Acta—General Subjects*, vol. 1770, no. 6, pp. 989–996, 2007.

[29] E. K. Kim and E.-J. Choi, "Pathological roles of MAPK signaling pathways in human diseases," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1802, no. 4, pp. 396–405, 2010.

[30] T. Luedde and R. F. Schwabe, "NF-κB in the liver—linking injury, fibrosis and hepatocellular carcinoma," *Nature Reviews Gastroenterology & Hepatology*, vol. 8, no. 2, pp. 108–118, 2011.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3529149, 12 pages
http://dx.doi.org/10.1155/2016/3529149

Review Article

Is Liver Enzyme Release Really Associated with Cell Necrosis Induced by Oxidant Stress?

Martha Lucinda Contreras-Zentella and Rolando Hernández-Muñoz

Departamento de Biología Celular y Desarrollo, Instituto de Fisiología Celular, UNAM, 04510 Mexico City, DF, Mexico

Correspondence should be addressed to Rolando Hernández-Muñoz; rhernand@correo.ifc.unam.mx

Received 19 July 2015; Accepted 11 October 2015

Academic Editor: Karina R. Gordillo

Copyright © 2016 M. L. Contreras-Zentella and R. Hernández-Muñoz. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Hepatic diseases are a major concern worldwide. Increased specific plasma enzyme activities are considered diagnostic features for liver diseases, since enzymes are released into the blood compartment following the deterioration of the organ. Release of liver mitochondrial enzymes is considered strong evidence for hepatic necrosis, which is associated with an increased production of ROS, often leading to greater hepatic lipid peroxidation. Lipotoxic mediators and intracellular signals activated Kupffer cells, which provides evidence strongly suggesting the participation of oxidant stress in acute liver damage, inducing the progression of liver injury to chronic liver damage. Elevated transaminase activities are considered as an index marker of hepatotoxicity, linked to oxidant stress. However, a drastic increase of serum activities of liver enzyme markers ought not necessarily to reflect liver cell death. In fact, increased serum levels of cytoplasmic enzymes have readily been observed after partial hepatectomy (PH) in the regenerating liver of rats. In this regard, we are now showing that *in vitro* modifications of the oxidant status affect differentially the release of liver enzymes, indicating that this release is a strictly controlled event and not directly related to the onset of oxidant stress of the liver.

1. Introduction

Every organ can elicit a specific pattern of enzyme release, which remains not elucidated. Specifically, above-normal plasma enzyme activities are considered as diagnostic features for several diseases [1]. Release of enzymes usually follows their respective concentration gradients between an organ, such as the liver, and the blood compartments [2–4]. In fact, values of serum enzymes activities ("released") are much higher than the apparent disappearance rate constants and they are also consistent with disappearance rates from plasma to lactate dehydrogenase (LDH) and aspartate (AST) and alanine (ALT) aminotransferases, after acute liver injury [5]. However, the mechanisms controlling cellular enzyme release remain poorly understood. Moreover, a drastic increase of serum activities of "liver enzyme markers" ought not necessarily to reflect liver cell death. Therefore, pathological elevations of the plasma activities of liver enzymes do not seem to be simply related to the quantitative release of such enzymes from the liver. Consequently, several enzymatic indices may be determined by differences in the time course of hepatic enzyme release, rather than reflecting true differences in the released quantities of various enzymes [5]. However, the quantitative use of enzymatic data is hampered by the fact that the fractional catabolic rate constants for the elimination of enzyme activities from plasma are unknown [5].

Release of mitochondrial enzymes from the liver is considered to provide strong evidence for hepatic necrosis [6, 7] and is also associated with specific forms of liver disease. It has been shown, for instance, that glutamate dehydrogenase (GDH) correlates well with the presence and extent of necrosis in alcoholic liver disease [8]. Furthermore, the ratio of mitochondrial and total AST (mAST) has been proposed as a marker for chronic alcoholism [9]. However, both GDH and mAST are widely distributed in various organs and lack specificity as a marker of liver injury. Despite the fact that it was reported that cumulative release of various cytosolic enzymes occurred in proportion to the corresponding activities in human control livers, the mechanisms that govern the release of liver enzymes into the bloodstream are practically unknown.

2. Liver Damage

Hepatic diseases are a major concern worldwide. Since the liver is a primary organ involved in biotransformation of food and drugs, hepatic disorders are very often [10]. These disorders are mainly caused by toxic chemicals, xenobiotics, and anticancer, immunosuppressant, analgesic anti-inflammatory, and antitubercular drugs [10]. Additionally, other biological agents, as well as exposure to radiations, heavy metals, mycotoxins, galactosamine, and so forth, constitute predisposing factors to develop liver damage and hepatopathy. Moreover, additional risk factors for hepatic injury include age, gender, alcoholism, and nutrition, and genetic polymorphisms of cytochrome P_{450} enzymes have also been emphasized [10]. Nutritional deficiency may predispose to drug-induced liver injury as reported in patients with HIV, tuberculosis, or alcoholism. This is largely due to the reduced hepatic glutathione in liver tissues [11]. Indeed, alcohol is believed to be one of the most important risk factors for this type of liver damage, although its exact role is not fully understood. Despite the fact that the chronic use of alcohol, particularly with malnutrition, depletes the glutathione stores, the exact link between alcoholism and liver injury is missing [12].

Chronic hepatitis B and hepatitis C are now considered to enhance the risk of drug-induced liver injury, particularly from drugs used in the treatment of tuberculosis and HIV [13]. Furthermore, a strong dose response relationship exists between drugs and hepatotoxicity. Authors further stated that drugs administered in doses of >50 mg of oral medications have an enhanced risk of this pathology [14]. In this context, the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) is strongly associated with hepatotoxicity, as it is the case for nimesulide [15], diclofenac [16], and sulindac [17]. The NSAIDs, which inhibit cyclooxygenase enzymes (COX), are associated with idiosyncratic hepatotoxicity, showing symptoms ranging from elevation of serum transaminases to hepatocellular or cholestatic injury and occasionally to fatal fulminant hepatitis [18]. The mechanisms responsible for NSAID-induced liver injury may involve mitochondrial dysfunction and endoplasmic reticulum (ER) stress [19]. In addition, generation of the ER stress response induces cytochrome c (a marker of mitochondria-mediated cell death) release leading to mitochondria-mediated cell death (apoptosis). This mechanism is proposed as a major source for liver injury [20].

3. Liver Damage: Human Hepatic Steatosis

Liver fat deposition related to systemic insulin resistance is defined as a nonalcoholic fatty liver disease (NAFLD) which, when associated with oxidative hepatocellular damage, inflammation, and activation of fibrogenesis, can progress towards cirrhosis and hepatocellular carcinoma [21]. Due to increased onset for obesity, NAFLD is now the most frequent liver disease and the leading cause of altered liver enzymes in Western countries [21]. The NAFLD is a condition associated with obesity in which there is ectopic accumulation of triglycerides in the liver parenchyma [22]. NAFLD is used to describe a spectrum defined by liver biopsy findings ranging from accumulation of triglycerides as lipid droplets in the cytoplasm of hepatocytes, namely, simple steatosis, to the more aggressive form of nonalcoholic steatohepatitis (NASH).

Although simple steatosis appears to follow a nonprogressive course, there are still a large number of patients with NASH, some of which may also develop end-stage complications including cirrhosis [23] and hepatocellular carcinoma [24]. Considering the rapid increase in the prevalence of obesity in children globally, NAFLD is now also recognized as the most common cause of liver disease in the pediatric population [25].

As to NASH (term used in human), studies in animal models have revealed several molecular processes that recapitulate the cardinal features of NASH [24]: hepatocyte damage, inflammation, and fibrosis are the most remarkable findings in this pathology. Inflammation is a component of the wound healing process that leads to the deposition of extracellular matrix and fibrosis in the liver. A growing body of evidence supports a central role for proinflammatory cytokines, particularly tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), in the development of NASH [26].

Patients with NASH present elevated levels of TNF-α and IL-6 in the liver and blood, and inhibition of these cytokines has improved NAFLD in rodents [27]. A second potential mechanism is ER stress, resulting from improperly folded proteins accumulating in the ER, which elicits the unfolded protein response (UPR). The UPR activates nuclear factor κB, c-Jun N-terminal kinase, and oxidative stress pathways, all of which have been implicated in progression of steatosis to NASH [28]. Studies of humans with rare inherited disorders demonstrate that hepatic TG accumulation from dietary intake, changes in the distribution of TG from adipose tissue to the liver, and/or increased de novo lipogenesis result in hepatic steatosis [29].

Hepatic steatosis is often self-limited, but it can progress to NASH (nonalcoholic steatohepatitis). NASH is distinguished from simple steatosis by the presence of hepatocyte injury (hepatocyte ballooning and cell death), an inflammatory infiltrate, and/or collagen deposition (fibrosis) [24]. It is not known whether steatosis always precedes NASH or steatosis and NASH are distinct disorders [24]. NASH, in turn, can progress to cirrhosis. In cirrhosis, hepatocytes are replaced by scared tissue composed primarily of type 1 collagen [24], produced by specialized cells called stellate cells, which are activated by liver injury and play a key role in liver regeneration. Cirrhosis can ultimately progress to liver cancer (hepatocellular carcinoma) [30]. Although obesity and insulin resistance are the most prevalent risk factors for NAFLD, hepatic fat content varies substantially among individuals with equivalent adiposity, indicating that other factors contribute to this condition [24].

In the case of alcoholic fatty liver disease (AFLD), there is an increase of NADH/NAD+ value promoted by liver alcohol oxidation, which will induce the disorder of fat metabolism, resulting in triglyceride accumulation in hepatocytes [31]. Thereby, hepatic steatosis is the early manifestation of alcohol liver disease (ALD) and is believed to be the fundamental

pathological change of other more severe alcoholic liver diseases [32]. In addition, insulin pretreatment could exert a significant protective effect on oxidative damage and inflammatory reaction in the liver against ethanol exposure, but insulin can also exacerbate hepatic steatosis in mice exposed to ethanol [33]. Fatty acid synthesis in liver is mainly regulated by sterol regulatory elements binding protein-1c (SREBP-1c). Ethanol exposure can significantly activate SREBP-1c, which is responsible for the formation of fatty liver [34]. Insulin could also lead to the activation of SREBP-1c to increase the triglycerides in hepatocytes [35]. Indeed the expression of SREBP-1c can be regulated upwards by the administration of insulin and ethanol, suggesting that SREBP-1c activation might contribute to the deteriorative effects of insulin preadministration on hepatic steatosis in mice exposed to ethanol [33]. According to the "two-hit" hypothesis for ALD, even though steatosis is reversible, it might be the basis of other serious liver diseases and pathologies including steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma [32].

4. Oxidant Stress in the Generation of Liver Damage

Overproduction of reactive oxygen species (ROS) results in oxidative stress, a state in which tissue and cellular redox balance is altered towards a more oxidizing environment [36]. ROS lead to a cumulative damage to protein, lipids, DNA, carbohydrates, and membranes. The prime functions of antioxidative defenses are suppressors of the generation of ROS, scavenging them, besides repairing and promoting reconstitution of damage, and inducing the expression of antioxidant proteins and enzymes [37, 38].

In the NAFLD, the molecular and cellular mechanisms underlying hepatic injury are not well defined. However, multiple mechanisms have been suggested, including enhanced flow of free fatty acids and release of adipocytokines from the adipose tissue [39]. In the liver, mitochondrial dysfunction, oxidative stress, and hepatocyte apoptosis are key contributors to hepatocellular injury. In addition, lipotoxic mediators and intracellular signals activate Kupffer cells, which initiate and perpetuate the inflammatory response and development of fibrosis [39]. In the development of NAFLD, there is an increased production of ROS, often leading to a greater hepatic lipid peroxidation [40, 41]. In fact, a hypercholesterolemic diet increases liver TBARS, indicating increased oxidative stress. It is known that oxidative stress can occur by increasing of prooxidant systems and/or by lowering antioxidant enzymes. Increased NADPH oxidase activity has been reported in animal models of NASH, in which dietary antioxidants or NADPH oxidase inhibitors ameliorated the progression of the disease [42, 43]. In mice, the presence of triacylglycerol and cholesterol in the diet is needed for the development of hepatic histological abnormalities of NASH and its metabolic abnormalities [44].

On the other hand, in the pathogenesis of AFLD, there is an increase in NADPH oxidase activity and predominance of prooxidant agents, exceeding the capacity of the organic antioxidant defense [45]. Under these circumstances, intracellular homeostasis in the redox status is interrupted and, sometimes, induces cell damage. This results in apoptosis or necrosis, potentially contributing to the devastating injury and dysfunction of liver tissue [46, 47]. Total body deficiency in $p47^{phox}$ subunit of NADPH oxidase complex protects mice from alcohol-induced liver steatosis [48]. However, mice on a methionine-choline-deficient (MCD) diet develop NASH with similar pathology as the wild type, despite the lack of a functional NADPH oxidase enzyme [49]. Nevertheless, the role of this enzymatic complex in other animal models of NAFLD has not been investigated, but a role for the NADPH oxidases in chronic liver diseases related to chronic inflammation, such as fibrosis and viral hepatitis, has been proposed [50, 51].

Therefore, the present evidence strongly suggests the participation of oxidant stress in acute liver damage which, appearing to be in an accumulative effect, induces the progression of liver injury to chronic liver damage.

5. Effects of Vitamins and Other Antioxidants on Liver Damage

Markers for lipid peroxidation are increased in both liver and blood of patients with advanced ALD in concomitance with the lowering of antioxidant defenses [52]. Additionally supplementation with antioxidants reduced hepatic injury in alcohol-fed rodents [53].

There is evidence suggesting that the activation of AMP-activated protein kinase (AMPK) is associated with the hypoglycemic actions of metformin [54], a dimethylbiguanide, which is a commonly used antidiabetic drug [55]. Although the precise pharmacological mechanisms of metformin have not been fully elucidated, the anti-inflammatory effects of metformin involving both AMPK-dependent and AMPK-independent pathways have been mentioned [56–58]. Also, it has been suggested that metformin might have antioxidative effects both *in vivo* and *in vitro* [59–61]; metformin actually attenuates endotoxin-induced fulminant hepatitis in mice [62]. Moreover, this biguanide significantly reduces the CCl_4-induced elevation of serum aminotransferases and hepatic histological abnormalities, which seem to be associated with decreased hepatic contents of oxidized glutathione (GSSG) and malondialdehyde (MDA) [63].

Furthermore, the Nrf2 has emerged as an indispensable regulator of both constitutive and inducible expression of detoxifying phase II and antioxidant enzyme genes in various tissues and cell types [64]. Nrf2-null mice are particularly susceptible to oxidative stress, contributing to increased hepatotoxicity by ethanol [65] and acetaminophen [66]. In rats treated with CCl_4, there were depletion of cytoplasmic Nrf2 and suppression of Nrf2 nuclear translocation, accompanied by a dramatic downregulation of liver Nrf2 target genes, NQO1, HO-1, and GSTα. On the other hand, increased Nrf2 expression represses the genes involved in fatty acids synthesis and, therefore, may play a crucial role in the development of NASH [67]. The activation of Nrf-2 is important for maintaining intracellular and mitochondrial

GSH balance and for increasing the activities of antioxidant enzymes to protect cells from oxidative damage mediated by ethanol [68]. For instance, the treatment with α-lipoic acid (a vitamin) induced an early nuclear accumulation of Nrf2, resulting in a strong protection against apoptosis induced by palmitic acid [69].

Concerning vitamins, vitamin D may have a role in NAFLD pathogenesis via its effects on insulin resistance and metabolic syndrome [70]. Improvement of vitamin D status led to amelioration in serum high sensitive-CRP and MDA in patients with NAFLD. Therefore, vitamin D could be considered as an adjunctive therapy to attenuate systemic inflammation and lipid peroxidation along with other treatments administered to patients with NAFLD [71]. It is also known that another vitamin, vitamin E, a potent antioxidant that protects against oxidative stress induced liver damage *in vitro* and *in vivo*, has beneficial effects on histological outcomes in patients with NAFLD. This vitamin decreases serum levels for ALT activity in patients with HCV genotype 3, suggesting that vitamin E has a protective effect against HCV-induced liver cells necrosis [72].

In the same way, the diallyl disulfide, primarily derived from the garlic, effectively ameliorates CCl_4-induced oxidative hepatic injury and inflammatory responses in rats [64]. The hepatoprotective effects of diallyl disulfide may be due to its ability to induce antioxidant or detoxifying enzyme activities through activation of Nrf2 and to suppress the production of inflammatory mediators by inhibiting NF-κB activation. These properties confer to this molecule a useful protective effect against various hepatic injuries caused by oxidative stress and inflammatory response [64].

The effects of diverse antioxidants protecting or ameliorating liver injury also emphasize the important role of oxidant stress in the generation of acute and chronic liver damage.

6. Serum ("Marker") Enzyme Activities and Liver Damage

Several hepatotoxins such as chemicals, drugs, lipopolysaccharides, heavy metals, and mycotoxins elicit a wide variety of hepatic injuries. Numerous enzymes are produced in the liver and are normally distributed within the cells of the liver [10]. Elevation of serum enzyme is taken as the sensitive biomarker of liver toxicity. The determination of various liver enzymes in serum, as ALT, AST, alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GGTP), lactate dehydrogenase (LDL) in serum, and serum lipid profile, cholesterol, triacylglycerides, and lipoproteins, are used to evaluate the functional status of the liver and to detect liver injury. An elevation in transaminase in conjunction with a rise in bilirubin level to more than double is considered as a marker index of hepatotoxicity [73]. Other sensitive biomarkers of liver function are albumin concentration, total protein (TP), and prothrombin time (PT). These biomarkers can serve as an index of liver biosynthetic capacity [10]. Therefore, ALT and AST activities in serum are the most frequently used indicators for evaluation of liver injury [74], meeting drastic increases under these conditions [75]. The levels of cholesterol were similar in patients control and with NAFLD, but those with NAFLD had higher triglyceride levels [21]. A hypercholesterolemic diet causes liver damage and increased oxidative stress and cholesterol levels in female rats. The resultant liver injury was characterized by hepatomegaly and accompanied by increased activities of AST and ALT enzymes [76]. Even more, a large proportion of patients with chronic inflammatory liver diseases and of patients with metabolic syndrome complications had impaired glucose tolerance [70].

Alcoholic subjects having moderate/severe hepatic steatosis usually present an increase in the levels of triglycerides, cholesterol, glucose, γ-GGTP, ALT, bilirubin, α-1 and β-2 globulins, and iron and a decrease in the levels of AST [77]. In this regard, it has been found that in alcoholic subjects the AST/ALT ratio is significantly increased and it has been considered that the AST/ALT ratio could be a marker playing a role for alcoholic liver disease progression [77].

However, there exist discrepancies when matching changes of assumed "liver enzymes" in serum and other markers for liver integrity. For instance, increased levels of γ-GGTP, a liver enzyme, play an independent role in the pathogenesis and clinical evolution of cardiovascular disease induced by atherosclerosis and are associated with increased cardiovascular disease mortality [78]. Moreover, other authors reported similar results in the association of high γ-GGTP levels with fatal and nonfatal cardiovascular disease, independently of the metabolic risk factors and alcohol consumption [78]. On the other hand, some studies also reported the association of increased ALT levels and cardiovascular disease. Moreover, between patients without viral hepatitis or excessive alcohol consumption, those with elevated ALT level had a higher calculated risk of cardiovascular disease than those with normal ALT activity [78].

Therefore, several situations arise where there is evident loss of correlation between serum levels of liver enzymes and tissue necrosis and in the specificity of possible tissue markers.

7. Fluctuations of Serum "Marker" Enzymes in the Model of Liver Regeneration Induced by Partial Hepatectomy in Rats

In clinical practice, net enzyme release could be indicative of liver damage, even though hepatic enzyme activities can remain normal [79] or even elevated in the organ [80]. In CCl_4-induced hepatic injury, serum and liver enzyme activities vary according to the enzyme studied [81], but frequently the appearance of mitochondrial enzymes in the serum is delayed as compared to the cytoplasmic enzymes [80, 82]. MDH and AST activities found in perfusates from isolated livers are mainly derived from their cytoplasmic isozymes [2, 80].

Hence, enzyme release might depend on alterations in plasma membranes, mitochondrial dysfunction, and/or changes in cellular volume regulation [1, 83, 84]. In addition,

the level of increased serum enzyme activities would also depend on the susceptibility of the liver cell type being damaged [85, 86].

However, a discrepancy exists between a remarkable increase in serum enzyme activities and structural and functional characteristics found after hepatic resection. Remaining hepatocytes can restore the original mass of the organ, through a process widely known as liver regeneration [87]. Partial hepatectomy- (PH-) induced liver regeneration and enzyme release have been described in detail in the literature over the past 30 to 40 years. From this information, augmented levels of serum transaminases have been found in rats subjected to PH [88], while increased serum activity of ornithine carbamoyltransferase (OCT), a liver mitochondrial enzyme, was also found after PH [89]. Similarly, patients subjected to partial removal of the organ showed a "selective" release of liver enzymes, with the serum activity of OCT being the most enhanced in these patients [90]. Despite the regenerative capacity of the remnant liver, and independently of the extent of the liver resection, increased serum levels of cytoplasmic enzymes have readily been observed after PH in rats [91]. Increases in serum levels of liver enzymes were greater and more prolonged after 85% PH, which is accompanied by a marked mortality rate in rats suffering the largest liver mass loss [91].

While the latter has been interpreted as a consequence of progressive necrosis and liver failure after massive PH, in other models of liver injury and regeneration, increased serum ALT and AST did not correlate with cell necrosis. For example, liver injury and regeneration induced by acute carbon tetrachloride administration to rats occur irrespective of the extent of the increase in serum activities for these aminotransferases [83]. These findings support the suggestion that enhanced serum enzymes could be distinctly separable from prior elevations induced by tissue damage produced by carbon tetrachloride [81]. Therefore, the reason for a substantial increase in serum activities for liver enzymes is controversial in the case of PH-induced liver regeneration in rats.

A drastic increase of serum activities of "liver enzyme markers" does not necessarily have to reflect liver cell death. Indeed, we demonstrated recently that an important fraction of the released hepatic enzymes depends largely on hemodynamic changes in the rat liver [92].

Taking advantage of the model of two-thirds partial hepatectomy- (PH-) induced rat liver regeneration ("small-for-size liver"), we showed that liver cell proliferation occurs accompanied by a selective PH-induced elevation of serum enzymes, not related to hepatocellular necrosis [93] nor to mitochondrial dysfunction [94]. Indeed, the PH induction of specific enzymes (predominantly those from mitochondria) is partly regulated by flow-bearing physical forces and is independent of extrahepatic factors [92]. Similarly, patients subjected to partial removal of the organ, who were candidates for liver transplantation, showed a "selective" release of liver enzymes, where serum activity for OCT was the most enhanced [92]. Currently, it is known that mechanical forces can be converted into a sequence of intracellular biochemical signals targeting cells, as it occurs in the endothelial layer [95]. Hence, the physicochemical interactions within cells have become a fascinating field in the study of cell functioning, and the release of enzymes by vascular organs might constitute another event regulated by hemodynamic forces.

A number of intracellular events triggered by fluid shear stress have been elucidated and mechanisms causing these events have been proposed [96]. These include direct stimulation of luminal surface transmembrane proteins, activation of ion channels affecting intracellular Ca^{++} $[Ca^{2+}]_i$ mobilization [97] which has been postulated as a likely regulator of cell proliferation [98, 99], and production of nitric oxide (NO) [100]. These mechanisms allow the transduction of stress along cytoskeletal elements to other regions of the cell. Changes in the endothelial cell membrane may act as primary mechanoreceptors in response to shear stress. We have recently suggested a possible role for cell-mediated mechanotransduction in liver enzyme release mediated by increasing shear stress, which selectively affected the release of liver enzymes. Therefore, we demonstrated that flow-induced shear stress can control the amount of hepatic enzymes released into the bloodstream, which is largely regulated through modifications in cell calcium mobilization and production of liver NO. These events were markedly elevated in the proliferating rat liver [101].

8. The Effect of Pro- and Antioxidant Environments on *In Vitro* Liver Enzyme Release

The liver is capable of recovering from damage or loss of up to 90% of its mass by means of proliferative activity, restoring it to normal size. This process, known as liver regeneration, involves the endocrine and paracrine actions of growth factors and the activation of specific protooncogenes and of transcription factors [102]. However, the understanding of the delicate coordination that triggers, modulates, and stops this process is still not well understood.

The experimental model of cell proliferation and growth regulation was examined regarding the production of free radicals and the rate of lipid peroxidation (LP). The model showed that LP, promoted by PH and CCl_4 administration, is qualitatively distinct among subcellular fractions and may indeed be a normal cell event of physiological importance in the regenerating liver. Thus, LP plays a role in the early steps of liver regeneration [103].

8.1. Release of Liver Enzymes by Liver Slices. We recently made experiments to assess the *in vitro* impact of pro- and antioxidant conditions on enzyme release from control and regenerating rat livers. We used male Wistar rats (230–280 g of body weight) fed *ad libitum* and maintained under a 12 h light/dark period, which were subjected to two-thirds PH, while sham-operated (laparotomy) animals provided a control for surgical conditions [103]. Twenty hours after surgery, liver slices were obtained and incubated under basal (B) conditions described by Díaz-Juárez et al. [92]. Then, the oxidant status of the liver slices was changed by adding 400 μmol/L hydrogen peroxide (H_2O_2), as prooxidant, or

400 μmol/L butylated hydroxytoluene (BHT), as antioxidant. The liver slices were incubated under basal conditions in sealed flasks at 37°C for one hour in the presence of 5 mmol/L glucose and after 15 min of oxygenation with a 95% O_2 : 5% CO_2 mixture.

As shown in Figure 1, we found that LDH and ALT (cytoplasmic enzymes) were influenced by the oxidant status. In control (sham-operated) rats, LDH and ALT release was significantly diminished by the addition of the BHT antioxidant. Liver slices from PH rats released significantly more ALT into the incubation medium, which was also inhibited by BHT. Additionally, the regenerating liver had a lower LDH release, which was increased by prooxidant conditions given by the added hydrogen peroxide (Figure 1(a)). As to AST and malate dehydrogenase (MDH) (sharing cytoplasmic and mitochondrial compartments) release was increased under prooxidant conditions in liver slices from both control and hepatectomized rats (Figure 1(b)). The release for the mitochondrial enzymes, OCT and GDH, was unaffected by the use of pro- and antioxidants agents (Figure 1(c)).

The results indicate that modifications of the oxidant status affected differentially the enzymes tested, cytoplasmic, mitochondrial, or sharing cytoplasmic and mitochondrial compartments. Therefore, the data obtained through the experiments would suggest that release of hepatic enzymes is a strictly controlled event, which is not linearly related to the changes in the oxidant status of the liver.

8.2. Release of Enzymes by Isolated Liver Mitochondria. Although 70% PH in rats induces the release of mitochondrial matrix proteins into the cytosol [104], liver mitochondrial function is efficiently preserved [94] and necrotic or apoptotic events have not been conclusively found in the rat regenerating liver [105].

To study the release of enzymes from isolated mitochondria, a mitochondria pellet was obtained by differential centrifugation from livers obtained from control and PH rats (24 hs after treatments). Mitochondrial respiration and phosphorylation were measured as previously described in detail [94]. When incubated in a protein-free medium in the absence of substrates, isolated liver mitochondria were able to release enzymes contained at the mitochondrial matrix. The maximal release was reached during the first 15 min at 37°C (Figure 2). In control preparations, the release of OCT, a mitochondrial enzyme, was not affected by addition of substrates for the electron transport chain (glutamate-malate and succinate), but addition of ADP (phosphorylating condition) enhanced OCT release. Under phosphorylating conditions, prooxidant (with hydrogen peroxide) or antioxidant (BHT) environments had no significant effects on OCT release. On the contrary, isolated liver mitochondria from PH rats, incubated with the substrates, greatly increased the OCT release, whereas the addition of ADP returned the release of the enzyme to the basal condition (Figure 2(a)). There are significant differences in the release of OCT between control and mitochondrial preparations from PH rats in the prooxidant condition. In this condition more OCT was released and this effect was surprisingly more accentuated in the presence of BHT (Figure 2(a)). As in OCT, the addition of substrates plus ADP also elicited an opposite profile in the mitochondrial GDH release; the comparison between control and HP rats showed that, in controls, GDH release was significantly increased under phosphorylating conditions, while in PH rats GDH release was significantly inhibited (Figure 2(b)). Whereas changes in the oxidant status did not affect GDH release in control mitochondria, a significant increase of GDH release was noted after addition of either hydrogen peroxide or BHT to isolated mitochondria from PH-animals (Figure 2(b)). The MDH release (as AST, localized both in cytosol and in the mitochondria), under phosphorylating conditions, followed a distinct pattern: in control preparations, incubation with substrates enhanced MDH release, whereas under phosphorylating conditions, this release returned to that found in the basal conditions. In mitochondria from PH rats, neither addition of substrates nor addition of ADP had a significant effect on MDH release (Figure 2(c)). Moreover, changes in the oxidant status did not have significant effects on MDH release in mitochondrial preparations from either control or PH-animals (Figure 2(c)). Finally, release of AST from isolated mitochondria from both experimental groups was increased only after the addition of substrates (Figure 2(d)). Both, the prooxidant condition and the addition of BHT significantly reduced the AST release only in mitochondrial preparations isolated from control animals (Figure 2(d)). We observed again that the oxidant status affects in a differential manner the release of liver mitochondria enzymes and that there was no constant pattern of changes in this parameter in function of fluctuations imposed *in vitro* in the oxidant status.

9. Conclusions

The liver is a primary organ involved in biotransformation of food and drugs. Moreover, the increased specific enzyme activities in the blood are considered as diagnostic features for liver diseases. However, a drastic increase of serum activities of liver enzyme markers ought not necessarily to reflect liver cell death. Release of mitochondrial enzymes from the liver is considered to provide strong evidence for hepatic necrosis and also is associated with specific forms of liver disease.

