SUN,QIAN, Ph.D. Hepatic Zinc Deficiency in Alcoholic Liver Disease: Dysregulation of Zinc Transporters and Activation of Mitochondrial Apoptotic Cell Death Pathway.(2015) Directed by Dr. Zhanxiang Zhou. 139 pp.

Excessive alcohol consumption exerts health concerns, and alcoholic liver disease (ALD) is a major cause of morbidity and mortality in the United States. However, there is still lack of an effective treatment. Zinc deficiency has been found in alcoholics over a half century ago. Experimental animals chronically exposed to alcohol exhibit decreased zinc level in the liver as well. Therefore, it is critical to determine the role of zinc deficiency in the pathogenesis of ALD.

Our laboratory has repeatedly demonstrated that alcohol feeding significantly decreased hepatic zinc levels in rodents. In addition, clinical studies have shown that the severity of zinc deficiency is positively correlated with the stage of ALD. However, the mechanism that underlies alcohol-induced hepatic zinc deficiency is still unclear. Therefore, we hypothesized that dysregulation of hepatic zinc transporters, ZIP and ZnT, result in alcohol-induced zinc deficiency in the liver and decreased zinc levels in the liver enhanced intrinsic apoptotic cell death pathway via endoplasmic reticulum (ER) and mitochondrial stress. Aim 1 of this project investigated the expression of zinc transporters in alcohol- and pair-fed mice at different time points, along with the effect of reactive oxygen species (ROS) on the expression of zinc transporters. The results indicate that increased ROS due to chronic alcohol exposure altered the expression of zinc transporters, which account for the decrease in hepatic zinc level.

Our laboratory also demonstrated that zinc deficiency contributes to ALD by decreasing β-oxidation and blunting very low density lipoprotein (VLDL) secretion.

Furthermore, zinc deficiency is associated with increased oxidative stress. Zinc supplementation protects against alcohol-induced liver injury. Given the fact that increased oxidative stress and impaired mitochondrial functions are closely linked with the pathogenesis of apoptosis and increased hepatic apoptosis plays a critical role in the development of ALD, the role of zinc deficiency in the pathogenesis of apoptosis was assessed in Aims 2 and 3. In Aim 2, increased endoplasmic reticulum (ER) stress indicated by C/EBP homologous protein (CHOP) expression after zinc deprivation was linked with the activation of mitochondrial apoptotic cell death pathway, which was partially dependent on oxidative stress. Data obtain in Aim 3 demonstrated that mitochondrial biogenesis regulators were downregulated by zinc deprivation, which resulted in defect of the mitochondrial electron transport chain. As a result, overproduction of ROS and decreased mitochondrial membrane potential occurred.

Collectively, these findings provided critical insights into the molecular mechanisms of alcohol consumption-induced zinc deficiency and zinc deficiency-induced activation of apoptosis. However, we still need to investigate i) the direct effect of subcellular zinc deficiency on organelle functions, and ii) the beneficial effect of dietary zinc supplementation on subcellular zinc deficiency as well as organelle functions.

HEPATIC ZINC DEFICIENCY IN ALCOHOLIC LIVER DISEASE: DYS-REGULATION OF ZINC TRANSPORTERS AND ACTIVATION OF MITOCHONDRIAL APOPTOTIC CELL DEATH PATHWAY

by

Qian Sun

A Dissertation Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
2015

Approved by

_____
Committee Chair

APPROVAL PAGE

This dissertation written by Qian Sun has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____
Zhanxiang Zhou

Committee Members _____
Michael K. McIntosh

_____
Ron F. Morrison

_____
Keith Erikson

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to expression my deepest appreciation to my parents, Yongshun Sun and Zhonglu Yang, for giving me life and letting me receive good education. All that hard work and determination that they have given me, allowed me to obtain my today's accomplishment. I would also like to express my appreciation to my beloved husband, Yuchuan Zhou, for being so understanding and encouraging when I am in bad mood and feel frustrated about the negative experimental results. I appreciate all the love, care, and supportive that I have received from my family.

I am gratitude that I have the chance to pursue my doctoral study with a wisdoms mentor, Dr. Zhanxiang Zhou. He is knowledgeable and open to new ideas. He is a principal investigator and love to help out with experiments whenever we are short-handed. He is a teacher and also a friend. His earnest guidance and constant encouragement helped me get through difficulties and stress while doing my dissertation project in the last three and one half years. I would also like to thank my committee members, Dr.Keith Erikson, Dr. Michael McIntosh, and Dr. Ron Morrison. They are my exceptional resources for references supports.

I would like to thank the members of our research team, Dr. Wenliang Zhang, Dr. Wei Zhong, Dr. Xiuhua Sun, Dr. Xiaobing Tan, Dr. Qiong Li, Dr. Daoyin Dong and Xinguo Sun for providing outstanding training and valuable advice for my experiments and manuscripts. I would also like to thank Donna Gentry and Cherie Turner for their wonderful administrative assistance. I would like to extend my thanks to the research

group that collaborated on my dissertation: Dr. Xiang Zhang, Xinmin Yin, and Jiayang Zhang (Department of Chemistry, University of Louisville). Without the tremendous help from all of them, I could not accomplish my dissertation research.

Last but not the least, I would like to extend my appreciation to all my dear friends in the Department of Nutrition as well as at the North Carolina Research Campus especially Fuli He. They are not only my good source for research ideas and techniques, but they also make my life colorful in Kannapolis.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Overview

Alcoholism is a major health problem in the United States [1]. Alcoholic liver disease (ALD) is one of the major causes of alcohol-induced morbidity and mortality [2]. Since alcohol contributes to calories, excessive and prolonged alcohol consumption results in malnutrition by decreasing intake of nutritional food [3]. In addition, increased production of toxicants and reactive oxygen species (ROS), as well as altered redox state while metabolizing alcohol also contribute to alcohol-induced nutrient imbalance [4]. Among all the nutrients affected by alcohol consumption, zinc is the one showing the most consistent reduction after prolonged alcohol consumption [5-13]. Base on the importance of zinc as a cofactor in a large number of protein/enzymes, zinc deficiency would serve as a crucial pathological factor in the development of ALD. This project aimed at understanding the mechanisms of how alcohol induced haptic zinc deficiency and how zinc deficiency mediates alcohol-induced apoptotic cell death.

Our laboratory has previously demonstrated that chronic alcohol consumption-induced liver injury is associated with reduction of hepatic zinc levels in mice and rats, and zinc supplementation, at least partially, reversed hepatic lipid accumulation in association with improvement of alcohol-induced zinc levels [14-16]. Mechanistic study

demonstrated that zinc supplementation accelerated β-oxidation by upregulating gene expression of Cpt1α and acdl and VLDL secretion by upregulating gene expression of Mttp and Apob [14]. Additionally, the activity of hepatocyte nuclear factor 4 alpha (HNF-4α) [14, 17] and peroxisome proliferator-activated receptor alpha (PPAR-α) [14],were both improved. These results suggested that zinc dyshomeostasis critically mediates alcohol-induced pathogenesis. However, the molecular mechanisms of how alcohol causes zinc dyshomeostasis in the liver remain unclear, which will be determined in Aim 1 of this project by measuring zinc transporters. Alcohol consumption-induced cell death plays an important role in the progression of ALD [19]. Both clinical and experimental ALD studies revealed a positive correlation between the number of apoptotic cells and the severity of ALD [20]. Although activation of TNFα/TRNR1 and FasL/Fas pathway are believed to play a role in the pathogenesis of apoptosis in ALD [18, 21], the precise mechanism has not been fully understood. Therefore, Aim 2 and Aim 3 study aimed to determine the role of zinc deficiency in the pathogenesis of apoptosis. Aim 2 focused on the role of zinc deficiency in ER stress-induced apoptosis. It has been reported that alcohol consumption altered mitochondrial ultrastructure, along with increased oxidative stress [16]. Mitochondrial electron transport chain (ETC) is a critical source of ROS production secondary to ethanol administration [22, 23], and antioxidant treatment protected mitochondrial function and reduced apoptosis [24]. Therefore, Aim 3 aimed to determine the role of zinc deficiency in mitochondrial dysfunction-induced apoptosis.

Central Hypothesis and Specific Objectives

The *long-term goal* of the current project is to develop effective strategies for prevention and treatment for ALD. The *central hypothesis* of my dissertation research is that 1) dysregulation of hepatic zinc transporters, ZIP and ZnT, result in alcohol-induced zinc deficiency in the liver, and 2) decreased zinc levels enhanced intrinsic apoptotic cell death pathway via ER and mitochondrial stress.

In order to achieve the long-term goal, I assessed the central hypothesis and accomplished the overall objective of the current project by pursing the following specific aims: Aim 1) To determine the effect of alcohol exposure on zinc homeostasis and hepatic zinc transporters at different time points; 2) To define the link between zinc deficiency and ER stress/CHOP mediated intrinsic apoptosis; 3) To determine the link between zinc deficiency and mitochondrial stress-induced intrinsic apoptosis.

References

1. Moghe A, Joshi-Barve S, Ghare S, Gobejishvili L, Kirpich I, McClain CJ, et al. Histone modifications and alcohol-induced liver disease: are altered nutrients the missing link? World journal of gastroenterology : WJG. 2011;17(20):2465-72.

2. Purohit V, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. Alcoholism, clinical and experimental research. 2009;33(2):191-205.

3. Lieber CS. Relationships between nutrition, alcohol use, and liver disease. Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism. 2003;27(3):220-31.

4. Lieber CS. ALCOHOL: its metabolism and interaction with nutrients. Annual review of nutrition. 2000;20:395-430.

5. Vallee BL, Wacker WE, Bartholomay AF, Hoch FL. Zinc metabolism in hepatic dysfunction. II. Correlation of metabolic patterns with biochemical findings. The New England journal of medicine. 1957;257(22):1055-65.

6. Kahn AM, Helwig HL, Redeker AG, Reynolds TB. Urine and serum zinc abnormalities in disease of the liver. American journal of clinical pathology. 1965;44(4):426-35.

7. Sullivan JF, Heaney RP. Zinc metabolism in alcoholic liver disease. The American journal of clinical nutrition. 1970;23(2):170-7.

8. Walker BE, Dawson JB, Kelleher J, Losowsky MS. Plasma and urinary zinc in patients with malabsorption syndromes or hepatic cirrhosis. Gut. 1973;14(12):943-8.

9. Kiilerich S, Dietrichson O, Loud FB, Naestoft J, Christoffersen P, Juhl E, et al. Zinc depletion in alcoholic liver diseases. Scandinavian journal of gastroenterology. 1980;15(3):363-7.

10. McClain CJ, Su LC. Zinc deficiency in the alcoholic: a review. Alcoholism, clinical and experimental research. 1983;7(1):5-10.

11. Kang YJ, Zhou Z. Zinc prevention and treatment of alcoholic liver disease. Molecular aspects of medicine. 2005;26(4-5):391-404.

12. Wang J, Pierson RN, Jr. Distribution of zinc in skeletal muscle and liver tissue in normal and dietary controlled alcoholic rats. The Journal of laboratory and clinical medicine. 1975;85(1):50-8.

13. Barak AJ, Beckenhauer HC, Kerrigan FJ. Zinc and manganese levels in serum and liver after alcohol feeding and development of fatty cirrhosis in rats. Gut. 1967;8(5):454-7.

14. Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, et al. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. Hepatology. 2009;50(4):1241-50.

15. Zhong W, Zhao Y, Sun X, Song Z, McClain CJ, Zhou Z. Dietary zinc deficiency exaggerates ethanol-induced liver injury in mice: involvement of intrahepatic and extrahepatic factors. PloS one. 2013;8(10):e76522.

16. Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, Kang YJ. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. The American journal of pathology. 2005;166(6):1681-90.

17. Kang X, Song Z, McClain CJ, Kang YJ, Zhou Z. Zinc supplementation enhances hepatic regeneration by preserving hepatocyte nuclear factor-4alpha in mice subjected to long-term ethanol administration. The American journal of pathology. 2008;172(4):916-25.

18. Zhou Z, Liu J, Song Z, McClain CJ, Kang YJ. Zinc supplementation inhibits hepatic apoptosis in mice subjected to a long-term ethanol exposure. Experimental biology and medicine. 2008;233(5):540-8.

19. Ishii H, Adachi M, Fernandez-Checa JC, Cederbaum AI, Deaciuc IV, Nanji AA. Role of apoptosis in alcoholic liver injury. Alcoholism, clinical and experimental research. 2003;27(7):1207-12.

20. Natori S, Rust C, Stadheim LM, Srinivasan A, Burgart LJ, Gores GJ. Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. Journal of hepatology. 2001;34(2):248-53.

21. Lambert JC, Zhou Z, Kang YJ. Suppression of Fas-mediated signaling pathway is involved in zinc inhibition of ethanol-induced liver apoptosis. Experimental biology and medicine. 2003;228(4):406-12.

22. Bailey SM, Cunningham CC. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. Free radical biology & medicine. 2002;32(1):11-6.

23. Cahill A, Cunningham CC, Adachi M, Ishii H, Bailey SM, Fromenty B, et al. Effects of alcohol and oxidative stress on liver pathology: the role of the mitochondrion. Alcoholism, clinical and experimental research. 2002;26(6):907-15.

24. Fernandez-Checa JC, Garcia-Ruiz C, Colell A, Morales A, Mari M, Miranda M, et al. Oxidative stress: role of mitochondria and protection by glutathione. BioFactors. 1998;8(1-2):7-11.

CHAPTER II

REVIEW OF LITERATURE

Alcoholic Liver Disease

Heavy alcohol consumption is well-known to bring health concerns. Alcohol abuse contributes to 100,000 to 200,000 deaths annually in the United States, of which over 20,000 deaths are caused by hepatic cirrhosis [1]. Although a variety of dysfunctions are associated with excessive alcohol consumption, alcoholic liver disease (ALD) has the greatest health impact [2]. Previous studies suggest that the amount and duration of an individual patient exposed to alcohol correlate closely with the progression of ALD, and the data suggest that an average daily intake of 30g of alcohol for 10 years significantly increased the risk of developing to hepatic cirrhosis [3-5].

The spectrum of ALD includes alcoholic fatty liver, alcoholic hepatitis, and alcoholic cirrhosis [6, 7]. Alcoholic fatty liver is manifested as accumulation of lipid droplets in hepatic tissue with prominent in perivenular region; hepatitis is shown as hepatocyte ballooning and neutrophil infiltration in the liver; and cirrhosis is the end stage of ALD, in which scar tissue replaces normal parenchyma leading to irreversibly loss of the liver function [8, 9]. It is not necessary that ALD evolves from one stage to another, but rather multiple stages may represent simultaneously in a giving individual. It is noteworthy to mention that among heavy drinkers, over 90% develops fatty liver, 10-35% develops hepatitis, and 8-20% evolves to hepatic cirrhosis [1, 9].

The pathogenesis of ALD arises from the shift in ethanol oxidation enzyme and accumulation of toxic metabolites after excessive and/or prolonged alcohol intake. Under physiological conditions, small amount of alcohol generated from sugar fermentation is oxidized by cytosolic alcohol dehydrogenase (ADH) [10]. When ethanol level in the body over the ability of ADH to convert to acetaldehyde, the expression of microsomal ethanol oxidizing system (MEOS), mainly cytochrome P450 2E1 (CYP 2E1), is induced [11]. Induction of CYP 2E1 enhances ethanol oxidation , while brings harm to the body by promote ROS production [11]. Studies showed that mice knockout of CYP 2E1 gene attenuated alcohol-induced overproduction of oxidative stress and liver injury, on the contrary overexpression of CYP 2E1 gene exaggerated oxidative stress and liver injury [12]. Another enzyme, catalase, also contribute to ethanol oxidation, however the level of which is negligible in the liver [13]. Regardless of the involved pathway, the highly toxic compound, acetaldehyde, is further oxidized to acetate by acetaldehyde dehydrogenase (ALDH), mainly $ALDH_2$, in mitochondria [10, 14]. Since the reaction is catalyzed by NAD^+/NADH, acetaldehyde oxidation results in increased NADH to NAD^+ ratio, which favoring lipid accumulation and ROS production in the liver. The pathway of ethanol oxidation is summarized in Figure 2.1 [15].

Zinc

Zinc is the second most abundant trace element found in our body [16]. Given the importance of mediating normal functions, zinc is widely distributed in the body. Liver, kidney, bone, muscle and skin have higher amount of zinc levels compare to the other organs or tissues. In the United States, the Recommended Dietary Allowance (RDA) for

zinc is 11 mg/day and 8 mg/day for adult men and women, respectively, to maintain the zinc level. The amount of zinc in the body is estimated to be 2-3g [17].

The absorption of zinc mainly occurs in jejunum. Once food rich in zinc reaches the stomach, hydrochloric acid releases zinc from the food. In the small intestine, protease and nuclease hydrolyze the still bounded zinc from the food before absorption [18]. Zinc absorption is mainly achieved by zinc transporters. Two families of zinc transporters are ZIP (SLC39A) and ZnT (SLC30A). ZIP family transports zinc from outside of the cells or subcellular compartments into cytosol, contrarily, ZnT family removes zinc from cytosol to extracellular part and subcellular organelles [19]. It has been reported that ZIP 4 and ZnT 1 are located on the apical and basolateral membranes of enterocyte, respectively, and they work together to transport dietary zinc into blood [20]. Zinc is loosely bound to albumin in the blood and most of the zinc is taken by the liver, where the metal is initially concentrated. After leaving the liver, zinc is transport in the blood still bound to albumin and to be utilized by other tissues.

The storage of zinc is accomplished by protein thionein [21]. When thionien is bounded with minerals, it is known as metallothionein (MT). MT is found in the most tissue of the body, for instance liver, intestine, pancreas, kidney and red blood cells. There are four isoforms of MT, which are designated as MT 1 through MT 4. MT 1 and MT 2 are the most common form among tissues and which are also the main form found in the liver [22]. MT is composed of two zinc/thiolate clusters. The maximum zinc binding capacity of MT is seven, with one cluster binding three zinc ions and the other binding four zinc ions [23]. MT plays an important role in zinc trafficking, by which the

level of free zinc is tightly regulated and the activity of zinc proteins are maintained. In addition, MT participant in scavenge radicals [21]. It has been reported that zinc is released from MT to form thionien in oxidizing environment, when gluthione (GSH)/ glutathione disulfide (GSSG) is low [24]. The released zinc can then be redistributed and coordinated into the apo zinc enzymes to stimulate the corresponding cellular response.

The excretion of zinc is mainly through gastrointestinal track. Zinc in the sloughed intestinal cells account for the most of the zinc found in the fecal material [25]. Although zinc filtered in the glomerulus and secreted by the proximal tubule can be effectively reabsorbed in the distal nephron, small amount of zinc can be found in urine. The urine zinc is believed to be derived from the small percent of plasma zinc which is bounded to histidine and cysteine [26]. Zinc transporters are thought to be responsible for the resorption of zinc in the kidney, and ZnT 1, ZnT 2, ZnT 4 and ZIP 1 were found in the kidney [27]. In addition, the endogenous zinc is also secreted by the salivary glands, intestinal mucosa, pancreas, and liver.

The function of zinc includes maintaining the structural integrity of zinc proteins, acting as second messenger through free zinc, participant in antioxidant defense and enzymatic function, promotion of cell growth, development, and proliferation [28]. Except for the role of second messenger, the other functions of zinc are achieved through coordinate zinc into zinc proteins. For the antioxidant role, zinc saves the level of vitamin E, by which zinc protects cell membrane from lipid peroxidation [29]. A more direct role of zinc in scavenging free radicals is through formation of an antioxidant enzyme, Cu/Zn-SOD, found in cytosol [30]. There are more than 200 metalloenzymes require zinc for

their function, including ADH, carbonic anhydrase, alkaline phosphatase, enzymes of protein, carbohydrate, and lipid metabolism, and nucleic acid synthesis [31-34]. Given the fact that several zinc proteins involve in DNA repair, replication, and transcription, it is expected that zinc is essential in cell growth. During transcription and replication, zinc finger protein, 3-dimensional finger structure of the transcriptional regulatory protein stabilized by zinc, binds in the helical groove of DNA at a specific target gene regulatory site to regulate expression of the genes [31]. Disassociation of zinc from the protein could partially or completely inactivate the enzyme.

Accordingly, zinc homeostasis is achieved via a balance of absorption, storage, and excretion. As zinc plays a critical role in so many biological activities, zinc deficiency associates with the pathogenesis of disease.

Zinc Deficiency in ALD

Occurrence of zinc deficiency in ALD

Over half a century ago researchers have found that ALD patients had decreased zinc levels in the serum and liver biopsy [35]. Clinical study showed that serum zinc levels in alcoholic patients decreased to about 60% of that of healthy controls [36]. In addition, studies further demonstrated that the severity of zinc deficiency closely correlate with the advanced stage of ALD. It has been shown that ALD patients with no cirrhosis had a hepatic zinc level of 76% of that of the healthy controls, while with cirrhosis the hepatic zinc levels drop to 63% of that of the healthy controls. Another study showed that serum zinc level significantly decreased in patients with hepatic cirrhosis

compared to that of controls, and patients with encephalopathy had a further reduction of serum zinc levels compared to patients without encephalopathy [37].

In order to establish the link between zinc deficiency and ALD, animal models were utilized. Studies showed that chronic alcohol exposure decreased hepatic zinc levels in rodents, and the reduction of zinc level in the liver occurred as early as two weeks after alcohol consumption [38-40]. But one study reported elevation of plasma zinc levels in alcoholic with fatty liver [41]. Thus, it is likely that plasma zinc level declined after decrease of hepatic zinc level [40]. However, it is still unclear when plasma zinc levels begin to decline after chronic alcohol exposure.

Zinc dyshomeostasis in ALD

Food intake is compromised in alcoholic patients. Zinc is found in food bound with amino acids and nucleic acid, food rich in protein are good source of zinc, for instance red meat and sea food. Pork, poultry, and dairy products are also good source of zinc [42]. Given the fact that alcohol contributes calories to body, studies investigated the effect of alcohol consumption on food intake, as well as body weight and fat mass. It has been reported that with the percentage of energy derived from alcohol increase, the consumption of carbohydrate, protein, fat, as well as vitamin and minerals decreased [43]. Along with that was dramatically decreased body mass index (BMI) among heavy drinkers [44]. The more alcoholics drink, the less nutritional quality of food they consume, with the nutritional status even worse among heavy drinkers. In addition, considering the progression of ALD, impaired gastrointestinal and liver function would further reduce food intake, which cause severe zinc deficiency.