It has been frequently reported that in the development of liver diseases there is an increased production of ROS, often leading to greater hepatic lipid peroxidation. Lipotoxic mediators and intracellular signals activate Kupffer cells, which initiate and perpetuate the inflammatory response and development of fibrosis. This evidence strongly suggests the participation of oxidant stress in acute liver damage, probably inducing the progression of liver injury to chronic liver damage. It is known that elevated transaminase activities in conjunction with a rise in bilirubin level to more than double are considered as a marker index of hepatotoxicity, linked to oxidant stress. However, there exist discrepancies when matching changes of assumed "liver enzymes" in serum and other markers for liver integrity. In fact, there are several situations where an evident lack of correlation exists between serum levels of liver enzymes and tissue necrosis and in specificity as tissue marker. Despite the regenerative capacity of the remnant liver after PH, and independently

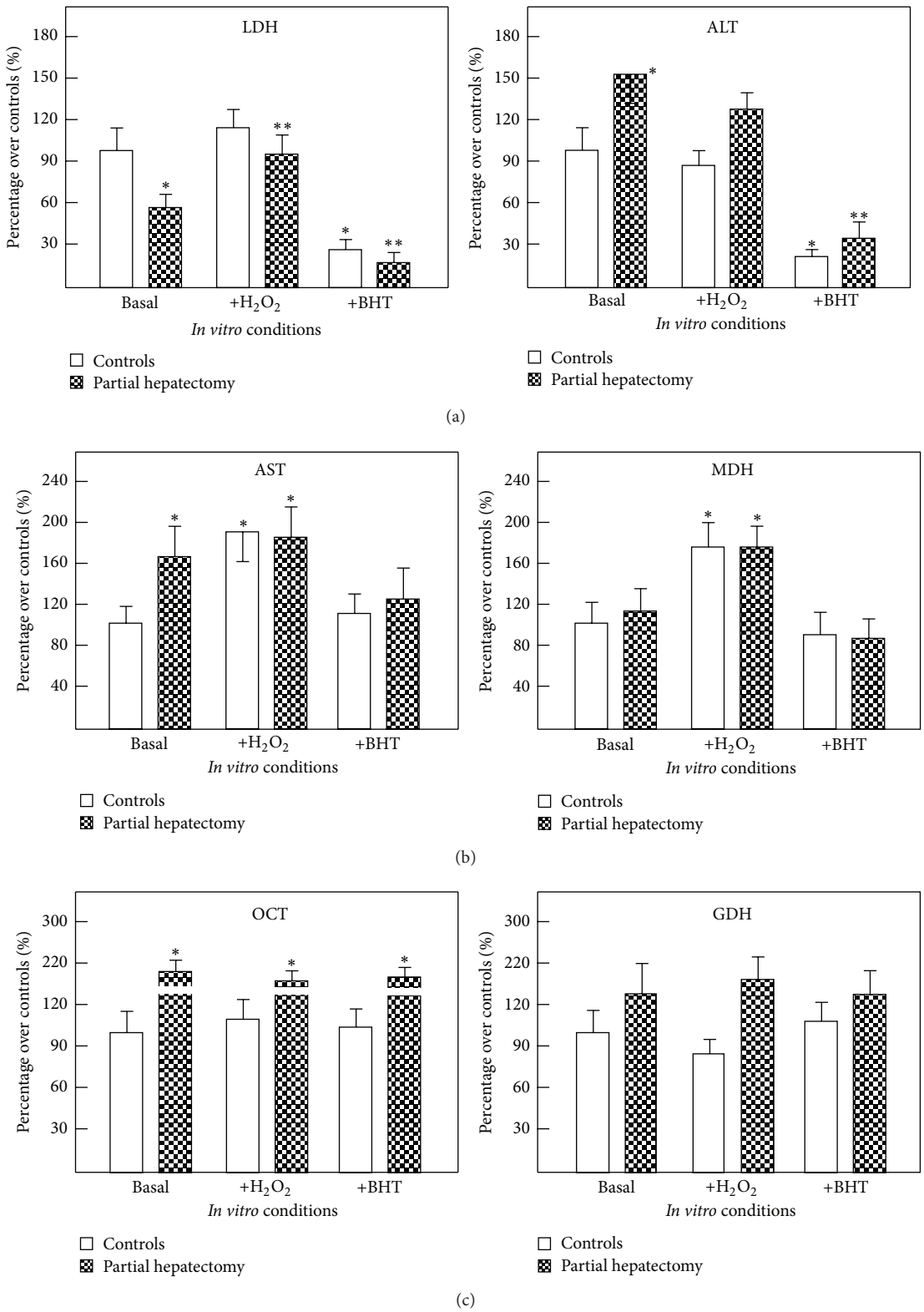


Figure 1: Effects of changing the oxidative status on cytoplasmic and mitochondrial enzymes from liver slices from control and PH rats. In panel (a), results of released ALT and LDH (cytoplasmic enzymes) are expressed as mean ± SE of six individual observations per experimental point. In panel (b), results of release of MDH and AST (enzymes sharing cytoplasmic and mitochondrial localization) are expressed and, in panel (c), those of the release of OCT and GDH activities (mitochondrial enzymes) are expressed. Enzyme release was tested under basal conditions. Abbreviations for the compounds used: hydrogen peroxide (H_2O_2) and butylated hydroxytoluene (BHT). Statistical significance: $^{*}p < 0.01$ against the group of sham-operated controls (basal conditions); $^{**}p < 0.01$ versus PH rats group (basal conditions).

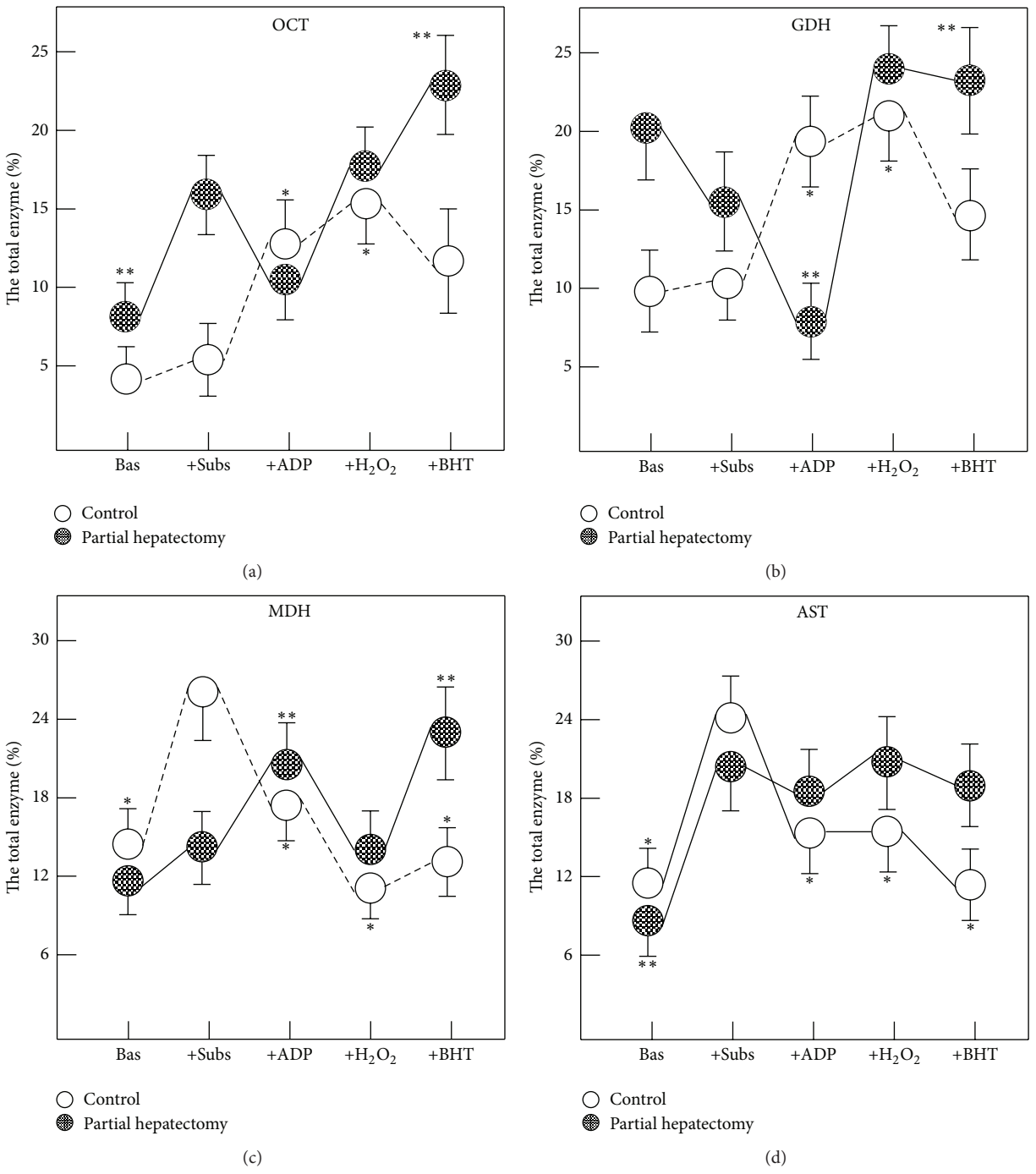


Figure 2: Effects of changing the oxidative status on enzyme release from isolated liver mitochondria from control and PH rats. Results are expressed as mean ± SE of four individual observations per experimental point and correspond to the percentage of the released enzyme in respect to the total mitochondrial activity for each enzyme. Bas: basal, Subs: substrates, H_2O_2: hydrogen peroxide, and BHT: butylated hydroxytoluene. Statistical significance: $^{*}p < 0.01$ against the group of sham-operated controls (incubated with substrates); $^{**}p < 0.01$ versus PH rats group (incubated with substrates).

of the extent of liver resection, increased serum levels of cytoplasmic enzymes have readily been observed after PH in rats. Similarly, patients subjected to partial removal of the organ showed a "selective" release of liver enzymes, serum activity of OCT being the most enhanced in these patients. Taking advantage of the model of PH-induced rat liver regeneration ("small-for-size liver"), we showed that liver cell proliferation occurs accompanied by a selective PH-induced elevation of serum enzymes. Here, we additionally demonstrated that *in vitro* modifications of the oxidant status differentially affected the enzymes tested in our laboratory in cytoplasmic, mitochondrial, or sharing cytoplasmic and

mitochondrial compartments. Therefore, the data obtained would suggest that the release of hepatic enzymes is an event strictly controlled and not directly related to the onset of oxidant stress of the liver.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Lorena Rivera-Valerdi and Aurora Castañeda-Arroyo for their valuable technical assistance and Martha Gabriela Alatriste-Contreras for careful corrections in English grammar and style. The present study was partially supported by a grant from PAPIIT-DGAPA, UNAM (IN 210611).

References

[1] H. L. Verrill, N. A. Pickard, and H. D. Gruemer, "Mechanisms of cellular enzyme release. I. Alteration in membrane fluidity and permeability," *Clinical Chemistry*, vol. 23, no. 12, pp. 2219–2225, 1997.

[2] E. Schmidt and F. W. Schmidt, "Release of enzymes from the liver," *Nature*, vol. 213, no. 5081, pp. 1125–1126, 1967.

[3] A.-M. Batt and L. Ferrari, "Manifestations of chemically induced liver damage," *Clinical Chemistry*, vol. 41, no. 12, pp. 1882–1887, 1995.

[4] D. R. Dufour, J. A. Lott, F. S. Nolte, D. R. Gretch, R. S. Koff, and L. B. Seeff, "Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests," *Clinical Chemistry*, vol. 46, no. 12, pp. 2027–2049, 2000.

[5] H. G. Peltenburg, W. T. Hermens, G. M. Willems, J. G. Flendrig, and E. Schmidt, "Estimation of the fractional catabolic rate constants for the elimination of cytosolic liver enzymes from plasma," *Hepatology*, vol. 10, no. 5, pp. 833–839, 1989.

[6] E. Schmidt and F. W. Schmidt, "Aspekte der Enzym-Diagnostik," *Die Medizinische Welt*, vol. 21, pp. 805–816, 1970.

[7] W. M. Frederiks, I. M. C. Vogels, and G. M. Fronik, "Plasma ornithine carbamyl transferase level as an indicator of ischaemic injury of rat liver," *Cell Biochemistry and Function*, vol. 2, no. 4, pp. 217–220, 1984.

[8] L. Van Waes and C. S. Lieber, "Glutamate dehydrogenase: a reliable marker of liver cell necrosis in the alcoholic," *British Medical Journal*, vol. 2, no. 6101, pp. 1508–1510, 1977.

[9] B. Nalpas, A. Vassault, S. Charpin, B. Lacour, and P. Berthelot, "Serum mitochondrial aspartate aminotransferase as a marker of chronic alcoholism: diagnostic value and interpretation in a liver unit," *Hepatology*, vol. 6, no. 4, pp. 608–614, 1986.

[10] D. K. Ingawale, S. K. Mandlik, and S. R. Naik, "Models of hepatotoxicity and the underlying cellular, biochemical and immunological mechanism(s): a critical discussion," *Environmental Toxicology and Pharmacology*, vol. 37, no. 1, pp. 118–133, 2014.

[11] R. Singla, S. K. Sharma, A. Mohan et al., "Evaluation of risk factors for antituberculosis treatment induced hepatotoxicity," *Indian Journal of Medical Research*, vol. 132, no. 7, pp. 81–86, 2010.

[12] C. Gronhagen-Riska, P.-E. Hellstrom, and B. Froseth, "Predisposing factors in hepatitis induced by isoniazid-rifampin treatment of tuberculosis," *American Review of Respiratory Disease*, vol. 118, no. 3, pp. 461–466, 1978.

[13] M. Den Brinker, F. W. N. M. Wit, P. M. E. Wertheim-van Dillen et al., "Hepatitis B and C virus co-infection and the risk for hepatotoxicity of highly active antiretroviral therapy in HIV-1 infection," *AIDS*, vol. 14, no. 18, pp. 2895–2902, 2000.

[14] C. Lammert, S. Einarsson, C. Saha, A. Niklasson, E. Bjornsson, and N. Chalasani, "Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals," *Hepatology*, vol. 47, no. 6, pp. 2003–2009, 2008.

[15] G. Traversa, C. Bianchi, R. Da Cas, I. Abraha, F. Menniti-Ippolito, and M. Venegoni, "Cohort study of hepatotoxicity associated with nimesulide and other non-steroidal anti-inflammatory drugs," *British Medical Journal*, vol. 327, no. 7405, pp. 18–22, 2003.

[16] A. T. Banks, H. J. Zimmerman, K. G. Ishak, and J. G. Harter, "Diclofenac-associated hepatotoxicity: analysis of 180 cases reported to the food and drug administration as adverse reactions," *Hepatology*, vol. 22, no. 3, pp. 820–827, 1995.

[17] E. M. Tarazi, J. G. Harter, H. J. Zimmerman, K. G. Ishak, and R. A. Eaton, "Sulindac-associated hepatic injury: analysis of 91 cases reported to the Food and Drug Administration," *Gastroenterology*, vol. 104, no. 2, pp. 569–574, 1993.

[18] G. P. Aithal and C. P. Day, "Nonsteroidal anti-inflammatory drug-induced hepatotoxicity," *Clinics in Liver Disease*, vol. 11, no. 3, pp. 563–575, 2007.

[19] S. Somasundaram, G. Sigthorsson, R. J. Simpson et al., "Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat," *Alimentary Pharmacology & Therapeutics*, vol. 14, no. 5, pp. 639–650, 2000.

[20] U. A. Boelsterli, M. R. Redinbo, and K. S. Saitta, "Multiple NSAID-induced hits injure the small intestine: underlying mechanisms and novel strategies," *Toxicological Sciences*, vol. 131, no. 2, pp. 654–667, 2013.

[21] P. Dongiovanni, Q. M. Anstee, and L. Valenti, "Genetic predisposition in NAFLD and NASH: impact on severity of liver disease and response to treatment," *Current Pharmaceutical Design*, vol. 19, no. 29, pp. 5219–5238, 2013.

[22] C. P. Day, "From fat to inflammation," *Gastroenterology*, vol. 130, no. 1, pp. 207–210, 2006.

[23] J. D. Browning, L. S. Szczepaniak, R. Dobbins et al., "Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity," *Hepatology*, vol. 40, no. 6, pp. 1387–1395, 2004.

[24] J. C. Cohen, J. D. Horton, and H. H. Hobbs, "Human fatty liver disease: old questions and new insights," *Science*, vol. 332, no. 6037, pp. 1519–1523, 2011.

[25] J. E. Lavine and J. B. Schwimmer, "Nonalcoholic fatty liver disease in the pediatric population," *Clinics in Liver Disease*, vol. 8, no. 3, pp. 549–558, 2004.

[26] A. E. Feldstein, "Novel insights into the pathophysiology of nonalcoholic fatty liver disease," *Seminars in Liver Disease*, vol. 30, no. 4, pp. 391–401, 2010.

[27] H. Tilg, "The role of cytokines in non-alcoholic fatty liver disease," *Digestive Diseases*, vol. 28, no. 1, pp. 179–185, 2010.

[28] G. S. Hotamisligil, "Endoplasmic reticulum stress and the inflammatory basis of metabolic disease," *Cell*, vol. 140, no. 6, pp. 900–917, 2010.

[29] A. J. Hooper, L. A. Adams, and J. R. Burnett, "Genetic determinants of hepatic steatosis in man," *Journal of Lipid Research*, vol. 52, no. 4, pp. 593–617, 2011.

[30] B. Q. Starley, C. J. Calcagno, and S. A. Harrison, "Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection," *Hepatology*, vol. 51, no. 5, pp. 1820–1832, 2010.

[31] T. M. Donohue Jr., "Alcohol-induced steatosis in liver cells," *World Journal of Gastroenterology*, vol. 13, no. 37, pp. 4974–4978, 2007.

[32] S. K. Mantena, A. L. King, K. K. Andringa, H. B. Eccleston, and S. M. Bailey, "Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity-induced fatty liver diseases," *Free Radical Biology and Medicine*, vol. 44, no. 7, pp. 1259–1272, 2008.

[33] J. Liu, X. Wang, Z. Peng et al., "The effects of insulin preadministration in mice exposed to ethanol: alleviating hepatic oxidative injury through anti-oxidative, anti-apoptotic activities and deteriorating hepatic steatosis through SRBEP-1c activation," *International Journal of Biological Sciences*, vol. 11, no. 5, pp. 569–586, 2015.

[34] M. You, M. Fischer, M. A. Deeg, and D. W. Crabb, "Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP)," *The Journal of Biological Chemistry*, vol. 277, no. 32, pp. 29342–29347, 2002.

[35] I. Shimomura, Y. Bashmakov, S. Ikemoto, J. D. Horton, M. S. Brown, and J. L. Goldstein, "Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 24, pp. 13656–13661, 1999.

[36] J. Medina and R. Moreno-Otero, "Pathophysiological basis for antioxidant therapy in chronic liver disease," *Drugs*, vol. 65, no. 17, pp. 2445–2461, 2005.

[37] A. K. Tiwari, "Imbalance in antioxidant defence and human diseases: multiple approach of natural antioxidants therapy," *Current Science*, vol. 81, no. 9, pp. 1179–1187, 2001.

[38] S. R. Naik, V. N. Thakare, and S. R. Patil, "Protective effect of curcumin on experimentally induced inflammation, hepatotoxicity and cardiotoxicity in rats: evidence of its antioxidant property," *Experimental and Toxicologic Pathology*, vol. 63, no. 5, pp. 419–431, 2011.

[39] M. A. Ibrahim, M. Kelleni, and A. Geddawy, "Nonalcoholic fatty liver disease: current and potential therapies," *Life Sciences*, vol. 92, no. 2, pp. 114–118, 2013.

[40] J. Ludwig, D. B. McGill, and K. D. Lindor, "Review: nonalcoholic steatohepatitis," *Journal of Gastroenterology and Hepatology*, vol. 12, no. 5, pp. 398–403, 1997.

[41] S. Morán-Ramos, A. Avila-Nava, A. R. Tovar, J. Pedraza-Chaverri, P. López-Romero, and N. Torres, "Opuntia ficus indica (nopal) attenuates hepatic steatosis and oxidative stress in obese Zucker (fa/fa) rats," *Journal of Nutrition*, vol. 142, no. 11, pp. 1956–1963, 2012.

[42] M. Carmiel-Haggai, A. I. Cederbaum, and N. Nieto, "A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats," *The FASEB Journal*, vol. 19, no. 1, pp. 136–138, 2005.

[43] P. Sancho, P. Martín-Sanz, and I. Fabregat, "Reciprocal regulation of NADPH oxidases and the cyclooxygenase-2 pathway," *Free Radical Biology and Medicine*, vol. 51, no. 9, pp. 1789–1798, 2011.

[44] C. Savard, E. V. Tartaglione, R. Kuver et al., "Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis," *Hepatology*, vol. 57, no. 1, pp. 81–92, 2013.

[45] Y. H. Paik and D. A. Brenner, "NADPH oxidase mediated oxidative stress in hepatic fibrogenesis," *The Korean Journal of Hepatology*, vol. 17, no. 4, pp. 251–257, 2011.

[46] J. L. Evans, I. D. Goldfine, B. A. Maddux, and G. M. Grodsky, "Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes," *Endocrine Reviews*, vol. 23, no. 5, pp. 599–622, 2002.

[47] W. Zhu, Q. Jia, Y. Wang, Y. Zhang, and M. Xia, "The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: involvement of a cAMP-PKA-dependent signaling pathway," *Free Radical Biology and Medicine*, vol. 52, no. 2, pp. 314–327, 2012.

[48] I. Levin, J. Petrasek, and G. Szabo, "The presence of p47phox in liver parenchymal cells is a key mediator in the pathogenesis of alcoholic liver steatosis," *Alcoholism: Clinical and Experimental Research*, vol. 36, no. 8, pp. 1397–1406, 2012.

[49] A. dela Peña, I. A. Leclercq, J. Williams, and G. C. Farrell, "NADPH oxidase is not an essential mediator of oxidative stress or liver injury in murine MCD diet-induced steatohepatitis," *Journal of Hepatology*, vol. 46, no. 2, pp. 304–312, 2007.

[50] J. X. Jiang, S. Venugopal, N. Serizawa et al., "Reduced nicotinamide adenine dinucleotide phosphate oxidase 2 plays a key role in stellate cell activation and liver fibrogenesis in Vivo," *Gastroenterology*, vol. 139, no. 4, pp. 1375–1384, 2010.

[51] N. S. R. De Mochel, S. Seronello, S. H. Wang et al., "Hepatocyte NAD(P)H oxidases as an endogenous source of reactive oxygen species during hepatitis C virus infection," *Hepatology*, vol. 52, no. 1, pp. 47–59, 2010.

[52] E. Albano, "Alcohol, oxidative stress and free radical damage," *Proceedings of the Nutrition Society*, vol. 65, no. 3, pp. 278–290, 2006.

[53] S.-J. Lee, S.-Y. Kim, and H. Min, "Effects of vitamin C and E supplementation on oxidative stress and liver toxicity in rats fed a low-fat ethanol diet," *Nutrition Research and Practice*, vol. 7, no. 2, pp. 109–114, 2013.

[54] G. L. Russo, M. Russo, and P. Ungaro, "AMP-activated protein kinase: a target for old drugs against diabetes and cancer," *Biochemical Pharmacology*, vol. 86, no. 3, pp. 339–350, 2013.

[55] C. J. Bailey and R. C. Turner, "Metformin," *The New England Journal of Medicine*, vol. 334, no. 9, pp. 574–579, 1996.

[56] A. Nerstedt, A. Johansson, C. X. Andersson, E. Cansby, U. Smith, and M. Mahlapuu, "AMP-activated protein kinase inhibits IL-6-stimulated inflammatory response in human liver cells by suppressing phosphorylation of signal transducer and activator of transcription 3 (STAT3)," *Diabetologia*, vol. 53, no. 11, pp. 2406–2416, 2010.

[57] C. S. Park, B.-R. Bang, H.-S. Kwon et al., "Metformin reduces airway inflammation and remodeling via activation of AMP-activated protein kinase," *Biochemical Pharmacology*, vol. 84, no. 12, pp. 1660–1670, 2012.

[58] Y. Kato, N. Koide, T. Komatsu et al., "Metformin attenuates production of nitric oxide in response to lipopolysaccharide by inhibiting MyD88-independent pathway," *Hormone and Metabolic Research*, vol. 42, no. 9, pp. 632–636, 2010.

[59] A. A. Alhaider, H. M. Korashy, M. M. Sayed-Ahmed, M. Mobark, H. Kfoury, and M. A. Mansour, "Metformin attenuates streptozotocin-induced diabetic nephropathy in rats through modulation of oxidative stress genes expression," *Chemico-Biological Interactions*, vol. 192, no. 3, pp. 233–242, 2011.

[60] X. Hou, J. Song, X.-N. Li et al., "Metformin reduces intracellular reactive oxygen species levels by upregulating expression of the antioxidant thioredoxin via the AMPK-FOXO3 pathway," *Biochemical and Biophysical Research Communications*, vol. 396, no. 2, pp. 199–205, 2010.

[61] I. Onaran, G. S. Guven, S. B. Ozdaş, G. Kanigur, and S. Vehid, "Metformin does not prevent DNA damage in lymphocytes despite its antioxidant properties against cumene hydroperoxide-induced oxidative stress," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 611, no. 1-2, pp. 1–8, 2006.

[62] H. Yuan, L. Li, W. Zheng et al., "Antidiabetic drug metformin alleviates endotoxin-induced fulminant liver injury in mice," *International Immunopharmacology*, vol. 12, no. 4, pp. 682–688, 2012.

[63] J. Dai, M. Liu, Q. Ai et al., "Involvement of catalase in the protective benefits of metformin in mice with oxidative liver injury," *Chemico-Biological Interactions*, vol. 216, no. 1, pp. 34–42, 2014.

[64] I.-C. Lee, S.-H. Kim, H.-S. Baek et al., "The involvement of Nrf2 in the protective effects of diallyl disulfide on carbon tetrachloride-induced hepatic oxidative damage and inflammatory response in rats," *Food and Chemical Toxicology*, vol. 63, pp. 174–185, 2014.

[65] J. Lamlé, S. Marhenke, J. Borlak et al., "Nuclear factor-erythroid2-related factor 2 prevents alcohol-induced fulminant liver injury," *Gastroenterology*, vol. 134, no. 4, pp. 1159–1168, 2008.

[66] S. A. Reisman, I. I. Csanaky, L. M. Aleksunes, and C. D. Klaassen, "Altered disposition of acetaminophen in Nrf2-null and keap1-knockdown mice," *Toxicological Sciences*, vol. 109, no. 1, pp. 31–40, 2009.

[67] N. R. Kitteringham, A. Abdullah, J. Walsh et al., "Proteomic analysis of Nrf2 deficient transgenic mice reveals cellular defence and lipid metabolism as primary Nrf2-dependent pathways in the liver," *Journal of Proteomics*, vol. 73, no. 8, pp. 1612–1631, 2010.

[68] D. Yan, J. Dong, K. K. Sulik, and S.-Y. Chen, "Induction of the Nrf2-driven antioxidant response by tert-butylhydroquinone prevents ethanol-induced apoptosis in cranial neural crest cells," *Biochemical Pharmacology*, vol. 80, no. 1, pp. 144–149, 2010.

[69] M. P. Valdecantos, P. L. Prieto-Hontoria, V. Pardo et al., "Essential role of Nrf2 in the protective effect of lipoic acid against lipoapoptosis in hepatocytes," *Free Radical Biology and Medicine*, vol. 84, pp. 263–278, 2015.

[70] N. C. Bozkurt, E. Cakal, M. Sahin, E. C. Ozkaya, H. Firat, and T. Delibasi, "The relation of serum 25-hydroxyvitamin-D levels with severity of obstructive sleep apnea and glucose metabolism abnormalities," *Endocrine*, vol. 41, no. 3, pp. 518–525, 2012.

[71] N. Sharifi, R. Amani, E. Hajiani, and B. Cheraghian, "Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial," *Endocrine*, vol. 47, no. 1, pp. 70–80, 2014.

[72] C. Bunchorntavakul, T. Wootthananont, and A. Atsawarungruanqkit, "Effects of vitamin E on chronic hepatitis C genotype 3: a randomized, double-blind, placebo-controlled study," *Journal of the Medical Association of Thailand*, vol. 97, supplement 11, pp. S31–S40, 2014.

[73] A. Reuben, "Hy's law," *Hepatology*, vol. 39, no. 2, pp. 574–578, 2004.

[74] L. E. Johansson, U. Lindblad, C. A. Larsson, L. Råstam, and M. Ridderstråle, "Polymorphisms in the adiponutrin gene are associated with increased insulin secretion and obesity," *European Journal of Endocrinology*, vol. 159, no. 5, pp. 577–583, 2008.

[75] C. J. Weston, E. L. Shepherd, L. C. Claridge et al., "Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis," *Journal of Clinical Investigation*, vol. 125, no. 2, pp. 501–520, 2015.

[76] I. C. M. E. de Abreu, J. F. D. C. Guerra, R. R. Pereira et al., "Hypercholesterolemic diet induces hepatic steatosis and alterations in mRNA expression of NADPH oxidase in rat livers," *Arquivos Brasileiros de Endocrinologia & Metabologia*, vol. 58, no. 3, pp. 251–259, 2014.

[77] E. Grasselli, A. D. Compalati, A. Voci et al., "Altered oxidative stress/antioxidant status in blood of alcoholic subjects is associated with alcoholic liver disease," *Drug and Alcohol Dependence*, vol. 143, no. 1, pp. 112–119, 2014.

[78] J. Luo, L. Xu, J. Li, and S. Zhao, "Nonalcoholic fatty liver disease as a potential risk factor of cardiovascular disease," *European Journal of Gastroenterology and Hepatology*, vol. 27, no. 3, pp. 193–199, 2015.

[79] S. J. Curtis, M. Mortiz, and P. J. Snodgrass, "Serum enzymes derived from liver cell fractions. I. The response to carbon Liver flow/mass controls enzyme release 231 tetrachloride intoxication in rats," *Gastroenterology*, vol. 62, pp. 84–92, 1972.

[80] H. J. Zimmerman, Y. Kodera, and M. West, "Rate of increase in plasma levels of cytoplasmic and mitochondrial enzymes in experimental carbon tetrachloride hepatotoxicity," *The Journal of Laboratory and Clinical Medicine*, vol. 66, no. 2, pp. 315–323, 1965.

[81] B. D. Dinman and I. A. Bernstein, "Acute carbon tetrachloride hepatotoxicity. V. Enzymatic activity and structural concomitants during the regenerative phase," *Archives of Environmental Health*, vol. 16, no. 6, pp. 777–784, 1968.

[82] G. Ruggiero, "Rate of release of cytoplasmic and mitochondrial enzymes from the isolated and perfused rat liver treated with phalloidin," *The Journal of Laboratory and Clinical Medicine*, vol. 82, no. 5, pp. 695–703, 1973.

[83] J. Kanta and F. Bartos, "Relationship of liver damage and liver regeneration after carbon tetrachloride treatment in rats," *Sbornik Vedeckych Praci Lekarske Fakulty Karlovy University v Hradci Kralove*, vol. 34, no. 3, pp. 237–242, 1991.

[84] C. J. Fievet, M. P. Gigandet, and H. C. Ansel, "Hemolysis of erythrocytes by primary pharmacologic agents," *American Journal of Hospital Pharmacy*, vol. 28, no. 12, pp. 961–966, 1971.

[85] S. Miyake, "The mechanism of release of hepatic enzymes in various liver diseases. Altered activity ratios of GOT and GPT in serum and liver of patients with liver diseases," *Acta Medica Okayama*, vol. 33, pp. 343–358, 1979.