The absorption of zinc is impaired after chronic alcohol consumption. It has been reported that the zinc^{65} absorption rate, detected by dual isotope absorption technique, from intestine decreased from 56% in normal subjects to 37% in alcoholic subjects [45, 46]. Moreover, animal study showed that the absorption of zinc in the ileum remarkably decreased after 4 weeks of alcohol-feeding [47]. Perturbed zinc transporters are believed responsible for the decreased zinc absorption in the small intestine under disease conditions. It has been reported that patient with acrodermatitis enteropathica, have decreased zinc levels, and the mutation of ZIP 4 result in reduced absorption of zinc from small intestine and subsequent reduction of zinc levels [48]. However, few studies investigate the expression of zinc transporters in small intestine after chronic alcohol exposure. The effect of alcohol on the expression of zinc transporter remains unknown.

The urine excretion is increased after alcohol consumption. It is recognized that zinc excretion from urine is negligible under physiological condition. However, studies showed that urinary zinc excretion increased by about 150% in alcoholic cirrhosis patients compared to that of the healthy controls [49]. Another representative study further demonstrated the causality relationship between alcohol intake and urinary zinc excretion. In that study they included 40 alcoholics, and after 17 days of abstinence they were given alcohol every 2 hours for 18 hours per day for a total 17 days. The result illustrated that alcohol drinking caused nearly two times the elevation of urinary zinc levels compared to abstinence period [50]. Mechanistic study showed an elevated rate of both renal filtration and reabsorption of zinc in dog after alcohol intoxication [51]. In addition, study reported that zinc turnover and leakage in the tubular cells may relate to

the increased urinary zinc excretion in cirrhosis patients [52]. But the exact mechanism of the positive correlation between alcohol intake and urinary zinc excretion is not fully understood. Several zinc transporters have been found in the kidney, it will be interesting to investigate whether dysregulated zinc transporters in kidney responsible for the decreased reabsorption of zinc.

The level of zinc in the liver is decreased after alcohol consumption. It is well-known that MT is a zinc reservoir, and the level of MT is decreased after alcohol exposure [22, 53]. In order to determine the causality relationship between zinc deficiency and reduced MT level, our laboratory performed alcohol feeding experiment on MT-null mice. The study showed that MT-null and wild type (WT) mice fed with control diet have the similar hepatic zinc levels. However, alcohol-feeding significantly decreased hepatic zinc level in MT-null mice compared to WT mice. Furthermore, zinc supplementation to mice fed with alcohol diet increases hepatic zinc level in both MT-null mice and WT mice. These data demonstrated that hepatic zinc level is not dependent of MT level; rather decrease of MT level may sensitize alcohol-induced hepatic zinc deficiency. Another way to regulate zinc concentration is through zinc transporters. Studies confirmed the altered the expression of zinc transporters linked with increased or decreased zinc levels in the liver [54]. However the effect of alcohol consumption on the expression of zinc transporters in the liver is unclear.

As a whole, hepatic zinc deficiency is consistently observed in ALD, but the underlying mechanism is still unclear. In addition, controversial results were found in plasma zinc levels after alcohol exposure. Thus, Aim 1 will focus on the effect of alcohol

on disturbing zinc homeostasis, and explore the mechanism of hepatic zinc deficiency at different time point of alcohol consumption. The schematic picture of Aim is shown in Figure 2.2.

Apoptosis in ALD

Apoptosis

Apoptosis is also called programed cell death, the morphological change include blebbing, cell shrink, nuclear fragmentation, and DNA fragmentation [55]. In contrast to necrosis, apoptosis is highly regulated and controlled to remove damaged and senescent cells without damage to the cellular microenvironment [56]. The regulation of apoptosis is through Fas receptor, cytochrome C release and caspase. Increased apoptosis is linked with pathophysiological state, for instance aging, degenerative disorders, and autoimmune disease [56]. Increased apoptotic cell death has been found in alcoholic patients and animals [57, 58], and the number of apoptotic cells is positively associated with the duration of ethanol [57]. It has been reported that alcoholic hepatitis patients have significantly increased apoptosis in the liver compared to controls without liver disease[58]. Increasing evidences indicate that increased cell death trigger macrophage and neutrophil activation and fibrogenesis [59]. In addition, studies suggested that necrosis does not occur in the absence of apoptosis, as evidenced by inhibiting of apoptosis abolished necrosis and increase apoptosis with FasL agonist led to necrosis [60, 61]. Thus, it is likely that hepatic apoptosis is associated with the development of ALD.

Proinflammatory cytokine and oxidative stress in alcohol-induced apoptosis

The excessive release of cytokines after alcohol administration is caused by stimulating Kuffer cells with increased gut bacteria-derived lipopolysaccharide (LPS) via leaky gut, by which liver injury and necrosis occurs [13]. Among various proinflammatory cytokines, tumor necrosis factor α (TNF-α) has been proposed as a major player in alcohol consumption-induced apoptosis. It has been reported that alcohol exposure increased level of TNF-α/ tumor necrosis factor receptor-1 (TNF-R1) in alcoholics [62, 63]. Treatment with neutralizing antibodies to TNF-α or knockdown of TNF-R1 abrogated TNF-α signaling and diminished apoptosis [64]. Our laboratory has demonstrated that zinc supplementation attenuated expression of TNF-α/TNF-R1, and ameliorated apoptosis and liver damage in mice [53].

In addition to TNF-α, increased oxidative stress is also proposed as a mediator in activation of apoptotic cell death pathway. Alcohol consumption-induced oxidative stress arises from both increased ROS production and decreased capacity of endogenous antioxidant defense systems [65]. Previous study showed that depletion of glutathione exaggerated ethanol-induced oxidative stress in hepatocyte; meanwhile antioxidant treatment protected the hepatocyte from cell death [56]. In addition, studies showed that a positive correlation between apoptosis and the amount of hydrogen peroxide generation by TNF-α [66]. The transgenic mice with overexpression of endogenous antioxidant proteins, such as Cu/Zn SOD or Mn SOD blunted alcohol-induced apoptosis and liver injury [67, 68]. On the contrary, Cu/Zn SOD, or glutathione peroxidase-1, or metallothionein knockout mice showed increased number of apoptotic cells and more

severe liver damage compared to the WT mice after chronic alcohol exposure [22, 69-71]. Our laboratory showed that zinc supplementation attenuated alcohol-induced oxidative stress and partially inhibited apoptosis [53].

Role of endoplasmic reticulum and mitochondria in alcohol-induced apoptosis

Endoplasmic reticulum (ER) is an essential organelle for lipid synthesis, protein modification and oxidation of certain drugs [72]. Under physiological conditions, proteins undergo oxidative protein folding in the ER through protein disulfide isomerase (PDI) [73]. While forming the disulfide bond, the ROS is generated as byproduct [73]. The safety level of ROS is maintained by sufficient level of glutathione in the cytosol [73]. However, uncontrolled ROS generation by chronic alcohol intake could reduce glutathione level, which perturbs PDI mediated oxidative folding leading to increase utilization of glutathione [74]. Overproduction of ROS and depletion of glutathione trigger ER stress [74]. Unfolded protein response (UPR) is the adaptive response of ER stress, by which protein folding and degradation is enhanced and protein synthesis is reduced to restore homeostasis. However prolonged or sever UPR may induce fat accumulation, inflammation and eventually apoptosis, which are detrimental to cells [75]. UPR is mediated by three ER resident transmembrane sensor proteins, inositol requiring protein 1 (IRE1), ds-RNA-activated protein kinase (PKR) like ER kinase (PERK), and activating transcription factor 6 (ATF6) [73]. The three sensors are activated by dissociation from chaperon GRP78/BiP. The activation of IRE1 activate the transcription of sliced X-box binding protein-1 (XBP1), which increase the expression of chaperons and genes involves in ER associated degradation [75]. The activation of PERK pathway

leads to a global attenuation of protein translation via phosphorylation of eukaryotic initiation factor 2α-subunit (eIF2α) and subsequent activation of transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) [76]. Another sensor of UPR is ATF6, the activation of which triggers transcription of UPR target genes.

Although the precise mechanism of sustained ER stress induced apoptosis is not fully understood, the transcription factor CHOP, calcium and redox homeostasis, and caspase activation are all implicated [77]. Knockout of CHOP resulted in minimum alcohol-induced apoptosis in the liver of mouse [78]. In addition, antioxidant treatment has been shown to preserve the level of glutathione and function of ER [64].

Mitochondria are referred as the power plant of a cell by generating ATP to satisfy the energy need of the cell [79], meanwhile mitochondria could be dangerous due to their inherent quality of generating ROS while passing electrons to produce ATP [80]. Under physiological conditions, about 1-2% of the oxygen consumed in the mitochondria is leaked out as superoxide from complex I and complex III [80]. With intact cell functions, the superoxide will soon be scavenged by antioxidant system. After alcohol administration, increased NADH/NAD^+ ratio favor superoxide generation from complex I by increasing electron flow along the electron transport chain [80]. In addition, alcohol consumption decreased mitochondria membrane potential (MMP), by which increased cytochrome C release into cytosol and activation of caspase-3 led to apoptosis [56, 81, 82]. It has been reported that impaired MMP is associated with increased mitochondrial ROS production [81].

Collectively, apoptosis plays a crucial role in the progression of ALD. The role of zinc deficiency in activation of mitochondria-mediated apoptotic cell death pathway has been evaluated in Aim 2 and Aim 3. Aim 2 determined the role of zinc deficiency in ER stress-mediated activation of apoptotic cell death pathway. Aim 3 defined the role of zinc deficiency in mitochondrial stress induced activation of apoptotic cell death pathway. The schematic picture of Aim 2 and Aim 3 are shown in Figure 2.2.

References

1. Zhu H, Jia Z, Misra H, Li YR. Oxidative stress and redox signaling mechanisms of alcoholic liver disease: updated experimental and clinical evidence. Journal of digestive diseases. 2012;13(3):133-42.

2. Mann RE, Smart RG, Govoni R. The epidemiology of alcoholic liver disease. Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism. 2003;27(3):209-19.

3. Sorensen TI. Alcohol and liver injury: dose-related or permissive effect? British journal of addiction. 1989;84(6):581-9.

4. O'Shea RS, Dasarathy S, McCullough AJ. Alcoholic liver disease. The American journal of gastroenterology. 2010;105(1):14-32; quiz 3.

5. Anderson P. Alcohol consumption and all-cause mortality. Addiction. 1995;90(4):481-4; discussion 93-8.

6. O'Shea RS, Dasarathy S, McCullough AJ, Practice Guideline Committee of the American Association for the Study of Liver D, Practice Parameters Committee of the American College of G. Alcoholic liver disease. Hepatology. 2010;51(1):307-28.

7. Purohit V, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. Alcoholism, clinical and experimental research. 2009;33(2):191-205.

8. Theise ND. Histopathology of Alcoholic Liver Disease: AASLD; 2013. Available from: http://onlinelibrary.wiley.com/doi/10.1002/cld.172/epdf.

9. Breitkopf K, Nagy LE, Beier JI, Mueller S, Weng H, Dooley S. Current experimental perspectives on the clinical progression of alcoholic liver disease. Alcoholism, clinical and experimental research. 2009;33(10):1647-55.

10. Cederbaum AI. Alcohol metabolism. Clinics in liver disease. 2012;16(4):667-85.

11. Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. Free radical biology & medicine. 2008;44(5):723-38.

12. Wang X, Wu D, Yang L, Gan L, Cederbaum AI. Cytochrome P450 2E1 potentiates ethanol induction of hypoxia and HIF-1alpha in vivo. Free radical biology & medicine. 2013;63:175-86.

13. Louvet A, Mathurin P. Alcoholic liver disease: mechanisms of injury and targeted treatment. Nature reviews Gastroenterology & hepatology. 2015;12(4):231-42.

14. Zakhari S, Li TK. Determinants of alcohol use and abuse: Impact of quantity and frequency patterns on liver disease. Hepatology. 2007;46(6):2032-9.

15. Zakhari S. Overview: How is alcohol metabolized by the body? Available from: http://pubs.niaaa.nih.gov/publications/arh294/245-255.htm.

16. Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T. Zinc homeostasis and signaling in health and diseases: Zinc signaling. Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry. 2011;16(7):1123-34.

17. Devirgiliis C, Zalewski PD, Perozzi G, Murgia C. Zinc fluxes and zinc transporter genes in chronic diseases. Mutation research. 2007;622(1-2):84-93.

18. Rojas LX, McDowell LR, Cousins RJ, Martin FG, Wilkinson NS, Johnson AB, et al. Interaction of different organic and inorganic zinc and copper sources fed to rats. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements. 1996;10(3):139-44.

19. Lichten LA, Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. Annual review of nutrition. 2009;29:153-76.

20. Weaver BP, Dufner-Beattie J, Kambe T, Andrews GK. Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zip5). Biological chemistry. 2007;388(12):1301-12.

21. Cummings JE, Kovacic JP. The ubiquitous role of zinc in health and disease. Journal of veterinary emergency and critical care. 2009;19(3):215-40.

22. Zhou Z, Sun X, Lambert JC, Saari JT, Kang YJ. Metallothionein-independent zinc protection from alcoholic liver injury. The American journal of pathology. 2002;160(6):2267-74.

23. Zhou Z. Zinc and alcoholic liver disease. Digestive diseases. 2010;28(6):745-50.

24. Hogstrand C, Kille P, Nicholson RI, Taylor KM. Zinc transporters and cancer: a potential role for ZIP7 as a hub for tyrosine kinase activation. Trends in molecular medicine. 2009;15(3):101-11.

25. King JC, Shames DM, Woodhouse LR. Zinc homeostasis in humans. The Journal of nutrition. 2000;130(5S Suppl):1360S-6S.

26. Zlotkin SH. Nutrient interactions with total parenteral nutrition: effect of histidine and cysteine intake on urinary zinc excretion. The Journal of pediatrics. 1989;114(5):859-64.

27. Ranaldi G, Perozzi G, Truong-Tran A, Zalewski P, Murgia C. Intracellular distribution of labile Zn(II) and zinc transporter expression in kidney and MDCK cells. American journal of physiology Renal physiology. 2002;283(6):F1365-75.

28. Mohammad MK, Zhou Z, Cave M, Barve A, McClain CJ. Zinc and liver disease. Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition. 2012;27(1):8-20.

29. Bunk MJ, Dnistrian AM, Schwartz MK, Rivlin RS. Dietary zinc deficiency decreases plasma concentrations of vitamin E. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine. 1989;190(4):379-84.

30. Chausmer AB. Zinc, insulin and diabetes. Journal of the American College of Nutrition. 1998;17(2):109-15.

31. Berg JM, Shi Y. The galvanization of biology: a growing appreciation for the roles of zinc. Science. 1996;271(5252):1081-5.

32. Shankar AH, Prasad AS. Zinc and immune function: the biological basis of altered resistance to infection. The American journal of clinical nutrition. 1998;68(2 Suppl):447S-63S.

33. Ho E, Courtemanche C, Ames BN. Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. The Journal of nutrition. 2003;133(8):2543-8.

34. Frassinetti S, Bronzetti G, Caltavuturo L, Cini M, Croce CD. The role of zinc in life: a review. Journal of environmental pathology, toxicology and oncology : official organ of the International Society for Environmental Toxicology and Cancer. 2006;25(3):597-610.

35. Bartholomay AF, Robin ED, Vallee RL, Wacker WE. Zinc metabolism in hepatic dysfunction. I. Serum zinc concentrations in Laennec's cirrhosis and their validation by sequential analysis. The New England journal of medicine. 1956;255(9):403-8.

36. Goode HF, Kelleher J, Walker BE. Relation between zinc status and hepatic functional reserve in patients with liver disease. Gut. 1990;31(6):694-7.

37. Rahelic D, Kujundzic M, Romic Z, Brkic K, Petrovecki M. Serum concentration of zinc, copper, manganese and magnesium in patients with liver cirrhosis. Collegium antropologicum. 2006;30(3):523-8.

38. Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, et al. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. Hepatology. 2009;50(4):1241-50.

39. Barak AJ, Beckenhauer HC, Kerrigan FJ. Zinc and manganese levels in serum and liver after alcohol feeding and development of fatty cirrhosis in rats. Gut. 1967;8(5):454-7.

40. Wang J, Pierson RN, Jr. Distribution of zinc in skeletal muscle and liver tissue in normal and dietary controlled alcoholic rats. The Journal of laboratory and clinical medicine. 1975;85(1):50-8.

41. Hartoma TR, Sotaniemi EA, Pelkonen O, Ahlqvist J. Serum zinc and serum copper and indices of drug metabolism in alcoholics. European journal of clinical pharmacology. 1977;12(2):147-51.

42. Murphy EW, Willis BW, Watt BK. Provisional tables on the zinc content of foods. Journal of the American Dietetic Association. 1975;66(4):345-55.

43. Lieber CS. Relationships between nutrition, alcohol use, and liver disease. Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism. 2003;27(3):220-31.

44. Gearhardt AN, Corbin WR. Body mass index and alcohol consumption: family history of alcoholism as a moderator. Psychology of addictive behaviors : journal of the Society of Psychologists in Addictive Behaviors. 2009;23(2):216-25.

45. Dinsmore WW, Callender ME, McMaster D, Love AH. The absorption of zinc from a standardized meal in alcoholics and in normal volunteers. The American journal of clinical nutrition. 1985;42(4):688-93.

46. Valberg LS, Flanagan PR, Ghent CN, Chamberlain MJ. Zinc absorption and leukocyte zinc in alcoholic and nonalcoholic cirrhosis. Digestive diseases and sciences. 1985;30(4):329-33.

47. Antonson DL, Vanderhoof JA. Effect of chronic ethanol ingestion on zinc absorption in rat small intestine. Digestive diseases and sciences. 1983;28(7):604-8.

48. Dufner-Beattie J, Wang F, Kuo YM, Gitschier J, Eide D, Andrews GK. The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. The Journal of biological chemistry. 2003;278(35):33474-81.

49. McClain CJ, Antonow DR, Cohen DA, Shedlofsky SI. Zinc metabolism in alcoholic liver disease. Alcoholism, clinical and experimental research. 1986;10(6):582-9.

50. Atukorala TM, Herath CA, Ramachandran S. Zinc and vitamin A status of alcoholics in a medical unit in Sri Lanka. Alcohol and alcoholism. 1986;21(3):269-75.
51. Sargent WQ, Simpson JR, Beard JD. The effects of acute and chronic ethanol administration on divalent cation excretion. The Journal of pharmacology and experimental therapeutics. 1974;190(3):507-14.
52. Sullivan JF, Heaney RP. Zinc metabolism in alcoholic liver disease. The American journal of clinical nutrition. 1970;23(2):170-7.
53. Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, Kang YJ. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. The American journal of pathology. 2005;166(6):1681-90.
54. Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, et al. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(19):6843-8.
55. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer. 1972;26(4):239-57.
56. Kurose I, Higuchi H, Miura S, Saito H, Watanabe N, Hokari R, et al. Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication. Hepatology. 1997;25(2):368-78.
57. Goldin RD, Hunt NC, Clark J, Wickramasinghe SN. Apoptotic bodies in a murine model of alcoholic liver disease: reversibility of ethanol-induced changes. The Journal of pathology. 1993;171(1):73-6.

58. Natori S, Rust C, Stadheim LM, Srinivasan A, Burgart LJ, Gores GJ. Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. Journal of hepatology. 2001;34(2):248-53.

59. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology. 2014;147(4):765-83 e4.

60. Deaciuc IV, D'Souza NB, de Villiers WJ, Burikhanov R, Sarphie TG, Hill DB, et al. Inhibition of caspases in vivo protects the rat liver against alcohol-induced sensitization to bacterial lipopolysaccharide. Alcoholism, clinical and experimental research. 2001;25(6):935-43.

61. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, et al. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. The Journal of experimental medicine. 1995;182(5):1223-30.

62. Ribeiro PS, Cortez-Pinto H, Sola S, Castro RE, Ramalho RM, Baptista A, et al. Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. The American journal of gastroenterology. 2004;99(9):1708-17.

63. Garcia-Ruiz C, Morales A, Colell A, Ballesta A, Rodes J, Kaplowitz N, et al. Feeding S-adenosyl-L-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. Hepatology. 1995;21(1):207-14.

64. Ji C, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. Gastroenterology. 2003;124(5):1488-99.

65. Ishii H, Kurose I, Kato S. Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. Journal of gastroenterology and hepatology. 1997;12(9-10):S272-82.

66. Laster SM, Wood JG, Gooding LR. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. Journal of immunology. 1988;141(8):2629-34.

67. Wheeler MD, Nakagami M, Bradford BU, Uesugi T, Mason RP, Connor HD, et al. Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. The Journal of biological chemistry. 2001;276(39):36664-72.

68. Wheeler MD, Kono H, Yin M, Rusyn I, Froh M, Connor HD, et al. Delivery of the Cu/Zn-superoxide dismutase gene with adenovirus reduces early alcohol-induced liver injury in rats. Gastroenterology. 2001;120(5):1241-50.

69. Kessova IG, Ho YS, Thung S, Cederbaum AI. Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase. Hepatology. 2003;38(5):1136-45.

70. Curry-McCoy TV, Osna NA, Nanji AA, Donohue TM, Jr. Chronic ethanol consumption results in atypical liver injury in copper/zinc superoxide dismutase deficient mice. Alcoholism, clinical and experimental research. 2010;34(2):251-61.

71. Kim SJ, Lee JW, Jung YS, Kwon do Y, Park HK, Ryu CS, et al. Ethanol-induced liver injury and changes in sulfur amino acid metabolomics in glutathione peroxidase and catalase double knockout mice. Journal of hepatology. 2009;50(6):1184-91.

72. Ji C, Kaplowitz N. ER stress: can the liver cope? Journal of hepatology. 2006;45(2):321-33.

73. Ji C. Mechanisms of alcohol-induced endoplasmic reticulum stress and organ injuries. Biochemistry research international. 2012;2012:216450.

74. Ji C. Advances and New Concepts in Alcohol-Induced Organelle Stress, Unfolded Protein Responses and Organ Damage. Biomolecules. 2015;5(2):1099-121.

75. Hetz C, Martinon F, Rodriguez D, Glimcher LH. The unfolded protein response: integrating stress signals through the stress sensor IRE1alpha. Physiological reviews. 2011;91(4):1219-43.

76. Gika HG, Ji C, Theodoridis GA, Michopoulos F, Kaplowitz N, Wilson ID. Investigation of chronic alcohol consumption in rodents via ultra-high-performance liquid chromatography-mass spectrometry based metabolite profiling. Journal of chromatography A. 2012;1259:128-37.

77. Lluis JM, Colell A, Garcia-Ruiz C, Kaplowitz N, Fernandez-Checa JC. Acetaldehyde impairs mitochondrial glutathione transport in HepG2 cells through endoplasmic reticulum stress. Gastroenterology. 2003;124(3):708-24.

78. Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. Journal of hepatology. 2011;54(4):795-809.

79. Bailey SM, Cunningham CC. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. Free radical biology & medicine. 2002;32(1):11-6.

80. Murphy MP. How mitochondria produce reactive oxygen species. The Biochemical journal. 2009;417(1):1-13.

81. Higuchi H, Adachi M, Miura S, Gores GJ, Ishii H. The mitochondrial permeability transition contributes to acute ethanol-induced apoptosis in rat hepatocytes. Hepatology. 2001;34(2):320-8.

82. Zoratti M, Szabo I. The mitochondrial permeability transition. Biochimica et biophysica acta. 1995;1241(2):139-76.

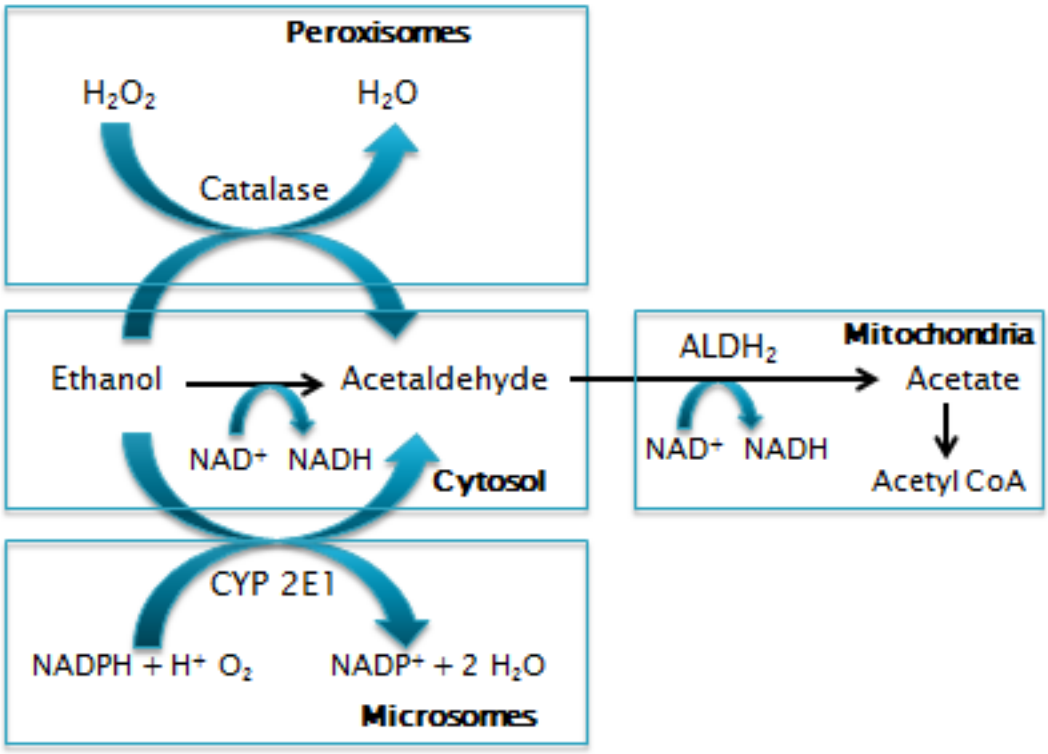


Figure 2.1. Oxidation Pathway of Alcohol Metabolism.

Ethanol is metabolized mainly by cytosolic alcohol dehydrogenase (ADH). However, prolonged and excessive alcohol consumption induce the proliferation of microsomal ethanol oxidizing system (MEOS), namely overexpress cytochrome P450 2E1 (CYP 2E1), by which the efficiency of ethanol oxidation as well as ROS production are both enhanced. The ethanol oxidation through catalase in peroxisomes is negligible. Regardless the pathway involved in ethanol oxidation, acetaldehyde is oxidized by acetaldehyde dehydrogenase (ALDH), mainly ALDH2, in mitochondria, with increase NADH/ NAD^+ ratio.

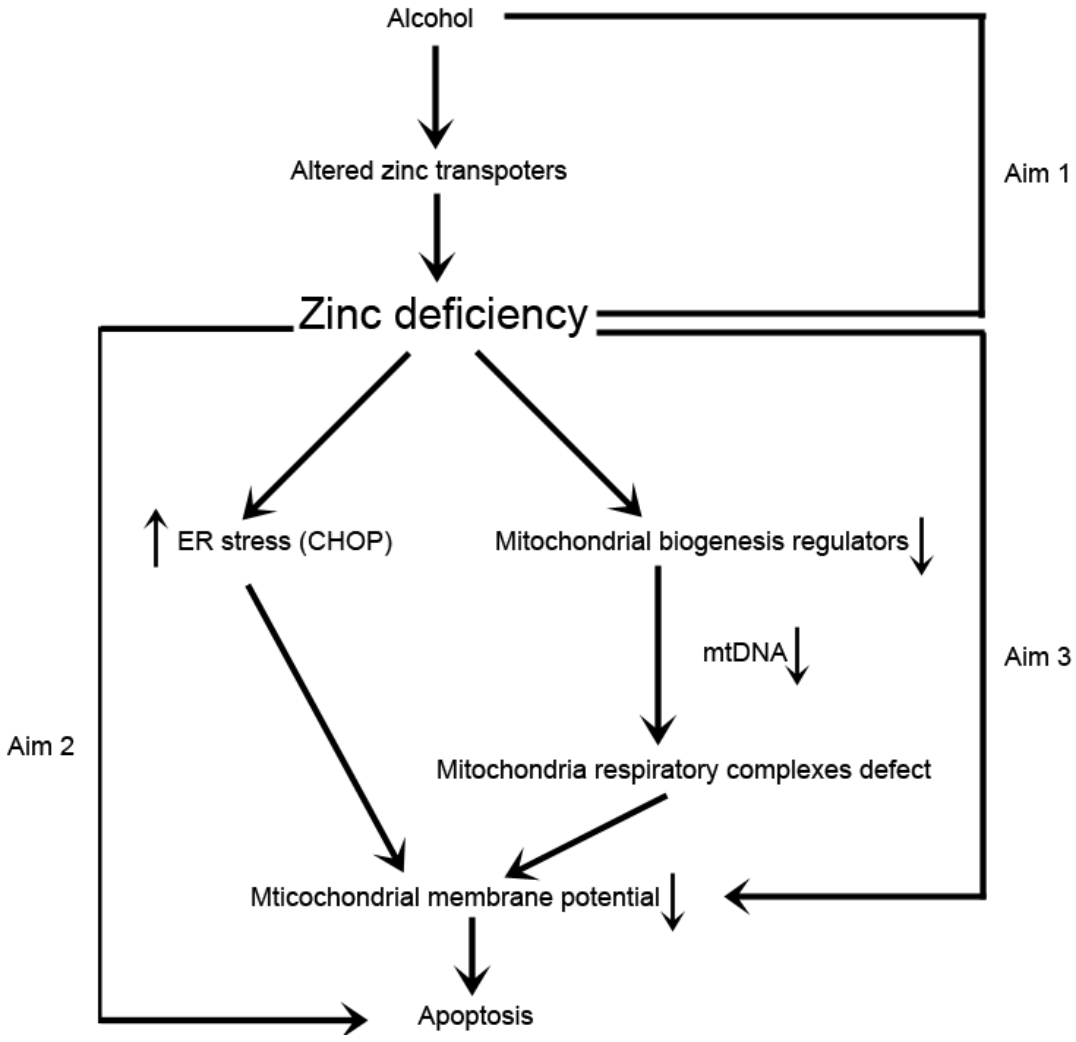


Figure 2.2. Research Aims**.**

The altered expression of zinc transporters by increases oxidative stress underlies the mechanism of alcohol-induced hepatic zinc deficiency (Proposed mechanism in Aim 1). Hepatic zinc deficiency enhances apoptosis through increase ER stress (CHOP) and consequently decrease mitochondrial membrane potential (Proposed mechanism in Aim 2). Zinc deficiency could directly activate mitochondrial mediated apoptotic cell death signaling through downregulate mitochondrial biogenesis regulators, by which the amount of mitochondrial DNA (mtDNA) is decreased, in turn, mitochondria respiratory

complexes is decreased at protein level. The defective mitochondrial respiratory chain contributes to loss of mitochondrial membrane potential, which leads to apoptosis (Proposed mechanism in Aim3).

CHAPTER III

DYSREGULATION OF HEPATIC ZINC TRANSPORTERS IN A MOUSE MODEL OF ALCOHOLIC LIVER DISEASE

Qian Sun, Qiong Li, Wei Zhong, Jiayang Zhang, Xiuhua Sun, Xiaobing Tan, XinminYin, Xinguo Sun, Xiang Zhang, Zhanxiang Zhou. American Journal Physiology Gastrointestinal and Liver Physiology 307 (3): G313-22), 2014.

Abstract

Zinc deficiency is a consistent phenomenon observed in patients with alcoholic liver disease, but the mechanisms have not been well defined. The objective of this study was to determine if alcohol alters hepatic zinc transporters in association with reduction of hepatic zinc levels and if oxidative stress mediates the alterations of zinc transporters. C57BL/6 mice were pair-fed with the Lieber-DeCarli control or ethanol diets for 2-, 4- or 8-week. Chronic alcohol exposure reduced hepatic zinc levels, but increased plasma and urine zinc levels, at all-time point. Hepatic zinc finger proteins, PPAR-α and HNF-4α, were down-regulated in ethanol-fed mice. Four hepatic zinc transporter proteins showed significant alterations in ethanol-fed mice compared to the controls. ZIP5 and ZIP 14 proteins were down-regulated, while ZIP7 and ZnT7 proteins were up-regulated, by ethanol exposure at all-time point. Immunohistochemical staining demonstrated that chronic ethanol exposure up-regulated cytochrome P450 2E1 and caused 4-hydroxynonenal accumulation in the liver. For the *in vitro* study, murine FL-83B

hepatocytes were treated with 5 μM 4-hydroxynonenal or 100 μM hydrogen peroxide for 72 hours. The results from *in vitro* studies demonstrated that 4-hydroxynonenal treatment altered ZIP5 and ZIP7 protein abundance, and hydrogen peroxide treatment changed ZIP7, ZIP14 and ZnT7 protein abundance. These results suggest that chronic ethanol exposure alters hepatic zinc transporters via oxidative stress, which might account for ethanol-induced hepatic zinc deficiency.

Introduction

Alcoholic liver disease (ALD) is a major cause of morbidity and mortality among alcoholic populations [34] . Zinc deficiency as a major nutritional defect has been well documented in ALD [29]. Clinical studies showed that patients with advanced ALD had lower zinc levels in serum and liver but higher zinc level in urine [2, 16, 20, 33]. Hepatic zinc levels in patients with hepatitis and cirrhosis were reduced to 49-54% of the healthy subjects [2]. Chronic ethanol exposure significantly decreased hepatic zinc levels and induced production of reactive oxygen species (ROS) in mice [38]. Dietary zinc supplementation to ethanol-fed mice improved hepatic zinc status, attenuated oxidative stress and liver injury in association with increased antioxidant capacity. [17, 38].

Zinc is an essential trace element that plays an important role in maintaining normal cellular functions [22]. Zinc serves as a catalytic cofactor for more than hundreds of enzymes [4, 7]. Zinc is also required for stabilizing structure of thousands of proteins [4, 7, 21]. Zinc deficiency is linked with impaired immune function, growth retardation, and dermatologic lesion [4, 35]. On the other hand, high level of free zinc is cytotoxic [7]. Therefore, tight regulation of intracellular zinc concentrations is required, which is

achieved mainly by two families of zinc transporters, Zn^{2+}-regulated metal transporter (Zrt) and Iron-regulated metal transporter (Irt)-like protein (ZIP) and zinc transporter (ZnT) [25].

ZIP family has fourteen members from ZIP1 to ZIP14, and they function in transporting zinc from extracellular space or intracellular organelles into cytosol. ZnT family contains ten members from ZnT1 to ZnT10, and they are responsible for exporting zinc from cytosol to extracellular space or intracellular organelles. The cytosolic zinc concentration is regulated positively by ZIPs and negatively by ZnTs, while the organelle zinc levels are regulated positively by ZnTs and negatively by ZIPs. Dysregulation of zinc transporters has been implicated in the pathogenesis of human diseases [6, 13, 26]. Given the evidence that hepatic zinc deficiency is consistently observed in alcoholic patients and chronic ethanol-fed animals, and given the fact that ZIP and ZnT are two major zinc transporters in controlling extracellular and intracellular zinc trafficking, we postulated that dysregulation of zinc transporters would be responsible for alcohol-induced hepatic zinc deficiency. The present study was undertaken to determine whether or not chronic ethanol feeding alters hepatic expression of zinc transporters in mice. Because oxidative stress is a feature of ALD, the mechanistic link between oxidative stress and dysregulation of zinc transporters was also evaluated.

Methods and Materials

Animal and alcohol feeding experiments

Male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were treated according to the experimental procedures approved by Institutional

Animal Care and Use Committee. The mice were pair-fed a modified Lieber-DeCarli ethanol or control liquid diet for 2-week, 4-week or 8-week, respectively. The ethanol provided 28% of total calories in the diet. Urine samples were collected at 9:00am one day before sacrificing. The mice were anesthetized with isofluorane, and blood and liver tissues were harvested for assays.

Blood parameter assay

Blood glucose was determined by OneTouch Ultra2 blood glucose meter (Life Scan, Milpitas, CA). Plasma β-hydroxybutyrate was measured by Cayman Chemical assay kit (Ann Arbor, MI). Activity of alanine aminotransferase (ALT) was measured by Infinity ALT reagent (Thermo Scientific, Waltham, MA).

Determination of zinc concentrations in the liver, plasma and urine

Zinc concentrations in the liver, plasma and urine were determined by atomic absorption spectrophotometry (AAS). The frozen livers were air-dried in the hood overnight, and then each sample was digested with 1 ml concentrated nitrite acid for approximately 12 h. Each test tube with digested sample was then incubated in water bath at 100^0C for an hour. After cooling, each sample was diluted with deionized water and measured with AAS. One hundred and twenty microliter of plasma or urine was directly measured with AAS. The urinary creatinine concentrations were measured with a urinary creatinine assay kit (Cayman Chemical company, Anna Arbor, MI) to normalize urine zinc levels. The zinc concentrations in the liver, plasma and urine were calculated as μg/g dry liver weight, μg/dL and μg/mg creatinine, respectively.

Immunohistochemical detection of hepatic CYP2E1, 4-hydroxynonenal, ZIP5, ZIP7 and ZnT7

Hepatic CYP2E1, 4-hydroxynonenal (4-HNE), ZIP5, Zip7 and ZnT7 levels were detected by immunohistochemical staining. Briefly, liver tissue paraffin sections were incubated with 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases. The endogenous mouse IgG was blocked by incubation with a mouse-to-mouse blocking reagent (ScyTek Laboratories, Logan, UT). Tissue sections were then incubated with a polyclonal rabbit anti-CYP2E1 antibody (Abcam, Cambridge, MA), or a monoclonal mouse anti-4-HNE antibody (Northwest Life Science Specialties, Vancouver, WA), or a polyclonal rabbit anti-ZIP5 antibody (Novus biological, Littleton, CO), or polyclonal goat anti-ZIP7 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) or a polyclonal rabbit anti-ZnT7 antibody (Proteintech, Chicago, IL) at 4 °C overnight, followed by incubation with EnVision+ Labled Polymer-HRP-conjugated anti-rabbit IgG or anti-mouse IgG (DAKO, Carpinteria, CA) or HRP-conjugated goat anti-rabbit IgG (Thermo Scientific, Waltham, MA) at room temperature for 30 minutes. Diaminobenzidine (DAB) was used as HRP substrate for visualization.

qPCR

The total RNA was isolated and reverse transcribed with TaqMan reverse transcription reagents (Life Technologies, Carlsbad, CA). The forward and reverse primers of all twenty-four zinc transporters and β-actin were purchased from Integrated DNA Technologies (Coralville, IA). Primer sequences for the qPCRs are provided in Table 1. qPCR analysis with SYBR green PCR Master Mix (Qiagen, Valencia, CA) was

performed on the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems). The data were normalized to β-actin and expressed as relative fold-changes, with the value of control of ZIP1 or ZnT1 setting as one, respectively.

Immunoblotting analysis

Liver tissue proteins were extracted by T-PER tissue extraction reagent (Thermo scientific) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO). Aliquots containing 80 μg proteins were loaded onto an 8%-15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with polyclonal antibodies against hepatocyte nuclear factor 4 (HNF-4α), superoxide dismutase 1 (SOD1), ZIP7, ZnT5, ZnT6, ZnT10 (Santa Cruz Biotechnologies), peroxisome proliferator-activated receptor alpha (PPAR-α), ZIP1, ZIP4, ZIP5, ZIP14, ZnT4 (Novus biological), ZIP8 or ZnT7 (Proteintech), respectively. The membrane was then incubated with HRP-conjugated goat anti-rabbit IgG, or goat anti-mouse IgG, or rabbit anti-goat IgG antibody. The protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis.

SOD1 activity assay

The liver tissues were homogenized with 200 mM HEPES buffer (pH 7.2, containing 1mM EDTA, 210mM mannitol, and 70mM sucrose). Cytosolic fraction was isolated by centrifuging tissue homogenates at 1,500g for 5 minutes at 4^0C, and then the supernatant was centrifuged at 10,000g for 15 minutes at 4^0C. The supernatant was the crust cytosol of mice liver tissues. Superoxide dismutase assay kit (Cayman Chemical

Company, Anna Arbor, MI) was used to assess the activity of SOD1 by measuring the amount of superoxide radicals, which were generated from xanthine oxidase and hypoxanthine quenched by SOD1.

Cell culture and treatment

Murine FL-83B cells obtained from the American Type Culture Collection (Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin (100U/ml) streptomycin sulfate (100µg/mL) (Invitrogen, Carlsbad, CA). To evaluate the role of ROS on alterations of zinc transporters, 1×10^6 cells were seeded to 10-cm dish to reached 70-80% confluence. The cells were treated with 5 µM 4-HNE or 100 µM hydrogen peroxide (H_2O_2) for 72 h. Cells were then washed with ice cold PBS and harvested with M-PER mammalian protein extraction (Thermo scientific). Cell lysates were then stored at -80^0C until immunoblotting analysis.

Statistical analysis

Results are expressed as mean± standard deviation. Differences among multiple groups were analyzed by analysis of variance (ANOVA) followed by Tukey's test. Differences between two groups were analyzed by two-tail Student's *t*-test. The significance between groups was defined as $P < 0.05$.

Results

Effects of ethanol exposure on body weight, liver weight and plasma parameters

As shown in Table 2, the body weight did not show significant difference between ethanol-fed mice and the controls at all three-time point. The liver weight showed a

remarkable increase at 4-week and 8-week in ethanol-fed mice compared to the controls. The ratio of liver weight to body weight was also significantly higher in ethanol-fed mice at all-time point. The activity of plasma ALT and the level of plasma β-hydroxybutyrate were elevated, whereas the plasma glucose levels were reduced in ethanol-fed mice compared to the controls at 8-week.

Ethanol exposure impaired zinc homeostasis

In order to investigate zinc status in mice after alcohol exposure, zinc levels in the liver, plasma, and urine were measured. As illustrated in Figure 1, hepatic zinc levels were significantly lower at all three-time point in ethanol-fed mice compared to the controls. However, zinc levels in plasma and urine were remarkably increased in ethanol-fed mice compared to the controls at all three-time point.

Ethanol exposure suppressed hepatic zinc proteins

The major function of zinc is achieved through coordination to zinc proteins. To determine if decreased hepatic zinc levels were associated with inactivation of zinc binding proteins, the protein levels of HNF-4α, PPAR-α and SOD1 were measured. As shown in Figure 2, ethanol exposure significantly reduced the protein level of PPAR-α at 4-week and 8-week, and HNF-4α at all three-time point. Although the protein level of cytosolic SOD1 was not affected by ethanol exposure, the activity of SOD1 was decreased at 2-week in ethanol-fed mice compared to the controls.

Ethanol exposure altered hepatic zinc transporters

To determine how ethanol exposure affects hepatic zinc transporters, the gene expression levels of the full panel of 14 ZIPs and 10 ZnTs were measured, and results are

shown in Figure 3. In the control mice, the expressions of ZIP1, ZIP4, ZIP5, ZIP7, ZIP8, ZIP9, ZIP13, ZIP14, ZnT1, ZnT4, ZnT5, ZnT6, ZnT7 and ZnT10 were relatively abundant at all three-time point. The major effect of ethanol exposure on zinc transporter genes was down-regulation. ZIP8, ZIP9, ZnT4, ZnT5, ZnT6, ZnT7 and ZnT10 genes were down-regulated by ethanol exposure at 2-week. ZIP1, ZIP5, ZIP7, ZIP8, ZIP9, ZIP13, ZnT1, ZnT4, ZnT5, ZnT6, and ZnT7 genes were down-regulated by ethanol at 4-week. Ethanol down-regulated genes at 8-week include ZIP5, ZIP13, ZnT3, ZnT4, ZnT6, and ZnT7. However, ZIP14 gene was up-regulated by ethanol exposure at 2-and 4-week.

The protein abundance of zinc transporters with higher mRNA levels was then measured by immunoblotting, including ZIP1, ZIP4, ZIP5, ZIP7, ZIP8, ZIP13, ZIP14, ZnT1, ZnT4, ZnT5, ZnT6, ZnT7 and ZnT10. The protein levels of 7 ZIPs and 6 ZnTs are shown in Figure 4A, and 4B, respectively. Ethanol exposure significantly reduced the protein levels of ZIP5 and ZIP14 at all three-time point, but significantly increased ZIP4 protein abundance at 4-week and ZIP7 protein abundance at all three-time point (Figure 4A). Among the 6 ZnT proteins (Figure 4B), ethanol exposure significantly reduced ZnT4 protein abundance at 8-week, but significantly increased ZnT7 protein abundance at all three-time point.

Distribution of ZIP5, ZIP7 and ZnT7 in the liver of mice fed ethanol for 8-week was detected by immunohistochemistry. As showed in Figure 6A, chronic ethanol exposure reduced the staining intensity of ZIP5 in the liver. On the contrary, the staining intensity of hepatic ZIP7 (Figure 6B) and ZnT7 (Figure 6C) was increased by chronic

alcohol-fed, particularly in the area around the portal vein and central vein, compared to the controls.