[86] W. G. Guder, A. Habicht, J. Kleissl, U. Schmidt, and O. H. Wieland, "The diagnostic significance of liver cell inhomogeneity: serum enzymes in patients with central liver necrosis and the distribution of glutamate dehydrogenase in normal human liver," *Zeitschrift fur Klinische Chemie und Klinische Biochemie*, vol. 13, no. 7, pp. 311–318, 1975.

[87] G. K. Michalopoulos and M. C. DeFrances, "Liver regeneration," *Science*, vol. 276, no. 5309, pp. 60–66, 1997.

[88] K. F. Aronsen, B. Ericsson, and B. Pihl, "Metabolic changes following major hepatic resection," *Annals of Surgery*, vol. 169, no. 1, pp. 102–110, 1969.

[89] S. Bengmark, L. Engevik, and R. Olsson, "Changes in ornithine carbamoyl transferase activity in serum and in liver after partial hepatectomy in rats," *Scandinavian Journal of Gastroenterology*, vol. 3, no. 3, pp. 264–266, 1968.

[90] O. Almersjö, S. Bengmark, L. O. Hafström, and R. Olsson, "Enzyme and function changes after extensive liver resection in man," *Annals of Surgery*, vol. 169, no. 1, pp. 111–119, 1969.

[91] Y. Panis, D. M. McMullan, and J. C. Emond, "Progressive necrosis after hepatectomy and the pathophysiology of liver failure after massive resection," *Surgery*, vol. 121, no. 2, pp. 142–149, 1997.

[92] J. Díaz-Juárez, L. Rivera-Valerdi, D. E. Bernal-Cerrillo, and R. Hernández-Muñoz, "Predominance of released mitochondrial enzymes by partial hepatectomy-induced rat regenerating liver is controlled by hemodynamic changes and not related to mitochondrial damage," *Scandinavian Journal of Gastroenterology*, vol. 41, no. 2, pp. 223–233, 2006.

[93] J. A. Morales-González, J. Gutiérrez-Salinas, L. Yánez, C. Villagómez-Rico, J. Badillo-Romero, and R. Hernández-Munoz, "Morphological and biochemical effects of a low ethanol dose on rat liver regeneration: role of route and timing of administration," *Digestive Diseases and Sciences*, vol. 44, no. 10, pp. 1963–1974, 1999.

[94] R. Hernández-Muñoz, L. Sánchez-Sevilla, A. Martínez-Gómez, and M. A. R. Dent, "Changes in mitochondrial adenine nucleotides and in permeability transition in two models of rat liver regeneration," *Hepatology*, vol. 37, no. 4, pp. 842–851, 2003.

[95] M. Chachisvilis, Y.-L. Zhang, and J. A. Frangos, "G protein-coupled receptors sense fluid shear stress in endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 42, pp. 15463–15468, 2006.

[96] C. R. White and J. A. Frangos, "The shear stress of it all: the cell membrane and mechanochemical transduction," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 362, no. 1484, pp. 1459–1467, 2007.

[97] N. Adamek, L. M. Coluccio, and M. A. Geeves, "Calcium sensitivity of the cross-bridge cycle of Myo1c, the adaptation motor in the inner ear," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 15, pp. 5710–5715, 2008.

[98] H. Rubin and T. Koide, "Mutual potentiation by magnesium and calcium of growth in animal cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 73, no. 1, pp. 168–172, 1976.

[99] J. F. Whitfield, A. L. Boynton, J. P. MacManus et al., "The roles of calcium and cyclic AMP in cell proliferation," *Annals of the New York Academy of Sciences*, vol. 339, pp. 216–240, 1980.

[100] M. Y. Pahakis, J. R. Kosky, R. O. Dull, and J. M. Tarbell, "The role of endothelial glycocalyx components in mechanotransduction of fluid shear stress," *Biochemical and Biophysical Research Communications*, vol. 355, no. 1, pp. 228–233, 2007.

[101] J. Díaz-Juárez and R. Hernández-Muñoz, "The role of calcium and nitric oxide during liver enzyme release induced by increased physical forces as evidenced in partially hepatectomized rats," *Liver Transplantation*, vol. 17, no. 3, pp. 334–343, 2011.

[102] N. Fausto and E. M. Webber, "Control of liver growth," *Critical Reviews in Eukaryotic Gene Expression*, vol. 3, no. 2, pp. 117–135, 1993.

[103] I. Aguilar-Delfín, F. López-Barrera, and R. Hernández-Muñoz, "Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: partial hepatectomy and acute CCl_4 administration," *Hepatology*, vol. 24, no. 3, pp. 657–662, 1996.

[104] M. Greco, L. Moro, G. Pellecchia, S. Di Pede, and F. Guerrieri, "Release of matrix proteins from mitochondria to cytosol during the prereplicative phase of liver regeneration," *FEBS Letters*, vol. 427, no. 2, pp. 179–182, 1998.

[105] I. Oikawa and P. M. Novikoff, "Catalase-negative peroxisomes: transient appearance in rat hepatocytes during liver regeneration after partial hepatectomy," *American Journal of Pathology*, vol. 146, no. 3, pp. 673–687, 1995.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8327410, 15 pages
http://dx.doi.org/10.1155/2016/8327410

Review Article

Oxidative Stress in the Healthy and Wounded Hepatocyte: A Cellular Organelles Perspective

Tommaso Mello,[1,2] Francesca Zanieri,[1] Elisabetta Ceni,[1] and Andrea Galli[1,2]

[1]*Department of Biomedical Clinical and Experimental Sciences "Mario Serio", University of Florence, 50134 Florence, Italy*
[2]*Careggi University Hospital, 50134 Florence, Italy*

Correspondence should be addressed to Tommaso Mello; tommaso.mello@unifi.it

Received 25 July 2015; Accepted 10 September 2015

Academic Editor: Pablo Muriel

Copyright © 2016 Tommaso Mello et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Accurate control of the cell redox state is mandatory for maintaining the structural integrity and physiological functions. This control is achieved both by a fine-tuned balance between prooxidant and anti-oxidant molecules and by spatial and temporal confinement of the oxidative species. The diverse cellular compartments each, although structurally and functionally related, actively maintain their own redox balance, which is necessary to fulfill specialized tasks. Many fundamental cellular processes such as insulin signaling, cell proliferation and differentiation and cell migration and adhesion, rely on localized changes in the redox state of signal transducers, which is mainly mediated by hydrogen peroxide (H_2O_2). Therefore, oxidative stress can also occur long before direct structural damage to cellular components, by disruption of the redox circuits that regulate the cellular organelles homeostasis. The hepatocyte is a systemic hub integrating the whole body metabolic demand, iron homeostasis and detoxification processes, all of which are redox-regulated processes. Imbalance of the hepatocyte's organelles redox homeostasis underlies virtually any liver disease and is a field of intense research activity. This review recapitulates the evolving concept of oxidative stress in the diverse cellular compartments, highlighting the principle mechanisms of oxidative stress occurring in the healthy and wounded hepatocyte.

1. Introduction

1.1. Redox Homeostasis and Oxidative Stress. Accurate control of the cell redox state, which is mandatory for maintaining the structural integrity and physiological functions, is achieved both by a fine-tuned balance between prooxidant and anti-oxidant molecules and by spatial and temporal confinement of the oxidative species. This tight regulation is mainly achieved by controlling the steady-state production and the subcellular compartmentalization of reactive oxygen (ROS) and reactive nitrogen species (RNS), prooxidant enzymes such as NADH/NAPDH oxidases (NOX) and glutathione peroxidases (Gpx) and that of several antioxidant systems such as reduced/oxidized glutathione (GSH/GSSG), reduced/oxidized cysteine (Cys/CySS), thioredoxin (Trx), peroxiredoxin (Prx), superoxide dismutase (SOD), and catalase.

While it has long been recognized that an imbalance between pro- and anti-oxidants is harmful to cells and is a central mechanism in the development of several pathologies including neurodegeneration, atherosclerosis, diabetes, cancer, and aging, the importance of ROS as second messengers in the cell physiology is a relatively recent acquisition. Indeed, many fundamental cellular processes such as insulin signaling, cell proliferation and differentiation, and cell migration and adhesion, just to name a few, rely on localized changes in the redox state of signal transducers mainly mediated by hydrogen peroxide (H_2O_2) [1].

The widespread notion of oxidative stress is that an excessive production of prooxidants or exhaustion of the cellular anti-oxidant defenses can lead to oxidative damage to proteins, nucleic acids, carbohydrates, and lipids, in which radical ROS or RNS are generally thought to play a major role. However, since the activities of many proteins involved in the cellular signaling are regulated by the redox state of their oxidizable thiol residues, which act as redox-sensitive molecular-switches [2], oxidative stress can also occur in the absence of direct structural damage by disruption of

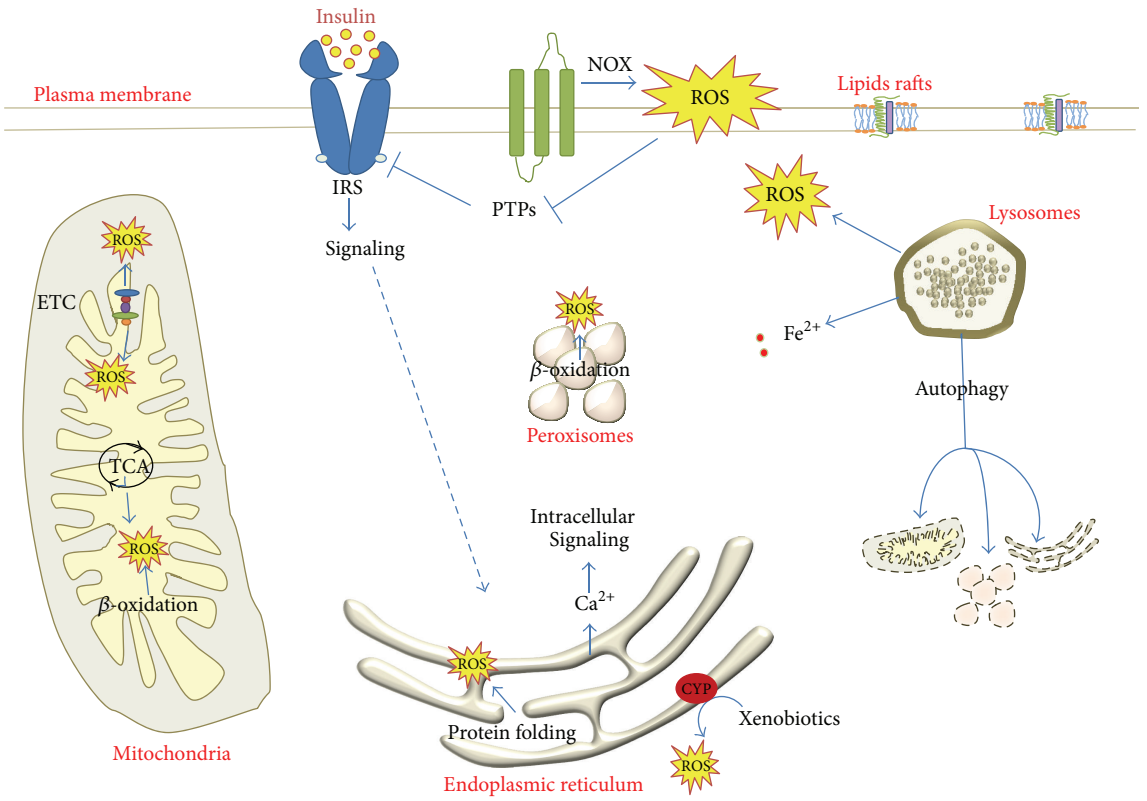


Figure 1: Sites of physiologically produced ROS. Plasma membrane localized ROS bursts deactivate PTPs and allow signal transduction (i.e., by insulin or IGF-1) after tyrosine kinase receptor activation. Mitochondria produce ROS during cellular respiration and metabolic activity. ROS are produced in the ER during protein folding and detoxification by the cytochrome P450 systems. Lysosomes are required for iron metabolism and the removal of damaged cellular components through autophagy. Peroxisomes produce ROS during metabolic or detoxification activities.

the redox circuits that regulate many signaling pathways [3]. Among ROS, hydrogen peroxide is supposed to play a major role either directly or indirectly, in the regulation of the thiol/disulphide redox switches [4], because (i) these reactions typically require a two-electron transfer, (ii) H_2O_2 is kinetically restricted and thus can be highly selective in substrate oxidation, and (iii) H_2O_2 is generated following growth factor, cytokine, or hormone signaling. However, the detailed molecular mechanisms leading to selective thiol oxidation in redox-sensitive proteins by H_2O_2 are still mostly obscure and are the focus of intense research activity. A growing body of data suggests that altered redox signaling precedes and contributes substantially more than direct radical damage to the development of several human pathologies.

The concept of "oxidative stress," introduced 30 years ago [5], evolved over time from the original oxidative damage to the cell structure and subsequent stress response to include that of alteration of signaling pathways, redox homeostasis, and redox adaptation to stress [6, 7].

Consequently, oxidative stress is not necessarily harmful and antioxidants are not utterly beneficial. In fact, many clinical trials failed to prove the efficacy of low-molecular weight antioxidants in the treatment of several pathologies, and the use of the antioxidants selenium, beta-carotene, and vitamin E was even found to increase overall mortality in a large meta-analysis [8].

Our understanding of the redox landscape of the cell is rapidly evolving and thanks to the recent development of specific redox probes [9–12] we are beginning to unravel a complex spatial and temporal organization of the redox fluxes in the living cells. Compartmentalization of the redox circuitry is crucial to maintain physiology and is a key to understand the alterations of the redox homeostasis occurring in disease.

The liver is the main metabolic organ and plays a fundamental role in whole body detoxification and blood stream filtering. Most detoxification processes (drugs, alcohol, and endo- and xenobiotics) are carried out through oxidative reactions by the cytochrome P450 (CYP) isoenzymes, which generate superoxide anion ($O_2^{\bullet -}$) (Figure 1). Derangement of the liver metabolic processes, such as those occurring by fatty acids overload in NAFLD, results in increased ROS production by increased electron transfer during mitochondrial β-oxidation, as well as increased CYP2E1 expression and activity [13]. Strong induction of CYP2E1 also occurs due to excessive consumption of ethanol, whose toxic metabolite acetaldehyde (AcCHO) generates oxidative stress through a number of direct and indirect mechanisms [6, 14,

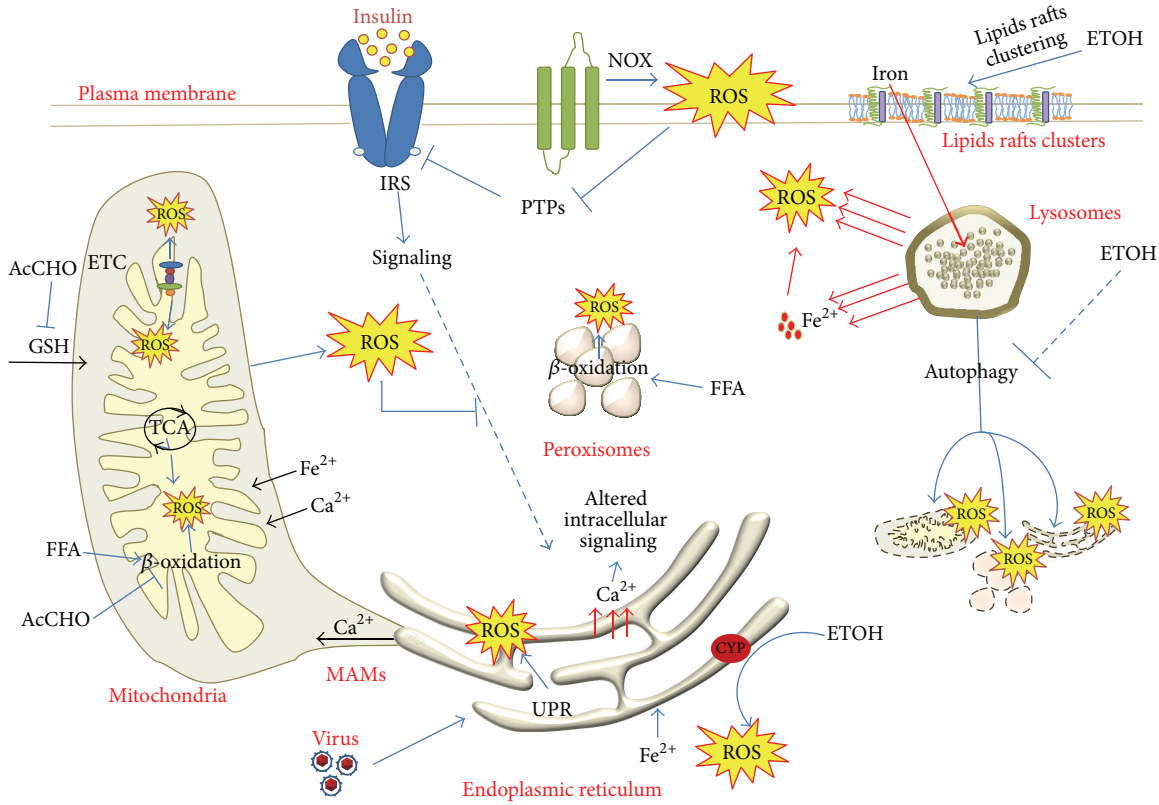


FIGURE 2: Mechanisms of enhanced ROS production during hepatocyte damage. Ethanol metabolism promotes strong ROS production in the ER by the inducible CYP. It impairs GSH import in the mitochondria, preventing ROS removal. It also impairs β-oxidation promoting lipid accumulation. ETOH induces lipid-raft clustering and increases iron uptake, promoting Fe^{2+} leakage from lysosomes and increased Fe^{2+} loads in mitochondria and ER, resulting in ROS production. Ethanol also reduced the autophagic removal of damaged cellular components. Viral infection challenges the ER protein folding process leading to ROS production and Ca^{2+} leakage in the cytosol and mitochondria. Increased MAMs formation promotes Ca^{2+} efflux from ER into mitochondria, increasing mitochondrial ROS production.

15] (Figure 2). Hepatotropic viruses cause direct oxidative damage to the endoplasmic reticulum (ER) of hepatocytes, triggering an inflammatory response which propagates the oxidative stress-induced damage to neighboring cells. Activation of the inflammatory signaling pathways in the hepatocyte (i.e., due to translocation of bacterial toxins from the gut or inflammatory cytokines release from visceral fat in obese subjects) is a trait d'union molecular mechanism that synergistically propagates the oxidative stress within different cellular compartments, regardless of the initiating agent.

The liver also plays a central role in heme catabolism and iron recycling, which poses an additional threat since iron catalyzes the formation of ROS through the Fenton and Haber-Weiss reactions (see Section 1.2). Iron accumulation in the liver is associated with increased oxidative stress and can occur as a consequence of genetic disorders (as in hemochromatosis) but also secondarily to other liver diseases, such as NAFLD [16–18] or HCV infection [19, 20], significantly contributing to the progression of disease.

Therefore, the liver has an enormous potential for the generation of oxidative species, and virtually any noxia targeting the liver results in elevated oxidative stress that, when chronic, promotes the fibroproliferative response and progression through fibrosis, cirrhosis, and eventually hepatocellular carcinoma. The onset and progression of chronic liver disease require a complex interplay among different cellular components of the liver, hepatocytes, cholangiocytes, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells, mostly orchestrated through a proinflammatory and profibrogenic crosstalk in which oxidative stress mediators such as H_2O_2 or nitric oxide ($^{\bullet}NO$) are active players.

As we become more and more aware of the complexity of the redox signaling underlying crucial metabolic regulations, cell fate decision mechanisms, and intercellular communication, it is easy to foresee that the "redox hepatology" field will shape the liver biology research in the next future.

This review recapitulates the evolving concept of oxidative stress in diverse cellular compartments, highlighting the principle mechanisms of oxidative stress occurring in the healthy and wounded hepatocyte.

1.2. Mechanism of Reactive Oxygen and Nitrogen Species (RONS) Mediated Toxicity. Several ROS ($O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2) and RNS ($^{\bullet}NO$, $ONOO^-$) are generated inside the cells under physiological and pathological conditions. The biological activity of RONS toward cellular substrate is not

equivalent: the hydroxyl radical ($^{\bullet}OH$) has an indiscriminate reactivity toward most biological substrates and is the most relevant ROS involved in oxidative DNA damage while $O_2^{\bullet-}$, the most abundant mitochondrial ROS, preferentially reacts with iron-sulfur clusters in target proteins and is effectively converted to H_2O_2, which is the principal oxidant of low pKa cysteine residues (Cys) acting as sulfur switches in redox-sensitive proteins [21].

Nitric oxide is a highly diffusible signaling molecule generated by Nitric Oxide Synthases (eNOS, iNOS, and mNOS) in the cytoplasm, extracellular space, and possibly mitochondria [22] and can react with redox-sensitive cysteine residues in proteins forming nitrosothiols, a mechanism of redox sensing analog to hydrogen peroxide (H_2O_2) [23]. $^{\bullet}NO$ does not appear to be toxic at physiological concentrations [24] but can readily react with superoxide anion and generate peroxynitrite ($ONOO^-$), a spontaneous reaction occurring at such a fast rate that outperforms SOD capability of removing $O_2^{\bullet-}$. Peroxynitrite is therefore formed whenever $^{\bullet}NO$ and $O_2^{\bullet-}$ are produced simultaneously [25]; it is highly reactive toward iron-sulfur clusters (present in several metabolic enzymes like mitochondrial aconitase and alcohol dehydrogenase), can oxidize protein thiols, and promote tyrosine nitration in target proteins (i.e., Complexes I, II, III, and V of the mitochondrial Electron Transport Chain (ETC)), thereby impairing both the redox- and phosphorylation-dependent cellular signaling. A major mechanism of peroxynitrite toxicity is mediated by lipid peroxidation that causes the degradation of membranes through radical reactions, leading to changes in membrane permeability and fluidity. Finally, oxidative damage to DNA is a hallmark of high-level oxidative stress not only in the nuclei but also in mitochondria [25].

Moreover, while highly reactive radical ROS and RNS are generally diffusion-limited, some ROS and RNS can easily diffuse through biological membranes thanks to their non-/low-polar nature (H_2O_2, $^{\bullet}NO$, and peroxynitrous acid ONOOH) and to dedicated transporter such as aquaporins (for H_2O_2) or the HCO_3^-/Cl^- anion exchanger (for $ONOO^-$). The pKa for the couple ONOO/ONOOH is 6.8, very close to physiological pH, thus implying that both mechanisms of peroxynitrite diffusion are relevant in vivo [26]. Therefore, depending on the given RONS involved, oxidative stress can elicit localized alteration of the redox state, localized structural damage, or spread among different cellular compartments and neighboring cells. An elucidating mechanism of such event is the ROS-Induced ROS Release (RIRR) [27], that is, the temporarily opening of mitochondria permeability transition pore (mPTP) that elicit an amplified ROS production after an oxidative challenge. ROS released during RIRR may spread to neighboring mitochondria and, depending on the level of ROS release, either promote mitophagy and removal of nonfunctional mitochondria or trigger a ROS avalanche that can lead to cell death. Of importance, RIRR is considered to be the main mechanism of hepatocyte damage during ischemia/reperfusion injury that occurs following hepatic surgery or transplantation.

Iron overload constitutes a source of oxidative stress of particular relevance in the liver, since hepatocytes and Kupffer cells are the main cell type devoted to iron storage in the body. Iron is an essential component of oxygen sensing proteins, oxygen transport systems, and iron-sulfur containing enzymes [28]; it is a transition metal readily converted between the reduced ferrous (Fe^{2+}) and the oxidized ferric (Fe^{3+}) forms. The majority of iron in biological complexes is kept as Fe^{3+}, while iron reduction to Fe^{2+} is crucial for its mobilization and transport through membranes, loading on ferritin and heme synthesis [28].

In the hepatocyte, iron is stored in the cytoplasm, ER, mitochondria, and lysosomes largely as ferritin-bound Fe^{3+}. About 0.2%–5% of the total cellular iron is considered as intracellular transiently mobile "labile pool," either "free" iron or loosely bound "chelatable" iron, both mainly in the form of redox-active Fe^{2+} [29]. The "labile pool" iron is potentially toxic, since it can catalyze the formation of dangerous $^{\bullet}OH$ radical through the Fenton (1) and Haber-Weiss (3) reactions:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (1)$$

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2 \quad (2)$$

$$\text{The net reaction: } O_2^{\bullet-} + H_2O_2 \longrightarrow OH^- + O_2 + {}^{\bullet}OH \quad (3)$$

Therefore, leakage of Fe^{2+} from the lysosome due to altered membrane permeability, as well as reduction of Fe^{3+} by superoxide (2), can catalyze the production of ROS and promote lipid peroxidation and severe cellular damage (Figure 2). Mitochondria are particularly susceptible to iron-mediated oxidative stress due to the high production rate of $O_2^{\bullet-}$ and its dismutation product H_2O_2 during cellular respiration in close proximity to several Fe-S containing enzymes [30].

2. Mitochondria

Among cellular organelles, mitochondria account for the largest amount of electron transfer to oxygen thanks to the electron transfer chain (ETC) complexes I–V. ETC complex I (NADH ubiquinone oxidoreductase) and complex II (ubiquinone cytochrome c oxidoreductase), as well as other mitochondrial enzymes such as α-ketoglutarate dehydrogenase, pyruvate phosphate dehydrogenase, fatty acyl CoA dehydrogenase, and glycerol 3-phosphate dehydrogenase [31], can produce $O_2^{\bullet-}$ as byproduct [32], releasing it within the mitochondrial matrix. Moreover, H_2O_2 is produced by the monoamine oxidases (MAOs) located in the outer mitochondrial membrane [33] (Figure 1). Therefore, mitochondria are often referred to as a major ROS production site, although in fact whether high ROS leakage occurs in the mitochondria, at least in a physiological setting, is still highly debated [34, 35]. Estimates of H_2O_2 production, as a measure of $O_2^{\bullet-}$ leakage, vary from 2% [36] to 0.1-0.2% [31, 32, 34] of total O_2 consumption and vary largely depending on tissue origin, experimental settings, and the specific substrate fed to the mitochondria. For liver

mitochondria, the rate of ROS leakage could be even lower than 0.1% [32]. In fact, since mitochondria are physiologically prone to produce high ROS levels due to the oxidative phosphorylation process, they are also well equipped with a large array of antioxidant systems and radical scavengers, such as Mn-Superoxide Dismutase (Mn-SOD), CuZn-SOD, GSH, glutathione peroxidase, tioredoxin-2, peroxiredoxins, glutaredoxins, and also catalase [37]. Mn-SOD (SOD2) in the mitochondrial matrix readily catalyzes the dismutation of $O_2^{\bullet -}$ to H_2O_2, which in turn is eliminated by glutathione peroxidase using reduced glutathione (GSH) as hydrogen donor. Oxidized glutathione (GSSG) is then reduced by NADPH-dependent glutathione reductase. Superoxide released in the intermembrane space by the ETC complex III is scavenged by CuZn-SOD (SOD1), followed again by GPx and GSH to eliminate H_2O_2. Since GSH is only synthetized in the cytosol [38] and the mitochondrial pool of GSH (mGSH) is replenished by importing GSH produced in the cytoplasm [39], the GSH/GSSG redox state inside the mitochondria is heavily controlled by GSH import through the 2-oxoglutarate carrier and the dicarboxylate carrier [40–43].

Two major enzymatic antioxidant systems collaborate in the mitochondrial matrix: the GSH-dependent glutathione peroxidase and the NADPH-dependent thioredoxin-2 systems, each with specific cofactors.

It must be noted that although abundant, GSH has very limited spontaneous antioxidant activity but very high affinity for GPx. Within the mitochondria, GPx1 [44, 45] and Gpx4 are the most abundant with GPx1 representing over one-third of total GPx activity in the liver [46]. Gpx1 is the major isoform localized both in the mitochondrial matrix and in the intermembrane space and is mainly devoted to H_2O_2 detoxification, while GPx4 preferentially reduces lipid peroxides thereby preventing membrane damage to mitochondria [47].

Nevertheless, a number of molecular mechanisms promote mitochondrial ROS overproduction or decreased antioxidant defense under nonphysiological condition [48]. The alteration of the redox homeostasis of mitochondria is well documented in several human pathologies such as NAFLD, viral infection, and toxic events (Figure 2).

Chronic alcohol feeding depletes the mGSH in several animal models [49–51], leading to enhanced ROS production and mitochondrial damage. The mechanism underlying mGSH depletion involves cholesterol accumulation in the inner mitochondrial membrane that results in excess membrane rigidity and impaired GSH carriers functionality, thus disrupting GSH import from the cytosol (Figure 2). In fact, restoring the membrane fluidity, but not increasing cytoplasmic levels of GSH by N-acetylcysteine administration (NAC), recovers mGSH pool and ameliorates liver damage in alcohol-fed rats [45, 50]. The importance of GSH import in the mitochondria can be appreciated considering that several antioxidant systems depend upon mGSH and that the mitochondrial GSH/GSSG redox state is even maintained in a more reduced steady-state redox potential than in the cytoplasm [52], thus requiring energy expenditure for GSH import.

The absence of protective histones, incomplete DNA repair mechanisms, and the close proximity to ROS production site renders mitochondrial DNA (mDNA) sensitive to oxidative damage, increasing the risk of double-strand breaks and somatic mutations with increased ROS production [53]. Indeed, a single dose of alcohol proved effective in inducing massive mitochondrial DNA degradation through a ROS-dependent pathway [54]. The acute degradation of mDNA is then followed by an overshoot of mDNA synthesis as a compensatory mechanism. However, repeated administration of alcohol (binge drinking) accumulated DNA damage and blocked the adaptive response of mDNA resynthesis, resulting in prolonged hepatic mDNA depletion [55]. mDNA encodes 13 proteins involved in the ETC, two rRNA, and all the tRNA necessary for translating the 13 encoded proteins. Mutations in the mDNA therefore may produce dysfunctional ETC complexes, increase ROS production, and expose the mitochondria to new damage in a vicious circle [53]. Indeed, mitochondrial DNA depletion and mutation have been described in patients with alcoholic and nonalcoholic steatohepatitis [56, 57].

Acetaldehyde (AcCHO) produced by ethanol metabolism is readily detoxified by aldehyde dehydrogenase 1 (ALDH1) in the cytosol and by ALDH2 in the mitochondria. Acetaldehyde oxidation to acetate generates NADH and reduces the NAD^+/NADH ratio, possibly impairing mitochondrial β-oxidation which requires NAD^+ (Figure 2).

Chronic alcohol administration reduces ALDH activity therefore promoting AcCHO accumulation and inducing adduct formation with lipids, proteins, and mDNA [58, 59].

Failure to efficiently remove AcCHO exposes mitochondria to protein, lipid, and DNA adduct formation such as MDA, 4-NHE, and mixed MAA adducts [15]. Moreover, 4-HNE, a lipid peroxidation derivative, can directly inhibit ALDH2, thus promoting AcCHO accumulation in the mitochondrion in a endangering loop [60].