Ethanol exposure induced hepatic CYP2E1 expression and caused lipid peroxidation

Hepatic CYP2E1 protein and lipid peroxidation product, 4-HNE, were detected by immunohistochemistry. As shown in Figure 5A, the CYP2E1 staining was mostly found around central vein area, and the staining was weak in the liver of controls, although a slight increase was found at 4- and 8-week compared to 2-week. Ethanol exposure increased hepatic CYP2E1 as early as 2-week, and further increased at 4- and 8-week. The immunohistochemical staining of 4-HNE is shown in Figure 5B. While only weak staining was found in the control mice at 8-week, ethanol exposure increased 4-HNE staining at all three-time point, particularly at 4- and 8-week, compared to the controls.

Treatment with 4-HNE or H_2O_2 altered expression of zinc transporters in murine FL-83B cells

Oxidant molecules, 4-HNE and H_2O_2, are generated in association with ethanol metabolism. In order to determine if these cytotoxic molecules mediate ethanol-induced alterations of zinc transporters, murine FL-83B hepatocytes were treated with these molecules for 72 h. As shown in Figure 6A, the treatment with 5 μM 4-HNE significantly decreased the protein levels of ZIP5, whereas increased ZIP7 protein level compared to the controls. The treatment with 100 μM H_2O_2 significantly increased the protein levels of ZIP7 and ZnT7 but remarkably decreased ZIP14 protein level compared to the control.

Discussion

The present study demonstrated that chronic alcohol exposure disturbed zinc homeostasis as indicated by decreased hepatic zinc levels and increased plasma and urinary zinc levels. The alcohol-induced alteration in hepatic and urinary zinc levels from this study was consistent with previous reports (31, 38). However, controversial data on the effect of alcohol exposure on serum zinc levels have been reported from human studies. Decreased serum zinc levels has been shown in patients with alcoholic hepatitis and cirrhosis [31, 34, 37]. Long-term of alcohol exposure (16 weeks) also reduce the plasma zinc levels in a mouse model of ALD. On the other hand, Hartoma *et al*. [10] reported that the serum zinc levels were elevated in alcoholics with normal liver or fatty liver, while reduced serum zinc levels were found in patients with alcoholic hepatitis or cirrhosis. These clinical studies suggest that serum zinc levels could be elevated at early stage of ALD, but decreased at advanced stage. In the mouse model of ALD showing reduced plasma zinc levels [17], the duration of alcohol exposure and dietary alcohol concentration were different from the present study. In this report, mice were exposed to ethanol for 16 weeks, and the dietary ethanol content was increased to make up 30% of total calories. In the present study, the longest ethanol exposure time was eight weeks, and ethanol contributed to 28% of total calories, which generated very early stage of alcoholic liver injury. These animal data also support the idea that plasma zinc levels may be increased at the early stage of alcoholic liver injury. Overall, all the observations suggest that zinc dyshomeostasis consistently exists in ALD, but the stage of ALD determines the redistribution pattern of zinc in body. In addition, plasma zinc level has

been used as an indicator for dietary zinc deficiency [22]. Our mouse model of ALD used a liquid diet containing adequate zinc, but this zinc adequate liquid diet differentially affected plasma and liver zinc levels. These results suggest that plasma zinc level is not always a good indicator for assessing dietary zinc status and organ zinc status at different pathophysiological conditions.

Zinc participates in diverse physiological activities via binding to proteins, therefore decreased availability of zinc leads to inactivation of zinc proteins. The present study demonstrated that zinc proteins including PPAR-α, HNF-4α, and SOD1 were inactivated, and previous reports have shown that the suppression of the three proteins correlates to the pathogenesis of ALD [3, 5, 11, 18, 19, 32]. This study also shows that alcohol exposure differentially affects PPAR-α, HNF-4α, and SOD1. While alcohol exposure reduced PPAR-α and HNF-4α protein levels, it did not affect the protein level of SOD1. However, the activity of SOD1 was significantly decreased by alcohol exposure for 2-week. It is known that zinc does not coordinate to proteins with equal affinity [28, 30]. Therefore, zinc proteins with lower zinc affinity will be affected by alcohol exposure at earlier time point. The data suggests that alcohol exposure induced alteration of hepatic zinc levels may associate with zinc binding protein inactivation, which is manifested as decreased protein expressions or impaired protein activities.

Zinc transporters are major players in regulation of hepatic zinc homeostasis, but a whole picture of hepatic expression of zinc transporters is lacking. The present study first analyzed the expression of zinc transporter genes (14 ZIPs and 10 ZnTs) and found that 8 ZIP and 6 ZnT genes are relatively abundant in the liver. Then the protein levels of these

abundant zinc transporters were further analyzed. Among the 14 zinc transporter proteins measured by immunoblotting, we detected 7 ZIP proteins and 5 ZnT proteins in the liver. However, ZIP9 and ZnT5 proteins were not detected, probably due to the limit of either antibody reactivity or the protein expression levels. Moreover, the results of mRNA and protein indicate that different zinc transporters might undergo various regulations. According to the results, we conclude that ZIP5 and ZnT1 might be regulated at transcriptional level, but ZIP7, ZIP8, ZIP13, ZIP14, ZnT4, ZnT6 and ZnT7 might undergo a posttranscriptional regulation. Among which, ZIP7, ZIP8, ZIP13, ZnT4, ZnT6 and ZnT7 might have a stabilized protein life; ZIP14 might undergo degradation process.

The altered expression of hepatic zinc transporters by ethanol exposure may lead to zinc dyshomeostasis in liver. Beker *et al.* showed that ZIP14 was located on the plasma membrane of hepatocytes, and increased level of ZIP14 could sequester zinc from plasma into liver under infection [1]. The ZIP5 protein is predicted located on plasma membrane of hepatocyte as well. Therefore, decreased ZIP5 and ZIP14 protein abundance by ethanol exposure may be the cause of decreased zinc levels in the liver. The ZIP7 and ZnT7 were reported located on organelles, such as ER and Golgi apparatus [23, 36]. It is well known that ER stress is accompanied with alcohol abuse [14, 15]. Previous studies indicate that zinc deficiency [8] or dysregulation of zinc transporters in ER [9] would induce ER stress in Saccharomyces Cerevisiae and Drosophila. Therefore, altered expression of these zinc transporters might not only cause organelle zinc dyshomeostasis but also lead to organelle dysfunction. Collectively, ethanol may alter the expression of zinc transporters through distinctive, translational or post-translational, regulatory

mechanisms. Furthermore, dysregulated zinc transporters might lead to organelle zinc dyshomeostasis and dysfunction of the organelles.

Oxidative stress is a feature of ALD. The present study demonstrates the induction of CYP2E1 and the generation of lipid peroxidation product, 4-HNE, in the liver occurred as early as the second week of alcohol exposure. Immunohistochemical staining of ZIP5, ZIP7 and ZnT7 suggested that altered expression of the zinc transporters are spatially correlated with the expression of CYP2E1 and 4-HNE. In order to demonstrate the causal relationship between oxidative stress and zinc transporters, H_2O_2 , one of the ROS generated by CYP2E1[27] , and 4-HNE were selected for *in vitro* study. We observed that 5 μM 4-HNE significantly decreased protein abundance of ZIP5 and increased the protein abundance of ZIP7. We also found that 100 μM H_2O_2 remarkably reduced production of ZIP14 protein and elevated the production of ZIP7 and ZnT7 proteins. These results indicate that the effects of alcohol on zinc transporters may be mediated by the oxidative molecules, which are generated in association with alcohol metabolism. The results also indicate that different oxidative molecules may not affect zinc transporters in the same way. The ZIP7 protein was affected by both H_2O_2 and 4-HNE treatment, suggesting that ZIP7 was susceptible to oxidative stress. We assume that oxidative stress induced by ethanol metabolism might directly affect the zinc transporters at transcriptional levels or/and enhance its stabilization or degradation at protein levels, but we do not exclude that oxidative stress may indirectly cause alteration of the zinc transporters. Maret *et al.* [28] suggested that oxidants might induce zinc dissociation from zinc proteins and concurrently alter the availability of zinc ion. Consequently, altered

labile zinc would affect the expression of zinc transporters by up-regulation or down-regulation of zinc sensing transcription factors, such as metal response element-binding transcription factor (MTF-1) [12, 24]. However, the precise molecular mechanisms have not been defined. The current study provides solid evidence that oxidative stress could affect the expression of zinc transporters. Therefore, a combined dietary intervention with antioxidant and zinc might produce a better improvement in alcohol-induced zinc deficiency and liver injury.

In summary, ethanol-induced hepatic zinc reduction occurred as early as the second week of ethanol exposure in mice. Accompanying with that, zinc proteins were affected either by decreased expression at protein levels (PPAR-α and HNF-4α) or impaired activity (SOD1). Zinc transporter screening detected 23 (except ZnT2) out of 24 zinc transporters at mRNA levels in the mouse liver. Among the zinc transporters with relative abundant gene expression, twelve zinc transporters were detected at protein levels, for the first time, in the mouse liver. Alcohol exposure consistently affected the protein levels of 4 zinc transporters (ZIP5, ZIP7, ZIP14 and ZnT7) out of 12 zinc transporters tested. The results from *in vitro* study demonstrated that the protein levels of ZIP5, ZIP7, ZIP14 and ZnT7 were affected by 4-HNE and H_2O_2. These results demonstrated that hepatic zinc transporters are remarkably altered by alcohol abuse via an oxidative stress-dependent mechanism, which might account for alcohol-induced hepatic zinc deficiency.

Acknowledgement

Qian Sun conducted all of the assays, western blot, qPCR, and cell culture studies. Dr. Qiong Li, Dr. Wei Zhong, Dr. Xiaobing Tan, Dr. Xiuhua Sun and Xinguo Sun

performed animal study. Jiayang Zhang, Xinmin Yin, and Dr. Xiang Zhang conducted atomic absorption spectrophotometry (AAS). Qian Sun,Xinguo Sun and Dr.Zhanxiang Zhou performed immunohistochemical staining. Dr. Zhanxiang Zhou and Qian Sun designed the studies; Qian Sun analyzed the data and draft the manuscript. All authors read and approval the final manuscript. This study was supported by a grant from the NIH National Institute on Alcohol Abuse and Alcoholism (R01AA1020212 and R01AA1018844 to Z.Z)

References

1. Beker Aydemir T, Chang SM, Guthrie GJ, Maki AB, Ryu MS, Karabiyik A, and Cousins RJ. Zinc transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the innate immune response (endotoxemia). *PLoS One* 7: e48679, 2012.

2. Bode JC, Hanisch P, Henning H, Koenig W, Richter FW, and Bode C. Hepatic zinc content in patients with various stages of alcoholic liver disease and in patients with chronic active and chronic persistent hepatitis. *Hepatology* 8: 1605-1609, 1988.

3. Crabb DW, Galli A, Fischer M, and You M. Molecular mechanisms of alcoholic fatty liver: role of peroxisome proliferator-activated receptor alpha. *Alcohol* 34: 35-38, 2004.

4. Cummings JE, and Kovacic JP. The ubiquitous role of zinc in health and disease. *J Vet Emerg Crit Care (San Antonio)* 19: 215-240, 2009.

5. Curry-McCoy TV, Osna NA, Nanji AA, and Donohue TM, Jr. Chronic ethanol consumption results in atypical liver injury in copper/zinc superoxide dismutase deficient mice. *Alcohol Clin Exp Res* 34: 251-261, 2010.

6. Desouki MM, Geradts J, Milon B, Franklin RB, and Costello LC. hZip2 and hZip3 zinc transporters are down regulated in human prostate adenocarcinomatous glands. *Mol Cancer* 6: 37, 2007.

7. Devirgiliis C, Zalewski PD, Perozzi G, and Murgia C. Zinc fluxes and zinc transporter genes in chronic diseases. *Mutat Res* 622: 84-93, 2007.

8. Ellis CD, Wang F, MacDiarmid CW, Clark S, Lyons T, and Eide DJ. Zinc and the Msc2 zinc transporter protein are required for endoplasmic reticulum function. *J Cell Biol* 166: 325-335, 2004.

9. Groth C, Sasamura T, Khanna MR, Whitley M, and Fortini ME. Protein trafficking abnormalities in Drosophila tissues with impaired activity of the ZIP7 zinc transporter Catsup. *Development* 140: 3018-3027, 2013.

10. Hartoma TR, Sotaniemi EA, Pelkonen O, and Ahlqvist J. Serum zinc and serum copper and indices of drug metabolism in alcoholics. *Eur J Clin Pharmacol* 12: 147-151, 1977.

11. Hayhurst GP, Lee YH, Lambert G, Ward JM, and Gonzalez FJ. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 21: 1393-1403, 2001.

12. Hogstrand C, Zheng D, Feeney G, Cunningham P, and Kille P. Zinc-controlled gene expression by metal-regulatory transcription factor 1 (MTF1) in a model vertebrate, the zebrafish. *Biochem Soc Trans* 36: 1252-1257, 2008.

13. Jayaraman AK, and Jayaraman S. Increased level of exogenous zinc induces cytotoxicity and up-regulates the expression of the ZnT-1 zinc transporter gene in pancreatic cancer cells. *J Nutr Biochem* 22: 79-88, 2011.

14. Ji C, and Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* 124: 1488-1499, 2003.

15. Ji C, and Kaplowitz N. Hyperhomocysteinemia, endoplasmic reticulum stress, and alcoholic liver injury. *World J Gastroenterol* 10: 1699-1708, 2004.

16. Kahn AM, Helwig HL, Redeker AG, and Reynolds TB. Urine and serum zinc abnormalities in disease of the liver. *Am J Clin Pathol* 44: 426-435, 1965.

17. Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, and Zhou Z. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. *Hepatology* 50: 1241-1250, 2009.

18. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, and Wahli W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* 103: 1489-1498, 1999.

19. Kessova IG, Ho YS, Thung S, and Cederbaum AI. Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase. *Hepatology* 38: 1136-1145, 2003.

20. Kiilerich S, Dietrichson O, Loud FB, Naestoft J, Christoffersen P, Juhl E, Kjems G, and Christiansen C. Zinc depletion in alcoholic liver diseases. *Scand J Gastroenterol* 15: 363-367, 1980.

21. King JC. Zinc: an essential but elusive nutrient. *Am J Clin Nutr* 94: 679S-684S, 2011.

22. King JC, Shames DM, and Woodhouse LR. Zinc homeostasis in humans. *J Nutr* 130: 1360S-1366S, 2000.

23. Kirschke CP, and Huang L. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. *J Biol Chem* 278: 4096-4102, 2003.

24. Langmade SJ, Ravindra R, Daniels PJ, and Andrews GK. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. *J Biol Chem* 275: 34803-34809, 2000.

25. Lichten LA, and Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. *Annu Rev Nutr* 29: 153-176, 2009.

26. Lopez V, and Kelleher SL. Zip6-attenuation promotes epithelial-to-mesenchymal transition in ductal breast tumor (T47D) cells. *Exp Cell Res* 316: 366-375, 2010.

27. Lu Y, and Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free Radic Biol Med* 44: 723-738, 2008.

28. Maret W, and Li Y. Coordination dynamics of zinc in proteins. *Chem Rev* 109: 4682-4707, 2009.

29. McClain CJ, Antonow DR, Cohen DA, and Shedlofsky SI. Zinc metabolism in alcoholic liver disease. *Alcohol Clin Exp Res* 10: 582-589, 1986.

30. Mocchegiani E, Muzzioli M, Cipriano C, and Giacconi R. Zinc, T-cell pathways, aging: role of metallothioneins. *Mech Ageing Dev* 106: 183-204, 1998.

31. Mohammad MK, Zhou Z, Cave M, Barve A, and McClain CJ. Zinc and liver disease. *Nutr Clin Pract* 27: 8-20, 2012.

32. Nagy LE. Molecular aspects of alcohol metabolism: transcription factors involved in early ethanol-induced liver injury. *Annu Rev Nutr* 24: 55-78, 2004.

33. Rodriguez-Moreno F, Gonzalez-Reimers E, Santolaria-Fernandez F, Galindo-Martin L, Hernandez-Torres O, Batista-Lopez N, and Molina-Perez M. Zinc, copper, manganese, and iron in chronic alcoholic liver disease. *Alcohol* 14: 39-44, 1997.

34. Schwartz JM, and Reinus JF. Prevalence and natural history of alcoholic liver disease. *Clin Liver Dis* 16: 659-666, 2012.

35. Solomons NW. Update on zinc biology. *Ann Nutr Metab* 62 Suppl 1: 8-17, 2013.

36. Taylor KM, Morgan HE, Johnson A, and Nicholson RI. Structure-function analysis of HKE4, a member of the new LIV-1 subfamily of zinc transporters. *Biochem J* 377: 131-139, 2004.

37. Wu CT, Lee JN, Shen WW, and Lee SL. Serum zinc, copper, and ceruloplasmin levels in male alcoholics. *Biol Psychiatry* 19: 1333-1338, 1984.

38. Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, and Kang YJ. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. *Am J Pathol* 166: 1681-1690, 2005.

Table 3.1 Primer Sequence Used for qPCR Analysis

Origin.	Name	Forward Primer 5'-3'	Reverse Primer 5'-3'
Mouse NM_013901	ZIP1	AGGTCAGGTGCTAACCATGAA	CTGTTCCTTGTAAGCCAGCGT
Mouse NM_001039676	ZIP2	CATATGACTGCTGAAGCTCTGG	CGAGAAGAATTTCCCTTACTTCC
Mouse NM_134135	ZIP3	GGTGGCGTATTCCTGGCTAC	CTGCTCCACGAACACAGTGA
Mouse NM_028064	ZIP4	ATGCTCCCAAAGTCGGTCAC	CAGCGTATTTAACAGGCCGTC
Mouse NM_028092	ZIP5	ATCATCTGCTGACTGGCCTAT	CAGTGTCCCGTTCTCTCCATA
Mouse NM_139143	ZIP6	GTCACACGGTTGCTGGTAAAA	GGGCGAGATCCTTTCCCTAGA
Mouse NM_008202	ZIP7	TGAAAGCATCTGGCATGGG	TGGAGGCTATCGTGGGAGTG
Mouse NM_026228	ZIP8	GCCAAGCTCATGTACCTGTCT	AAGATGCCCCAATCGCCAA
Mouse NM_026244	ZIP9	TGTTGGTGGGATGTTACGTGG	GATGACCGCCAGTGCAGTT
Mouse NM_172653	ZIP10	TCATCGCCATCGTTTGCATCA	CTCTGGTGAAGGGCTGTGAC
Mouse NM_027216	ZIP11	GTTAGCGGCTTCCTACTGGTC	TCCCCAACAGTGGTGTTTTCG
Mouse NM_001012305	ZIP12	CCTGCTCCATCTCATACCTC	TCCCAACAGCTTCCAAATAGG
Mouse NM_026721	ZIP13	TGCCTGTCGCCTGGATAATAA	ACTGAGCCCAACCATGAGAGA
Mouse NM_144108	ZIP14	GTGTCTCACTGATTAACCTGGC	AGAGCAGCGTTCCAATGGAC
Mouse NM_009579	ZnT1	GGAAGCGGAAGACAACAGGG	CAAGGCATTCACGACCACG
Mouse NM_001039677	ZnT2	CACCTGCTCACGGATTTTG	AGATGGAAAGCACGGACAAC
Mouse NM_011773	ZnT3	GACCTCTCTCTCTCTCCATCTT	AGGCACAGGCACACATAAA
Mouse NM_011774	ZnT4	AAGCGCCTCAAATCCCTGC	CCACCACGACTCGAAGTTTATT
Mouse NM_022885	ZnT5	TGGACCACTAAGGACCTTGCT	CAGCCCCTCTTGTCTTTGC
Mouse NM_144798	ZnT6	ATGGGGACGATTCATCTCTTTCG	CACAGCACGTTGATTGCACC
Mouse NM_023214	ZnT7	GGATGATGAATACAAACCACCCA	AAAGCGAAAGAGAGGTTCAGG
Mouse NM_172816	ZnT8	TGAGCGCCTTTTGTATCCTG	GTTGTAGCCAAAGTTCCGTTG
Mouse NM_178651	ZnT9	ACCAATGGAATCCCTGCTATG	ATTGCCTGTTATGGAGGTAAGG
Mouse NM_001033286	ZnT10	TCTGAAGCACTCAATATCAGAGG	AGAATATGATAGCCGTGATGACC
Mouse NM_007393	β-actin	TGAGCGCAAGTACTCTGTGTGGAT	GTTTGCTCCAACCAACTGCTGTC

Table 3.2 Weight, Liver Weight, and Blood Parameters

	2-week		4-week		8-week	
	Ctrl	EtOH	Ctrl	EtOH	Ctrl	EtOH
Body weight (g)	27.14±2.04	24.72±3.16	28.97±2.11	29.34±1.94	31.15±2.02	29.63±3.39
Liver weight (g)	1.09±0.1	1.18±0.1	1.10±0.1	1.4±0.2*	1.18±0.1	1.42±0.2*
Liver/body weight	4.01±0.2	4.80±0.5*	3.79±0.12	4.47±0.38*	3.80±0.14	4.78±0.36*
Blood parameters						
ALT activity (U/L)	26.19±8.03	25.06±7.3	17.53±4.08	30.06±13.73	17.37±7.72	48.02±18.21*
β-hydroxybutyrate (mg/dL)	6.6±2.4	9.7±3.0	4.8±1.2	6.9±0.69	4.5±1.2	10.6±3.6*
Glucose (mg/dL)	247±60.7	202.5±71.6	273.7±53.2	269.5±43.6	306.2±25.3	246.4±33.9*

Mice were pair-fed control or ethanol liquid diets for 2-week, 4-week, or 8-week. Data are means ± SD (n=6-8). Significant differences (*$P < 0.05$) between control- and ethanol-fed mice are determined by Student's *t*-test. Ctrl, control; EtOH, ethanol;

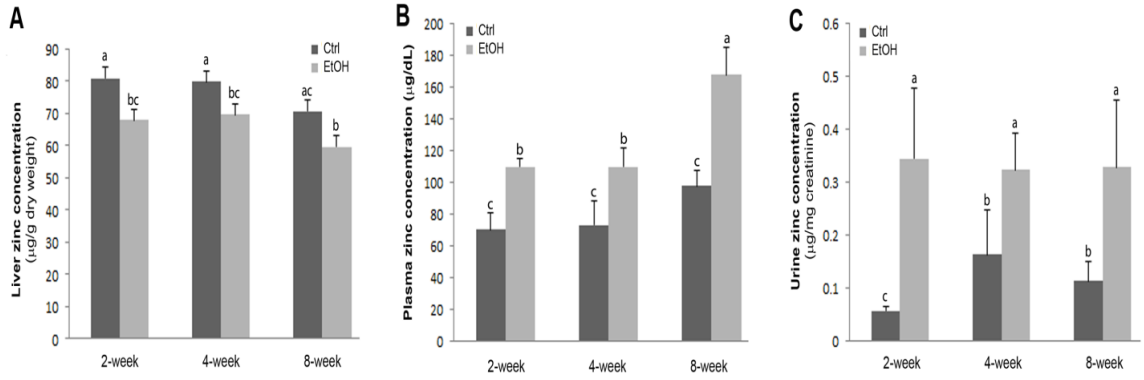


Figure 3.1. Zinc Levels in Liver, Plasma and Urine Samples in Mice Chronically Fed Ethanol or Control Diet for 2-week, 4-week or 8-week. Zinc levels in the liver (Figure 1A), plasma (Figure 1B), and urine (Figure 1C), were measured by atomic absorption spectrophotometry (AAS). Urine zinc was normalized by urine creatinine levels. Results are expressed as means ± SD (n =6-8). Results for bars that do not share a letter differed significantly among groups (P <0.05). Significant differences among groups are determined by ANOVA followed by Tukey's test. Ctrl, control; EtOH, ethanol.

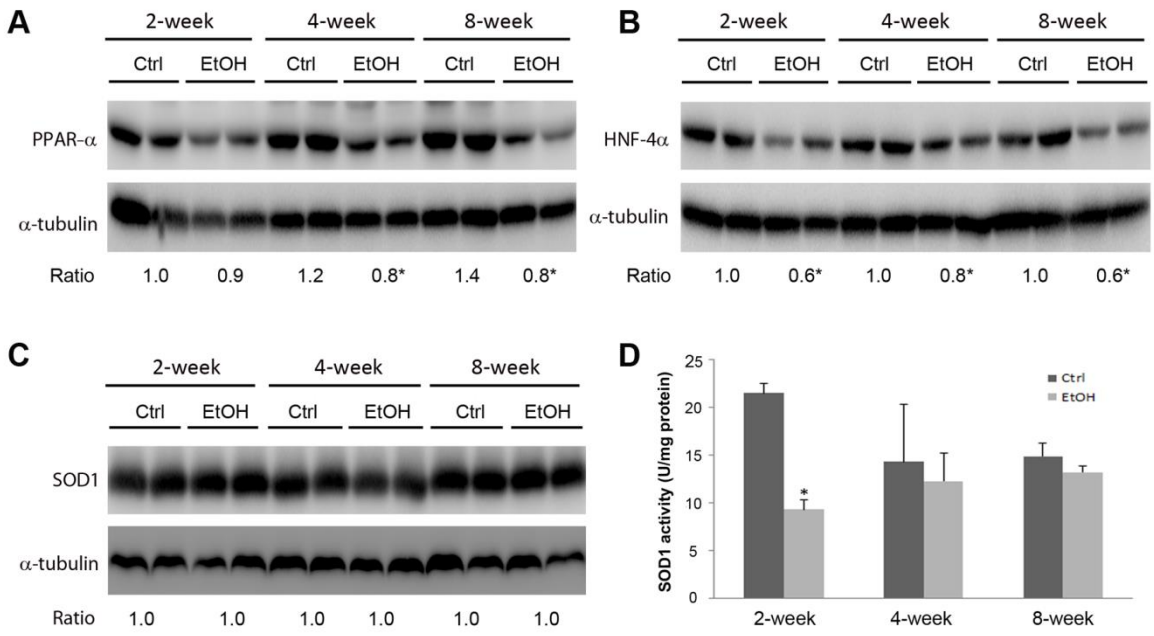


Figure 3.2. Protein Levels of Zinc-binding Proteins in Mouse Livers. Mice were chronically fed an ethanol or control diet for 2-week, 4-week or 8-week. The immunoblot bands of PPAR-α (A), HNF-4α (B) and SOD1 (C) were quantified by densitometry. The ratio to α-tubulin was calculated by setting the value of 2-week of control as one. The activity of SOD1 (D) was determined by SOD1 assay kit. Results are expressed as means ± SD (n =4). Significant differences (*$P < 0.05$) between groups are determined by Student's *t*-test. Ctrl, control; EtOH, ethanol.

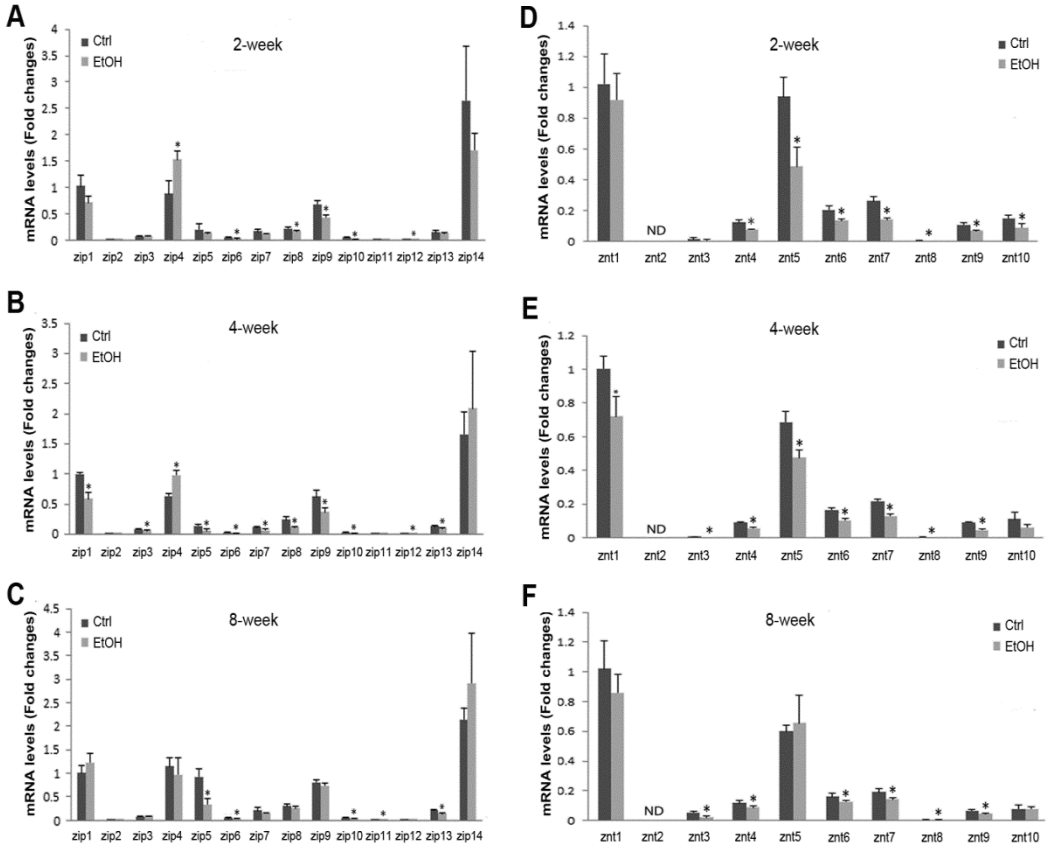


Figure 3.3. The Gene Expression Levels of Zinc Transporters in Mouse Livers. Mice were fed a liquid control diet or ethanol containing diet for 2-week, 4-week or 8-week. The gene expression of zinc transporters was measured by qPCR. The expression levels of hepatic ZIP at 2-week (A), 4-week (B), and 8-week (C). Hepatic ZnT mRNA levels at 2-week (D), 4-week (E), and 8-week (F). Significant differences (*$P < 0.05$) between control- and ethanol-fed mice are determined by Student's *t*-test. Ctrl, control; EtOH, ethanol.

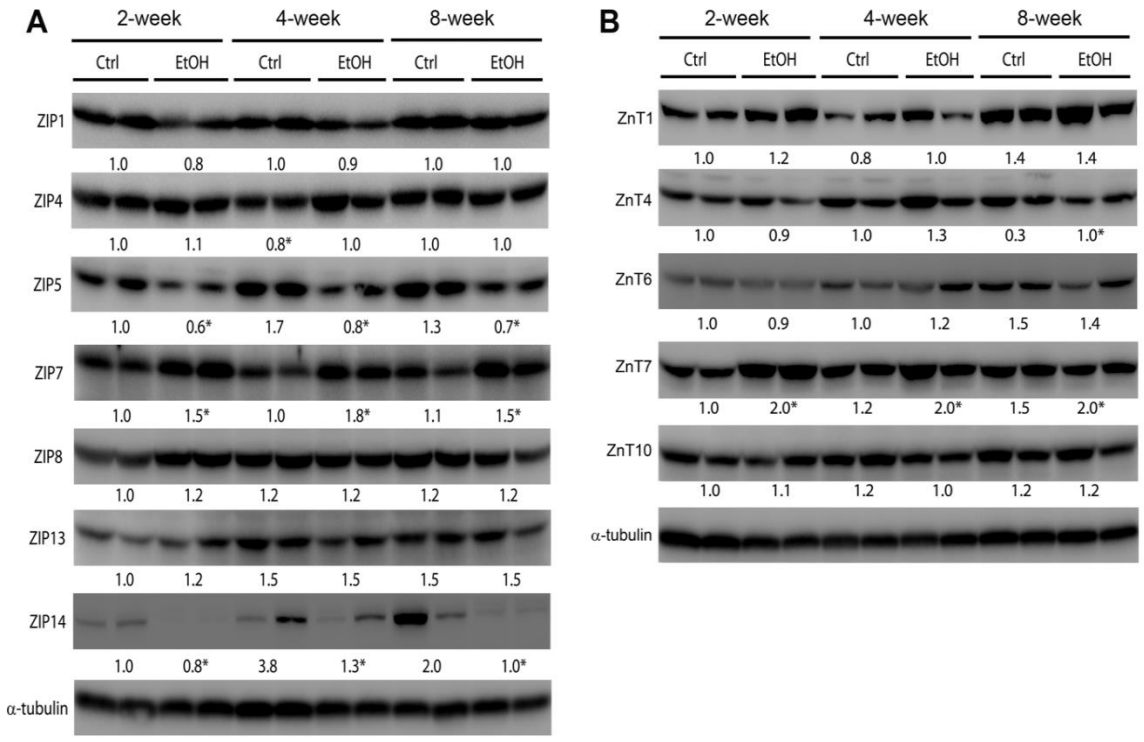


Figure 3.4. Protein Levels of Zinc Proteins in Mouse Livers. Mice were chronically fed ethanol or control diet for 2-week, 4-week or 8-week. The immunoblot bands of ZIP (A) and ZnT (B) were quantified by densitometry analysis. The ratio to α-tubulin was calculated by setting the value of 2-week of control as one. Significant differences (*P < 0.05) between groups are determined by Student's *t*-test. Ctrl, control; EtOH, ethanol.

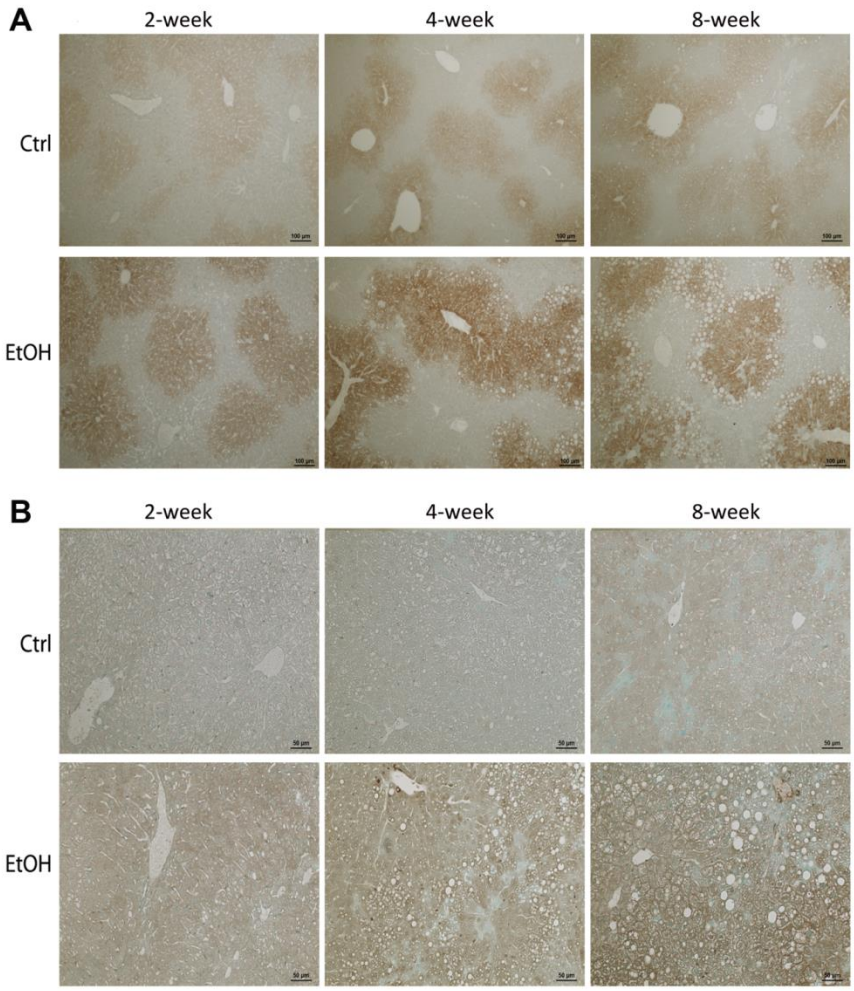


Figure 3.5. Immunohistochemical Staining of Cytochrome P450 2E1 (CYP2E1) and Hepatic 4-hydroxynonenal (4-HNE) in Mouse Liver. Mice were chronically fed with ethanol or control diet for 2-week, 4-week, or 8-week. (A) Representative images of CYP2E1 immunostaining. (B). Representative images of 4-HNE immunostaining. Scale car = 100 μm. Ctrl, control; EtOH, ethanol.

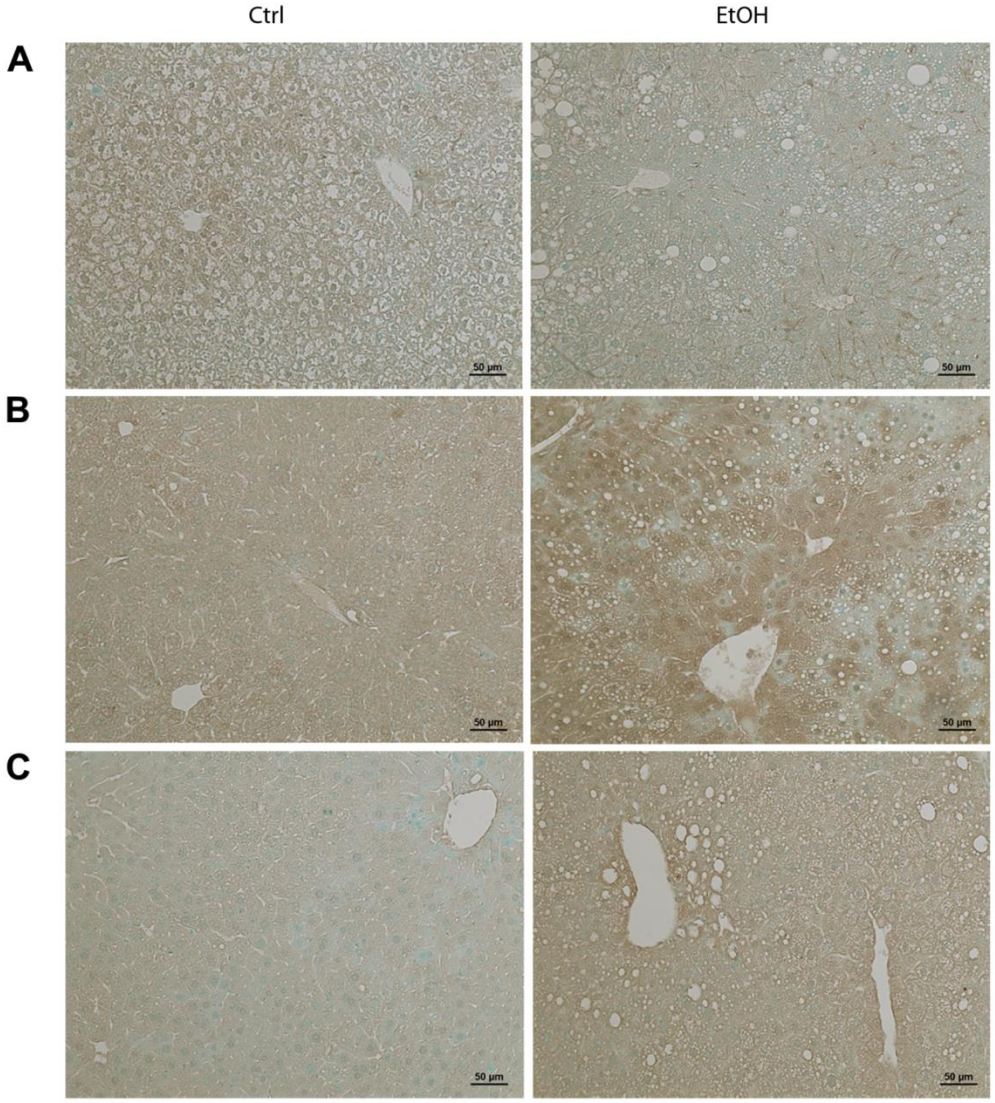


Figure 3.6. Immunohistochemical Staining of ZIP5, ZIP7 and ZnT7 in Mouse Liver. Mice were chronically fed with ethanol or control diet for 8-week. (A) Representative images of ZIP5 immunostaining. (B) Representative images of ZIP7 immunostaining. (C) Representative images of ZnT7 immunostaining. Scale car = 50µm. Ctrl, control; EtOH, ethanol.

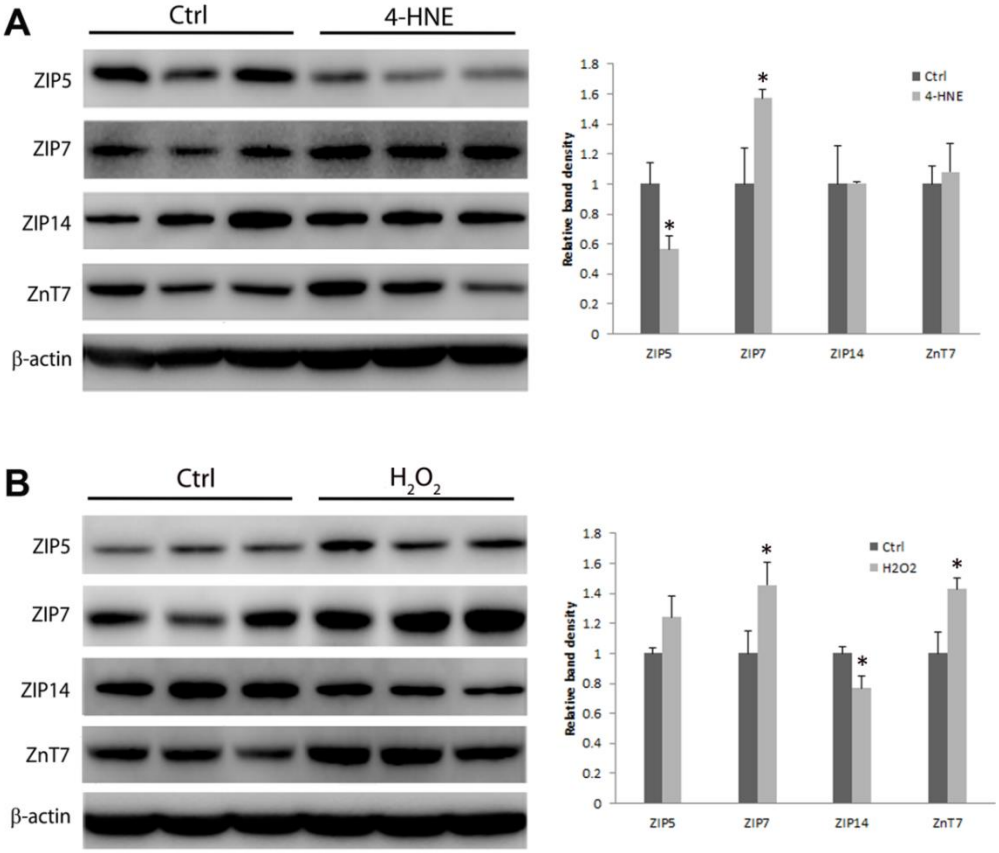


Figure 3.7. Effects of 4-hydroxynonenal (4-HNE) and Hydrogen peroxide (H_2O_2) on The Expression of ZIP5, ZIP7, ZIP14 and ZnT7 in Murine FL-83B Cells. FL-83B cells were treated with 5μM 4-HNE, or 100μM H_2O_2 for 72 h. Four zinc transporters were analyzed by immunoblotting. The bands were quantified by densitometry analysis. (A) Immunoblotting of four zinc transporters by 4-HNE treatment. (B) Immunoblotting of four zinc transporters by H_2O_2 treatment. Results are expressed as means ± SD (n = 3). Significant differences (*P < 0.05) between control and treatment are determined by Student's *t*-test. Ctrl, control.

CHAPTER IV

ZINC DEFICIENCY MEDIATES ALCOHOL-INDUCED APOPTOTIC CELL DEATH IN THE LIVER OF RATS THROUGH ACTIVATING ER AND MITOCHONDRIAL CELL DEATH PATHWAYS

Qian Sun, WeiZhong, Wenliang Zhang, Qiong Li, Xiuhua Sun, XiaobingTan, Xinguo Sun, Daoyin Dong, Zhanxiang Zhou. American Journal Physiology Gastrointestinal and Liver Physiology 308 (9): G757-66), 2015.

Abstract

Hepatic zinc deficiency has been well documented in alcoholic patients, but the mechanisms by which zinc deficiency mediates cell death have not been well defined. The objectives of this study were to determine if alcohol perturbs subcellular zinc homeostasis and how organelle zinc depletion may link with cell death pathways. Wistar rats were pair-fed with the Lieber-DeCarli control or ethanol diet for 5 months. Chronic alcohol exposure significantly reduced hepatic and hepatocyte ER and mitochondria zinc levels. Among the detected zinc transporters, ER ZIP 13 and mitochondrial ZIP 8, which transport zinc from ER and mitochondria to cytosol, were significantly increased. However, mitochondrial ZnT4, which transport zinc from cytosol to mitochondria, was also increased. Hepatic p-eIF-2α, ATF4, and Chop were significantly upregulated, mitochondria cytochrome C release and Bax insertion were detected in association with caspase-3 activation and apoptotic cell death. To define the role of zinc deficiency in ER

and mitochondrial stress, H4IIEC3 cells were treated with 3 μM TPEN for 6 hours with or without supplementation with zinc or NAC. The results demonstrated that zinc deprivation induced caspase-3 activation and apoptosis in association with ER and mitochondria dysfunction, which were inhibited by zinc as low as 10 μM but not by 2 mM NAC. These results suggest that chronic ethanol exposure induced ER and mitochondria zinc deficiency might activate intrinsic cell death signaling pathway, which could not be effectively rescued by antioxidant treatment.