Consistently, the inactivating polymorphism ALDH2*2, common in East Asia, confers reduced alcohol tolerance and is associated with increased risk of gastrointestinal cancer. Very recently, by the use of a knock-in mice harboring the ALDH2 (E487K) mutation, Jin and colleagues recapitulated the ALDH2*2 human phenotype including intolerance to acute or chronic alcohol administration, impaired clearance of AcCHO, increased DNA damage, and susceptibility to cancer development [61].

Many of the abovementioned findings also apply to the mitochondria of NASH patients, which have altered morphology [62, 63], reduced or mutated mDNA content [57], reduced oxidative phosphorylation [64], and increased ROS production.

However, the molecular mechanisms initiating the mitochondrial dysfunction in NASH are different and originate by an overwhelming induction of mitochondrial β-oxidation rather than its inhibition as in ASH. This is consistent with the increased expression of UCP-2 observed in the mitochondria of several obesity and NASH animal models and in the expansion of peroxisomal β-oxidation found in humans. The increased electron flux through the ETC produces oxidative

stress, which is strongly associated with the severity of NASH (Figure 2).

Depletion of mGSH occurs in NASH animal models, similar to ASH [65], and in NASH patients that have reduced levels of GSH, SOD, and catalase and increased protein oxidation, a hallmark of increased oxidative stress [66]. In principle, targeting oxidative stress is potential therapeutic option for oxidative liver diseases [67]. Of notice, mGSH depletion can affect also the outcome of potential therapeutic antioxidant treatments, such as the use of SOD mimetics in steatohepatitis. Indeed, the use of SOD2 mimetics in a context of mGSH depletion results in increased H_2O_2 levels and increases liver injury in animal models of steatohepatitis, highlighting the importance of a combinatory strategy in the targeting of oxidative stress mechanisms [68].

3. Endoplasmic Reticulum

Endoplasmic reticulum (ER) is a master intracellular organelle responsible for protein synthesis, folding, modification, and trafficking. In addition, the ER plays a crucial role in calcium homeostasis and in regulating the biosynthesis of steroids, lipids, and carbohydrates [69].

During the folding process, a protein may be oxidized to form disulfide bonds, isomerized to allow polypeptide rearrangement or reduced to allow unfolding and subsequent degradation [70].

The ER lumen has a high ratio of oxidized to reduced glutathione (GSSG/GSH) (145), which creates an oxidizing environment that promotes disulfide bond formation. The electron transport required for this process is driven by a protein pathway that involves two ER-located enzymes: protein disulphide isomerase (PDI) and ER oxidoreductin 1 (ERO1) [71]. PDI directly accepts electrons, leading to the oxidation of cysteine residues and the formation of disulphide bonds. In turn, ERO1 oxidizes PDI through a flavin-dependent reaction and transfers electrons to molecular oxygen as final acceptor. The use of molecular oxygen as the terminal electron recipient leads to the production of ROS, mainly hydrogen peroxide, contributing to cellular oxidative stress [72].

It has been estimated that about 25% of the ROS generated in a cell derive from ER disulfide bond formation during oxidative protein folding, thus making ER the major site of ROS production [73] (Figure 1).

Furthermore, additional oxidative stress can result from the depletion of reduced glutathione that is consumed during the reduction of unstable and improperly formed disulphide bonds [74]. Therefore, an increase in the protein-folding load in the ER can lead to the accumulation of ROS [75]. Cells have evolved several strategies to oppose the ER accumulation of unfolded and misfolded proteins, which are collectively referred to as the UPR (unfolded protein response).

Under normal physiological conditions, the unfolded or misfolded proteins are directed to degradative pathways to restore the ER homeostasis; however, if the unfolded protein production overwhelm the ER buffering capacity, the UPR can activate a cascade of intracellular events resulting in cell death [76, 77]. The UPR is of major importance in hepatocytes, which are rich in ER content and responsible for the synthesis of proteins, cholesterol, bile acids, and phospholipids [78]. And it is characterized by the activation of three distinct signal transduction pathways: the inositol requiring 1 (IRE1) pathway, the protein kinase RNA-like ER kinase (PERK) pathway, and the activating transcription factor 6 (ATF6) pathway. Under nonstressed condition, these three proteins are kept inactive by binding to a chaperone protein, BiP/GRP78, which is the master regulator of the UPR. Under stressed condition (due to, for example, accumulation of misfolded or unfolded proteins, depletion of ER calcium content, or increase of free cholesterol in the ER lumen) BiP/GRP78 dissociates from the UPR transducers resulting in activation of their respective signaling pathways.

Briefly, the activated IRE1α removes a 26-bp intron from the XBP1 mRNA, resulting in the production of a spliced XBP1 protein (XBP1s). XBP1s is a transcription factor that regulates the expression of several genes involved in UPR and ER-assisted degradation (ERAD) to help restore ER homeostasis [79]. The IRE1α/Xbp1 pathway is also critical for hepatic lipid homeostasis, since it activates the transcription of master adipogenic regulators such as PPARγ and C/EBPs [80]. In addition, IRE1α induces the activation of stress kinases, JNK and p38 MAPK, that promote apoptosis [81].

The PERK pathway activates an antioxidant program focused on ATF4 and nuclear factor-erythroid-derived 2-(NF-E2-) related factor 2 (NRF2) [82, 83]. NRF2 is a key player in antioxidant response. After PERK-mediated phosphorylation, NRF2 translocates to the nucleus and activates the transcription of a set of antioxidant and oxidant-detoxifying enzymes, including NAD(P)H-quinone oxidoreductase (NQ01), heme oxygenase 1 (HO1), and glutathione S-transferase (GST) [84, 85]. In addition, NRF2 and ATF4 induce the transcription of genes whose products are involved in the maintenance of glutathione cellular level, the main redox buffer in the cell [82, 83, 86, 87]. The overall antioxidant effect of the PERK pathway is supported by the finding that a potent ER-stress-inducing chemical, tunicamycin, induces only weak accumulation of ROS in wild-type cells, whereas this treatment induces a toxic accumulation of ROS in cells that lack PERK [75].

Dissociation of BiP/GRP78 from ATF6α leads to its translocation to the Golgi, where this protein is processed into its active form [88]. The activated ATF6 translocates to the nucleus and functions as a transcription factor, promoting the expression of downstream target genes involved in ER stress including XBP1, GADD153 (also known as CHOP, a proapoptotic transcription factor that plays a critical role in ER stress-mediated apoptosis), and ER chaperones [89, 90]. ATF6α is also a regulator of gluconeogenesis [91].

All together, these three pathways mitigate the ER stress by reducing global protein synthesis, increasing misfolded or unfolded protein degradation, and, simultaneously, increasing the specific expression of proteins that help maintaining the protein folding process in the ER lumen as well as ER integrity [92–94].

Under pathological and/or stressful conditions, in which the demand of protein synthesis, folding, and/or repair is

increased, the UPR efficiency decreases, resulting in the accumulation of unfolded protein and misfolded proteins and ER damage [93, 95–98].

Moreover, an overactivation of the UPR leads to a sustained activity of ERO1 as well as induction of ERO1β expression [99], resulting in an increased H_2O_2 production [100] that is found in several liver diseases such as NASH, ASH, and viral infection (Figure 2).

As a first-line response during UPR activation, ER-related PERK pathway attenuates general mRNA translation and activates the Nrf2 transcription factor [71, 83, 92, 101, 102] that translocates to the nucleus and activates antioxidant responsive element-dependent gene expression [71, 75, 92, 93, 101]. However, in NASH, the UPR-induced Nrf2-mediated response is downregulated [75]. Impaired Nrf2 activity is associated with mitochondrial depolarization/dysfunction, as well as increased hepatic free fatty acid levels, fatty liver, and NASH development [102–104]. Moreover, NASH-related accumulation of misfolded proteins, and related unmitigated ER stress, also induces increased ROS production and macromolecules oxidation in the ER lumen through PDI, leading to intracellular depletion of reduced glutathione [71, 72, 105]. Indeed, when oxidized, PDI with ERO1 acts in the oxidative folding of proteins by allowing proper disulfide bond formation. When reduced, PDI breaks and rearranges disulfides in the nascent proteins until the reduced glutathione pool is depleted [71, 73]. Furthermore, both ER stress and oxidative damage prompt calcium leak from the ER, leading to mitochondrial calcium accumulation, which in turn promotes exacerbated mitochondrial ROS production, further amplifying ER stress [72, 104, 106] (Figure 2). It has been recently suggested that elevated levels of palmitic acid would compromise the ER ability to maintain calcium stores, resulting in the stimulation of mitochondrial oxidative metabolism, ROS production, and, ultimately, cellular dysfunction [75]. Therefore, it appears that ER stress may occur earlier than the onset of mitochondrial dysfunction, ROS accumulation, and apoptosis [107, 108]. Moreover, SREBP-1, the master regulator of triglycerides and cholesterol synthesis, is kept inactive at the ER by interaction with insulin induced gene proteins (INSIGs). During ER stress, proteolytic degradation of Insig-1 releases SREBP [96], which is subsequently processed in the Golgi and finally directed to the nuclei where it activates the transcription of the lipogenic program. In turn, excess fatty acids and cholesterol promote ER stress; thus, the reinforced cycle of ER stress, oxidative stress, and lipogenesis-induced lipotoxicity fuels the pathogenesis of NASH [78].

Alcoholic liver disease (ALD) is certainly related to an excessive production of ROS from ethanol metabolism and the consequent oxidative stress within the hepatocytes [109, 110]. Two metabolic pathways are involved in the degradation of ethanol. First, ethanol is oxidized into acetaldehyde by alcohol dehydrogenase (ADH), followed by production of acetate by means of acetaldehyde dehydrogenase (ALDH). Acetaldehyde mediates most of the toxic effect of alcohol [15, 111, 112]. The second pathway of ethanol degradation, which is largely inducible, operates through the microsomal ethanol-oxidizing system (MEOS) cytochrome P450. CYP2E1, the main cytochrome P450 isoform induced by ethanol consumption, is located at the membrane of ER [113–116], making it the master mechanism of ER ethanol-induced ROS production. Ethanol oxidation by CYP2E1 generates $O_2^{\bullet-}$ and H_2O_2 promoting membrane lipoperoxidation. Moreover, ethanol administration and ROS production increase free iron, which catalyzes the production of strong oxidants, such as hydroxyl radical ($OH^{\bullet}$), ferrous oxide (FeO), and hydroxyethyl radical (CH_3CHOH). This damaging mechanism is also common to lysosomes and mitochondria (Figure 2).

The UPR overactivation and ROS production occur also in Hepatitis C and B, but the process that induces these responses is different from other liver diseases.

Hepatitis C virus (HCV) replication in infected host cells is dependent on several viral proteins that are folded in the ER and synthesized in ribonucleoprotein complexes in association with the ER [117]. HCV replication has been shown to cause ER stress and its gene products such as Core, E2, NS5A, and NS4B have also been demonstrated to induce UPR and ROS production [118, 119].

The protein aggregates that are formed in the viral replication induce all three different pathways of UPR which sustain viral replication alleviating ER stress [118, 119]. ROS production during HCV infection also occurs due to altered intracellular Ca^{+2} homeostasis. For example, Core viral protein perturbs the intracellular calcium both by inducing ER Ca^{2+} release and by stimulating the Ca^{2+} uniporter in mitochondria, increasing ROS production in mitochondria and opening of the mPTP [120]. Similarly, NS5A perturbs Ca^{2+} signaling and elevates ROS production in mitochondria leading to activation of transcription factors such as NF-κB and STAT that are involved in HCV-mediated hepatocarcinogenesis [121]. NF-κB activation is also stimulated by NS4B, a mechanism requiring Ca^{2+}-induced ROS production [122].

Similar mechanisms of UPR activation and ROS production also occur in HBV infection [123] (Figure 2).

4. Lysosomes

Autophagy is a fundamental mechanism of cell adaptation to stress, allowing removal of damaged molecules and cellular components by degradation in the lysosomal compartment, which is of particular importance for the removal of nonfunctional mitochondria (mitophagy) (Figure 1). Alterations of the autophagic pathway play a major role in the onset and perpetuation of several chronic diseases, including neurodegenerative and metabolic disorders as well as cancer chemoresistance. Most of the processes associated with RONS production also stimulate autophagy [124]. For instance, starvation, through inhibition of mTOR pathway, stimulates autophagy and increases mitochondrial ROS production. H_2O_2 oxidizes a redox-sensitive thiol of Atg4, which then promotes LC3-I conversion to LC3-II and maturation of the autophagosome [125]. Consistently, autophagy is stimulated in vivo by mitochondrial superoxide production, as seen by experimental downregulation of MnSOD, which increases $O_2^{\bullet-}$ and reduces H_2O_2 [126]. However, it is not

clear whether $O_2^{\bullet -}$ directly stimulates autophagy or more likely induces lipid peroxidation and mitochondrial damage which in turn activate autophagy. This second line of thought is supported by the observation that, in nutrient-deprived hepatocytes, mitochondrial membrane depolarization precedes the formation of the autophagosome [127] and defective mitophagy results in accumulation of dysfunctional mitochondria, increases oxidative stress, and promotes tissue liver damage and cancer [128]. Chronic ethanol feeding is associated with decreased intralysosomal hydrolases content and reduced proteasomal activity due to impaired cathepsin L trafficking in the lysosome [129, 130]. Oxidative stress can also harm lysosomal membranes resulting in elevated cytosolic levels of cathepsin B due to lysosomal leakage [131].

The effect of ethanol metabolism on autophagy is quite debated, and controversial results have been reported [132]. Using LC3-GFP transgenic mice, two groups described that binge- [133], acute- or chronic-ethanol administration [134] promoted autophagosome formation in vivo. The increase in autophagosome formation was paralleled by inhibition of mTORC1 signaling pathway [133].

In contrast, using a CYP2E1 knock-out or knock-in mice and parallel in vitro model, Wu and colleagues showed that binge ethanol administration reduced macroautophagy in CYP2E1 KI mice and cells but not in KO mice [135]. Despite the different autophagy status, which could be due to the use of different transgenic models and different binge drinking protocols, inhibition of autophagy enhanced ethanol toxicity while pharmacological promotion of macroautophagy by carbamazepine, as well as rapamycin, was shown to have a therapeutic potential in animal models of acute ethanol-induced toxicity [133] as well as ASH [135] and NASH [136].

Since ethanol is known to reduce the proteolytic activity of lysosomes, it is possible that the degradative part of autophagy, that is, the autophagolysosome formation, may be impaired in chronic ethanol feeding despite increased autophagosome formation, leading to defective removal of damaged cellular components [137] (Figure 2).

This mechanism could be relevant also for the pathogenesis of NAFLD. In vitro experiments showed that autophagy was increased in hepatoma cells exposed to FFA, mimicking the "first hit" of NASH pathogenesis. However, after a "second hit" of H_2O_2 or TNFα (oxidative damage and inflammation), the autophagic flux diminished despite enhanced autophagosome formation [138].

An important mechanism of ROS induced liver injury is mediated by the release of "labile" iron (Fe^{2+}) that occurs in damaged lysosomes during ischemia/reperfusion [139].

Lysosomes are a major storage site of iron, which enters the hepatocyte through transferrin mediated endocytosis, subsequent endosome acidification, iron reduction, and release from transferrin. Fe^{2+} is then released in the cytosol or delivered to mitochondria in controlled amount (Figure 1). Moreover, lysosomes can significantly increase their iron content by reparative autophagic uptake of damaged mitochondria, peroxisomes, or cytosolic iron-loaded ferritin [29]. Labile iron is transported to mitochondria, where it catalyzes the Fenton reaction with H_2O_2 (see Section 1.2), producing the deleterious hydroxyl ($OH^{\bullet}$) and hydroxyperoxyl ($OOH^{\bullet}$) radicals which damage mitochondria membranes and induce the opening of the permeability transition pore, triggering the RIRR response and eventually cell death (Figure 2). Fe^{2+} induced ROS formation can represent a potent mechanism of damage amplification not only within the cellular compartments but also at the cell and organ level. In fact, many human diseases, especially liver diseases, are associated with increased serum ferritin levels that arise due to hepatocyte death. Increased ferritin uptake in hepatocytes is associated with abnormal endosome clustering and induces lysosomal membrane permeability and promotes lipid peroxidation, depletion of GSH, and reduction of GSH/GSSG ratio. Ferritin accumulation triggers macroautophagy which is abolished by Fe chelation, confirming the mechanistic role of Fe-induced ROS formation in the onset of ferritin toxicity. Also in this model, pharmacological inhibition of macroautophagy strongly enhanced ferritin toxicity, further substantiating the concept that induction of autophagy is a generalized defense mechanism against ROS-mediated cellular damage [140].

5. Peroxisomes

Peroxisomes are ubiquitous organelles involved in catabolic oxidative reactions, xenobiotic detoxification, and bile salt synthesis. In mammals, peroxisomes carry on the α-oxidation of very-long chain fatty acids that cannot directly enter the mitochondria for β-oxidation[141].

Several peroxisomal oxidases produce ROS, primarily H_2O_2, but also $^{\bullet}NO$, since iNOS was detected in peroxisomes of hepatocytes [142]. Peroxisomes are not only a source of ROS but also a powerful ROS disposal compartment, thanks to a large array of antioxidant enzymes, mainly catalase, but also GPx, Mn-SOD, and CuZn-SOD, and peroxyredoxin-1 and peroxyredoxin-5 [143] (Figure 1).

Peroxisomes are highly dynamic structures that undergo enlargement, elongation, and increase in number in response to several xenobiotic or physiologic stimuli.

Peroxisomes can support mitochondria in the β-oxidation of fatty acids [144], a process mediated by the activation of the nuclear receptor PPARα [145]. Accordingly, peroxisomes are elevated in several mice models of NAFLD [146] and proliferation and enlargement of peroxisomes are also described in patients with fatty liver [147] (Figure 2).

Activation of PPARα and subsequent induction of peroxisomal β-oxidation is a potent inducer of H_2O_2 production which is not paralleled by an equal increase in antioxidant enzymes, mainly catalase, leading to oxidative stress and substantially contributing to the hepatocarcinogenic effects of PPARα activators observed in mice, but not in humans [148].

Disruption of the peroxisomal structure and function in the hepatocyte, as in the PDX5 knock-out mice, resulted in structural alteration and reduced functionality of mitochondria, increased proliferation of ER and lysosomes, and accumulation of lipid droplets. Surprisingly, no sign of significant oxidative stress was found, although metabolic rearrangement toward a more glycolytic setting was observed [149].

6. Plasma Membrane

At the plasma membrane interface, key signal transduction events originate following the ligand-receptor interaction. Signal transduction is biologically encoded by phosphorylation of key Tyr or Ser/Thr residues that induce conformational and functional changes in transducing proteins. The coordinated activities of protein kinases and protein phosphatases ensure the substrate, temporal, and spatial confinement required to obtain specificity for signal transduction events. Signal transduction occurs not only by means of the well-known "phosphorylation code," but also by regulation of redox-sensitive Cys residues in signaling proteins. Such "redox-code" [21] adds a level of complexity and flexibility to signaling circuitry.

The best characterized redox regulation of kinase signaling, and likely the most relevant to the hepatocyte, is the insulin pathway. Insulin binding to its receptor activates intrinsic kinase activity and initiates a phosphorylation cascade through the PI3K/AKT/mTOR pathway. In parallel, insulin promotes the deactivation of protein phosphatases (PTEN, PP2A, and SH2P), a mechanism requiring localized H_2O_2 burst at the plasma membrane generated by extracellular NOX [150–153] (Figure 1).

Adiponectin, which potentiates insulin action, activates 5-LOX H_2O_2 bursts and deactivates PTP1B [154].

Exogenous H_2O_2 administration can therefore modulate insulin signaling through inhibition of Protein Tyrosine Phosphatases (PTP), as recently demonstrated by Iwakami et al. [155].

The ROS-mediated inactivation of PTP is a general physiological mechanism necessary for efficient signal transduction of several, if not all, MAPK family members [2], including p38 [9, 156], JNK [157], and ERK1/2 [158] (Figure 1).

Removal of signaling H_2O_2 and reduction of oxidized Cys in PTP are ensured by cytoplasmic antioxidants, such as GSH, thioredoxin 1 [159] and peroxiredoxin-2 [152], thus terminating the signaling activity.

Although H_2O_2 is required for normal signal transduction, excess oxidative stress can activate stress-sensitive Ser/Thr kinase such as JNK, ERK1 and IKKb (inhibitor of NF-κB), and p70S6k, all of which are client of the redox-regulated protein phosphatase PP2A. Stress sensitive kinases take part in insulin resistance by inhibitory phosphorylation of IRS-1 [160] (Figure 2).

Clearly, redox signaling is highly intertwined with phosphorylation signaling, and a high level of spatial and temporal confinement must be fulfilled to ensure the proper biological response.

Very recently, Hua and colleagues uncovered the coordinated redox mechanisms initiating and terminating hepatocyte proliferation during liver ontogenesis or regeneration after partial hepatectomy [161]. They found that sustained elevated H_2O_2 levels are required for the activation of ERK signaling and trigger a shift from quiescence to proliferation, while sustained decreased H_2O_2 levels activate p38 signaling and trigger a shift from proliferation to quiescence. Pharmacological lowering of H_2O_2 levels reduces the extent of fetal hepatocyte proliferation and delays the onset of liver regeneration. Chemical augmentation of H_2O_2 levels in adult hepatocytes triggers proliferation and delays the termination of liver regeneration. Although the authors did not map the precise cellular location of H_2O_2 production, the mechanism triggering the H_2O_2-induced hepatocyte proliferation during embryogenesis and liver regeneration involves early induction of NADPH Oxidase 4 (NOX4) and the adaptor protein p66shc [161], which is known to be necessary for NOX assembly at the plasma membrane [162]. Interestingly, NOX4 recruitment at the plasma membrane and localized H_2O_2 production are also required for IGF-I signal transduction to Src, a mechanism mediated by the scaffold protein SHPS-1 [163], suggesting that NOX4 assembly at the plasma membrane could promote specific signal transduction by redox regulation of different client effectors.

In hepatocytes, membrane fluidity and lipid-raft clustering have been described to mediate ethanol-induced oxidative stress. ROS produced by ethanol metabolism increase plasma membrane fluidity and determine an increase in low-molecular weight iron, triggering massive ROS production and membrane peroxidation [164]. These event are associated with lipid-raft clustering, intraraft disulfide-bond and peroxidation, phospholipase C recruitment inside the lipid-raft, and activation of a signaling pathway that modulate iron content in the lysosomal compartment [165] (Figure 2). Consistently, reducing lipid-raft clustering and oxidation by DHA administration [166] or increasing plasma membrane fluidity by benzo(a)pyrene administration [167], respectively, alleviated or worsened the ethanol toxicity mediated by increased lysosomal iron leakage. Since membrane lipid rafts are required for peroxisome biogenesis [168], it would be interesting to assess the effect of ethanol-induced lipid-raft clustering on peroxisome structure and function.

7. Concluding Remarks

Redox homeostasis in the cell is achieved by accurate compartmentalization of prooxidants and RONS buffering systems. Each cellular compartment differs in the overall redox state as well as in the concentration and oxidative steady-state of its antioxidant pairs. Lysosomes and ER are relatively more oxidizing than cytoplasm, while more reducing conditions are found in the nuclei and mitochondria. Each of these compartments is actively maintained in a nonequilibrium redox state to fulfill specific tasks. At the same time, cellular organelles are highly interconnected by a complex network of physical connections, signaling pathways, intracellular trafficking routes, and metabolic activities.

Oxidative stress can arise by different mechanism in any cellular compartment and spread to other cellular organelles through any of the physical or functional connections.

For instance, mild alterations of plasma membrane permeability induced by ethanol metabolism promote clustering of lipid raft and increase ferritin uptake that promote membrane lipid peroxidation and free iron leakage from the lysosome. Free iron is eventually transported to

the mitochondria where it triggers a burst of oxidative stress and opening of the mPTP, propagating the oxidative damage through the RIRR mechanism (Figure 2).

Perturbation of the network of physical and functional connections between cellular organelles is not only a consequence of pathological oxidative stress but can be indeed the primary causal mechanism of oxidative stress and morbidity. In a recent paper, Arruda and colleagues, by using an elegant in vivo strategy, demonstrated that artificially increasing the physical interaction between mitochondria and ER (MAMs: mitochondrial-associated ER membranes) in the liver led to altered calcium load in the mitochondria, increased oxidative stress, and metabolic alterations, promoting obesity and metabolic dysfunction [169]. Rearrangement of ER around mitochondria seems to be an early event after HFD treatment and likely reflects a transient adaptation of this physiologically highly dynamic process (Figure 2).

The recent development of genetically encoded ROS sensor paved the way for a new era of exploration in the redox cell biology, allowing real-time detection and measure of the redox dynamics in living cells. These technological advances are contributing to unravel the complex spatial and temporal dynamics of H_2O_2 bursts that, acting as switches, can activate or deactivate signaling components, thus interconnecting the redox-based and kinase-based signaling pathways.

As we deepen the understanding of the redox homeostasis in the different cellular compartment, the concept of "oxidative stress" evolves and alterations of redox mechanisms are increasingly being recognized in human diseases, revealing the potential for new therapeutic opportunities.

Abbreviations

IRS: Insulin receptor substrate
NOX: NADPH Oxidase
PTPs: Protein Tyrosine Phosphatases
ETC: Electron Transport Chain
TCA: Tricarboxylic acid cycle
FFA: Free fatty acids
GSH: Reduced Glutathione
CYP: Cytochrome P450 system
UPR: Unfolded protein response
MAMs: Mitochondria associated membranes
ETOH: Ethanol
AcCHO: Acetaldehyde.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work is supported by Italian Ministry of Health (Grant GR-2009-1600315) and Ente Cassa di Risparmio di Firenze (Grant 2013.0673).

References

[1] D. P. Jones, "Radical-free biology of oxidative stress," *The American Journal of Physiolog—Cell Physiology*, vol. 295, no. 4, pp. C849–C868, 2008.

[2] P. D. Ray, B.-W. Huang, and Y. Tsuji, "Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling," *Cellular Signalling*, vol. 24, no. 5, pp. 981–990, 2012.

[3] D. P. Jones and Y.-M. Go, "Redox compartmentalization and cellular stress," *Diabetes, Obesity and Metabolism*, vol. 12, supplement 2, pp. 116–125, 2010.

[4] C. C. Winterbourn, "Are free radicals involved in thiol-based redox signaling?" *Free Radical Biology and Medicine*, vol. 80, pp. 164–170, 2015.

[5] H. Sies, "Oxidative stress: a concept in redox biology and medicine," *Redox Biology*, vol. 4, pp. 180–183, 2015.

[6] L. L. Ji, M.-C. Gomez-Cabrera, and J. Vina, "Exercise and hormesis: activation of cellular antioxidant signaling pathway," *Annals of the New York Academy of Sciences*, vol. 1067, no. 1, pp. 425–435, 2006.

[7] M. Schieber and N. S. Chandel, "ROS function in redox signaling and oxidative stress," *Current Biology*, vol. 24, no. 10, pp. R453–R462, 2014.

[8] G. Bjelakovic, D. Nikolova, L. L. Gluud, R. G. Simonetti, and C. Gluud, "Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis," *Journal of the American Medical Association*, vol. 297, no. 8, pp. 842–857, 2007.

[9] R.-M. Liu, J. Choi, J.-H. Wu et al., "Oxidative modification of nuclear mitogen-activated protein kinase phosphatase 1 is involved in transforming growth factor β1-induced expression of plasminogen activator inhibitor 1 in fibroblasts," *The Journal of Biological Chemistry*, vol. 285, no. 21, pp. 16239–16247, 2010.

[10] M. Benčina, "Illumination of the spatial order of intracellular pH by genetically encoded pH-sensitive sensors," *Sensors*, vol. 13, no. 12, pp. 16736–16758, 2013.

[11] K. A. Lukyanov and V. V. Belousov, "Genetically encoded fluorescent redox sensors," *Biochimica et Biophysica Acta*, vol. 1840, no. 2, pp. 745–756, 2014.

[12] S. G. Rhee, T.-S. Chang, W. Jeong, and D. Kang, "Methods for detection and measurement of hydrogen peroxide inside and outside of cells," *Molecules and Cells*, vol. 29, no. 6, pp. 539–549, 2010.

[13] V. Zámbó, L. Simon-Szabó, P. Szelényi, É. Kereszturi, G. Bánhegyi, and M. Csala, "Lipotoxicity in the liver," *World Journal of Hepatology*, vol. 5, no. 10, pp. 550–557, 2013.

[14] E. Ceni, T. Mello, and A. Galli, "Pathogenesis of alcoholic liver disease: role of oxidative metabolism," *World Journal of Gastroenterology*, vol. 20, no. 47, pp. 17756–17772, 2014.

[15] T. Mello, E. Ceni, C. Surrenti, and A. Galli, "Alcohol induced hepatic fibrosis: role of acetaldehyde," *Molecular Aspects of Medicine*, vol. 29, no. 1-2, pp. 17–21, 2008.

[16] P. Dongiovanni, A. L. Fracanzani, S. Fargion, and L. Valenti, "Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target," *Journal of Hepatology*, vol. 55, no. 4, pp. 920–932, 2011.

[17] N. Fujita and Y. Takei, "Iron overload in nonalcoholic steatohepatitis," *Advances in Clinical Chemistry*, vol. 55, pp. 105–132, 2011.

[18] J. E. Nelson, H. Klintworth, and K. V. Kowdley, "Iron metabolism in Nonalcoholic Fatty Liver Disease," *Current Gastroenterology Reports*, vol. 14, no. 1, pp. 8–16, 2012.

[19] M. Arciello, M. Gori, and C. Balsano, "Mitochondrial dysfunctions and altered metals homeostasis: new weapons to counteract HCV-related oxidative stress," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 971024, 10 pages, 2013.

[20] K. Hino, S. Nishina, and Y. Hara, "Iron metabolic disorder in chronic hepatitis C: mechanisms and relevance to hepatocarcinogenesis," *Journal of Gastroenterology and Hepatology*, vol. 28, no. 4, pp. 93–98, 2013.

[21] D. P. Jones and H. Sies, "The redox code," *Antioxidants & Redox Signaling*, 2015.

[22] P. Venkatakrishnan, E. S. Nakayasu, I. C. Almeida, and R. T. Miller, "Absence of nitric-oxide synthase in sequentially purified rat liver mitochondria," *Journal of Biological Chemistry*, vol. 284, no. 30, pp. 19843–19855, 2009.

[23] J. S. Stamler, D. J. Singel, and C. A. Piantadosi, "SNO-hemoglobin and hypoxic vasodilation," *Nature Medicine*, vol. 14, no. 10, pp. 1008–1009, 2008.

[24] A.-L. Levonen, R. P. Patel, P. Brookes et al., "Mechanisms of cell signaling by nitric oxide and peroxynitrite: from mitochondria to MAP kinases," *Antioxidants and Redox Signaling*, vol. 3, no. 2, pp. 215–229, 2001.

[25] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.

[26] A. Denicola, J. M. Souza, and R. Radi, "Diffusion of peroxynitrite across erythrocyte membranes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3566–3571, 1998.

[27] D. B. Zorov, C. R. Filburn, L.-O. Klotz, J. L. Zweier, and S. J. Sollott, "Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes," *Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1001–1014, 2000.

[28] L. Tandara and I. Salamunic, "Iron metabolism: current facts and future directions," *Biochemia Medica*, vol. 22, no. 3, pp. 311–328, 2012.

[29] N. Bresgen and P. M. Eckl, "Oxidative stress and the homeodynamics of iron metabolism," *Biomolecules*, vol. 5, no. 2, pp. 808–847, 2015.

[30] R. Lill, B. Hoffmann, S. Molik et al., "The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1823, no. 9, pp. 1491–1508, 2012.

[31] E. B. Tahara, F. D. T. Navarete, and A. J. Kowaltowski, "Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation," *Free Radical Biology and Medicine*, vol. 46, no. 9, pp. 1283–1297, 2009.

[32] J. St-Pierre, J. A. Buckingham, S. J. Roebuck, and M. D. Brand, "Topology of superoxide production from different sites in the mitochondrial electron transport chain," *The Journal of Biological Chemistry*, vol. 277, no. 47, pp. 44784–44790, 2002.

[33] N. Kaludercic, A. Carpi, T. Nagayama et al., "Monoamine oxidase B prompts mitochondrial and cardiac dysfunction in pressure overloaded hearts," *Antioxidants and Redox Signaling*, vol. 20, no. 2, pp. 267–280, 2014.

[34] K. Staniek and H. Nohl, "Are mitochondria a permanent source of reactive oxygen species?" *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1460, no. 2-3, pp. 268–275, 2000.

[35] G. C. Brown and V. Borutaite, "There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells," *Mitochondrion*, vol. 12, no. 1, pp. 1–4, 2012.

[36] A. Boveris, N. Oshino, and B. Chance, "The cellular production of hydrogen peroxide," *Biochemical Journal*, vol. 128, no. 3, pp. 617–630, 1972.

[37] H. Bakala, M. Hamelin, J. Mary, C. Borot-Laloi, and B. Friguet, "Catalase, a target of glycation damage in rat liver mitochondria with aging," *The Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1822, no. 10, pp. 1527–1534, 2012.

[38] O. W. Griffith and A. Meister, "Origin and turnover of mitochondrial glutathione," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 14, pp. 4668–4672, 1985.

[39] K. Kurosawa, N. Hayashi, N. Sato, T. Kamada, and K. Tagawa, "Transport of glutathione across the mitochondrial membranes," *Biochemical and Biophysical Research Communications*, vol. 167, no. 1, pp. 367–372, 1990.

[40] Z. Chen and L. H. Lash, "Evidence for mitochondrial uptake of glutathione by dicarboxylate and 2-oxoglutarate carriers," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 285, no. 2, pp. 608–618, 1998.

[41] Z. Chen, D. A. Putt, and L. H. Lash, "Enrichment and functional reconstitution of glutathione transport activity from rabbit kidney mitochondria. Further evidence for the role of the dicarboxylate and 2-oxoglutarate carriers in mitochondrial glutathione transport," *Archives of Biochemistry and Biophysics*, vol. 373, no. 1, pp. 193–202, 2000.

[42] O. Coll, A. Colell, C. García-Ruiz, N. Kaplowitz, and J. C. Fernández-Checa, "Sensitivity of the 2-oxoglutarate carrier to alcohol intake contributes to mitochondrial glutathione depletion," *Hepatology*, vol. 38, no. 3, pp. 692–702, 2003.

[43] H. M. Wilkins, K. Marquardt, L. H. Lash, and D. A. Linseman, "Bcl-2 is a novel interacting partner for the 2-oxoglutarate carrier and a key regulator of mitochondrial glutathione," *Free Radical Biology and Medicine*, vol. 52, no. 2, pp. 410–419, 2012.

[44] J. Legault, C. Carrier, P. Petrov, P. Renard, J. Remacle, and M.-E. Mirault, "Mitochondrial GPx1 decreases induced but not basal oxidative damage to mtDNA in T47D cells," *Biochemical and Biophysical Research Communications*, vol. 272, no. 2, pp. 416–422, 2000.

[45] M. Marí, A. Morales, A. Colell, C. García-Ruiz, and J. C. Fernández-Checa, "Mitochondrial glutathione, a key survival antioxidant," *Antioxidants and Redox Signaling*, vol. 11, no. 11, pp. 2685–2700, 2009.

[46] B. Chance, H. Sies, and A. Boveris, "Hydroperoxide metabolism in mammalian organs," *Physiological Reviews*, vol. 59, no. 3, pp. 527–605, 1979.

[47] P. Cole-Ezea, D. Swan, D. Shanley, and J. Hesketh, "Glutathione peroxidase 4 has a major role in protecting mitochondria from oxidative damage and maintaining oxidative phosphorylation complexes in gut epithelial cells," *Free Radical Biology and Medicine*, vol. 53, no. 3, pp. 488–497, 2012.

[48] P. H. Willems, R. Rossignol, C. E. Dieteren, M. P. Murphy, and W. J. Koopman, "Redox homeostasis and mitochondrial dynamics," *Cell Metabolism*, vol. 22, no. 2, pp. 207–218, 2015.

[49] J. C. Fernández-Checa, C. García-Ruiz, M. Ookhtens, and N. Kaplowitz, "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*, vol. 87, no. 2, pp. 397–405, 1991.

[50] J. C. Fernandez-Checa, M. Ookhtens, and N. Kaplowitz, "Effects of chronic ethanol feeding on rat hepatocytic glutathione. Relationship of cytosolic glutathione to efflux and mitochondrial sequestration," *The Journal of Clinical Investigation*, vol. 83, no. 4, pp. 1247–1252, 1989.

[51] T. Hirano, N. Kaplowitz, H. Tsukamoto, S. Kamimura, and J. C. Fernandez-Checa, "Hepatic mitochondrial glutathione depletion and progression of experimental alcoholic liver disease in rats," *Hepatology*, vol. 16, no. 6, pp. 1423–1427, 1992.

[52] Y.-M. Go and D. P. Jones, "Redox compartmentalization in eukaryotic cells," *Biochimica et Biophysica Acta*, vol. 1780, no. 11, pp. 1273–1290, 2008.

[53] C. Ricci, V. Pastukh, J. Leonard et al., "Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis," *American Journal of Physiology—Cell Physiology*, vol. 294, no. 2, pp. C413–C422, 2008.

[54] A. Mansouri, I. Gaou, C. De Kerguenec et al., "An alcoholic binge causes massive degradation of hepatic mitochondrial DNA in mice," *Gastroenterology*, vol. 117, no. 1, pp. 181–190, 1999.

[55] C. Demeilliers, C. Maisonneuve, A. Grodet et al., "Impaired adaptive resynthesis and prolonged depletion of hepatic mitochondrial DNA after repeated alcohol binges in mice," *Gastroenterology*, vol. 123, no. 4, pp. 1278–1290, 2002.

[56] F. Chiappini, A. Barrier, R. Saffroy et al., "Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray," *Laboratory Investigation*, vol. 86, no. 2, pp. 154–165, 2006.

[57] H. Kawahara, M. Fukura, M. Tsuchishima, and S. Takase, "Mutation of mitochondrial DNA in livers from patients with alcoholic hepatitis and nonalcoholic steatohepatitis," *Alcoholism: Clinical and Experimental Research*, vol. 31, no. 1, pp. S54–S60, 2007.

[58] C. S. Lieber, "Ethanol metabolism, cirrhosis and alcoholism," *Clinica Chimica Acta*, vol. 257, no. 1, pp. 59–84, 1997.

[59] C. S. Lieber, "Metabolic effects of acetaldehyde," *Biochemical Society Transactions*, vol. 16, no. 3, pp. 241–247, 1988.

[60] J. A. Doorn, T. D. Hurley, and D. R. Petersen, "Inhibition of human mitochondrial aldehyde dehydrogenase by 4-hydroxynon-2-enal and 4-oxonon-2-enal," *Chemical Research in Toxicology*, vol. 19, no. 1, pp. 102–110, 2006.

[61] S. Jin, J. Chen, L. Chen et al., "ALDH2 (E487K) mutation increases protein turnover and promotes murine hepatocarcinogenesis," *Proceedings of the National Academy of Sciences*, vol. 112, no. 29, pp. 9088–9093, 2015.

[62] S. H. Caldwell, R. H. Swerdlow, E. M. Khan et al., "Mitochondrial abnormalities in non-alcoholic steatohepatitis," *Journal of Hepatology*, vol. 31, no. 3, pp. 430–434, 1999.

[63] S. Seki, T. Kitada, T. Yamada, H. Sakaguchi, K. Nakatani, and K. Wakasa, "In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases," *Journal of Hepatology*, vol. 37, no. 1, pp. 56–62, 2002.

[64] M. Pérez-Carreras, P. Del Hoyo, M. A. Martín et al., "Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis," *Hepatology*, vol. 38, no. 4, pp. 999–1007, 2003.

[65] L. Llacuna, A. Fernández, C. V. Montfort et al., "Targeting cholesterol at different levels in the mevalonate pathway protects fatty liver against ischemia-reperfusion injury," *Journal of Hepatology*, vol. 54, no. 5, pp. 1002–1010, 2011.

[66] L. A. Videla, R. Rodrigo, M. Orellana et al., "Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients," *Clinical Science*, vol. 106, no. 3, pp. 261–268, 2004.

[67] R. Gambino, G. Musso, and M. Cassader, "Redox balance in the pathogenesis of nonalcoholic fatty liver disease: mechanisms and therapeutic opportunities," *Antioxidants and Redox Signaling*, vol. 15, no. 5, pp. 1325–1365, 2011.

[68] C. von Montfort, N. Matias, A. Fernandez et al., "Mitochondrial GSH determines the toxic or therapeutic potential of superoxide scavenging in steatohepatitis," *Journal of Hepatology*, vol. 57, no. 4, pp. 852–859, 2012.

[69] N. Borgese, M. Francolini, and E. Snapp, "Endoplasmic reticulum architecture: structures in flux," *Current Opinion in Cell Biology*, vol. 18, no. 4, pp. 358–364, 2006.

[70] S. Chakravarthi and N. J. Bulleid, "Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway," *Journal of Biological Chemistry*, vol. 279, no. 38, pp. 39872–39879, 2004.

[71] A. Higa and E. Chevet, "Redox signaling loops in the unfolded protein response," *Cellular Signalling*, vol. 24, no. 8, pp. 1548–1555, 2012.

[72] J. D. Malhotra and R. J. Kaufman, "Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?" *Antioxidants and Redox Signaling*, vol. 9, no. 12, pp. 2277–2293, 2007.

[73] M. Csala, É. Margittai, and G. Bánhegyi, "Redox control of endoplasmic reticulum function," *Antioxidants & Redox Signaling*, vol. 13, no. 1, pp. 77–108, 2010.

[74] J. W. Cuozzo and C. A. Kaiser, "Competition between glutathione and protein thiols for disulphide-bond formation," *Nature Cell Biology*, vol. 1, no. 3, pp. 130–135, 1999.

[75] K. Zhang and R. J. Kaufman, "From endoplasmic-reticulum stress to the inflammatory response," *Nature*, vol. 454, no. 7203, pp. 455–462, 2008.

[76] X. Shen, K. Zhang, and R. J. Kaufman, "The unfolded protein response—a stress signaling pathway of the endoplasmic reticulum," *Journal of Chemical Neuroanatomy*, vol. 28, no. 1-2, pp. 79–92, 2004.

[77] P. I. Merksamer and F. R. Papa, "The UPR and cell fate at a glance," *Journal of Cell Science*, vol. 123, no. 7, pp. 1003–1006, 2010.

[78] E. Passos, A. Ascensão, M. J. Martins, and J. Magalhães, "Endoplasmic reticulum stress response in non-alcoholic steatohepatitis: the possible role of physical exercise," *Metabolism*, vol. 64, no. 7, pp. 780–792, 2015.

[79] D. Acosta-Alvear, Y. Zhou, A. Blais et al., "XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks," *Molecular Cell*, vol. 27, no. 1, pp. 53–66, 2007.

[80] K. Zhang, S. Wang, J. Malhotra et al., "The unfolded protein response transducer IRE1α prevents ER stress-induced hepatic steatosis," *The EMBO Journal*, vol. 30, no. 7, pp. 1357–1375, 2011.

[81] D. Ron and S. R. Hubbard, "How IRE1 reacts to ER stress," *Cell*, vol. 132, no. 1, pp. 24–26, 2008.

[82] H. P. Harding, Y. Zhang, H. Zeng et al., "An integrated stress response regulates amino acid metabolism and resistance to oxidative stress," *Molecular Cell*, vol. 11, no. 3, pp. 619–633, 2003.

[83] S. B. Cullinan, D. Zhang, M. Hannink, E. Arvisais, R. J. Kaufman, and J. A. Diehl, "Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival," *Molecular and Cellular Biology*, vol. 23, no. 20, pp. 7198–7209, 2003.

[84] J. Mathers, J. A. Fraser, M. McMahon, R. D. C. Saunders, J. D. Hayes, and L. I. McLellan, "Antioxidant and cytoprotective

responses to redox stress," *Biochemical Society Symposium*, vol. 71, pp. 157–176, 2004.

[85] D. D. Zhang, "Mechanistic studies of the Nrf2-Keap1 signaling pathway," *Drug Metabolism Reviews*, vol. 38, no. 4, pp. 769–789, 2006.

[86] S. B. Cullinan and J. A. Diehl, "PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress," *The Journal of Biological Chemistry*, vol. 279, no. 19, pp. 20108–20117, 2004.

[87] J. B. DuRose, D. Scheuner, R. J. Kaufman, L. I. Rothblum, and M. Niwa, "Phosphorylation of eukaryotic translation initiation factor 2α coordinates rRNA transcription and translation inhibition during endoplasmic reticulum stress," *Molecular and Cellular Biology*, vol. 29, no. 15, pp. 4295–4307, 2009.

[88] X. Chen, J. Shen, and R. Prywes, "The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the er to the Golgi," *Journal of Biological Chemistry*, vol. 277, no. 15, pp. 13045–13052, 2002.

[89] S. Oyadomari and M. Mori, "Roles of CHOP/GADD153 in endoplasmic reticulum stress," *Cell Death & Differentiation*, vol. 11, no. 4, pp. 381–389, 2004.

[90] S. J. Marciniak, C. Y. Yun, S. Oyadomari et al., "CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum," *Genes and Development*, vol. 18, no. 24, pp. 3066–3077, 2004.

[91] Y. Wang, L. Vera, W. H. Fischer, and M. Montminy, "The CREB coactivator CRTC2 links hepatic ER stress and fasting gluconeogenesis," *Nature*, vol. 460, no. 7254, pp. 534–537, 2009.

[92] G. S. Hotamisligil, "Endoplasmic reticulum stress and the inflammatory basis of metabolic disease," *Cell*, vol. 140, no. 6, pp. 900–917, 2010.

[93] D. Ron and P. Walter, "Signal integration in the endoplasmic reticulum unfolded protein response," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 7, pp. 519–529, 2007.

[94] S. Fu, S. M. Watkins, and G. S. Hotamisligil, "The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling," *Cell Metabolism*, vol. 15, no. 5, pp. 623–634, 2012.

[95] U. Özcan, Q. Cao, E. Yilmaz et al., "Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes," *Science*, vol. 306, no. 5695, pp. 457–461, 2004.

[96] J. Lee and U. Ozcan, "Unfolded protein response signaling and metabolic diseases," *The Journal of Biological Chemistry*, vol. 289, no. 3, pp. 1203–1211, 2014.

[97] X.-Q. Zhang, C.-F. Xu, C.-H. Yu, W.-X. Chen, and Y.-M. Li, "Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease," *World Journal of Gastroenterology*, vol. 20, no. 7, pp. 1768–1776, 2014.

[98] C. Hetz, "The unfolded protein response: controlling cell fate decisions under ER stress and beyond," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 2, pp. 89–102, 2012.

[99] C. X. C. Santos, L. Y. Tanaka, J. Wosniak, and F. R. M. Laurindo, "Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase," *Antioxidants & Redox Signaling*, vol. 11, no. 10, pp. 2409–2427, 2009.

[100] C. M. Haynes, E. A. Titus, and A. A. Cooper, "Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death," *Molecular Cell*, vol. 15, no. 5, pp. 767–776, 2004.

[101] S. B. Cullinan and J. A. Diehl, "Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway," *International Journal of Biochemistry and Cell Biology*, vol. 38, no. 3, pp. 317–332, 2006.

[102] J. D. Hayes and A. T. Dinkova-Kostova, "The Nrf2 regulatory network provides an interface between redox and intermediary metabolism," *Trends in Biochemical Sciences*, vol. 39, no. 4, pp. 199–218, 2014.

[103] S. Chowdhry, M. H. Nazmy, P. J. Meakin et al., "Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis," *Free Radical Biology and Medicine*, vol. 48, no. 2, pp. 357–371, 2010.

[104] H. Sugimoto, K. Okada, J. Shoda et al., "Deletion of nuclear factor-E_2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 298, no. 2, pp. G283–G294, 2010.

[105] R. J. Kaufman, S. H. Back, B. Song, J. Han, and J. Hassler, "The unfolded protein response is required to maintain the integrity of the endoplasmic reticulum, prevent oxidative stress and preserve differentiation in beta-cells," *Diabetes, Obesity and Metabolism*, vol. 12, no. 2, pp. 99–107, 2010.

[106] K. Zhang, "Integration of ER stress, oxidative stress and the inflammatory response in health and disease," *International Journal of Clinical and Experimental Medicine*, vol. 3, no. 1, pp. 33–40, 2010.

[107] R. A. Egnatchik, A. K. Leamy, D. A. Jacobson, M. Shiota, and J. D. Young, "ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload," *Molecular Metabolism*, vol. 3, no. 5, pp. 544–553, 2014.

[108] A. K. Leamy, R. A. Egnatchik, M. Shiota et al., "Enhanced synthesis of saturated phospholipids is associated with ER stress and lipotoxicity in palmitate treated hepatic cells," *Journal of Lipid Research*, vol. 55, no. 7, pp. 1478–1488, 2014.

[109] A. I. Cederbaum, Y. Lu, and D. Wu, "Role of oxidative stress in alcohol-induced liver injury," *Archives of Toxicology*, vol. 83, no. 6, pp. 519–548, 2009.

[110] H. Cichoz-Lach and A. Michalak, "Oxidative stress as a crucial factor in liver diseases," *World Journal of Gastroenterology*, vol. 20, no. 25, pp. 8082–8091, 2014.

[111] A. I. Cederbaum, "Alcohol metabolism," *Clinics in Liver Disease*, vol. 16, no. 4, pp. 667–685, 2012.

[112] A. Ambade and P. Mandrekar, "Oxidative stress and inflammation: essential partners in alcoholic liver disease," *International Journal of Hepatology*, vol. 2012, Article ID 853175, 9 pages, 2012.

[113] A. Louvet and P. Mathurin, "Alcoholic liver disease: mechanisms of injury and targeted treatment," *Nature Reviews Gastroenterology & Hepatology*, vol. 12, no. 4, pp. 231–242, 2015.

[114] C. S. Lieber, "Alcohol: its metabolism and interaction with nutrients," *Annual Review of Nutrition*, vol. 20, pp. 395–430, 2000.

[115] C. S. Lieber, "Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis," *Alcohol*, vol. 34, no. 1, pp. 9–19, 2004.

[116] Y. Lu and A. I. Cederbaum, "CYP2E1 and oxidative liver injury by alcohol," *Free Radical Biology and Medicine*, vol. 44, no. 5, pp. 723–738, 2008.

[117] T. Hügle, F. Fehrmann, E. Bieck et al., "The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein," *Virology*, vol. 284, no. 1, pp. 70–81, 2001.

[118] L. Kong, S. Li, M. Huang et al., "The roles of endoplasmic reticulum overload response induced by HCV and NS4B protein in human hepatocyte viability and virus replication," *PLOS ONE*, vol. 10, no. 4, Article ID e0123190, 2015.

[119] C. Vasallo and P. Gastaminza, "Cellular stress responses in hepatitis C virus infection: mastering a two-edged sword," *Virus Research*, 2015.

[120] Y. Li, D. F. Boehning, T. Qian, V. L. Popov, and S. A. Weinman, "Hepatitis C virus core protein increases mitochondrial ROS production by stimulation of Ca^{2+} uniporter activity," *The FASEB Journal*, vol. 21, no. 10, pp. 2474–2485, 2007.

[121] G. Gong, G. Waris, R. Tanveer, and A. Siddiqui, "Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-κB," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 17, pp. 9599–9604, 2001.

[122] S. Li, L. Ye, X. Yu et al., "Hepatitis C virus NS4B induces unfolded protein response and endoplasmic reticulum overload response-dependent NF-kappaB activation," *Virology*, vol. 391, no. 2, pp. 257–264, 2009.

[123] K. Awe, C. Lambert, and R. Prange, "Mammalian BiP controls posttranslational ER translocation of the hepatitis B virus large envelope protein," *FEBS Letters*, vol. 582, no. 21-22, pp. 3179–3184, 2008.

[124] M. Dodson, V. Darley-Usmar, and J. Zhang, "Cellular metabolic and autophagic pathways: traffic control by redox signaling," *Free Radical Biology and Medicine*, vol. 63, pp. 207–221, 2013.

[125] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, and Z. Elazar, "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4," *The EMBO Journal*, vol. 26, no. 7, pp. 1749–1760, 2007.

[126] Y. Chen, M. B. Azad, and S. B. Gibson, "Superoxide is the major reactive oxygen species regulating autophagy," *Cell Death and Differentiation*, vol. 16, no. 7, pp. 1040–1052, 2009.

[127] S. P. Elmore, T. Qian, S. F. Grissom, and J. J. Lemasters, "The mitochondrial permeability transition initiates autophagy in rat hepatocytes," *The FASEB Journal*, vol. 15, no. 12, pp. 2286–2287, 2001.

[128] A. Takamura, M. Komatsu, T. Hara et al., "Autophagy-deficient mice develop multiple liver tumors," *Genes and Development*, vol. 25, no. 8, pp. 795–800, 2011.

[129] K. K. Kharbanda, D. L. McVicker, R. K. Zetterman, and T. M. Donohue Jr., "Ethanol consumption reduces the proteolytic capacity and protease activities of hepatic lysosomes," *Biochimica et Biophysica Acta*, vol. 1245, no. 3, pp. 421–429, 1995.

[130] K. K. Kharbanda, D. L. McVicker, R. K. Zetterman, and T. M. Donohue Jr., "Ethanol consumption alters trafficking of lysosomal enzymes and affects the processing of procathepsin L in rat liver," *Biochimica et Biophysica Acta: General Subjects*, vol. 1291, no. 1, pp. 45–52, 1996.

[131] T. M. Donohue, T. V. Curry-McCoy, A. A. Nanji et al., "Lysosomal leakage and lack of adaptation of hepatoprotective enzyme contribute to enhanced susceptibility to ethanol-induced liver injury in female rats," *Alcoholism: Clinical and Experimental Research*, vol. 31, no. 11, pp. 1944–1952, 2007.

[132] Y. Li, S. Wang, H. Ni, H. Huang, and W. Ding, "Autophagy in alcohol-induced multiorgan injury: mechanisms and potential therapeutic targets," *BioMed Research International*, vol. 2014, Article ID 498491, 20 pages, 2014.

[133] W. X. Ding, M. Li, X. Chen et al., "Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice," *Gastroenterology*, vol. 139, no. 5, pp. 1740–1752, 2010.

[134] P. G. Thomes, C. S. Trambly, G. M. Thiele et al., "Proteasome activity and autophagosome content in liver are reciprocally regulated by ethanol treatment," *Biochemical and Biophysical Research Communications*, vol. 417, no. 1, pp. 262–267, 2012.

[135] D. Wu, X. Wang, R. Zhou, L. Yang, and A. I. Cederbaum, "Alcohol steatosis and cytotoxicity: the role of cytochrome P4502E1 and autophagy," *Free Radical Biology and Medicine*, vol. 53, no. 6, pp. 1346–1357, 2012.

[136] C.-W. Lin, H. Zhang, M. Li et al., "Pharmacological promotion of autophagy alleviates steatosis and injury in alcoholic and non-alcoholic fatty liver conditions in mice," *Journal of Hepatology*, vol. 58, no. 5, pp. 993–999, 2013.

[137] A. Dolganiuc, P. G. Thomes, W.-X. Ding, J. J. Lemasters, and T. M. Donohue Jr., "Autophagy in alcohol-induced liver diseases," *Alcoholism: Clinical and Experimental Research*, vol. 36, no. 8, pp. 1301–1308, 2012.

[138] P. Jiang, Z. Huang, H. Zhao, and T. Wei, "Hydrogen peroxide impairs autophagic flux in a cell model of nonalcoholic fatty liver disease," *Biochemical and Biophysical Research Communications*, vol. 433, no. 4, pp. 408–414, 2013.

[139] X. Zhang and J. J. Lemastersn, "Translocation of iron from lysosomes to mitochondria during ischemia predisposes to injury after reperfusion in rat hepatocytes," *Free Radical Biology and Medicine*, vol. 63, pp. 243–253, 2013.

[140] M. A. Krenn, M. Schürz, B. Teufl, K. Uchida, P. M. Eckl, and N. Bresgen, "Ferritin-stimulated lipid peroxidation, lysosomal leak, and macroautophagy promote lysosomal 'metastability' in primary hepatocytes determining in vitro cell survival," *Free Radical Biology and Medicine*, vol. 80, pp. 48–58, 2015.

[141] J. K. Ready and G. P. Mannaerts, "Peroxisomal lipid metabolism," *Annual Review of Nutrition*, vol. 14, pp. 343–370, 1994.

[142] P. A. Loughran, D. B. Stolz, Y. Vodovotz, S. C. Watkins, R. L. Simmons, and T. R. Billiar, "Monomeric inducible nitric oxide synthase localizes to peroxisomes in hepatocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 39, pp. 13837–13842, 2005.

[143] M. Schrader and H. D. Fahimi, "Peroxisomes and oxidative stress," *Biochimica et Biophysica Acta*, vol. 1763, no. 12, pp. 1755–1766, 2006.

[144] R. J. A. Wanders, P. Vreken, S. Ferdinandusse et al., "Peroxisomal fatty acid α- and β-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases," *Biochemical Society Transactions*, vol. 29, no. 2, pp. 250–267, 2001.

[145] J. K. Reddy, "Peroxisome proliferators and peroxisome proliferator-activated receptor α: biotic and xenobiotic sensing," *American Journal of Pathology*, vol. 164, no. 6, pp. 2305–2321, 2004.

[146] B. Knebel, S. Hartwig, J. Haas et al., "Peroxisomes compensate hepatic lipid overflow in mice with fatty liver," *Biochimica et Biophysica Acta*, vol. 1851, no. 7, pp. 965–976, 2015.

[147] J. C. Collins, I. H. Scheinberg, D. R. Giblin, and I. Sternlieb, "Hepatic peroxisomal abnormalities in abetalipoproteinemia," *Gastroenterology*, vol. 97, no. 3, pp. 766–770, 1989.

[148] S. Yu, S. Rao, and J. K. Reddy, "Peroxisome proliferator-activated receptors, fatty acid oxidation, steatohepatitis and hepatocarcinogenesis," *Current Molecular Medicine*, vol. 3, no. 6, pp. 561–572, 2003.

[149] R. Dirkx, I. Vanhorebeek, K. Martens et al., "Absence of peroxisomes in mouse hepatocytes causes mitochondrial and ER abnormalities," *Hepatology*, vol. 41, no. 4, pp. 868–878, 2005.

[150] N. Bashan, J. Kovsan, I. Kachko, H. Ovadia, and A. Rudich, "Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species," *Physiological Reviews*, vol. 89, no. 1, pp. 27–71, 2009.

[151] B. J. Goldstein, K. Mahadev, X. Wu, L. Zhu, and H. Motoshima, "Role of insulin-induced reactive oxygen species in the insulin signaling pathway," *Antioxidants and Redox Signaling*, vol. 7, no. 7-8, pp. 1021–1031, 2005.

[152] J. Kwon, S.-R. Lee, K.-S. Yang et al., "Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 47, pp. 16419–16424, 2004.

[153] S.-R. Lee, K.-S. Yang, J. Kwon, C. Lee, W. Jeong, and S. G. Rhee, "Reversible inactivation of the tumor suppressor PTEN by H_2O_2," *The Journal of Biological Chemistry*, vol. 277, no. 23, pp. 20336–20342, 2002.

[154] T. Fiaschi, F. Buricchi, G. Cozzi et al., "Redox-dependent and ligand-independent trans-activation of insulin receptor by globular adiponectin," *Hepatology*, vol. 46, no. 1, pp. 130–139, 2007.

[155] S. Iwakami, H. Misu, T. Takeda et al., "Concentration-dependent dual effects of hydrogen peroxide on insulin signal transduction in H4IIEC hepatocytes," *PLoS ONE*, vol. 6, no. 11, Article ID e27401, 2011.

[156] K. A. Robinson, C. A. Stewart, Q. N. Pye et al., "Redox-sensitive protein phosphatase activity regulates the phosphorylation state of p38 protein kinase in primary astrocyte culture," *Journal of Neuroscience Research*, vol. 55, no. 6, pp. 724–732, 1999.

[157] H. Kamata, S.-I. Honda, S. Maeda, L. Chang, H. Hirata, and M. Karin, "Reactive oxygen species promote TNFα-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases," *Cell*, vol. 120, no. 5, pp. 649–661, 2005.

[158] C. C. Wentworth, A. Alam, R. M. Jones, A. Nusrat, and A. S. Neish, "Enteric commensal bacteria induce extracellular signal-regulated kinase pathway signaling via formyl peptide receptor-dependent redox modulation of dual specific phosphatase," *The Journal of Biological Chemistry*, vol. 286, no. 44, pp. 38448–38455, 2011.

[159] S. R. Lee, K. S. Kwon, S. R. Kim, and S. G. Rhee, "Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor," *The Journal of Biological Chemistry*, vol. 273, pp. 15366–15372, 1998.

[160] S. Boura-Halfon and Y. Zick, "Phosphorylation of IRS proteins, insulin action, and insulin resistance," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 296, no. 4, pp. E581–E591, 2009.

[161] H. Bai, W. Zhang, X. Qin et al., "Hydrogen peroxide modulates the proliferation/quiescence switch in the liver during embryonic development and posthepatectomy regeneration," *Antioxidants & Redox Signaling*, vol. 22, no. 11, pp. 921–937, 2015.

[162] E. R. Galimov, "The role of p66shc in oxidative stress and apoptosis," *Acta Naturae*, vol. 2, no. 4, pp. 44–51, 2010.

[163] G. Xi, X.-C. Shen, C. Wai, and D. R. Clemmons, "Recruitment of Nox4 to a plasma membrane scaffold is required for localized reactive oxygen species generation and sustained Src activation in response to insulin-like growth factor-I," *Journal of Biological Chemistry*, vol. 288, no. 22, pp. 15641–15653, 2013.