Introduction

Alcohol consumption is associated with increased disease burden worldwide. Alcoholic liver disease (ALD) contributes to majority of the morbidity and mortality from alcohol abuse [20]. The spectrum of ALD is from hepatic steatosis to steatohepatitis, lastly fibrosis and cirrhosis [27]. The pathogenesis of ALD is derived from toxic metabolites of ethanol and a series of dysregulated signal transduction pathways. Ethanol is oxidized to acetaldehyde by cytosolic alcohol dehydrogenase (ADH) and microsomal cytochrome P450 2E1 (CYP2E1) [3]. Chronic alcohol intake induces CYP2E1 rather than ADH [3]. Acetaldehyde is converted to acetate by aldehyde dehydrogenase (ALDH) in mitochondria. In addition to the direct product of toxic acetaldehyde, ethanol metabolism also generates reactive oxygen species (ROS) [28]. Thus, ER and mitochondria as the major places for ethanol detoxification are the frontier organelles most easily being affected by chronic alcohol intake.

Zinc deficiency is a phenomenon consistently observed in ALD patients, especially alcoholic cirrhosis patients. Zinc supplementation to ALD patients reversed impaired

night vision, skin lesion and immune dysfunction [25]. Both animal and cell culture studies showed that zinc deprivation increased fat accumulation, inflammatory cell infiltration and greater number of apoptotic hepatocytes [15, 38, 39]. Accompanying with these abnormalities is increased ROS and decreased antioxidant components such as glutathione. Zinc supplementation to animals chronically fed alcohol increased ADH activity, suppressed CYP2E1 activity and prevented decrease in GSH [40]. Because zinc plays an important role in maintenance of normal structure and functions of large number of metalloenzymes and zinc proteins, zinc levels is tightly regulated by channels, zinc sensing molecules, such as metallothionein, metal –responsive-element-binding transcription factor-1, and zinc transporters [5]. Two families of zinc transporters are Zn transporter Zrt/Irt-like protein (ZIP) and Zinc transporter (ZnT) [17]. There are 14 members in ZIP family, and they are function in transporting zinc into cytosol from extracellular side or intracellular vesicles [11]. On the contrary, 10 members in ZnT family export zinc from cytosol to either outside of the cell or subcellular compartments [10]. Mechanistic study demonstrated that disrupted zinc transporters are linked with zinc dysregulation-associated pathogenesis [7, 30].

Alcohol induced hepatocyte apoptosis have been well documented in ALD patients and animal models [25]. Mechanistic study revealed that hepatic apoptosis is triggered by multiple signaling pathways, including ROS generation, cell membrane death receptor cascade, ER stress and dysfunction of mitochondria [8, 16, 19]. However, the importance of each mechanism may be different at a certain disease stage. Increasing evidence shows that ER stress plays a vital role in ALD [12]. ER stress induces unfolded protein response

(UPR) to restore ER homeostasis, but prolonged UPR leads to activation of inflammation, antioxidant defense, and/or insulin action signal pathways, and finally apoptosis [13]. The master regulator of ER stress induced apoptosis is C/EBP homologous protein (Chop), which upregulates pro-apoptotic protein expression, downregulates pro-survival protein expression and enhances oxidative stress [13, 14]. The unbalanced pro-apoptotic and pro-survival proteins could then affect mitochondria morphology and function and trigger mitochondrial mediated intrinsic apoptosis [2]. Therefore, we hypothesized that ethanol induced hepatic apoptosis is ROS dependent, which is caused by decreased subcellular zinc levels. In the present study zinc levels in hepatocyte organelles and hepatic apoptotic signaling activation were evaluated in rats. Mechanistic studies were conducted with rat hepatoma cells to establish the link between organelle zinc deficiency and apoptosis.

Materials and Methods

Animal and alcohol feeding experiments

Male Wistar rats were obtained from Harlan (Indianapolis, IN). The animal protocol was approved by the Institutional Animal Care and Use Committee of the North Carolina Research Campus. Eight-week-old male rats were pair-fed a modified Lieber-DeCarli alcohol or isocaloric maltose dextrin control liquid diet for 5 months (n= 6 for each group) with a stepwise feeding procedure The ethanol content (%, w/v) in the diet was 5.0 (36% of total calories) for the first 3 weeks, and increased by 0.25% every 2 weeks to reach 6.3 (44% of total calories), which was maintained for the last 8 weeks. The amount of food given to the pair-fed rats was that the alcohol-fed rats consumed in the previous day. At the end of 5-month feeding, rats were anesthetized with inhalational

isoflurane. Left lobe of liver was collected for organelle isolation process, and the rest of the liver were fixed for pathology or stored at -80°C.

Histopathology analysis of liver

Liver tissues were fixed in 10% formalin, and processed for paraffin embedding. Paraffin sections were cut at 5 μm and stained with hematoxylin and eosin (H&E) to assess the histological features of steatosis and inflammation.

Alanine Aminotransferase (ALT) and Aspartate aminotransferase (AST) activities

The ALT and AST activity in the serum were measured with an Infinity kit (Thermo Scientific, Waltham, MA).

Subcellular compartments isolation

Liver was perfused with buffer A (0.25 M sucrose, 10 mM Hepe-NaOH, 0.25 M KCL, pH 7.8) and the left lobe was removed and weighted (around 5 g). After minced with scissors, the left lobe of liver is homogenized with 40 ml dounce All-Glass tissue grinder (Kimble Chase, Vineland, NJ) in 20 ml of buffer A. The homogenate were centrifuged at 700 g for 5 min at 4 °C with Allegra X-22R centrifuge (Beckmen Coulter, Brea, CA). The pellets were crude nuclei, which were purified by differential centrifugation process. Briefly, 60 % Opti-prep (Sigm-Aldrich, St. Louis, MO) were mixed with crude nuclei samples to reach homogenate in 25% opti-prep. Thirty percent of opti-prep and 35 % opti-prep were prepared by mixing with diluent solution (0.25 M sucrose, 60 mM Hepe-NaOH, and pH 7.4). The solutions were carefully loaded in centrifuge tubes to three layers, and centrifuge at 13000 g for 1.5 h at 4°C with Sorvall RC6 plus centrifuge (Thermo Scientific). The supernatant from the first centrifugation

were then further centrifuged at 10,000 g for 15 min at 4 °C with Sorvall RC6 plus. The pellets were crude mitochondria, which was then homogenated and mixed with opti-prep to reach 36 % opti-prep. Ten percent and 30 % opti-prep were carefully loaded on top of it. The centrifuge process was carried out at 50,000 g for 4 h at 4 °C with Sorvall WX ultra series. Meanwhile supernatant of 10,000 g centrifugation were centrifuged again at 100,000 g for 1 h at 4°C with Sorvall WX ultra series. The supernantant was cysotol, and the pellets were microsome. The microsome were homogenized and mixed with 60 % opti-prep to prepare a homogenate in 20% opti-prep. The differential centrifugation process were conducted with the help of 15% and 30% opti-prep medium and centrifuge (Sorvall, WX ultra series) at 210,000 g for 2 h at 4 °C.

Determination of zinc concentrations in the liver, plasma and subcellular compartments

Zinc concentrations in the liver, plasma and subcellular compartments, namely ER, mitochondria, cytosol and nuclei, were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Twenty microgram of livers, one-hundred microliter of plasma, purified ER, mitochondria, cytosol and nuclei were frozen in liquid nitrogen and subsequently lyophilized for one and a half days. Dried samples were removed from the microcentrifuge tubes and added to microwave digester vessels. The mass of sample added to the digesters was recorded and used to normalize the concentration. The samples in the vessels were then digested with 5 ml of concentrated (69 %) nitric acid (HNO_3) and dried under a gentle flow of N_2 for 1 day. After drying, samples were reconstituted in 5 ml of 3 % HNO_3 in nanopure H_2O. An Agilent 7500cx ICP-MS platform was used to

develop standard curve and to subsequently analyze the digested samples. The zinc concentrations in samples were calculated as μg/g dry weight.

Immunoblotting analysis

Liver tissue, ER or mitochondria proteins were extracted by T-PER tissue extraction reagent (Thermo scientific) containing protease inhibitors (Sigma-Aldrich). Aliquots containing 30 μg proteins were loaded onto a 10 %-15 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and probed with polyclonal antibodies against Bax, ZIP7, ZnT5, ZnT6, ZnT10 (Santa Cruz Biotechnologies, Santa Cruz, CA), p-eIF-α, eIF-α, ATF-4, CHOP, ZIP1, ZIP4, ZIP5, ZIP14, ZnT4 (Novus biological, Littleton, CO), ZIP8, ZnT7 (Proteintech, Chicago, IL), cytochrome C (Abcam, Cambridge, MA), β-actin (Sigma-Aldrich) respectively. The membrane was then incubated with HRP-conjugated goat anti-rabbit IgG, or goat anti-mouse IgG, or rabbit anti-goat IgG antibody. The protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis.

Detection of hepatic and rat hepatoma cells cleaved caspase-3

Hepatic cleaved caspase-3 levels were detected by immunohistochemical staining. Briefly, liver tissue paraffin sections were incubated with 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases. Tissue sections were then incubated with a polyclonal rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA) at 4 °C overnight, followed by incubation with EnVision+ Labled Polymer-HRP-

conjugated anti-rabbit IgG (DAKO, Carpinteria, CA) at room temperature for 30 minutes. Diaminobenzidine (DAB) was used as HRP substrate for visualization. The positive staining area was quantified with Image J, and the data were expressed as percentage of positive staining area to the total area.

Rat H4IIEC3 hepatoma cells grown on slides chamber (Lab-Tek, Hatfield, PA) were detected by immunofluorescence staining. Briefly, cells were fixed with ice cold methanol for 10 min and then incubated with polyclonal rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology) at 4 °C overnight, followed by incubation with Alexa Fluor 594-conjugated anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) for 30 minutes.

Terminal Deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay

Apoptotic cell death in the liver and cell culture was assessed by detection of DNA fragmentation using an ApopTag peroxidase *in situ* Apoptosis Detection Kit (Millipore, Billerica, MA). Briefly, cell slides were fixed with 1% paraformaldehyde for 5min. Then liver tissue slides or cell slides were pretreated with proteinase K and H_2O_2 and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-conjugated dUTP for 1h at 37 °C. The labeled DNA was visualized with either HRP-conjugated anti-digoxigenin antibody with DAB as the chromagen followed by counterstaining with methyl green or Alexa Fluor 594-conjugated IgG anti-digoxigenin antibody (Jackson Immunoresearch) followed by DAPI counterstaining. The TUNEL- positive cells were counted under X20 objective, and the data were expressed as the average number of TUNEL-positive cells per view.

Cell culture and treatment

Rat H4IIEC3 hepatoma cells obtained from the American Type Culture Collection (Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml) streptomycin sulfate (100 μg/mL) (Invitrogen, Carlsbad, CA). H4IIEC3 hepatoma cells were seeded at 8×10^5 cells per well for 6-well plate, or 3×10^5 cells per well for 12-well plate, or 1×10^5 cells per well for 8-well chamber slide overnight. Then cells were treated with 3 μM N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Sigma-Aldrich), or 3 μM TPEN plus 10 μM zinc, or 3 μM TPEN plus 25 μM zinc, or 0.5 mM N-acetyl cysteine (Sigma-Aldrich), or 1 mM NAC, or 2 mM NAC, or 3 mM TPEN plus 0.5 mM NAC, or 3 μM TPEN plus 1 mM NAC, or 3 μM TPEN plus 2 mM NAC for six hours in absence of FBS.

Fluorescent Microplate

Dihydroethidium (Life technologies, Carlsbad, CA) is a superoxide indicator. After up-taken by cells, DHE was oxidized to ethidium, which then intercalates into DNA staining nucleus a bright fluorescent red. Live cells grown on 12-well plate were incubated with 5 μM DHE at 37 °C for 30 min in dark. The cells were trypsinized and washed twice with 1% BSA in PBS. The ROS generation was measured on microplate reader using excitation wavelength of 535 nm and an emission wavelength of 610 nm. The ROS production was expressed as the fluorescence ratio of the treated sample over the control.

Fluorescent Microscopy

Mitochondrial membrane potential is assessed in live cells by TMRE mitochondrial kit (Abcam). TMRE (tetramethylrhodamine, ethyl ester) is positively-charged red-orange dye which easily accumulates in active mitochondria. Depolarized or inactive mitochondria fail to sequester TMRE due to decreased membrane potential. Rat H4IIEC3 hepatoma cells were stained with 200 nM TMRE in culture medium at 37 °C for 20 min. The strength and the distribution pattern of red fluorescence reflect the alteration of mitochondria membrane potential among different treatments.

Cellular ROS was detected by CellROX Deep Red oxidative stress reagent (Life technologies) in live cells. The reagent is non-fluorescent while in a reduced state and upon oxidation showed strong fluorogenic signal in cytoplasm. ER was detected by ER-Tracker green dye (Life technologies), which is the drug conjugates glibenclamide BODIPY FL. Glibenclamide binds to the sulphonylurea receptor of ATP-sensitive K^+ channels, which are mainly on ER. Live cells were stained with 5 μM CellROX Deep Red at 37 °C for 30 min, and then cells were either counterstained with 1 μM ER-tracker at 37 °C for 20 min, or fixed with 3.7 % formaldehyde for 15 min and counterstained with DAPI (Life technologies).

Statistical analysis

Results are expressed as mean ± standard deviation. Differences among multiple groups were analyzed by analysis of variance followed by Tukey's test. Differences between two groups were analyzed by two-tail Student's *t*-test. The significance between groups was defined as $P < 0.05$.

Results

Body weight change and liver injury

The average body weight of rats increased from 303.36±8.21 g to 571.17±15.38 g in control groups and 303.42±8.52 g to 520.15±23.12 g in ethanol group, respectively, after 5 months of feeding (Figure 1A). The body weight did not show significant difference between control and ethanol groups at the time points of one month and two months, while significant difference was found at and after the time points of three months. Alcohol feeding also significantly increased the serum ALT activity (control 27.4 ± 6.3 U/L vs. ethanol 106.9 ± 80.6 U/L, $P < 0.05$) and AST activity (control 38.9 ± 6.4 U/L vs. ethanol 123.5 ± 85.3 U/L, $P < 0.05$) (Figure 1B). Light microscopy revealed that alcohol feeding caused formation of lipid droplets in hepatocytes and increased the number of neutrophil cells in the rat liver (Figure 1C).

Effect of alcohol feeding on subcellular zinc levels and zinc transporters

Chronic alcohol exposure significantly decreased total zinc levels in liver but differentially affect zinc levels in subcellular organelles. As shown in Figure 2A, the zinc levels in liver decreased from 82.6 ± 11.8 to 55.37 ± 2.08 μg/g dry weight after 5-month of alcohol-feeding. Alcohol feeding also reduced the zinc level in isolated ER and mitochondria from 2.66 ± 0.3 to 0.9 ± 0.08 and from 12.4 ± 1.4 to 5.3 ± 0.3 μg/g dry weights, respectively. However, alcohol feeding did not significantly affect the zinc level in cytosol or nuclei. In addition, plasma zinc level was not affected by alcohol feeding.

In order to determine which zinc transporters are localized in ER and mitochondria, and which ones are responsible for alcohol-feeding induced zinc dyshomeostasis, twelve

zinc transporters (ZIP 1, 4, 5, 7, 8, 13, 14 and ZnT 1, 4, 6, 7, 10) were examined by Western blot. As shown in Figure 2B, ZIP 7, ZIP 8, ZIP 13 and ZnT 7 were detected in ER, and only ZIP13 showed a significant increase after chronic alcohol-feeding. Meanwhile, three zinc transporters, ZIP 8, ZnT 1 and ZnT 4 were detected in mitochondria (Figure 2C). ZIP8 and ZnT4 were significantly upreguated at protein levels after alcohol exposure.

Alcohol feeding induced ER stress, mitochondria dysfunction and hepatocyte apoptosis

To determine whether cell death pathways were activated in association with zinc deficiency, ER and mitochondrial cell death pathway markers were examined. As shown in Figure 3A, alcohol-feeding significantly increased phosphorylated eukaryotic initiation factor 2α (p-eIF-2α) to eIF-2α ratio and protein levels of activating transcription factor 4 (ATF4) and Chop. Cytochrome C and Bcl-2-associated X protein (Bax) levels were determined in both mitochondria and cytosol. Figure 3B shows that alcohol-feeding remarkably increased mitochondrial Bax insertion and cytosolic cytochrome C release, which indicates activation of intrinsic apoptotic pathway.

Apoptosis were then evaluated by measuring cleaved caspase-3 and DNA fragmentation. Hepatic cleaved caspase-3 was determined by immunohistochemistry. As shown in Figure4A, the cleaved caspase-3 staining was faint in the liver of controls, but alcohol-feeding enhanced the staining, especially around the vein areas. The image quantification analysis (Figure 4B) shows that alcohol-feeding significantly increased expression of cleaved caspase-3 in the liver compared to controls. The number of hepatic apoptotic cells was evaluated by TUNEL assay. As shown in Figure 4C and D, alcohol

exposure remarkably increased the number of TUNEL positive cells (dark brown color) in the liver.

Experimental zinc deprivation induced apoptosis, mitochondria depolarization and ER stress in rat hepatoma cells

To determine the link of zinc deprivation and hepatocyte apoptosis, rat H4IIEC3 hepatoma cells were treated with zinc chelator, TPEN, for 6 h. Activation of cleaved caspase-3 and increased number of TUNEL positive cells were observed by fluorescence microscope (Figure 5A). Meanwhile, both 10 μM and 25 μM zinc treatment along with TPEN challenge inactivated cleaved caspase-3 and reduced the number of TUNEL positive cells (Figure 5A), indicating a specific chelation of zinc by TPEN.

Mitochondrial membrane potential and ER stress were then evaluated to determine whether zinc deprivation activates ER and mitochondria mediated intrinsic apoptotic pathway. As shown in Figure 5B, six hours of 3 μM TPEN treatment reduced mitochondrial membrane potentials indicated by decreased red fluorescence intensity, which was prevented by zinc supplementation. TPEN treatment significantly increased p-eIF-2α to eIF-2α ratio and the protein levels of ATF-4 and Chop, and zinc supplementation reversed the elevation of the ER stress makers (Figure 5C).

Zinc deprivation induced ROS accumulation in hepatoma cells

To examine whether zinc deprivation induces oxidative stress, ROS were detected by DHE immunofluorescence staining. Figure 6A shows that TPEN treatment significantly increased ROS production compared to control, and zinc supplementation at either 10 μM or25 μM prevented ROS accumulation. Localization of ROS in ER was

determined by double staining of ROS and ER marker. Figure 6B shows that TPEN induced oxidative stress in ER, which was inhibited by zinc supplementation.

ROS only partially mediated the pro-apoptotic effect of zinc deprivation in hepatoma cells

To determine whether ROS mediates zinc deprivation-activated ER and mitochondrial cell death signal pathways, rat hepatoma cells was treated with TPEN with or without antioxidant, NAC, at 0.5 mM, 1 mM, 2 mM. ROS were measured by fluorescent spectrometry (Figure 7A) and microscopy (Figure 7B). NAC at 2 mM completely blocked TPEN-induced ROS accumulation.

The ER stress and mitochondrial membrane potential were then assessed. As illustrated in Figure 7C, among the ER markers tested NAC at 2 mM only partially inhibited TPEN-increased Chop protein level. NAC treatment also partially reversed TPEN-induced loss of mitochondrial membrane potential (Figure 7D).

Lastly, caspase-3 activation and apoptotic cell death were measured to determine whether NAC treatment could inhibit TPEN induced apoptosis. As shown in Figure 7E, 2 mM NAC treatment partially decreased TPEN-induced activation of caspase-3 and the number of TUNEL positive cells. The results indicated that NAC could not completely rescue TPEN-induced ER and mitochondria mediated apoptosis in H4IIEC3 hepatoma cells.

Discussion

The present study not only presented the phenomenon that chronic alcohol exposure decreased hepatic zinc levels, but also for the first time showed that the

subcellular, ER and mitochondria, zinc levels were disturbed. The results indicate that comparing to nuclei and cytosol, ER and mitochondria are the two organelles most likely responsible for alcohol-consumption induced hepatic zinc deficiency. It is known that chronic alcohol drinking activates the microsomal ethanol oxidizing system (MEOS) to accelerate ethanol clearance. In addition, mitochondria are the main site for acetaldehyde oxidation. As a result, it is not surprising that ER and mitochondria are the most vulnerable organelles by prolonged alcohol-consumption.

Along with activation of MEOS and increased oxidation burden of mitochondria is the elevation of ROS production. It has been reported that alcohol drinking disturbed hepatic zinc transporters in mice, which might result from increased ROS [30]. Consistent with that observation, we found alteration of zinc transporters in hepatocyte ER and mitochondrial membranes in rats after alcohol-consumption. Four zinc transporters (ZIP7, ZIP8, ZIP13, and ZnT7) were detected in ER membrane, and ZIP13 significant increased by alcohol-consumption Three zinc transporters (ZIP8, ZnT1 and ZnT4) were detected in mitochondrial membrane, and ZIP8 and ZnT4 significantly increased by alcohol-consumption. The altered expression of ZIP13 and ZIP8 may be caused by increased oxidative stress due to alcohol metabolism [30]. But the upregulation of ZnT4 may be the adaptive response to reverse the reduction of zinc level in mitochondria. However, we did not find upregulation of any ZnT protein in ER to correct zinc level. Other mechanisms may also involve in decreasing zinc levels in ER and mitochondria, such as altered activity of zinc transporters and/or decreased zinc absorption from intestine. The alteration of spacial structure could decrease zinc binding

activity. It has been shown that alcohol- induced ROS inactivate zinc proteins such as hepatocyte nuclear factor-4 and peroxisome proliferator-activated receptor-α [15]. Therefore, further studies are required to confirm whether the spacial structure and activity of zinc transporters are affected by chronic alcohol exposure. Besides, our previous study has shown that chronic alcohol exposure induced intestinal barrier dysfunction and reduced zinc level of the ileum [36]. Thus, impaired intestinal zinc absorption may be another cause of hepatic zinc reduction and subsequent hepatocyte subcellular zinc dyshomeostasis. Although hepatic zinc deficiency has been well documented in ALD patients [1, 6], mechanisms of how alcohol interferes with hepatic zinc homeostasis are poorly understood. The current study not only revealed a differential effect of alcohol on zinc level in subcellular compartments, but also demonstrated dysregulation of subcellular zinc transporters after chronic alcohol exposure. To the best of our knowledge, this is the first report on subcellular distribution of zinc transporters in the liver regardless of alcohol exposure. Although ZIP4 has been detected in human liver at tissue level [31, 32], information on subcellular distribution of zinc transporters in human liver are not available. Therefore, one limitation of the study is that the data of hepatic zinc transporters are lack of support by human data. Our research group is very interested in obtaining the human data on subcellular distribution of zinc transporters and subcellular zinc concentrations at normal and ALD conditions in our future study.