[164] O. Sergent, M. Pereira, C. Belhomme, M. Chevanne, L. Huc, and D. Lagadic-Gossmann, "Role for membrane fluidity in ethanol-induced oxidative stress of primary rat hepatocytes," *Journal of Pharmacology and Experimental Therapeutics*, vol. 313, no. 1, pp. 104–111, 2005.

[165] P. Nourissat, M. Travert, M. Chevanne et al., "Ethanol induces oxidative stress in primary rat hepatocytes through the early involvement of lipid raft clustering," *Hepatology*, vol. 47, no. 1, pp. 59–70, 2008.

[166] F. Aliche-Djoudi, N. Podechard, A. Collin et al., "A role for lipid rafts in the protection afforded by docosahexaenoic acid against ethanol toxicity in primary rat hepatocytes," *Food and Chemical Toxicology*, vol. 60, pp. 286–296, 2013.

[167] A. Collin, K. Hardonnière, M. Chevanne et al., "Cooperative interaction of benzo[a]pyrene and ethanol on plasma membrane remodeling is responsible for enhanced oxidative stress and cell death in primary rat hepatocytes," *Free Radical Biology and Medicine*, vol. 72, pp. 11–22, 2014.

[168] J. Woudenberg, K. P. Rembacz, M. Hoekstra et al., "Lipid rafts are essential for peroxisome biogenesis in HepG2 cells," *Hepatology*, vol. 52, no. 2, pp. 623–633, 2010.

[169] A. P. Arruda, B. M. Pers, G. Parlakgül, E. Güney, K. Inouye, and G. S. Hotamisligil, "Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity," *Nature Medicine*, vol. 20, pp. 1427–1435, 2014.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9209825, 9 pages
http://dx.doi.org/10.1155/2016/9209825

Research Article

Cholesterol Enhances the Toxic Effect of Ethanol and Acetaldehyde in Primary Mouse Hepatocytes

Anayelly López-Islas,[1] Victoria Chagoya-Hazas,[2] Benjamin Pérez-Aguilar,[1] Mayrel Palestino-Domínguez,[1] Verónica Souza,[1,3] Roxana U. Miranda,[1,3] Leticia Bucio,[1,3] Luis Enrique Gómez-Quiroz,[1,3] and María-Concepción Gutiérrez-Ruiz[1,3]

[1]*Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana Iztapalapa, 09340 México, DF, Mexico*
[2]*Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, 04510 México, DF, Mexico*
[3]*Red Fisiopatología de las Enfermedades Hepáticas, PRODEP-SEP, México, DF, Mexico*

Correspondence should be addressed to María-Concepción Gutiérrez-Ruiz; mcgr@xanum.uam.mx

Received 23 July 2015; Accepted 15 September 2015

Academic Editor: Karina R. Gordillo

Copyright © 2016 Anayelly López-Islas et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Obesity and alcohol consumption are risk factors for hepatic steatosis, and both commonly coexist. Our objective was to evaluate the effect of ethanol and acetaldehyde on primary hepatocytes obtained from mice fed for two days with a high cholesterol (HC) diet. HC hepatocytes increased lipid and cholesterol content. HC diet sensitized hepatocytes to the toxic effect of ethanol and acetaldehyde. Cyp2E1 content increased with HC diet, as well as in those treated with ethanol or acetaldehyde, while the activity of this enzyme determined in microsomes increased in the HC and in all ethanol treated hepatocytes, HC and CW. Oxidized proteins were increased in the HC cultures treated or not with the toxins. Transmission electron microscopy showed endoplasmic reticulum (ER) stress and megamitochondria in hepatocytes treated with ethanol as in HC and the ethanol HC treated hepatocytes. ER stress determined by PERK content was increased in ethanol treated hepatocytes from HC mice and CW. Nuclear translocation of ATF6 was observed in HC hepatocytes treated with ethanol, results that indicate that lipids overload and ethanol treatment favor ER stress. Oxidative stress, ER stress, and mitochondrial damage underlie potential mechanisms for increased damage in steatotic hepatocyte treated with ethanol.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are the most common life-style liver diseases caused by obesity and alcohol intake, respectively [1, 2]. Epidemiological evidence shows that obese alcoholics have 2-3 times higher risk of developing steatohepatitis or cirrhosis as compared to nonobese alcoholics or obese nonalcoholics [3]. Obesity and alcohol consumption have some common mechanisms that may contribute to exacerbation of liver damage when these conditions coexit, including oxidative stress, cytochrome P450 (Cyp)2E1 induction, increased lipid synthesis, free fatty acids, and endoplasmic reticulum (ER) stress [4].

Epidemiological data connect an increase in cholesterol intake to the risk and severity of NAFLD and emerging experimental and human data suggest that disturbed hepatic cholesterol homeostasis and free cholesterol accumulation are relevant to the pathogenesis of NAFLD [5]. Free cholesterol accumulation damages hepatocytes by disturbing mitochondrial and ER membrane integrity, generating mitochondrial oxidative injury and ER stress [5]. ER controls cellular cholesterol levels through pathways that sense the level of cholesterol or cholesterol derivatives within the ER membrane itself. Hepatocyte lipid accumulation induces Cyp2E1, and the increase activity of Cyp2E1 in steatosis leads to tissue oxidative stress and production of reactive oxygen species.

Recent data from our lab show that lipid overloaded in VL-17A cells makes them more susceptible to cell damage and oxidative stress when treated with ethanol [6].

As mechanisms underlying synergistic hepatocyte injury caused by cholesterol and ethanol are not clear, our aim in this

study was to evaluate the effect of ethanol or its metabolite, acetaldehyde, on hepatocytes primary culture obtained from mice fed for two days with a high cholesterol diet determining the viability, oxidative stress, content and activity of Cyp2E1, and ER stress.

2. Materials and Methods

2.1. Mice, Hepatocytes Isolation, and Culture. Eight- to ten-week-old male C57BL/6 mice were maintained in pathogen-free housing and cared for in accordance with the Universidad Autonoma Metropolitana Guide for the Care and Use of Laboratory Animals.

Mice were fed with a high cholesterol diet (HC, 2% cholesterol + 0.5% of sodium cholate) for two days. Control mice were fed with regular standard chow (CW) rodent diet. After the two days under diets, mice were subjected to the two-step method of collagenase perfusion for hepatocyte isolation as described previously [7]. The viability was >90% as assessed by trypan blue exclusion. Primary hepatocytes from both HC and chow diet fed mice were seeded at 2.13×10^5 cells per cm^2 in 10 cm dishes (Nalge Nunc) in Ham's F-12/Dulbecco's modified Eagle's basal hepatocyte growth medium supplemented with 10% fetal bovine serum (Hyclone Lab Inc., Logan, UT), 1% antibiotics in a humidified atmosphere of 5% CO_2/95% air. All experiments were carried out using 225,000 cells/cm^2 seeded in 10 cm dishes in at least three independent experiments carried out in triplicate.

2.2. Treatments. After six h stabilization, culture media were exchanged for one without serum and containing ethanol (Et) 100 mM or acetaldehyde (Ac) 200 μM; both treatments lasted 24 h. In order to lessen Et and Ac evaporation, all dishes were wrapped with parafilm during time of treatment.

2.3. Oil Red O Staining. Cell lipid content was determined by oil red O staining as previously reported [6]. Briefly, cells were fixed in 4% formalin and stained with oil red O solution (0.35% in 60% isopropanol) for 10 min. Cells were counterstaining with hematoxylin.

The intracellular lipid droplets were stained by oil red O; the cell cultures were washed with PBS pH 7.4 and fixed for 20 min with 4% formalin in 0.05 M PBS; after washing with sterile double distilled water and 60% isopropanol for 2 min, the cells were stained with 0.35% oil red O solution in 60% isopropanol for 10 min at room temperature.

2.4. Cell Viability by Crystal Violet. Cell viability was determined by crystal violet staining method as we previously reported [8].

2.5. Transmission Electron Microscopy (TEM). After washing with PBS, samples were fixed with 2.5% glutaraldehyde plus paraformaldehyde in PBS (pH 7.4) for 2 h and washed three times for 30 min in PBS. After that, glutaraldehyde-fixed specimens were treated with 1% OsO_4 in PBS for 2 h, dehydrated in increasing concentrations of ethanol (50%–100%), infiltrated with propylene oxide, and embedded in an EPON mixture. Polymerized sections were then cut, stained, and examined using transmission electron microscopy (JEOL JEM 12000 EII).

2.6. Western Blot Analysis. Total protein was isolated from cells, with M-Per tissue protein extraction reagent (Pierce, Rockford, IL, USA), containing 1% halt protease inhibitor mixture (Pierce), 100 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 50 mM sodium orthovanadate. One hundred μg of total protein was separated on NuPAGE novex 4–20% gels (Invitrogen), transferred to polyvinylidene difluoride membranes (Invitrogen), and probed with anti-Cyp2E1 (Cell Signaling, Inc.) or anti-PERK (Abcam Inc.), followed by incubation with the specific horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Inc., Uppsala, Sweden). Immunoreactive bands were identified with ECL-Plus Western blotting detection reagent (GE Healthcare). Equal loading was demonstrated by probing the membrane with anti-actin.

2.7. Immunofluorescence and Filipin Staining. Immunofluorescence was carried out as we previously published [9]. Briefly, hepatocytes were fixed with 4% paraformaldehyde for 15 min. Coverslips were blocked using BSA 5% + triton 0.3% for 30 min at room temperature. Cells were incubated with a mouse anti-ATF6 monoclonal antibody (10 μg/mL, Abcam) overnight at 4°C, followed by incubation with a secondary anti-mouse IgG antibody, conjugated to Alexa Fluor 647 (Invitrogen) for 1 h at 25°C. DAPI was used to stain the cell nuclei.

Free cholesterol was identified by confocal microscopy using filipin (250 μg/mL, Sigma-Aldrich) as reported by Marí and coworkers [10]. Microphotography was recorded in a Carl Zeiss NLO 780 confocal microscopy.

2.8. Microsomal Fractions. Cell cultures were washed with PSB 24 h after treatment with Et or Ac. The cell pellet was resuspended vigorously using an insulin syringe for 5 cycles (4 times per cycle) in 600 μL of lysis buffer (20 mM HEPES, 2 mM KCl, 2 mM $MgCl_2$, 250 mM sucrose, and an inhibitor cocktail protease) to pH 7.4. Cells were always maintained on ice during this procedure. The suspension was centrifuged at 1,000 g for 5 min at 4°C. The supernatant was recovered and centrifuged at 8000 g for 10 min at 4°C. The resulting supernatant was transferred to ultracentrifuge tubes (Beckman Optima TLX) and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was discarded and the pellet was resuspended in 30 μL of storage buffer (it contains the same lysis buffer without sucrose).

2.9. Cyp2E1 Activity Assay. Cyp2E1 activity was assayed by the nitrocatechol method. 10 μL of *p*-nitrophenol (5 mM) and 25 μL of NADPH (20 mM) were added to 440 μL of assay buffer (50 mM potassium phosphate) to pH 7.4 and were incubated at 37°C for 15 min. After that, 100 μg of microsomal protein was added and the reaction was left for 1 h at 37°C. To stop the reaction, 100 μL of trichloroacetic acid was added (20%). The samples were placed on ice for 2 min.

The samples were centrifuged at 10,000 g for 5 min. 500 μL of the supernatant obtained was transferred to Eppendorf tubes and we added 250 μL of NaOH (2 M). The mixture was agitated with vortex and read at 535 nm in a multiplate reader spectrophotometer (Beckman DU640).

To determine the enzymatic activity, the molar extinction coefficient of the formed product (nitrocatechol, $9.53 \times 10^5\ M^{-1}\ CM^{-1}$) was considered, as well as the absorbance of each sample and the reaction time. Results were expressed as nmol of product per hour per microgram of microsomal protein.

2.10. Protein Oxidation. Carbonyl modification of proteins, a key biomarker for the identification of oxidative stress, was addressed by using Oxyblot Protein Oxidation Detection Kit (Millipore, Darmstadt, Germany).

2.11. Protein Content. The protein content was determined by using the bicinchoninic acid method (BCA kit, Pierce Inc.), following manufacturer's instructions.

2.12. Statistical Analysis. The data are presented as mean ± SEM for at least three independent experiments carried out in triplicate. Comparisons between groups were performed by one-way analysis of variance (ANOVA) with Bonferroni post hoc test using GraphPad Prism 5.0 for Mac OS X. Differences were considered significant at $^* p \leq 0.05$.

3. Results

3.1. A HC Diet Induces Lipid Overload in Mice Hepatocytes and Sensitizes to Alcohol and Acetaldehyde Damage. Mice fed with a HC or regular CW diet for two days were subjected to liver perfusion for the isolation of hepatocytes. After 6 h of seeding, and prior to treatments, HC cells clearly exhibited an overload of neutral lipids and free cholesterol, judged by ORO and filipin staining (Figure 1(a)), confirming the steatotic effect of the HC diet; these data were in agreement with our previous work in which we reported 3- and 2.2-fold increase of cholesterol and neutral lipids, respectively, in HC cells compared with CW [11].

HC and control CW cells were treated with Et (100 mM) or Ac (200 μM) for 24 h; HC cellular morphology was clearly affected in both treatments, observing that detached death cells were present in culture dishes, and many stressed cells remained adhered as depicted in Figure 1(e). To gain confidence cell viability was assayed by using crystal violet staining; Figure 1(g) shows that HC diet sensitized cells to the toxic effect of Et and Ac by decreasing cell viability; however no significant effect was observed in CW cells (Figure 1(f)).

3.2. HC Diet Increases Cyp2E1 Expression and Activity Inducing Oxidative Stress. In order to find a mechanism to explain the sensitization, we were focused to address the status of Cyp2E1, the main Et metabolism system with clearly toxic consequences [8]. Interestingly, HC diet promoted the overexpression of Cyp2E1 as Western blot result displayed (Figures 2(b)-2(c)), with no significant changes with Et and Ac treatments. CW cells did not present changes in Cyp2E1 protein content. We proceeded to study the Cyp2E1 activity in microsomes obtained from CW and HC cells treated with both toxics. Figure 2(c) shows a significant increment in the activity in HC nontreated cells compared with CW cells; Et treatment did not modify the activity; however, remarkably, Ac treatment considerably decreased the enzyme activity. Regarding CW cells, only Et induced an increment as expected.

Elevation in Cyp2E1 activity is directly associated with oxidative damage as we previously reported [8], and in order to confirm this phenomenon we performed an oxyblot to address the protein oxidation content; HC diet induced protein oxidation, with no significant differences in Et and Ac treatments. A slight increment was observed in CW cells treated with Et compared with CW nontreated cells; data are in agreement with Cyp2E1 activity (Figure 2(d)).

3.3. HC Diet Induces ER Stress. It is known that disturbances in lipid homeostasis induce cellular stress, particularly at mitochondria and endoplasmic reticulum level [10, 12]. An analysis by TEM pointed out that ER was clearly compromised in HC cells, presenting dramatic loss in ER architecture (Figure 3(d)) in comparison with CW cells (Figure 3(a)), seeing that Et treatment induced megamitochondria formation (Figures 3(b) and 3(e)), as we previously reported [13]. Interestingly we observed autophagosomes formation only in HC cells treated with Et (arrow, Figure 3(e)), suggesting that autophagy could play a prominent role. Ac treatment alone did not induce significant changes in the ER architecture, but we observed megamitochondria and some structures that suggest mitochondrial fission (Figure 3(f), asterisk). In order to support the idea that ER overloaded cholesterol, we assayed biochemically its content in microsomes isolated from CW and HC cells, confirming that certainly ER in HC cells presented an increment in cholesterol content.

Finally, to further address the ER stress we measure the content of PERK, a key regulator of the process. We found that HC diet exhibited an increment in the protein, effect observed in Et treatment in both CW and HC cells; interestingly, Ac did not exhibit any effect in PERK (Figures 4(a)-4(b)). Nuclear translocation of ATF6 is another key marker of ER stress; we identified nuclear ATF6 positive signal only in HC cells treated with Et, coinciding with less fluorescence signal in the cytosol (Figure 4(g)). The result supports the idea that cholesterol overload and Et treatment favor ER stress.

4. Discussion

Alcohol intake is one of the most important risk factors for liver disease [1], in addition to hepatitis B and hepatitis C virus infection; however liver lipid overload has been positioned as a clear risk factor for liver disease. We recently reported that lipid overload, predominantly cholesterol, induces oxidative stress and cell damage [14], and this could be a key determinant for initiation and progression of the disease, particularly when the steatotic phenotype is accompanying another risk factor such as alcohol. Previously,

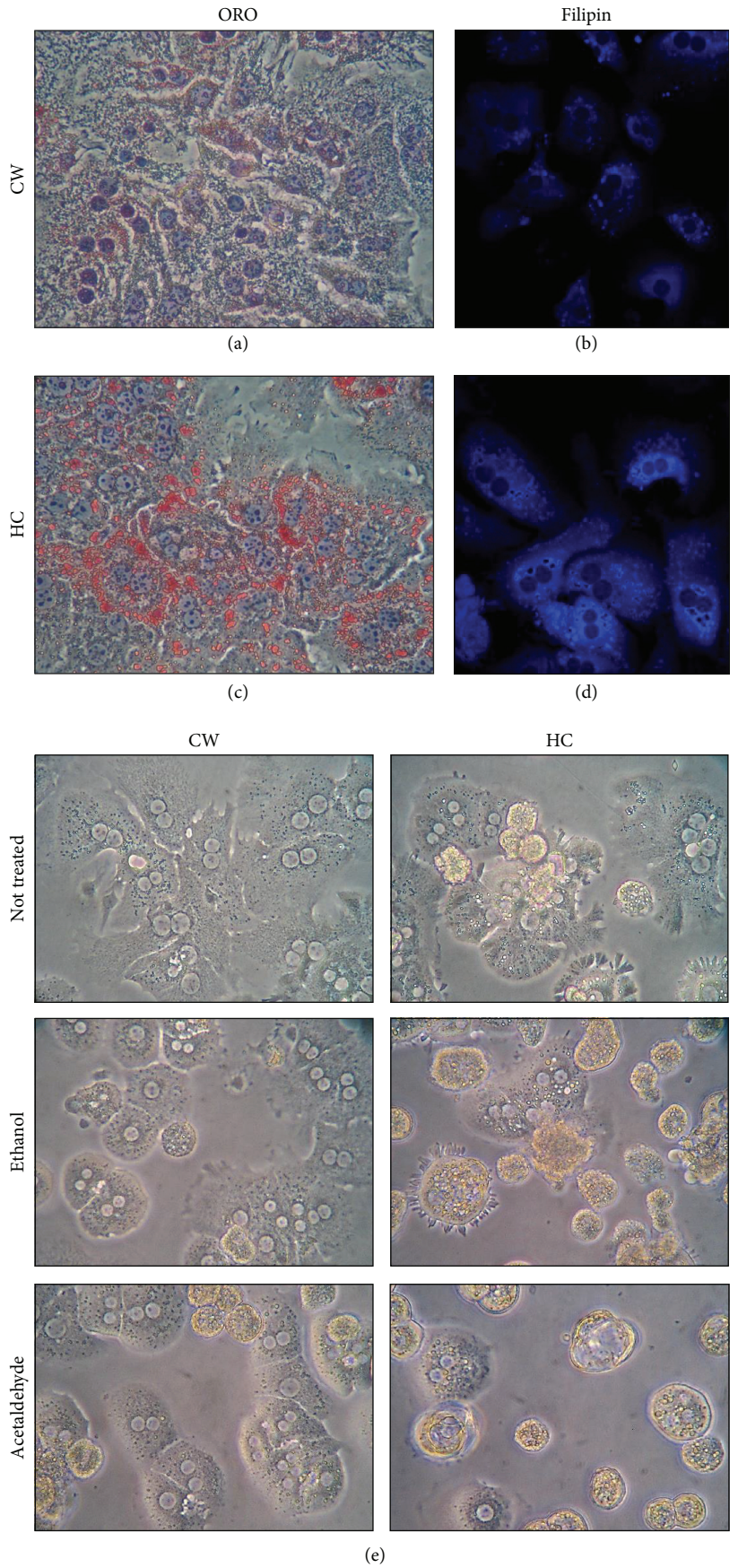


Figure 1: Continued.

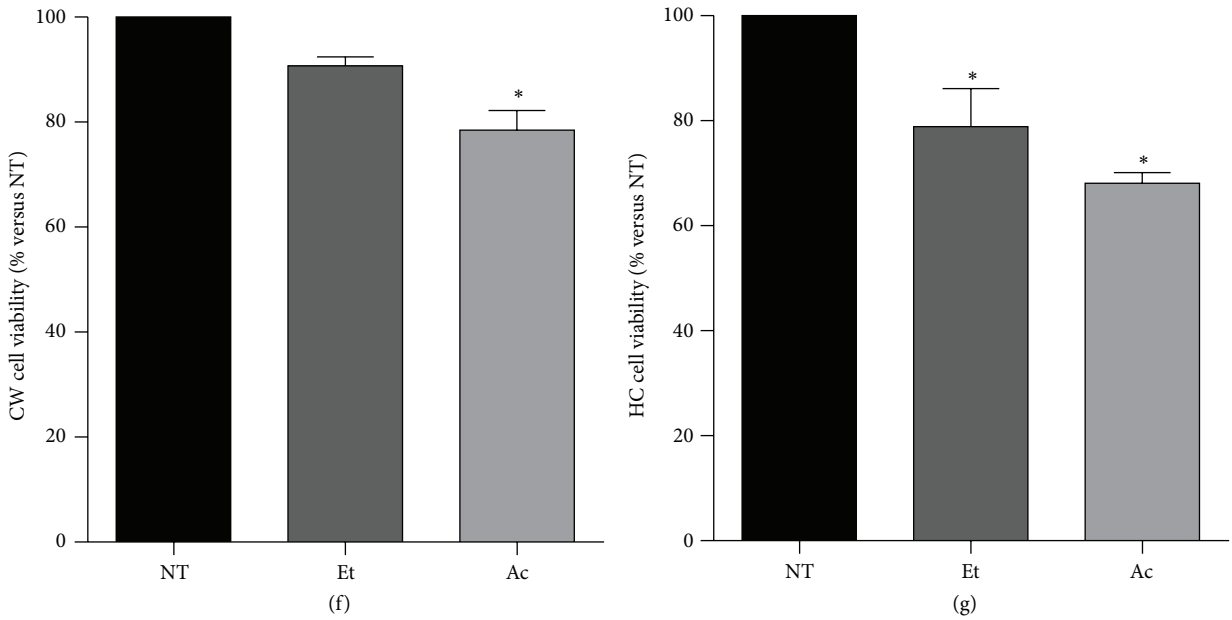


Figure 1: A high cholesterol diet induces neutral lipids and free cholesterol overload in mouse hepatocytes and decreases cell viability under ethanol and acetaldehyde treatments. Mice were fed with a high cholesterol diet (HC, 2% cholesterol) or regular standard chow (CW) for two days. Hepatocytes were isolated and plated. Oil red staining (ORO) in CW cells (a) and HC cells (c) for neutral lipids identification and filipin staining in CW cells (b) and HC cells (d) for free cholesterol determination were assayed. (e) Cell morphology determined by bright field microscopy of CW and HC cells under ethanol (100 mM) and acetaldehyde (200 μM) treatments. Cell viability assessed by crystal violet staining in (f) CW and (g) HC cells treated with ethanol (Et) and acetaldehyde (Ac). Each bar represents mean ± SEM of three independent experiments. Differences were considered significant at $^{*}p \leq 0.05$ versus NT cells. Images are representative of at least three independent experiments. Original magnification 320x.

we reported that cells under a free fatty acids overload are more susceptible to alcohol metabolism damage [6]. In the present work we focused on addressing the hepatic cell damage under high cholesterol content and the presence of Et and its toxic metabolite—Ac.

Our data show that HC cells are more susceptible to the toxic effects of Et and Ac, inducing important changes in cellular morphology, with no apparent effects in control CW cells. It is well known that oxidative stress induced by alcohol biotransformation is the main mechanism of toxicity, mainly by the activity of the Cyp2E1, as we previously probed [8]. However, ethanol metabolism toxicity reached significant differences at 48 h after treatment in cells that overexpress both Cyp2E1 and alcohol dehydrogenase (VL-17A) [8, 15]. Interestingly, in primary hepatocytes culture with high cholesterol content, we observed the toxic effects of both Et and Ac, at 24 h (Figures 1(e)–1(g)), suggesting that cholesterol could be inducing the expression or activity of the Cyp2E1 system.

It is well known that Cyp2E1 is also related to fatty acids biotransformation and it is positioned as a possible risk factor for the severity of NAFLD [16]. Our results indicate that the diet alone increased both expression and activity of Cyp2E1, possibly as an adaptive response; however the metabolism of fatty acids by this enzyme complex is related to the production of toxic intermediaries that could increase the damage [16]. Results depicted in Figure 2 clearly show that HC diet by itself increased both Cyp2E1 protein content and activity, which remained increased with both treatments. In CW cells did not increase the content, but Et induced a rise in the enzyme activity as expected; these results were in agreement with protein oxidation.

Changes in ER observed by TEM revealed significant changes in the structure of the cisternae, indicating ER stress. To confirm that ER is overloading cholesterol, we assayed the content of this lipid. The result indicated 1.8-fold the values of the CW cells. We observed megamitochondria formation in Et treatments, an undoubted effect due to oxidative stress and due also to heavy metals [17] and alcohol toxicity [18, 19]. Also, we detected autophagosomes in Et treated HC cells, but not in other treatments (Figure 3(e), arrow), indicating severe damage that is in agreement with other studies that pinpoint this effect in extensive injury, particularly by alcohol and hepatitis C virus infection [20]. This effect could serve as a protective mechanism in order to restore the normal function of the cell by eliminating compromised organelles such as mitochondria or misfolded proteins [21, 22].

Interestingly, Ac treatment induced mitochondrial fission, judged by TEM (Figure 3(f), asterisk) with modest changes in ER; although it is reported that Ac induces ER stress in HepG2 cells [23], we could not find significant changes in TEM studies.

Finally, the analysis of key markers of ER stress revealed that HC diet induced the expression of PERK, one of

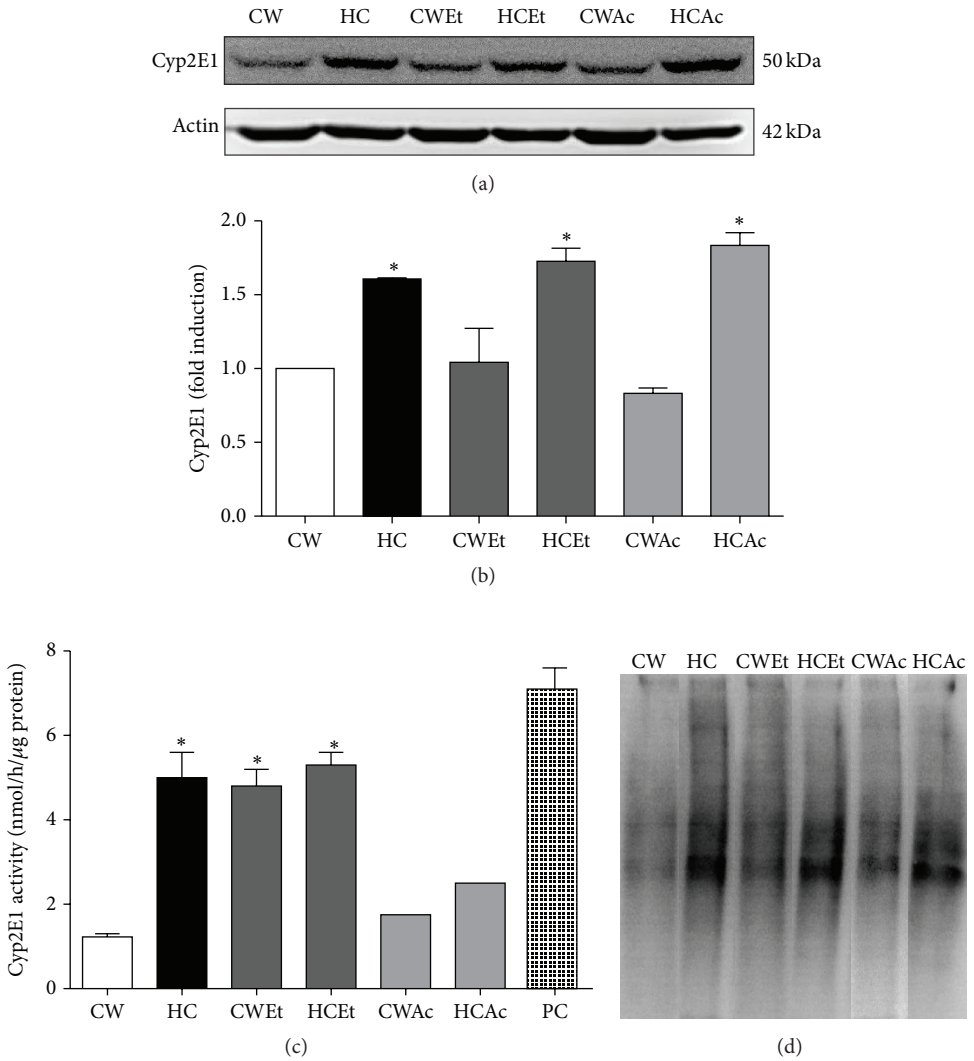


Figure 2: High cholesterol diet induces both Cyp2E1 overexpression and activity. Whole cell lysate was obtained from CW and HC cells treated or not with ethanol (Et, 100 mM) or acetaldehyde (Ac, 200 μM) and subjected to Western blotting. (a) Representative image of the Cyp2E1 immunoblot. (b) Densitometric analysis of protein content relative to actin used as loading control. (c) Cyp2E1 activity. (d) Protein oxidation determined by Oxyblot kit. Each column represents mean ± SEM of at least four independent experiments. Differences were considered significant at $^{*}P \leq 0.05$ versus NT cells. Images are representative of at least four independent experiments.

the sensors in ER stress that phosphorylates downstream substrates such as elF2α, which, in addition, impedes protein synthesis [24]. Et treatment sustained this increment, but remarkably Ac did not affect PERK expression. Ethanol by itself induced the elevation of PERK. The analysis of ATF6 nuclear localization showed positive signal only in ethanol treated HC cells, and once again we could not find changes in Ac treated cells. Data suggest that Ac induces predominantly damage by a mechanism dependent on mitochondria [10, 23] in presence and absence of cholesterol overload, but our results clearly show that cholesterol overload sensitizes cells to ethanol toxicity.

In conclusion, cholesterol overload is a determinant factor that could maintain or aggravate the alcohol-induced liver damage; a close surveillance of lipid overload is desired, particularly cholesterol, in the liver of those patients with alcohol abuse in order to avoid the exacerbation of the disease. Alcohol withdrawal is fundamental for the treatment of liver related disorders in obese patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Confocal Core Unit of the DCBS, Universidad Autónoma Metropolitana Iztapalapa, and Roberto

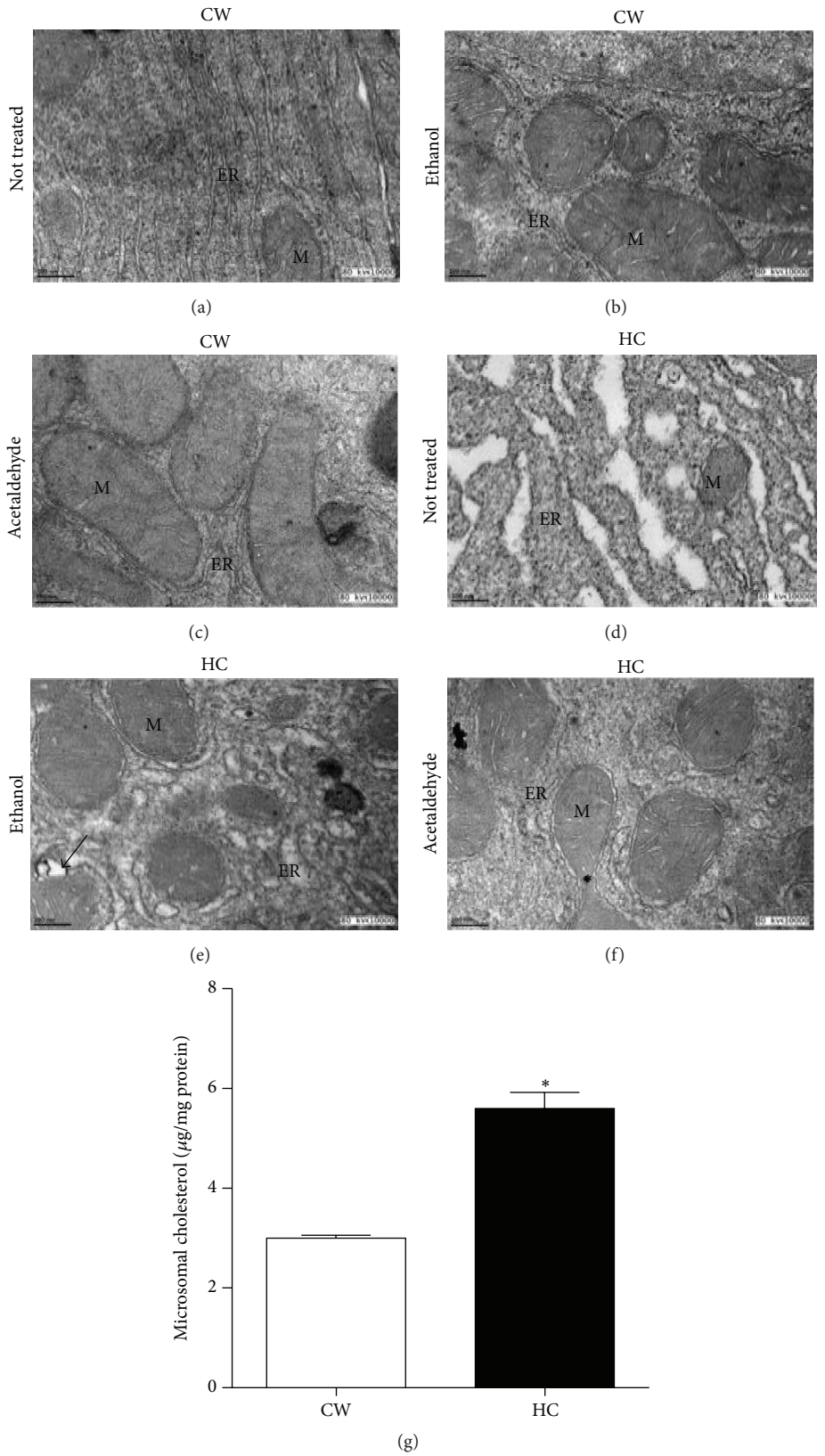


Figure 3: Cholesterol overload induces cell morphology changes. Cell morphology determined by transmission electron microscopy of CW and HC cells under ethanol (100 mM) and acetaldehyde (200 μM) treatments. M: mitochondria; ER: endoplasmic reticulum; $*$: mitochondria fission; black arrow: autophagosome. Bar 200 nm. Images are representative of at least three independent experiments. (g) Microsomal cholesterol determination. Each column represents mean ± SEM of at least four independent experiments. Differences were considered significant at $^{*}P \leq 0.05$ versus CW cells.

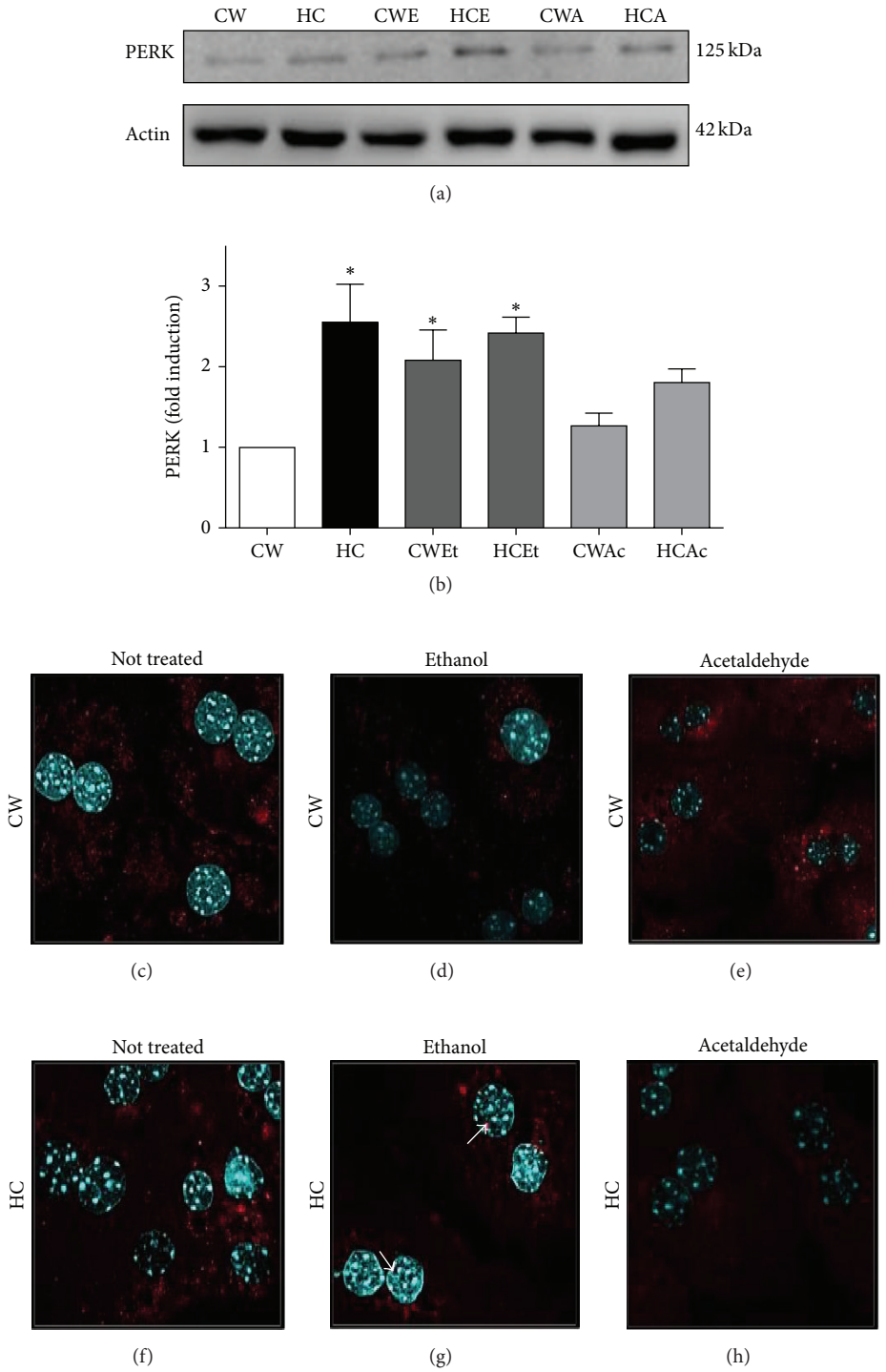


Figure 4: Cholesterol induces endoplasmic reticulum stress. Whole cell lysate was obtained from CW and HC cells treated or not with ethanol (Et, 100 mM) or acetaldehyde (Ac, 200 μM) and subjected to Western blotting. (a) Representative image of PERK immunoblot. (b) Densitometric analysis of protein content relative to actin used as loading control. (c–h) ATF6 nuclear translocation determined by confocal microscopy. ATF6 is in red and nucleus in blue. Images are representative of at least four independent experiments. Original magnification 320x.

Lazzarini-Lechuga for the assistance in confocal studies. This project was supported in part by National Science Council of Mexico (CONACYT) (nos. 166042 and 153902) and PRODEP 913026-14612111 and Universidad Autónoma Metropolitana Iztapalapa.

References

[1] P. Mathurin and R. Bataller, "Trends in the management and burden of alcoholic liver disease," *Journal of Hepatology*, vol. 62, no. 1, supplement, pp. S38–S46, 2015.

[2] C. D. Byrne and G. Targher, "NAFLD: a multisystem disease," *Journal of Hepatology*, vol. 62, no. 1, supplement, pp. S47–S64, 2015.

[3] J. Xu, K. K. Y. Lai, A. Verlinsky et al., "Synergistic steatohepatitis by moderate obesity and alcohol in mice despite increased adiponectin and p-AMPK," *Journal of Hepatology*, vol. 55, no. 3, pp. 673–682, 2011.

[4] S. Zakhari, "Bermuda Triangle for the liver: alcohol, obesity, and viral hepatitis," *Journal of Gastroenterology and Hepatology*, vol. 28, no. 1, pp. 18–25, 2013.

[5] G. Arguello, E. Balboa, M. Arrese, and S. Zanlungo, "Recent insights on the role of cholesterol in non-alcoholic fatty liver disease," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1852, no. 9, pp. 1765–1778, 2015.

[6] I. Hernández, M. Domínguez-Pérez, L. Bucio et al., "Free fatty acids enhance the oxidative damage induced by ethanol metabolism in an in vitro model," *Food and Chemical Toxicology*, vol. 76, pp. 109–115, 2015.

[7] L. E. Gómez-Quiroz, V. M. Factor, P. Kaposi-Novak, C. Coulouarn, E. A. Conner, and S. S. Thorgeirsson, "Hepatocyte-specific c-Met deletion disrupts redox homeostasis and sensitizes to Fas-mediated apoptosis," *The Journal of Biological Chemistry*, vol. 283, no. 21, pp. 14581–14589, 2008.

[8] A. Valdés-Arzate, A. Luna, L. Bucio et al., "Hepatocyte growth factor protects hepatocytes against oxidative injury induced by ethanol metabolism," *Free Radical Biology and Medicine*, vol. 47, no. 4, pp. 424–430, 2009.

[9] J. U. Marquardt, D. Seo, L. E. Gómez-Quiroz et al., "Loss of c-Met accelerates development of liver fibrosis in response to CCl_4 exposure through deregulation of multiple molecular pathways," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1822, no. 6, pp. 942–951, 2012.

[10] M. Marí, F. Caballero, A. Colell et al., "Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis," *Cell Metabolism*, vol. 4, no. 3, pp. 185–198, 2006.

[11] A. Lopez-Reyes, K. Martinez-Flores, D. Clavijo-Cornejo et al., "Cholesterol overload in hepatocytes affects nicotinamide adenine dinucleotide phosphate oxidase (NADPH) activity abrogating hepatocyte growth factor (HGF) induced cellular protection," *Gaceta Médica de México*, vol. 151, no. 4, pp. 456–464, 2015.

[12] Q. Zhang, Y. Li, T. Liang et al., "ER stress and autophagy dysfunction contribute to fatty liver in diabetic mice," *International Journal of Biological Sciences*, vol. 11, no. 5, pp. 559–568, 2015.

[13] M. C. Gutierrez-Ruiz, L. Bucio, V. Souza, and A. Carabez, "The effect of chronic and acute ethanol treatment on morphology, lipid peroxidation, enzyme activities and Na+ transport systems on WRL-68 cells," *Human & Experimental Toxicology*, vol. 14, no. 4, pp. 324–334, 1995.

[14] M. C. Gutierrez Ruiz, M. Dominguez Perez, S. Rodriguez Gonzalez, N. Nuno Lambarri, C. Licona Retama, and L. E. Gomez-Quiroz, "High cholesterol diet modifies the repairing effect of the hepatocyte growth factor," *Gaceta Médica de México*, vol. 148, no. 3, pp. 236–242, 2012.

[15] D. L. Clemens, A. Forman, T. R. Jerrells, M. F. Sorrell, and D. J. Tuma, "Relationship between acetaldehyde levels and cell survival in ethanol-metabolizing hepatoma cells," *Hepatology*, vol. 35, no. 5, pp. 1196–1204, 2002.

[16] A. K. Daly, "Relevance of CYP2E1 to non-alcoholic fatty liver disease," *Sub-Cellular Biochemistry*, vol. 67, pp. 165–175, 2013.

[17] L. Bucio, V. Souza, A. Albores et al., "Cadmium and mercury toxicity in a human fetal hepatic cell line (WRL-68 cells)," *Toxicology*, vol. 102, no. 3, pp. 285–299, 1995.

[18] T. Wakabayashi, "Megamitochondria formation—physiology and pathology," *Journal of Cellular and Molecular Medicine*, vol. 6, no. 4, pp. 497–538, 2002.

[19] T. Wakabayashi, M.-A. Teranishi, M. Karbowski et al., "Functional aspects of megamitochondria isolated from hydrazine- and ethanol-treated rat livers," *Pathology International*, vol. 50, no. 1, pp. 20–33, 2000.

[20] N. A. Osna, P. G. Thomes, and T. M. Donohue Jr., "Involvement of autophagy in alcoholic liver injury and hepatitis C pathogenesis," *World Journal of Gastroenterology*, vol. 17, no. 20, pp. 2507–2514, 2011.

[21] H.-I. Cho, J.-W. Choi, and S.-M. Lee, "Impairment of autophagosome-lysosome fusion contributes to chronic ethanol-induced liver injury," *Alcohol*, vol. 48, no. 7, pp. 717–725, 2014.

[22] C. Ji, "Advances and new concepts in alcohol-induced organelle stress, unfolded protein responses and organ damage," *Biomolecules*, vol. 5, no. 2, pp. 1099–1121, 2015.

[23] J. M. Lluis, A. Colell, C. García-Ruiz, N. Kaplowitz, and J. C. Fernández-Checa, "Acetaldehyde impairs mitochondrial glutathione transport in HepG2 cells through endoplasmic reticulum stress," *Gastroenterology*, vol. 124, no. 3, pp. 708–724, 2003.

[24] R. Sano and J. C. Reed, "ER stress-induced cell death mechanisms," *Biochimica et Biophysica Acta*, vol. 1833, no. 12, pp. 3460–3470, 2013.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1429835, 9 pages
http://dx.doi.org/10.1155/2016/1429835

Research Article

The Protective Effects of Trypsin Inhibitor on Hepatic Ischemia-Reperfusion Injury and Liver Graft Survival

Lianyue Guan,[1] Hongyu Liu,[1] Peiyao Fu,[1,2] Zhuonan Li,[1] Peidong Li,[3] Lijuan Xie,[4] Mingang Xin,[5] Zhanpeng Wang,[1] and Wei Li[1]

[1]*Department of Hepatobiliary-Pancreatic Surgery, China-Japan Union Hospital of Jilin University, Changchun 130033, China*
[2]*Department of Liver Surgery, Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, Zhongshan Hospital of Fudan University, Shanghai 200032, China*
[3]*Department of Tumor Therapy, Tumor Hospital of Jilin Province, Changchun 130033, China*
[4]*Department of Vascular Surgery, China-Japan Union Hospital of Jilin University, Changchun 130033, China*
[5]*Department of Anesthesiology, China-Japan Union Hospital of Jilin University, Changchun 130033, China*

Correspondence should be addressed to Wei Li; weili888@hotmail.com

Received 20 July 2015; Accepted 20 September 2015

Academic Editor: Pablo Muriel

Copyright © 2016 Lianyue Guan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The aim of this study was to explore the protective effects of ulinastatin (urinary trypsin inhibitor, UTI) on liver ischemia-reperfusion injury (IRI) and graft survival. We employed mouse liver cold IRI and orthotopic liver transplantation (OLTx) models. UTI was added to lactated Ringer's (LR) solution for liver perfusion and preservation *in vitro* or combined with UTI injection intraperitoneally to the liver graft recipient. Our results indicated that UTI supplementation protected the liver from cold IRI in a dose-dependent manner and prolonged liver graft survival from extended cold preserved liver donors significantly. The underlying mechanism of UTI on liver IRI may be mediated by inhibition of proinflammatory cytokine release, increasing the expression of the antiapoptotic gene *Bcl-2* and decreasing the expression of the proapoptosis genes of *Caspase-3* and *Bax*, and further protects hepatocytes from apoptotic death and improves liver function.

1. Introduction

Liver transplantation has become one of the most effective methods for the treatment of end-stage liver disease. However, hepatic ischemia-reperfusion injury (HIRI) remains a challenging issue for liver surgeons; moreover, the pathogenic mechanism of HIRI has not been elucidated completely. HIRI may be initiated by liver cell oxidative stress and may further induce primary graft nonfunction or function failure after liver transplantation. In addition, HIRI limits the applications of marginal donor livers. An effective therapeutic method for preventing HIRI is still lacking. Ulinastatin (urinary trypsin inhibitor, UTI), a trypsin inhibitor, plays an important role in inhibiting proinflammatory cytokine release. The application of UTI has been long-standing in clinical practice. It has been shown that UTI has certain protective effects against liver injury. However, the mechanisms are still not very clear and remain to be further investigated. The objectives of this study were to explore the protective effects of UTI against HIRI, the effects of UTI on liver cell oxidative stress, and the impact of UTI on graft survival, as well as the underlying mechanisms.

2. Materials and Methods

2.1. Animals. Male C57BL/6 (B6; H2b) mice, 8–14 weeks of age, were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained in a modified pathogen-free facility of Jilin Surgical Research Institute at China-Japan Union Hospital of Jilin University. The mice were provided with Purina Rodent Chow and tap water *ad libitum*. Animal care was in compliance with our institutional animal care and use committee-approved protocol and with the "Guide for the Care and

the Use of Laboratory Animals" published by the National Institutes of Health.

2.2. Reagents. UTI was purchased from Guangdong Techpool Biochemical Pharmaceutical Co., Ltd. (Guangzhou, China). Lactated Ringer's Solution was obtained from Baxter Healthcare Corporation (Deerfield, IL, USA). Rat anti-mouse IL-6, TNF-α, and IFN-γ antibodies were from BD Pharmingen (San Diego, CA, USA), rat anti-mouse IL-10 antibody was from Abcam (Cambridge, UK), Bcl-2, caspase-3, and Bax, β-actin were from Cell Signaling Technology, Inc., (Danvers, MA, USA), and the ApopTag Peroxidase In Situ Apoptosis Detection Kit was from the Millipore Corporation (Billerica, MA, USA).

2.3. UTI Treatment Protocol. The livers were flushed via the portal vein with 5 mL of cold LR or UW solution containing heparin 20 units/mL with or without UTI supplementation of 10–10000 U/mL and then were procured and stored in a sterile container containing 2 mL (equal mouse total body blood volume) perfusion solution for 6 hours. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) enzyme release from the preservative solution and the liver histology were examined, respectively. In the orthotopic liver transplantation study, the donor livers were preserved in the LR solution at an extended time of 1 hour, with or without UTI supplementation at 1000 U/mL, respectively. Liver biopsy and functional assays were performed at 1 hour, 6 hours, and 18 hours posttransplantation, respectively.

2.4. Orthotopic Liver Transplantation. Orthotopic liver transplantation (OLTx) with revascularization was accomplished with a combination of suture and cuff techniques performed between syngeneic strain combinations as described [1, 2]. The donor livers were preserved in the LR solution at an extended time of 1 hour, with or without UTI supplementation at 1000 U/mL, respectively. The liver graft recipients were also received subcutaneous injection of UTI 1000 U in 0.2 mL LR or an equal volume of LR solution immediate after complication of liver transplantation, respectively. Graft function, liver cell apoptosis, and expression of inflammatory cell factors were evaluated posttransplantation.

2.5. Liver Enzyme Assays. Samples from the liver preservation solutions at different concentrations of UTI supplementation at 6 hours in the LR solution and in the serum at 1, 6, and 18 hours posttransplantation were assayed for ALT, AST, and LDH levels using the Beckman Coulter Synchron UniCel DXC800 automatic analysis system, respectively.

2.6. Histological Analysis. Formalin-stored tissue specimens were embedded in paraffin and cut into 4 μm sections and were then examined by routine hematoxylin and eosin (H&E) staining. Three samples from each group and 10 high power-fields of each sample were analyzed. The histology scores of the liver tissue sections were determined by 2 independent persons in a blind manner according to the following scoring criteria: 0, no hepatocellular damage; 1, mild injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis; 2, moderate injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders; 3, moderate to severe injury with coagulative necrosis, abundant sinusoidal dilation, RBC extravasation into hepatic chords, and hypereosinophilia and margination of neutrophils; 4, severe necrosis with loss of hepatic architecture, disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. Criteria to specifically evaluate polymorphonuclear leukocyte (PMN) infiltration (0, zero; 1, minimal; 2, mild; 3, moderate; 4, severe) were also used [3].

2.7. TUNEL Staining. Apoptotic cells in paraffin sections (10 μm) were identified using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions, as described [1]. The sections were first deparaffinized, then fixed for 10 min at room temperature (RT) in 10% neutral buffered formalin (pH 7.4), followed by two washes (5 min each) in phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched in 2% H_2O_2, before exposure to terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 60 min. After washing in stop wash buffer (37°C, 30 min), anti-digoxigenin-peroxidase was added (RT; 30 min). Diaminobenzidine (DAB) (ScyTek Laboratories, Inc., Logan, UT, USA) was used for color development, and the sections were counterstained with hematoxylin. The numbers of apoptotic cells in the liver sections were counted under the light microscope by the numbers of apoptotic cells per 40 high-power fields in five sections per tissue per mouse (three mice per group).

2.8. Western Blot. The apoptosis-associated protein Bcl-2, caspase-3, and Bax expression in the liver tissues were determined by the Western blot assay. Proteins (50 μg/sample) extracted from the liver tissue in SDS loading buffer (50 mM Tris, pH 7.6, 10% glycerol, 1% SDS) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gel was then stained with Coomassie blue to document protein loading. The membrane was blocked with 5% dry milk and 0.1% Tween 20 (Bio-Rad) in PBS. The membrane was subsequently incubated with the primary antibodies at 4°C overnight. The primary antibodies were mouse monoclonal anti-human Bcl-2, caspase-3, and Bax antibody (Cell Signaling Technology). The membranes were developed according to the Amersham Enhanced Chemiluminescence protocol. Beta-actin was measured as a loading control.

2.9. Immunohistochemistry Staining. The paraffin sections of the liver tissues were stained for tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-10, and interferon-gamma (INF-γ) expression by the avidin-biotin-peroxidase complex (ABC) method, as described [1]. The paraffin sections were deparaffinized, followed by trypsin incubation to restore the antigen. Endogenous peroxidase activity was quenched in 2%

H_2O_2. Purified rat anti-mouse TNF-α, IL-6, IL-10, and IFN-γ antibodies (Abs) were applied (BD Pharmingen) overnight, followed by biotin-anti-rat immunoglobulin G (IgG). Rat IgG was used as isotype control. ABC (Vector Laboratories, Inc., Burlingame, CA, USA) was then added. DAB (ScyTek) was used as the substrate, and sections were counterstained with hematoxylin. The results were counted automatically under a high-power microscope (400x). The cytokine levels were analyzed using ECL Plus image lab software (Life Technology, GeneSnap, USA) and numerically expressed by the ratio of positive cells versus whole cells per high-power field. The data were averaged from 10 high-power fields for each section and 3 samples from each group.

2.10. Statistical Analysis. Statistical analysis was performed using SPSS version 22.0 software (IBM Corporation, Armonk, NY, USA). The results are expressed as mean ± SE. Comparison between groups was made by one-way analysis of variance (ANOVA), and data counts were analyzed by the chi-square test. Survival data were analyzed by the Kaplan-Mayer log-rank test. Probability (P) values < 0.05 were considered statistically significant.

3. Results

3.1. UTI Supplementation of the LR Perfusion and Preservation Solution Reduced the Liver Enzyme Release of ALT, AST, and LDH Significantly in a Dose-Dependent Manner. We first examined whether UTI supplementation of LR solution can protect the liver from cold ischemia injury. B6 mouse livers were perfused with 5 mL of cold Ringer's solution containing heparin 20 units/mL with or without the addition of UTI (10–10000 U/mL). The livers were then preserved in 2 mL of the above solution on ice for 6 hours. ALT, AST, and LDH levels from the preservation solution were tested from 3 livers pooled together in each group. The levels of ALT, AST, and LDH were detected to have significantly increased after 6 hours of cold preservation in the LR control group. The release of ALT, AST, and LDH was reduced significantly in the UTI-supplemented group compared to the LR-only group. The concentration with optimal efficacy was 1000 U/mL, while an excessive concentration (10000 U/mL) aggravated HIRI (Figure 1). Similarly, we tested UTI supplementation of UW solution at a concentration of 1000 U/mL. The release of ALT, AST, and LDH was reduced significantly in the UTI-supplemented group after 18 hours of preservation (data not shown), indicating that UTI has a uniformly protective effect on the liver.

3.2. UTI Supplementation of LR Solution Alleviates the Degree of Liver Tissue Damage and Prolongs Liver Graft Survival after an Extended Ischemia Time. To examine the protective role of UTI on hepatocytes during an extended time course of cold ischemia and the impact on liver graft survival from an extended time course of cold preservation, syngeneic orthotopic liver transplantation (OLTx) was performed in which the liver donors experienced an extended cold preservation time course in LR solution with or without the addition of

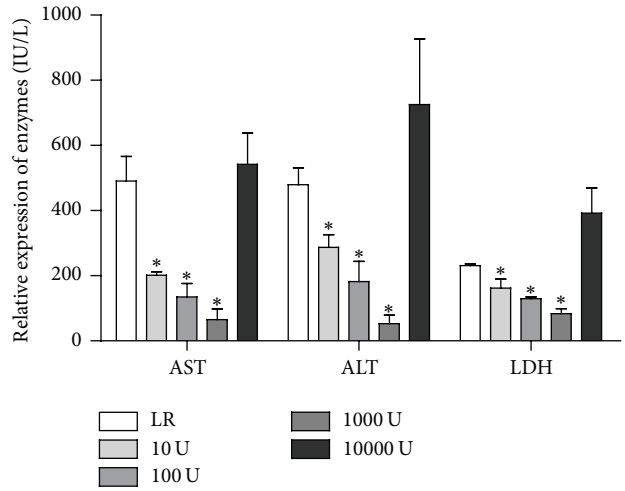


Figure 1: UTI supplementation to the perfusion and preservative LR solutions significantly inhibited liver enzyme release. UTI was added to LR solution at concentration of 10–10000 U/mL; the livers were perfused with 5 mL LR solution with or without UTI supplementation and were then procured and stored in a sterile container containing 2 mL (equal mouse total body blood volume) perfusion solution for 6 hours. The samples were pooled from 3 livers in each group; then, the ALT, AST, and LDH enzyme levels were measured. UTI concentration of 1000 U/mL showed maximal effects. Compared to LR control, $^*P < 0.05$. The results are representative of 3 separate experiments.

UTI for 1 hour. The survival rate of the liver grafts in both groups was significantly affected by an extended time course of cold preservation. However, in comparison with the LR group, the survival rate of the liver grafts was significantly improved in the group with UTI supplementation; the 3- and 7-day survival rates were 80% and 50% in the UTI-supplemented group versus 40% and 20% in the LR control group ($P < 0.05$) (Figure 2(a)). Histological examination of H&E sections of liver tissues harvested at 1, 6, and 18 hours posttransplantation revealed that hepatocyte swelling, increased cytoplasmic vacuolization, nuclear pyknosis, sinusoidal dilatation, and focal necrosis developed as early as 1 hour posttransplantation, became worst at 6 hours, and showed restoration at 18 hours posttransplantation. The addition of UTI to the LR preservation solution or combination treatment to the liver graft recipients gave the livers significant protection from cold ischemia injury during the extended preservation period. The liver morphology remained much better in those groups (Figure 2(b)). The overall pathological scores in the LR + UTI and LR + UTI + RT groups were much lower than the scores in the LR control group (Figure 2(c)).