Increased apoptosis is one of the major mechanisms involves in the progression of ALD. The present study showed that alcohol exposure increased the number of apoptotic cells in rat liver, and *in vitro* study demonstrates that zinc deprivation activated apoptotic

signaling. It is known that extrinsic and intrinsic signals can both initiate apoptotic mechanism. Extrinsic apoptotic pathway is activated by external death signals, namely through TNF-R1/TNF-α and Fas/FasL system, however, intrinsic apoptotic pathway activation depends on morphology change of mitochondria such as increased permeability and loss of membrane potential [2, 22]. Moreover, studies showed that ER stress induced upregulation of Chop is one way to initiate mitochondria mediated apoptotic pathway [14]. Therefore, ER could be the site in triggering intrinsic apoptosis. It has been reported that chronic alcohol drinking activated extrinsic apoptotic pathway [37, 39]. However, whether zinc deprivation played a role in activating ER and mitochondria mediated intrinsic apoptotic pathway is still unclear. In the present study, ER stress and mitochondria cytochrome C release were evaluated. The results indicated that the protein expression of Chop was increased and mitochondria cytochrome C was released into cytosol, meanwhile the caspase-3 was activated after 5 months of alcohol-feeding. *In vitro* zinc deprivation study showed Chop upregulation, loss of mitochondria membrane potential and increased cleaved caspase-3. Although in current study zinc supplementation was not given to rats to evaluate the protective effect of zinc on the expression of ER stress markers and mitochondrial dysfunction proteins. A previously study from our group has shown that zinc supplementation attenuates hepatic apoptosis through inhibition of cell death receptor (TNFR1 and Fas) mediated apoptotic pathway in mice chronically fed alcohol [39] In contrast, feeding mice a zinc deficient diet, in our previous study, exaggerated chronic alcohol exposure-induced liver injury in association with up-regulation of hepatic cell death receptors, TNFR1 and CD95 [37]. Another report

from our group further showed that zinc supplementation attenuates hepatic oxidative stress, leading to significant improvement of the ultrastructure of ER and mitochondria of hepatocytes in mice chronically fed alcohol [40]. These studies indicate that hepatic zinc homeostasis plays a critical role in regulation of alcohol-induced pathogenesis of hepatocellular apoptosis. Therefore it is predictable that protection of the integrity of mitochondrial membrane by zinc supplementation may lead to inhibition of alcohol induced Bax insertion to mitochondria and suppression of alcohol-induced cytochorome C release from mitochondria. Besides, ROS are important inducers of ER stress [13], and inhibition of oxidative stress by zinc supplementation would ameliorate alcohol-induced ER stress and consequent cell death signaling. Further investigations are required to confirm the protective effects of zinc supplementation on mitochondria and ER mediated cell death pathways. The results suggest that zinc deprivation induced ER stress could trigger mitochondria mediated intrinsic apoptotic pathway. However, we do not rule out the possibility that zinc deprivation directly affects mitochondria function, and the enhanced apoptotic process is the synergistic effect of ER and mitochondria dysfunction.

Decreased zinc level is associated with increased oxidative stress. The present study demonstrates that zinc deprivation increased the production of ROS, however antioxidant, NAC, treatment did not completely reverse zinc withdraw caused hepatoma cell apoptosis. It has been found that TPEN challenge decreased GSH level in rat primary hepatocyte [26]. In addition, Pelicci *et al.* reported that increased oxidative stress induced apoptosis through p66Shc stress adaptor protein [24]. Therefore, in the present study we tested whether zinc deprivation induced ROS is a key mechanism in apoptosis. The

results indicate that zinc deprivation induced apoptosis is partially dependent on oxidative stress. There are two possible explanations for the partial failure of antioxidant treatment in rescuing zinc deficiency induced hepatic cell apoptosis. First, zinc deprivation caused activation of caspase-3 is probably the key factor in apoptosis. Kaufmann *et al.* reported that in a cell free system the presence of cytochrome C and ATP activate caspase-3, -6,-7, and adding zinc to the system inhibited the activation of the above caspase species [23]. Besides, studies [18, 26, 29] measured cleaved caspase-3 activity after TPEN challenge and NAC treatment, and they found that NAC treatment did not reverse TPEN induced activation of caspase-3. Second, zinc deprivation induced ER stress not completely result from ROS. It is well-known that zinc plays role in maintenance of catalytic and structural activity of proteins, signal transduction, and antioxidant defense [5, 21, 33]. Therefore, it is most likely that zinc deprivation induced ER stress is caused by a series of events instead of a single factor. It has been reported that zinc-deficiency conditions affects structure of a zinc requiring enzyme, SOD1, which induce ER stress response [9]. In addition, Younce *et al.* demonstrated that MCP-1 induced protein (MCPIP), a zinc finger protein, involved in MCP-1 (monocyte chemotactic protein-1) caused ER stress in cardiomyoblast [35]. Therefore, zinc proteins may be involved in zinc deprivation induced ER stress and consequent apoptosis as well. The results suggest that other mechanisms may involve in zinc deprivation induced ER stress and mitochondria dysfunction besides increased oxidative stress.

In summary, for the first time we demonstrated that alcohol induced hepatic zinc deficiency might be from hepatocyte ER and mitochondria zinc reduction, and the

reduced zinc levels in ER and mitochondria caused organelle dysfunction, including ER stress, loss of mitochondrial membrane potential, and increase of mitochondria permeability. The interrupted subcellular compartments normal function led to activation of caspase-3 and excessive hepatocyte apoptosis. The pathogenic process was partially dependent on ROS.

Acknowledgement

Qian Sun conducted subcellular compartments isolation, all of the assays, western blot, cell culture and fluorescence staining studies. Qian Sun, Dr. Wei Zhong , Dr. Wenliang Zhang, Dr. Qiong Li,Dr. Xiaobing Tan, Dr. Xiuhua Sun, Dr.Daoyin Dong and Xinguo Sun performed animal study. Qian Sun, Xinguo Sun and Dr.Zhanxiang Zhou performed immunohistochemical staining. Dr. Zhanxiang Zhou and Qian Sun designed the studies; Qian Sun analyzed the data and draft the manuscript. All authors read and approval the final manuscript. This study was supported by a grant from the NIH National Institute on Alcohol Abuse and Alcoholism (R01AA1020212 and R01AA1018844 to Z.Z).

References

1. Bartholomay AF, Robin ED, Vallee RL, and Wacker WE. Zinc metabolism in hepatic dysfunction. I. Serum zinc concentrations in Laennec's cirrhosis and their validation by sequential analysis. *N Engl J Med* 255: 403-408, 1956.

2. Brenner D, and Mak TW. Mitochondrial cell death effectors. *Curr Opin Cell Biol* 21: 871-877, 2009.

3. Cederbaum AI. Alcohol metabolism. *Clin Liver Dis* 16: 667-685, 2012.

4. Dalton TP, He L, Wang B, Miller ML, Jin L, Stringer KF, Chang X, Baxter CS, and Nebert DW. Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. *Proc Natl Acad Sci U S A* 102: 3401-3406, 2005.

5. Fukada T, Yamasaki S, Nishida K, Murakami M, and Hirano T. Zinc homeostasis and signaling in health and diseases: Zinc signaling. *J Biol Inorg Chem* 16: 1123-1134, 2011.

6. Goode HF, Kelleher J, and Walker BE. Relation between zinc status and hepatic functional reserve in patients with liver disease. *Gut* 31: 694-697, 1990.

7. Groth C, Sasamura T, Khanna MR, Whitley M, and Fortini ME. Protein trafficking abnormalities in Drosophila tissues with impaired activity of the ZIP7 zinc transporter Catsup. *Development* 140: 3018-3027, 2013.

8. Hentze H, Kunstle G, Volbracht C, Ertel W, and Wendel A. CD95-Mediated murine hepatic apoptosis requires an intact glutathione status. *Hepatology* 30: 177-185, 1999.

9. Homma K, Fujisawa T, Tsuburaya N, Yamaguchi N, Kadowaki H, Takeda K, Nishitoh H, Matsuzawa A, Naguro I, and Ichijo H. SOD1 as a molecular switch for initiating the homeostatic ER stress response under zinc deficiency. *Mol Cell* 52: 75-86, 2013.

10. Huang L, and Tepaamorndech S. The SLC30 family of zinc transporters - a review of current understanding of their biological and pathophysiological roles. *Mol Aspects Med* 34: 548-560, 2013.

11. Jeong J, and Eide DJ. The SLC39 family of zinc transporters. *Mol Aspects Med* 34: 612-619, 2013.

12. Ji C. Mechanisms of alcohol-induced endoplasmic reticulum stress and organ injuries. *Biochem Res Int* 2012: 216450, 2012.

13. Ji C. New Insights into the Pathogenesis of Alcohol-Induced ER Stress and Liver Diseases. *Int J Hepatol* 2014: 513787, 2014.

14. Ji C, Mehrian-Shai R, Chan C, Hsu YH, and Kaplowitz N. Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. *Alcohol Clin Exp Res* 29: 1496-1503, 2005.

15. Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, and Zhou Z. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. *Hepatology* 50: 1241-1250, 2009.

16. Karbowski M, Kurono C, Wozniak M, Ostrowski M, Teranishi M, Nishizawa Y, Usukura J, Soji T, and Wakabayashi T. Free radical-induced megamitochondria formation and apoptosis. *Free Radic Biol Med* 26: 396-409, 1999.

17. Lichten LA, and Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. *Annu Rev Nutr* 29: 153-176, 2009.

18. Lopez JJ, Redondo PC, Salido GM, Pariente JA, and Rosado JA. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine induces apoptosis through the activation of caspases-3 and -8 in human platelets. A role for endoplasmic reticulum stress. *J Thromb Haemost* 7: 992-999, 2009.

19. Malhi H, and Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol* 54: 795-809, 2011.

20. Mann RE, Smart RG, and Govoni R. The epidemiology of alcoholic liver disease. *Alcohol Res Health* 27: 209-219, 2003.

21. Maret W, and Li Y. Coordination dynamics of zinc in proteins. *Chem Rev* 109: 4682-4707, 2009.

22. Martinou JC, and Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev Cell* 21: 92-101, 2011.

23. Mesner PW, Jr., Bible KC, Martins LM, Kottke TJ, Srinivasula SM, Svingen PA, Chilcote TJ, Basi GS, Tung JS, Krajewski S, Reed JC, Alnemri ES, Earnshaw WC, and Kaufmann SH. Characterization of caspase processing and activation in HL-60 cell cytosol under cell-free conditions. Nucleotide requirement and inhibitor profile. *J Biol Chem* 274: 22635-22645, 1999.

24. Migliaccio E, Giorgio M, and Pelicci PG. Apoptosis and aging: role of p66Shc redox protein. *Antioxid Redox Signal* 8: 600-608, 2006.

25. Mohammad MK, Zhou Z, Cave M, Barve A, and McClain CJ. Zinc and liver disease. *Nutr Clin Pract* 27: 8-20, 2012.

26. Nakatani T, Tawaramoto M, Opare Kennedy D, Kojima A, and Matsui-Yuasa I. Apoptosis induced by chelation of intracellular zinc is associated with depletion of cellular reduced glutathione level in rat hepatocytes. *Chem Biol Interact* 125: 151-163, 2000.

27. O'Shea RS, Dasarathy S, and McCullough AJ. Alcoholic liver disease. *Am J Gastroenterol* 105: 14-32; quiz 33, 2010.

28. Purohit V, Gao B, and Song BJ. Molecular mechanisms of alcoholic fatty liver. *Alcohol Clin Exp Res* 33: 191-205, 2009.

29. Rudolf E, and Cervinka M. Depletion of endogenous zinc stores induces oxidative stress and cell death in human melanoma cells. *Acta Medica (Hradec Kralove)* 47: 91-96, 2004.

30. Sun Q, Li Q, Zhong W, Zhang J, Sun X, Tan X, Yin X, Sun X, Zhang X, and Zhou Z. Dysregulation of hepatic zinc transporters in a mouse model of alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* 307: G313-322, 2014.

31. Weaver BP, Zhang Y, Hiscox S, Guo GL, Apte U, Taylor KM, Sheline CT, Wang L, and Andrews GK. Zip4 (Slc39a4) expression is activated in hepatocellular carcinomas and functions to repress apoptosis, enhance cell cycle and increase migration. *PLoS One* 5: 2010.

32. Xu X, Guo HJ, Xie HY, Li J, Zhuang RZ, Ling Q, Zhou L, Wei XY, Liu ZK, Ding SM, Chen KJ, Xu ZY, and Zheng SS. ZIP4, a novel determinant of tumor invasion in

hepatocellular carcinoma, contributes to tumor recurrence after liver transplantation. *Int J Biol Sci* 10: 245-256, 2014.

33. Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K, and Hirano T. Zinc is a novel intracellular second messenger. *J Cell Biol* 177: 637-645, 2007.

34. Yilmaz Y, Alahdab YO, Ozdogan O, and Dolar E. Non-alcoholic fatty liver disease and microalbuminuria in non-diabetic patients: role of insulin resistance. *Intern Med J* 39: 709-710, 2009.

35. Younce CW, and Kolattukudy PE. MCP-1 causes cardiomyoblast death via autophagy resulting from ER stress caused by oxidative stress generated by inducing a novel zinc-finger protein, MCPIP. *Biochem J* 426: 43-53, 2010.

36. Zhong W, McClain CJ, Cave M, Kang YJ, and Zhou Z. The role of zinc deficiency in alcohol-induced intestinal barrier dysfunction. *Am J Physiol Gastrointest Liver Physiol* 298: G625-633, 2010.

37. Zhong W, Zhao Y, Sun X, Song Z, McClain CJ, and Zhou Z. Dietary zinc deficiency exaggerates ethanol-induced liver injury in mice: involvement of intrahepatic and extrahepatic factors. *PLoS One* 8: e76522, 2013.

38. Zhou Z, Kang X, Jiang Y, Song Z, Feng W, McClain CJ, and Kang YJ. Preservation of hepatocyte nuclear factor-4alpha is associated with zinc protection against TNF-alpha hepatotoxicity in mice. *Exp Biol Med (Maywood)* 232: 622-628, 2007.

39. Zhou Z, Liu J, Song Z, McClain CJ, and Kang YJ. Zinc supplementation inhibits hepatic apoptosis in mice subjected to a long-term ethanol exposure. *Exp Biol Med (Maywood)* 233: 540-548, 2008.

40. Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, and Kang YJ. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. *Am J Pathol* 166: 1681-1690, 2005.

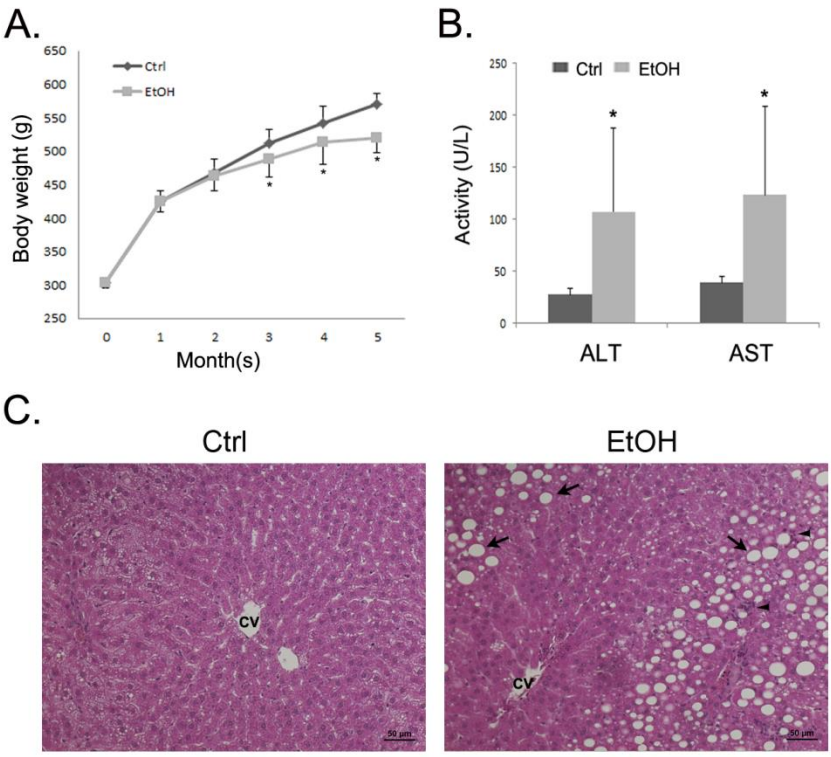


Figure 4.1. Body Weight Change, Plasma Markers of Liver Injury, and Liver Histopathology in Rats Chronically Fed Ethanol (EtOH) or Control (Ctrl) Liquid Diet for 5 Months. A. Body weight change. B. Plasma alanine aminotransferase (ALT) activities and aspartate aminotransferase (AST) activities. Plasma ALT and AST activities were measured using Infinity ALT and AST Reagents. C. Light microscopy with hematoxylin&eosin staining shows accumulation of lipid droplet (arrows) and neutrophil infiltration (arrow heads) in the liver of ethanol-fed rat. CV: central vein. Scale bar: 50 μM. Results are means ± SD (n=6). Significant differences (*$P < 0.05$) between control- and ethanol-fed rats are determined by Student's *t*-test.

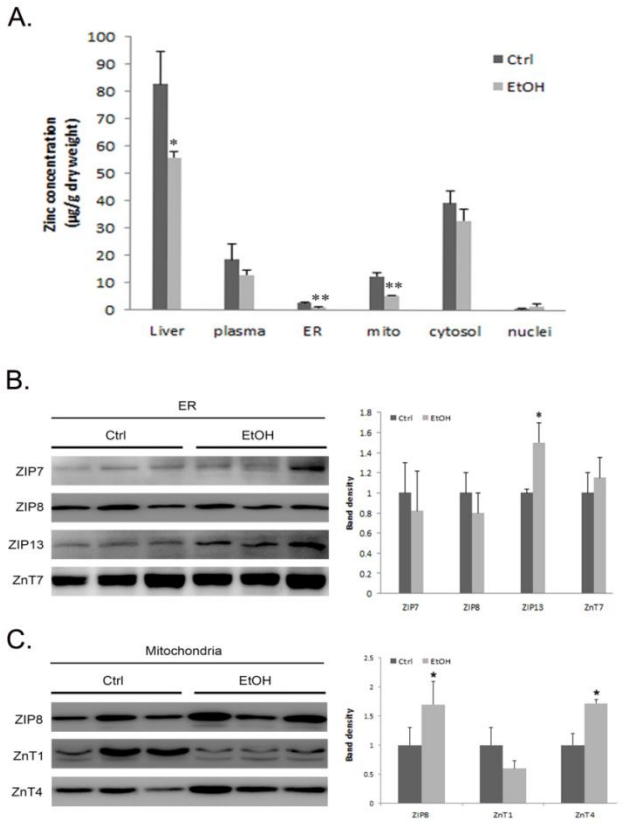


Figure 4.2. Subcellular Zinc Levels and Protein Levels of Zinc Transporters in Rats Chronically Fed Ethanol (EtOH) or Control (Ctrl) Liquid Diet for 5 Months. A. Zinc levels in the liver, plasma, and endoplasmic reticulum (ER), mitochondria, cytosol and nuclei of hepatocytes were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Results are expressed as means ± SD (n =6). B. Immunoblot of ER zinc transporter proteins. C. Immunoblot of mitochondrial zinc transporter proteins. The bands were quantified by densitometry analysis. The ratio to the total ER or mitochondrial proteins was calculated by setting the value of control as one. Significant differences (*$P < 0.05$, **$P < 0.01$) between control-and ethanol-fed rats are determined by Student's *t*-test.

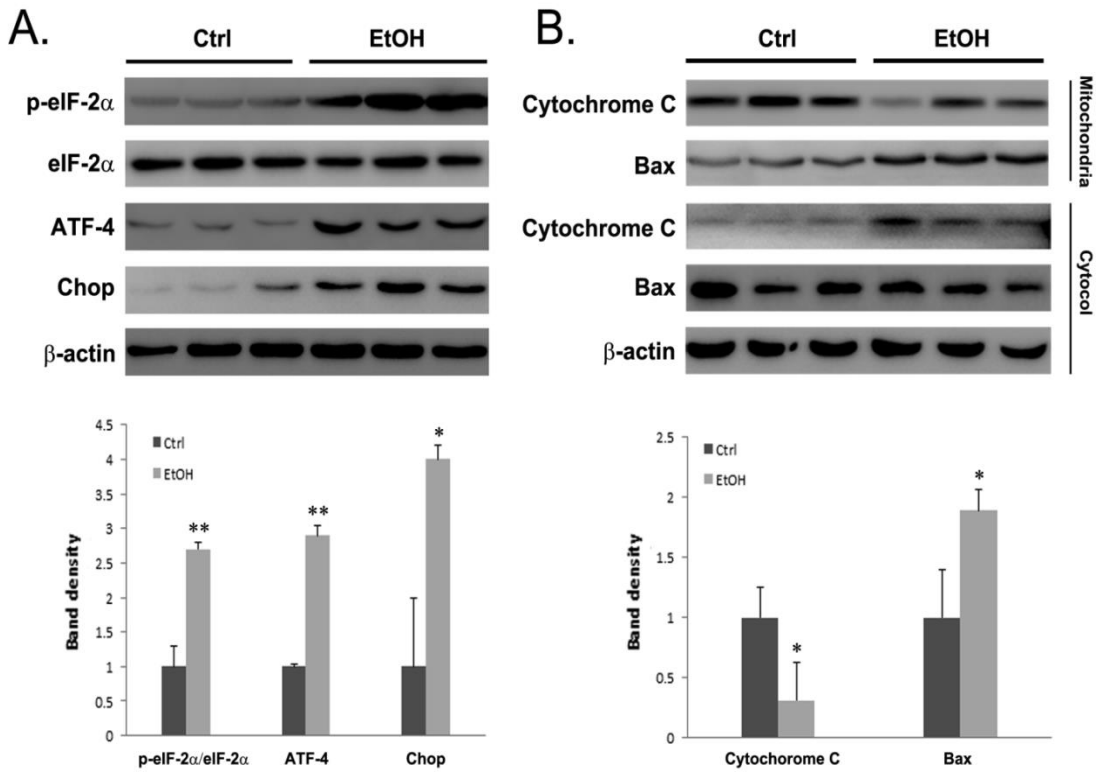


Figure 4.3. Protein Levels of ER Stress and Mitochondrial Apoptosis Markers. Rats were chronically fed ethanol (EtOH) or control (Ctrl) liquid diet for 5 months. A. Immunoblot of ER stress makers. B. Immunoblot of mitochondrial and cytosolic cytochrome C and Bax. The bands were quantified by densitometry analysis. The ratio to β-actin (A) or the ratio of mitochondrial proteins to cytosolic proteins (B) was calculated by setting the value of control as one. Significant differences (*$P < 0.05$, **$P < 0.01$) between control- and ethanol-fed rats are determined by Student's *t*-test.