3.3. UTI Significantly Decreased the Serum ALT, AST, and LDH Levels of Liver Graft Recipients with Extended Cold Preservation Time of Donor Livers. Liver grafts experience both cold and warm IRI during the process of transplantation. In order to prove the protective effects of UTI on liver grafts, we applied LR solution supplemented with UTI (1000 U/mL)

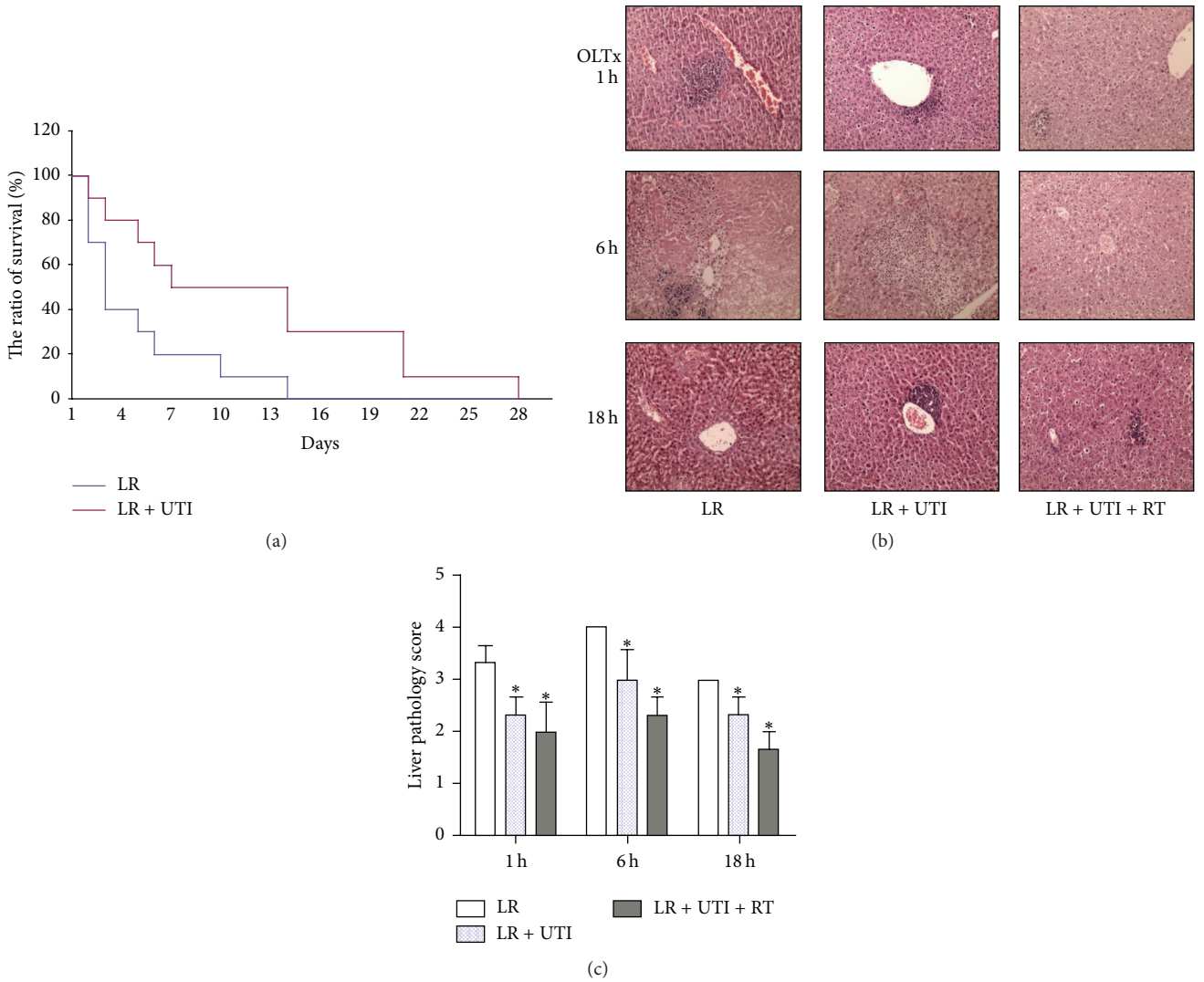


FIGURE 2: (a) Liver graft survival from extended cold-preserved donors. Syngeneic orthotopic liver transplantation was performed between inbred C57BL/6 strain mice. The liver donors were preserved *in vitro* in cold LR solution with or without UTI addition at 1000 U/mL for 1 hour. Liver graft survival was followed posttransplantation. Mean survival time was 9.8±2.44 days in the UTI-treated group ($n = 10$) versus 4.3±1.45 days in control ($n = 10$), $^{*}P < 0.05$. (b) Liver histology examination was performed by H&E staining at 1, 6, and 18 hours posttransplantation, respectively (200x). UTI alleviated the degree of liver injury and preserved the cell morphology of the extended-time cold-preserved grafts significantly. Increased hepatocyte swelling, increased cytoplasmic vacuolization, nuclear pyknosis, sinusoidal dilatation, and focal necrosis in the LR-only group with maximal injury at 6 h were observed. (c) Liver pathology scores were evaluated from the H&E sections of (b). The data are from 3 samples in each group and 3 separate experiments.

as the cold preservation solution for liver perfusion. The donor livers received extended time course cold preservation for 1 h in LR solution at 4°C, as well as intraoperative subcutaneous injection of UTI 1000 U (16 U/kg in 0.2 mL). The ALT, AST, and LDH levels in the recipient serum were detected, respectively, at 1 h, 6 h, and 18 h after transplantation. The results indicated that the serum enzyme indicators of each group increased significantly at all three time points and peaked at 6 h after transplantation. Release of AST, ALT, and LDH was remarkably reduced in the UTI preservation solution (LR + UTI) group and the combined UTI preservation plus recipient UTI administration (LR + UTI + RT) group compared to the LR control group ($P < 0.05$). In addition, the LR + UTI + RT group showed a minimum degree of enzyme increase, indicating that UTI is capable of protecting the structural integrity of hepatic cells and mitochondria and inhibiting the release of various enzymes of hepatic cells when HIRI occurs (Figure 3).

3.4. UTI Supplementation Inhibited Hepatocyte Apoptosis and Modulated Caspase-3, Bcl-2, and Bax Protein Expression. To determine the effects of UTI on liver cell apoptotic activities under the condition of cold ischemia, the liver tissue sections from the recipients with extended cold preserved liver grafts were examined at 6 h after transplantation by TUNEL staining. Our results revealed that the apoptosis

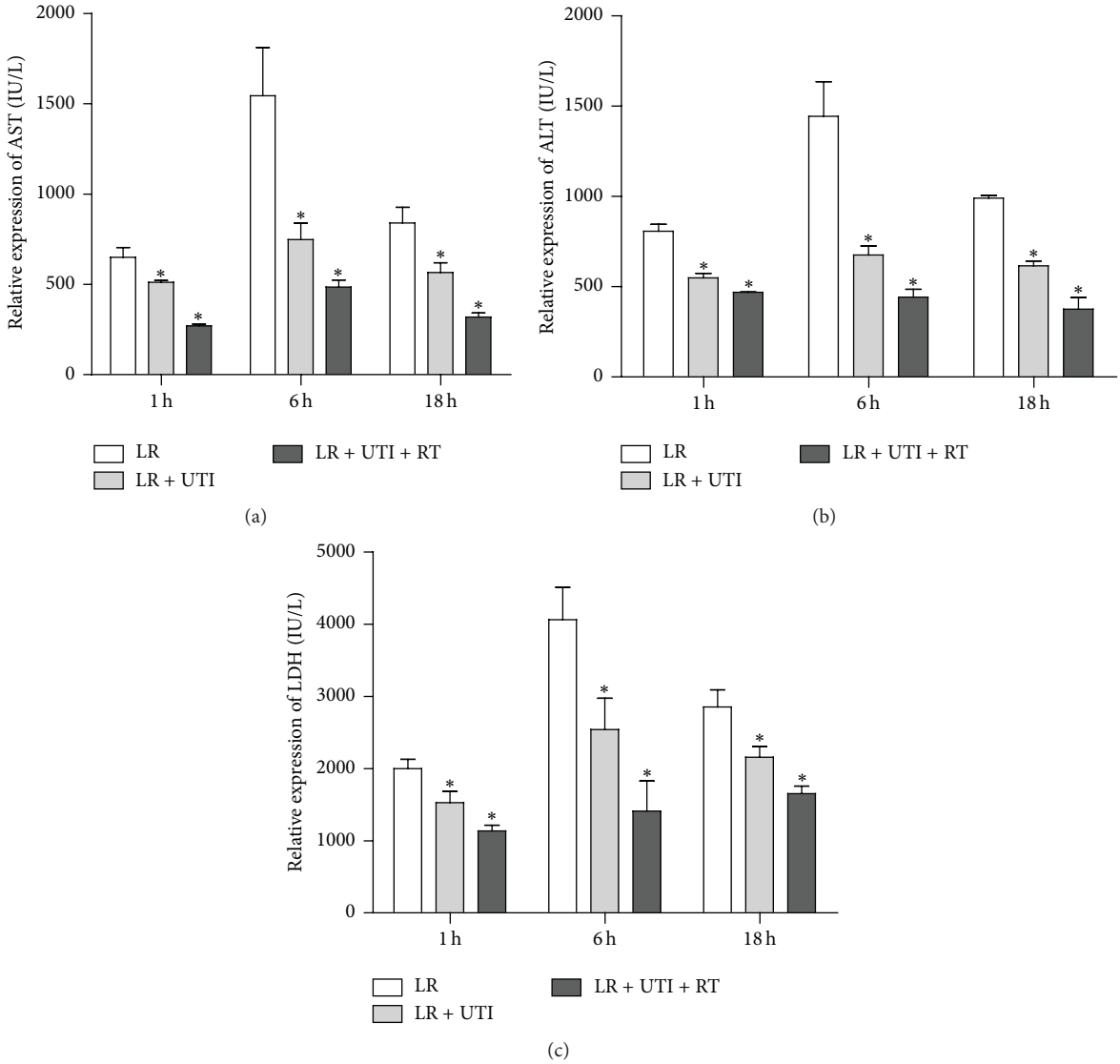


Figure 3: Change in serum enzymes of AST, ALT, and LDH after 1 h, 6 h, and 18 h from liver transplantation. The serum enzyme indicators of each group increased significantly and peaked at 6 h after transplantation. Release of AST, ALT, and LDH was remarkably reduced in the UTI preservation group and the UTI preservation combined with recipient UTI administration group, compared with the control group ($^*P < 0.05$).

rate of the hepatocytes in the UTI supplemented groups was markedly reduced compared with the LR control ($P < 0.05$) (Figures 4(a) and 4(b)). To further analyze the mechanism of UTI on reducing liver cell apoptosis, caspase-3, a cysteine protease critical for executing apoptosis, Bax, a factor to promote apoptosis, and Bcl-2, a family of proteins demonstrated to reduce apoptosis, were measured in liver tissue at 1, 6, and 18 hours after transplantation, respectively. The expression of the proapoptotic gene *Caspase-3* in the LR control group was significantly higher than that in the LR + UTI and LR + UTI + RT groups at all 3 time points after transplantation. In contrast, the anti-apoptotic gene *Bcl-2* showed relatively high expression in the two experimental groups. These results indicate that UTI effectively inhibits the apoptosis of liver cells through promoting the expression of *Bcl-2* and inhibiting the expression of the *Caspase-3* gene (Figures 4(c) and 4(d)).

3.5. UTI Significantly Inhibited the Production of Inflammatory Cytokines in the Liver Grafts. To better understand the mechanisms of UTI on HIRI, expression of proinflammatory and anti-inflammatory cytokines in extended cold-preserved liver grafts was examined by immunohistochemical staining at 1, 6, and 18 hours posttransplantation, respectively. Expression of the proinflammatory cytokines IL-6, TNF-α, and IFN-γ decreased significantly in the LR + UTI and LR + UTI + RT groups at all three time points posttransplantation compared to that in the LR control group (Figures 5(a)–5(c)). In contrast, the expression of the anti-inflammatory cytokine IL-10 was significantly higher in the LR + UTI and LR + UTI + RT

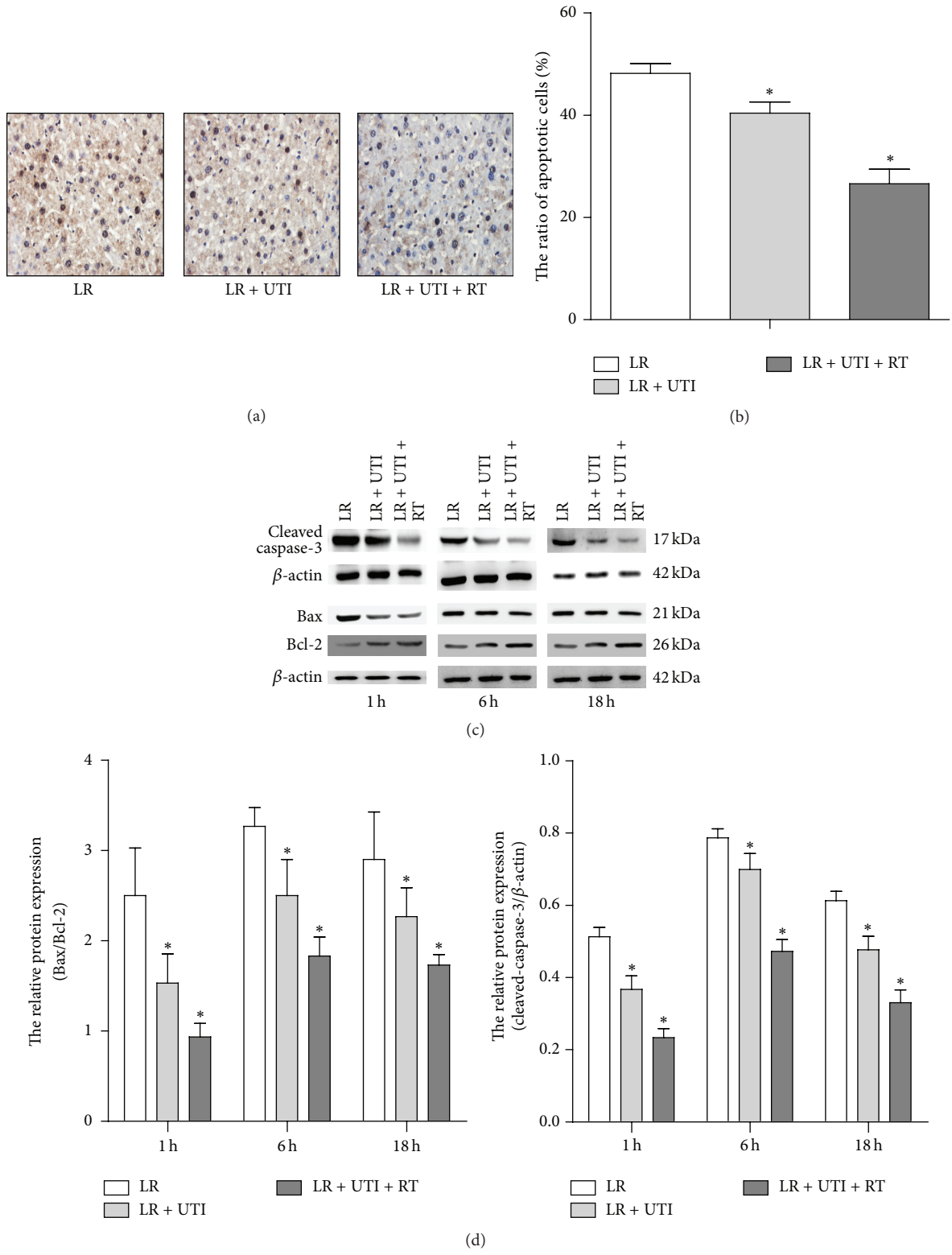


Figure 4: Hepatocyte apoptosis, *Caspase-3*, *Bcl-2,* and *Bax* gene expression on liver tissue. (a) Hepatocyte apoptosis TUNEL evaluation revealed a significantly reduced number of hepatocellular apoptotic nuclei treated with UTI at 6 hours, especially in the combined treatment group (48.2 ± 1.9 versus 40.4 ± 2.2 versus 26.6 ± 2.9), $^{*}P < 0.05$. (b) In terms of percentage calculation, three specimens in each group were stained under a 400x light microscope and 4–10 positive fields were observed in each section. The mean ratio of positive cells (brown-yellow stained cells) accounting for total cell count in each group was taken. (c) *Caspase-3* and *Bax* gene expression on liver tissue was reduced, but *Bcl-2* was increased significantly in the UTI-treated group, tested by Western blot. (d) The ratios of Bax/Bcl-2 were 1.5 ± 0.3 versus 0.9 ± 0.2 versus 2.5 ± 0.5, 2.5 ± 0.4 versus 1.8 ± 0.2 versus 3.3 ± 0.2, and 2.3 ± 0.3 versus 1.7 ± 0.1 versus 2.9 ± 0.5. Cleaved-caspase-3/beta-actin was 0.4 ± 0.04 versus 0.2 ± 0.02 versus 0.5 ± 0.02, 0.7 ± 0.04 versus 0.5 ± 0.03 versus 0.8 ± 0.03, and 0.5 ± 0.04 versus 0.3 ± 0.04 versus 0.6 ± 0.03 in the LR + UTI, LR + UTI + RT, and LR groups at 1 h, 6 h, and 18 hours after OLTx, respectively. ($^{*}P < 0.05$).

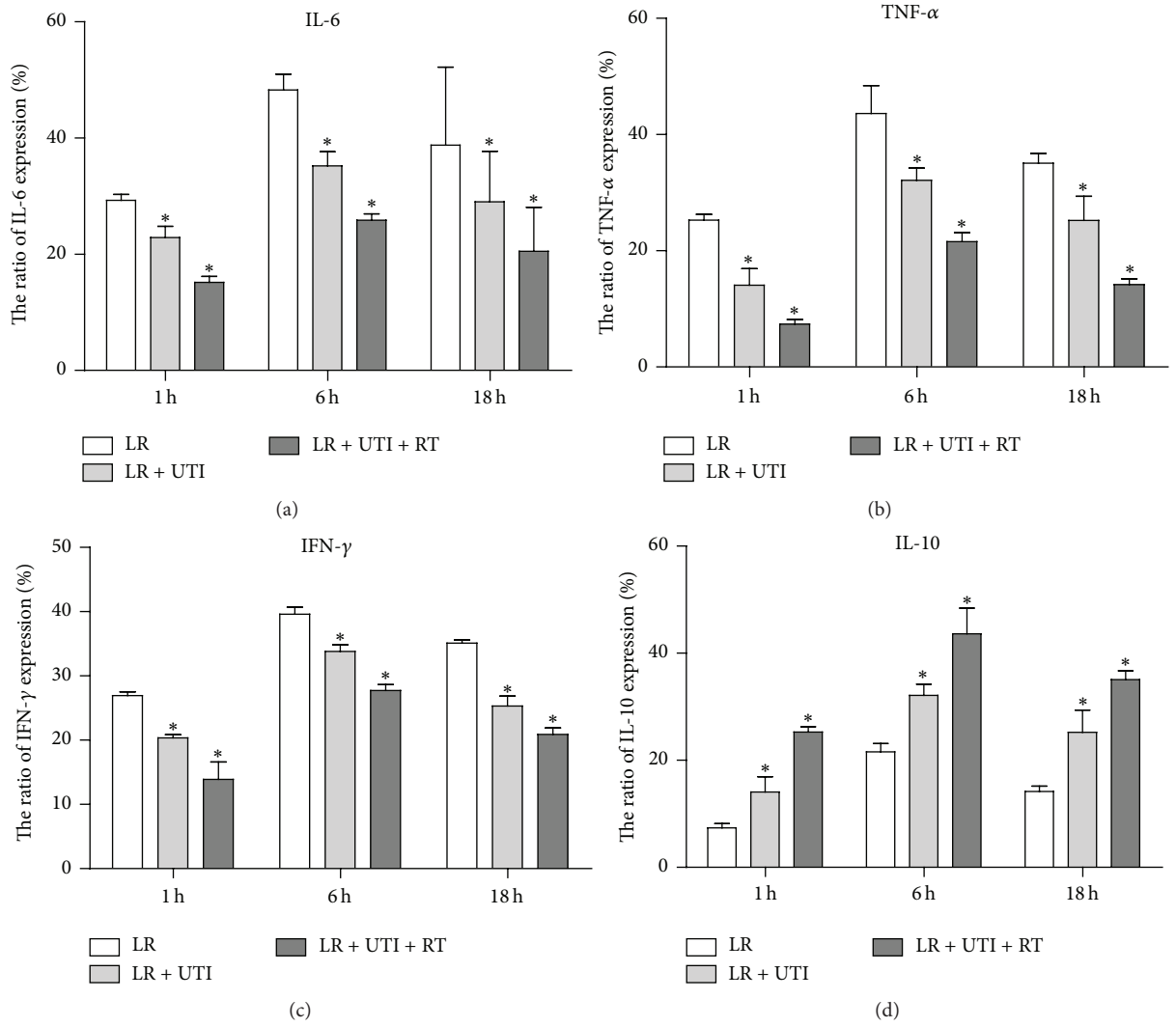


Figure 5: Cytokine levels in the liver grafts at 1 h, 6 h, and 18 h after OLTx. Production of proinflammatory cytokines IL-6, TNF-α, and IFN-γ in the liver tissues of the UTI-supplemented LR solution with extended cold preservation time was reduced, and IL-10 was increased significantly at 1, 6, and 18 hours after OLTx. $^{*}P < 0.05$. The data were pooled from 3 samples in each group.

groups than in the LR control group (Figure 5(d)). Overall, UTI inhibited the production and release of proinflammatory cytokines and promoted the expression of anti-inflammatory cytokines, which contributed at least in part to the protection of the liver against cold IRI.

4. Discussion

HIRI is a complicated pathophysiological process affected by multiple factors. UTI, as a broad-spectrum protease inhibitor, inhibits multiple proteases including trypsin, α-chymotrypsin, hyaluronidase, plasmin, and more [4]. UTI is also capable of inhibiting the release of inflammatory mediators, stabilizing lysosomal membrane, removing harmful free radicals, and inhibiting myocardial inhibitory factor [5]. UTI has been used clinically to treat acute or chronic pancreatitis, severe infection, and acute organ failure. It has also been proved to be an effective perioperative treatment in liver surgery [6–8]. However, the role of UTI in liver transplantation has been seldom reported. Therefore, it is worthwhile to investigate the effects of UTI on liver cold IRI and graft survival from extended cold-preserved donor livers.

The results of the present study showed that the levels of the liver enzymes AST, ALT, and LDH were significantly increased at 6 hours of cold preservation and that supplementation with UTI in either LR or UW preservation solution effectively reduced liver enzyme release. The best protective concentration of UTI was 1000 U/mL, indicating that UTI protects the liver from IRI in a dose-dependent manner. Similarly, in the liver transplantation experiments using the donor livers that experienced extended cold preservation time *in vitro*, the addition of UTI significantly reduced the serum enzyme levels of the liver graft recipients posttransplantation. The maximum reduction in enzyme levels was shown in the group of liver graft recipients who received the combined UTI treatment during the perioperative period. Moreover,

liver graft survival was significantly prolonged in the UTI-treated groups. Our results demonstrated that UTI plays a protective role against liver IRI, both in LR and in UW solution, suggesting that UTI may be a useful reagent to extend the cold preservation time of donor livers and offers therapeutic potential to recover more marginal donor livers for organ transplantation.

The protective effects of UTI on liver IRI were directly evidenced by histopathological changes. In comparison with the control group, UTI not only protected the liver cell morphology from cold ischemic injury but also reduced hepatocyte apoptosis and promoted the repair of liver tissue at 18 hours after reperfusion. This suggests that the protective effects of UTI may occur through stabilizing the lysosomal and cell membranes, maintaining the integrity of the vascular endothelial cells, reducing inflammatory cell infiltration, and preventing liver cell apoptosis.

The occurrence of apoptosis is regulated by several pathways, including the caspase-3 and Bcl-2 family members. Caspase-3 is the most important terminal shear enzyme in the process of cell apoptosis and is an important component of the killing mechanism of the cytotoxic T cells. Many studies have shown that the expression of caspase-3 increases significantly when the liver undergoes IRI, which could activate endonucleases and cause liver cell apoptosis by degrading nucleoproteins and activating the release of cytochrome C [9–11]. The Bcl-2 family includes many members, such as the *Mcl-1*, *Bcl-w*, *Bcl-x*, *Bax,* and *Bak* genes, which have either anti- or proapoptosis functions. *Bax* is one of the *Bcl-2* family members that are involved in cell apoptosis, while *Bcl-2* can reduce the release of cytochrome C induced by *Bax* and control apoptosis. Currently, the *Bax/Bcl-2* ratio is usually used to portray the relationship between cell survival and apoptosis; the lower the ratio is, the more significant the anti-apoptosis effects are [12]. The results from our experiments indicate that the *Bax/Bcl-2* and caspase-3/β-actin ratios were significantly decreased at the different time courses of 1, 6, and 18 hours after OLTx in the LR + UTI and LR + UTI + RT groups than in the LR control group, indicating that the mechanisms of UTI in protecting the liver from IRI may occur through upregulating the expression of *Bcl-2* and downregulating the expression of *Caspase-3*. The mechanism by which UTI protects the liver from IRI is also attributed to the functions of the antioxidative and stabilizing mitochondrial membrane of UTI [5].

The liver is an organ that produces many cytokines that are involved in a complex regulation network during HIRI. Our study has demonstrated that the cytokines IL-6, IL-10, IFN-γ, and TNF-α are all involved in the HIRI process. UTI treatment significantly inhibits the production of IL-6, IFN-γ, and TNF-α but increases the production of IL-10 in the liver at all time courses after liver transplantation. We presume that UTI regulates the production of inflammatory factors in liver tissue after HIRI, resulting in overexpression of anti-inflammatory cytokines, which provides protective effects against damage to the liver tissue. Other studies have also indicated that the protective effects of UTI against liver IRI are probably mediated by inhibiting TNF-α and IL-6 through inhibition of the oxidative stress response at the early stage of reperfusion and the activation of monocytes [13]. As we know, the serum cytokine level may more accurately reflect the degree of inflammation in transplant recipients. In order to appropriately elucidate the role of UTI in the regulation of inflammatory cytokines and chemokines, further investigation is needed on the recipient serum cytokine network.

The present study proves that UTI as a supplement added to cold preservation solution for donor livers and perioperative treatment to liver graft recipients both exert strong effects in protecting the liver from the deleterious effects of cold IRI and prolong liver graft survival in recipients who receive extended cold-preserved donor livers. Although our study had the limitations of small sample size and short follow-up time, the protective effects of UTI on HIRI are evidenced by inhibition of liver enzyme release, reduction of the liver pathology score, promotion of anti-inflammatory cytokine production, and decrease in liver cell apoptosis. Studies on other types of protease inhibitors, such as the ubiquitin-proteasome system (UPS) and secretory leukocyte protease inhibitor (SLPI), have shown that these protease inhibitors also have promising therapeutic potential of inhibiting cardiac and liver IRI through the inhibition of IL-1 and TNF-α, increase of NO generation, and control of neutrophil extracellular trap (NET) generation [14, 15].

Despite the limitations of the present study, the results are very encouraging. At the least, the study outcome demonstrates the great potential of future application of UTI in liver surgery and the use of marginal donor livers in living donor liver transplantation.

Abbreviations

Bcl: B-cell lymphoma
DC: Dendritic cell
IFN-γ: Interferon-gamma
IL: Interleukin
IRI: Ischemia-reperfusion injury
LR: Lactated Ringer's solution
OLTx: Orthotopic liver transplantation
TNF-α: Tumor necrosis factor-alpha
UTI: Ulinastatin.

Disclosure

This work was presented in part at the 8th Annual Meeting of the Chinese College of Surgeons and 19th Annual Meeting of the European Society of Surgery (May 15–17, 2015, Beijing, China), and the 6th Congress of The Federation of Immunological Societies of Asia Oceania (FIMSA 2015, June 30–July 3, 2015, Singapore).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lianyue Guan primarily drafted the paper and performed experiments; Hongyu Liu, Zhuonan Li, and Peidong Li performed the *in vivo* experiments in part; Peiyao Fu, Mingang Xin, Zhanpeng Wang, and Lijuan Xie performed the *in vitro* experiments in part and conducted the data collection and interpretation; Wei Li contributed to the concept design, critical revision, and finalization of the paper.

Acknowledgments

This paper was supported by Nature Science Foundation of China (NSFC) (81170416; 81273264); Jilin Province Science & Technology Bureau International Cooperation Fund (2011742); Techpool Research Fund (01201046).

References

[1] W. Li, C. S. Kuhr, X. X. Zheng et al., "New insights into mechanisms of spontaneous liver transplant tolerance: the role of Foxp3-expressing $CD25^+CD4^+$ regulatory T cells," *American Journal of Transplantation*, vol. 8, no. 8, pp. 1639–1651, 2008.

[2] X.-D. Shen, B. Ke, Y. Zhai et al., "Absence of toll-like receptor 4 (TLR4) signaling in the donor organ reduces ischemia and reperfusion injury in a murine liver transplantation model," *Liver Transplantation*, vol. 13, no. 10, pp. 1435–1443, 2007.

[3] J. D. Lang Jr., X. Teng, P. Chumley et al., "Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation," *The Journal of Clinical Investigation*, vol. 117, no. 9, pp. 2583–2591, 2007.

[4] C. Umeadi, F. Kandeel, and I. H. Al-Abdullah, "Ulinastatin is a novel protease inhibitor and neutral protease activator," *Transplantation Proceedings*, vol. 40, no. 2, pp. 387–389, 2008.

[5] Y. Koga, M. Fujita, R. Tsuruta et al., "Urinary trypsin inhibitor suppresses excessive superoxide anion radical generation in blood, oxidative stress, early inflammation, and endothelial injury in forebrain ischemia/reperfusion rats," *Neurological Research*, vol. 32, no. 9, pp. 925–932, 2010.

[6] H. Yang, Y. Mao, X. Lu et al., "The effects of urinary trypsin inhibitor on liver function and inflammatory factors in patients undergoing hepatectomy: a prospective, randomized, controlled clinical study," *The American Journal of Surgery*, vol. 202, no. 2, pp. 151–157, 2011.

[7] H. Takano, K.-I. Inoue, A. Shimada, H. Sato, R. Yanagisawa, and T. Yoshikawa, "Urinary trypsin inhibitor protects against liver injury and coagulation pathway dysregulation induced by lipopolysaccharide/D-galactosamine in mice," *Laboratory Investigation*, vol. 89, no. 7, pp. 833–839, 2009.

[8] C.-W. Jeong, C.-S. Lee, S.-H. Lee, H. J. Jeung, and S.-H. Kwak, "Urinary trypsin inhibitor attenuates liver enzyme elevation after liver resection," *Korean Journal of Anesthesiology*, vol. 63, no. 2, pp. 120–123, 2012.

[9] S. Hassan-Khabbar, M. Vamy, C.-H. Cottart et al., "Protective effect of post-ischemic treatment with trans-resveratrol on cytokine production and neutrophil recruitment by rat liver," *Biochimie*, vol. 92, no. 4, pp. 405–410, 2010.

[10] P. Georgiev, F. Dahm, R. Graf, and P.-A. Clavien, "Blocking the path to death: anti-apoptotic molecules in ischemia/reperfusion injury of the liver," *Current Pharmaceutical Design*, vol. 12, no. 23, pp. 2911–2921, 2006.

[11] D. Angoulvant, F. Ivanes, R. Ferrera, P. G. Matthews, S. Nataf, and M. Ovize, "Mesenchymal stem cell conditioned media attenuates in vitro and ex vivo myocardial reperfusion injury," *Journal of Heart and Lung Transplantation*, vol. 30, no. 1, pp. 95–102, 2011.

[12] S. J. Korsmeyer, J. R. Shutter, D. J. Veis, D. E. Merry, and Z. N. Oltvai, "Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death," *Seminars in Cancer Biology*, vol. 4, no. 6, pp. 327–332, 1993.

[13] Y.-Z. Cao, Y.-Y. Tu, X. Chen, B.-L. Wang, Y.-X. Zhong, and M.-H. Liu, "Protective effect of ulinastatin against murine models of sepsis: inhibition of TNF-α and IL-6 and augmentation of IL-10 and IL-13," *Experimental and Toxicologic Pathology*, vol. 64, no. 6, pp. 543–547, 2012.

[14] M. A. Zaouali, F. Bardag-Gorce, T. Carbonell et al., "Proteasome inhibitors protect the steatotic and non-steatotic liver graft against cold ischemia reperfusion injury," *Experimental and Molecular Pathology*, vol. 94, no. 2, pp. 352–359, 2013.

[15] K. Zabieglo, P. Majewski, M. Majchrzak-Gorecka et al., "The inhibitory effect of secretory leukocyte protease inhibitor (SLPI) on formation of neutrophil extracellular traps," *Journal of Leukocyte Biology*, vol. 98, no. 1, pp. 99–106, 2015.