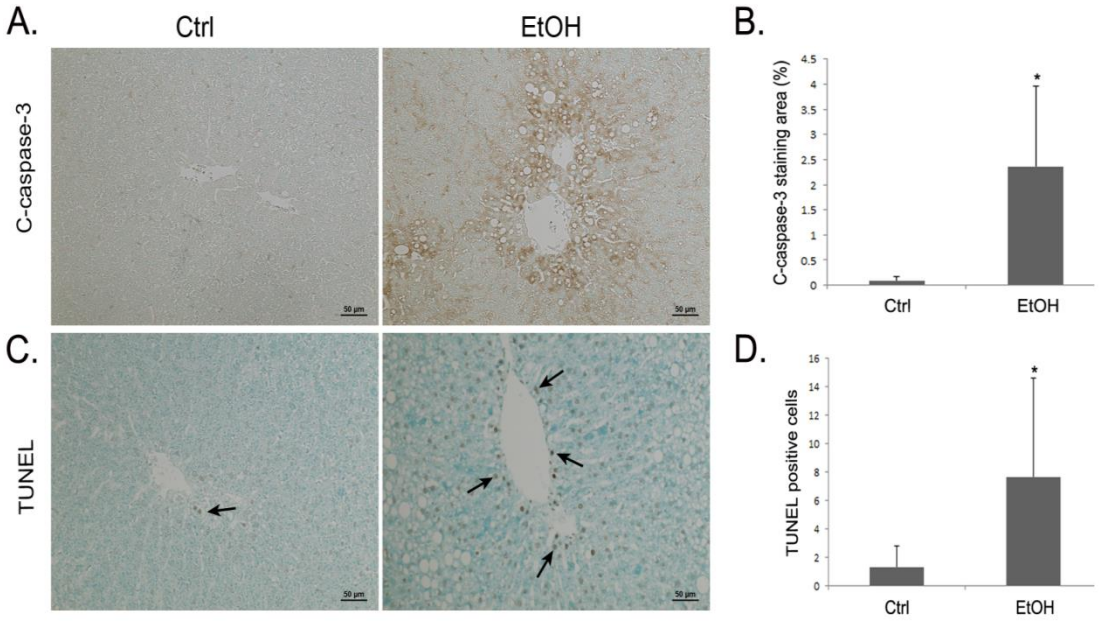


Figure 4.4. Immunohistochemical Staining of Cleaved Caspase-3 and TUNEL Assay in The Rat Liver. Rats were chronically fed with ethanol (EtOH) or control (Ctrl) liquid diet for 5 months. A. Representative image of immunostaining of cleaved caspase-3 (Arrows). B. Quantitative measurements of cleaved-caspase-3 in the liver. C. Representative image of TUNEL assay. Arrows indicate TUNEL-positive cells. D. Quantitative measurements of TUNEL-positive cells in liver. Scale bar: 50 μm. Results are mean ± SD (n=6). Significant differences (*$P < 0.05$) between control and ethanol-fed rats are determined by Student's *t*-test.

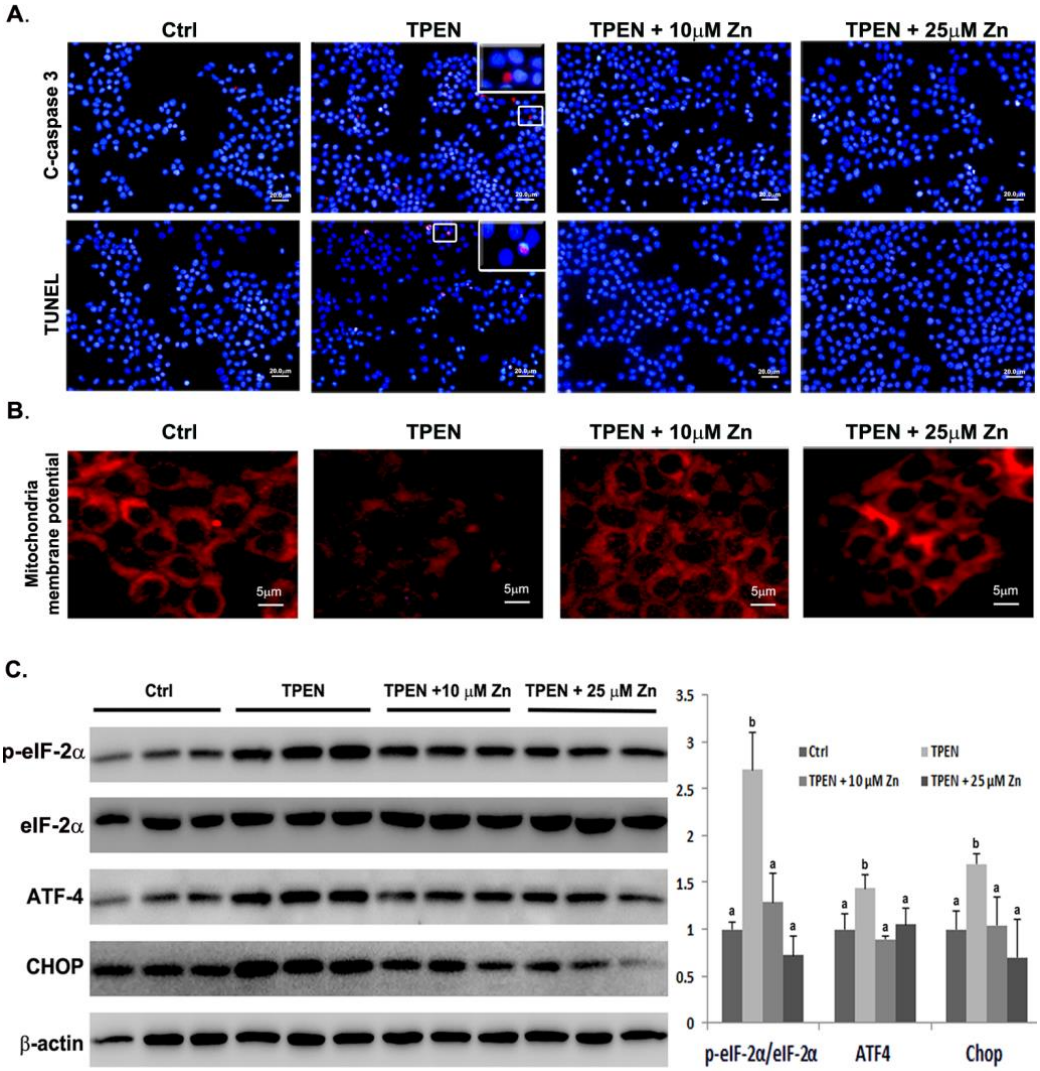


Figure 4.5. Effects of TPEN and Zinc Supplementation on ER and Mitochondrial Cell Death Signaling in Rat Hepatoma Cells. H4IIEC3 cells were treated with 3 μM TPEN with or without 10 μM or 25 μM Zn for 6 h. A. Representative images of immunofluorescence staining of cleaved caspase-3 and TUNEL assay of apoptosis. Inserts at the up-right corner are enlarged image of the framed area. B. Representative images of immunofluorescence staining of mitochondrial membrane potential. C. Immunoblot of ER stress markers. The bands density was quantified by densitometry

analysis. The ratio to β-actin was calculated by setting the value of control (ctrl) as one. Results for bars that do not share a letter differed significantly among groups (P <0.05). Significant differences among groups are determined by ANOVA followed by Tukey's test. Scale bar: 20 μm (A) Scale bar: 5 μm (B).

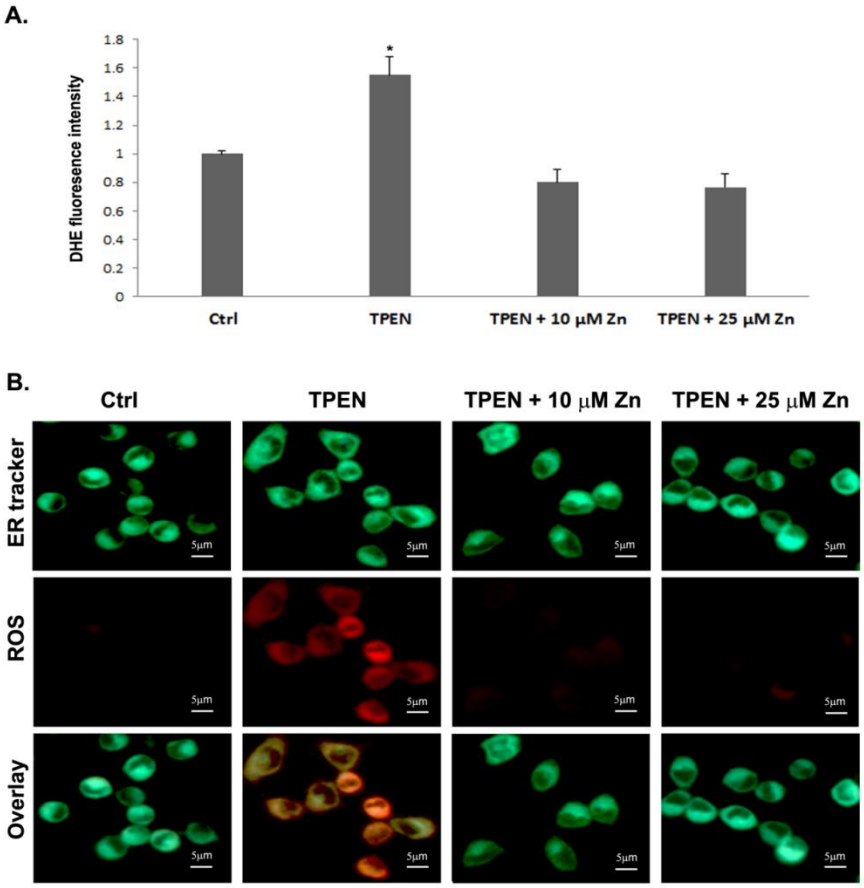
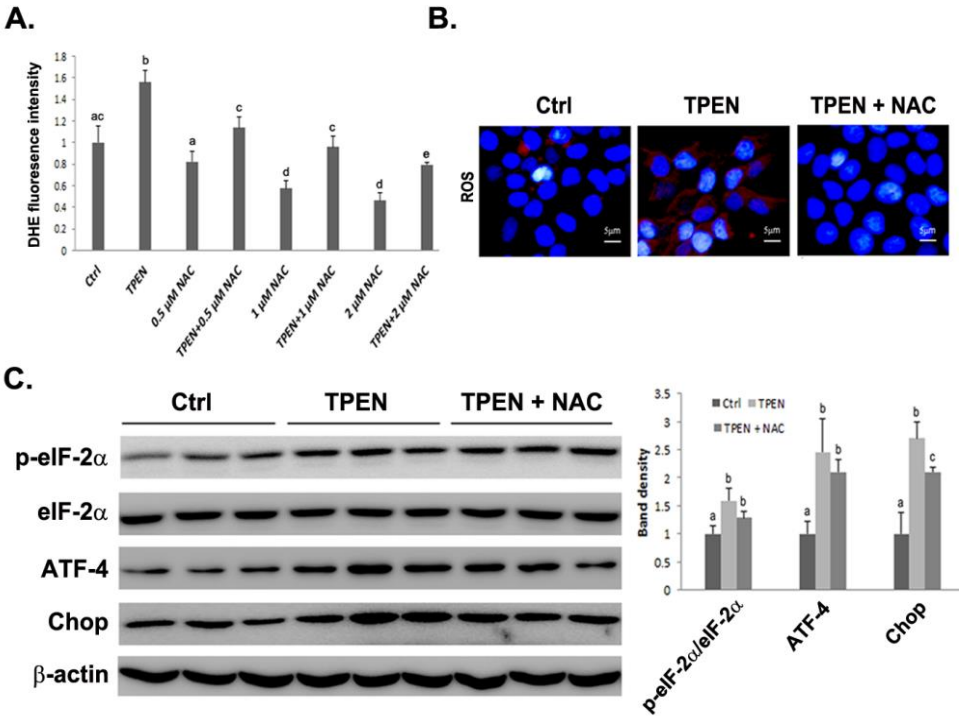
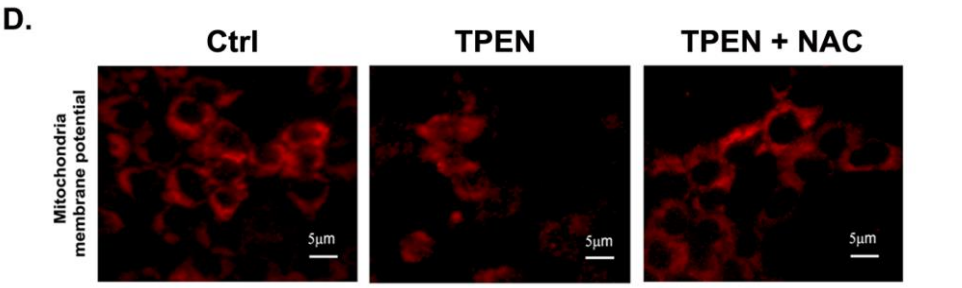


Figure 4.6. Effects of TPEN and Zinc Supplementation on Generation of Reactive Oxygen Species (ROS). H4IIEC3 cells were treated with 3 μM TPEN with or without 10 μM or 25 μM Zn for 6 h. A. The ROS was quantified by dihydroethidium fluorescence intensity. Results are expressed as means ± SD (n =4). Significant differences (*$P < 0.05$) among groups are determined by ANOVA followed by Tukey's test. B. Representative images of double immunofluorescence staining of ROS and ER. ROS was detected by CellROX Deep Red oxidative stress reagent, ER was detected by ER Tracker. Ctrl, control. Scale bar: 5 μm.


A.
DHE fluoresence intensity
Ctrl
TPEN
0.5 μM NAC
TPEN+0.5 μM NAC
1 μM NAC
TPEN+1 μM NAC
2 μM NAC
TPEN+2 μM NAC
B.
Ctrl
TPEN
TPEN + NAC
ROS
5μm
C.
Ctrl
TPEN
TPEN + NAC
p-eIF-2α
eIF-2α
ATF-4
Chop
β-actin
Band density
p-eIF-2α/eIF-2α
ATF-4
Chop


D.
Ctrl
TPEN
TPEN + NAC
Mitochondria membrane potential
5μm

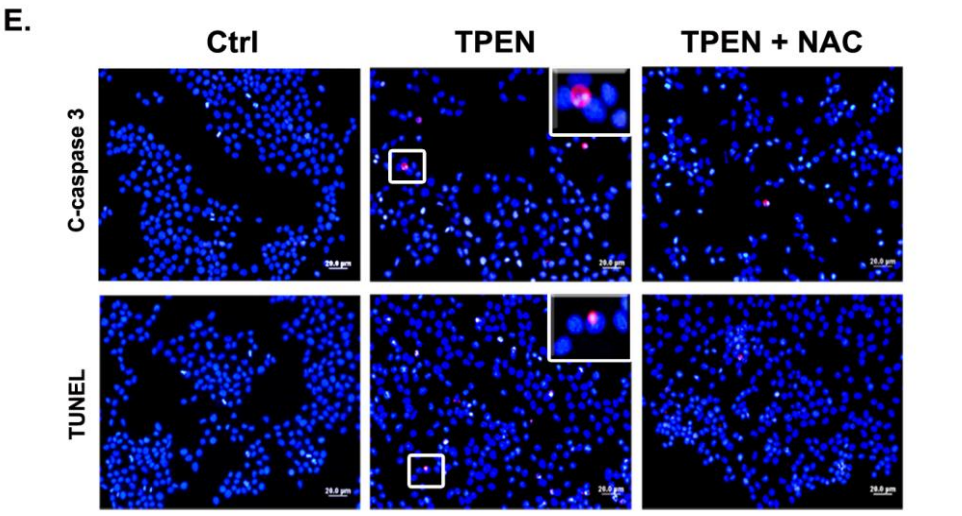

E.
Ctrl
TPEN
TPEN + NAC
C-caspase 3
TUNEL

Figure 4.7. Effects of NAC on TPEN Induced ER and Mitochondrial Cell Death Signaling. A. H4IIE3C cells were treated with 0 or 0.5 or 1 or 2 mM NAC with or without 3μM TPEN for 6 h. ROS production was measured by dihydroethidium fluorescence intensity. B. H4IIE3C cells were treated with 3μM TPEN with or without 2 mM NAC. Representative images of ROS staining. ROS was detected by CellROX Deep Red oxidative stress reagent. C. Immunoblot of ER stress markers. The bands were quantified by densitometry analysis. The ratio to β-actin was calculated by setting the value of control (ctrl) as one. Results for bars that do not share a letter differed significantly among groups (P <0.05). Significant differences among groups are determined by ANOVA followed by Tukey's test. D. Representative images of immunofluorescence staining of mitochondrial membrane potential. E. Representative images of immunofluorescence staining of cleaved caspase-3 and TUNEL assay. Scale bar: 5 μm (B, C), Scale bar: 20 μm (E).

CHAPTER V

DEFECT OF MITOCHONDRIAL RESPIRATORY CHAIN IS A MECHANISM OF ROS OVERPRODUCTION IN A RAT MODEL OF ALCOHOLIC LIVER DISEASE: ROLE OF ZINC DEFICIENCY

Qian Sun, Wei Zhong, Wenliang Zhang, Zhanxiang Zhou. American Journal PhysiologyGastrointestinal and Liver Physiology (in press).

Abstract

Morphological and functional alterations of hepatic mitochondria have been documented in patients with alcoholic liver disease (ALD). Our recent study demonstrated that zinc level was decreased in whole liver and mitochondria by chronic alcohol feeding. The present study was undertaken to determine if zinc deficiency mediates alcohol-induced mitochondrial electron transport chain (ETC) defect and if defected ETC function may lead to generation of reactive oxygen species (ROS). Male Wistar rats were pair-fed with the Lieber-DeCarli control or ethanol diet for 5 months. Chronic alcohol exposure increased hepatic triglyceride, free fatty acid, and 4-hydroxynonenal (4HNE) levels; meanwhile hepatic mitochondrial 4HNE level was also increased. Moreover, hepatic mitochondrial respiratory complex I, III, IV, and V and hepatic ATP production were decreased by chronic alcohol exposure. Chronic alcohol feeding decreased peroxisome proliferator-activated receptor gamma coactivator-1-alpha

(PGC1α), nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), and mitochondrial DNA. HepG2 cells were treated with N,N,N′,N′-tetrakis (2 pyridylmethyl) ethylenediamine (TPEN) for 6 h. Zinc deficiency significantly decreased mitochondrial respiratory complex I, III, and IV. In addition, PGC1α, NRF1 and TFAM levels as well as mitochondrial DNA were significantly decreased by TPEN treatment. Knockdown of mitochondrial respiratory complex I, III, or IV by shRNA caused a decrease in mitochondrial membrane potential and an increase in ROS production. These results suggest that alcohol exposure-induced hepatic zinc deficiency could inactivate mitochondrial biogenesis pathway and decrease mitochondrial DNA replication, which, in turn, decreases mitochondrial complex proteins expression. The defect of mitochondrial respiratory complexes may worsen alcohol-induced ROS production.

Introduction

Excessive alcohol consumption can exert serious adverse health effects, including the development of alcoholic liver disease (ALD). The spectrum of ALD includes alcoholic steatosis, alcoholic hepatitis, and alcoholic cirrhosis, which may ultimately progress to carcinoma [15]. The simple steatosis manifests as lipid accumulation and enlarged liver, and alcoholic hepatitis is characterized by inflammatory cell infiltration in the liver [22, 42]. Cirrhosis is the most severe stage of ALD, and at this stage normal liver tissue is replaced by scare tissue and proper function of liver is lost [24] . It has been proposed that alcohol-induced generation of reactive oxygen species (ROS) contributes to the transition of simple steatosis to advanced stage of ALD [48]. Studies demonstrated that alcohol induced massive production of ROS result in inflammasome formation, cell

injury and apoptotic signal activation [45, 46]. Meanwhile, deletion of genes that promote hydrogen peroxide generation ameliorated alcohol-induced steatosis and cell injury [19]. Mitochondrion is the organelle well-known for its role in energy generation from nutrients and alcohol oxidation. However, the generation of ATP accompanies with ROS production, which is the inevitable byproducts of oxidative phosphorylation (OXPHOS) [31]. Mechanistic studies demonstrated that defect in mitochondrial OXPHOS process results in further generation of ROS [2, 9]. In addition, mitochondria are dynamic organelle involved in biogenesis of mitochondria. The regulation of mitochondrial biogenesis is achieved by PGC1α, which promotes nuclear respiratory factor (NRF) mediated mitochondrial transcription factor A (TFAM) expression [3]. The translocation of TFAM to mitochondria stimulates mitochondrial biogenesis and mitochondria DNA (mtDNA) replication [3]. Alterations in morphology and function of mitochondria have been reported in the liver of patients with ALD [11].

Zinc deficiency is well-documented in patients with advanced ALD [26]. Zinc is required to maintain normal structure of proteins and for activities of more than three hundreds of enzymes [13]. In addition, zinc participates in antioxidant defense [26]. Thus, zinc deficiency links with severe health consequences. It has been documented that chronic alcohol feeding significantly decreases zinc levels in the plasma and liver of mice and rats [18, 33],while zinc supplementation to mice chronically fed alcohol diet ameliorates ROS generation in liver [18]. Our recent study demonstrated that zinc level is the most abundant in the mitochondria compared to other subcellular compartments, but significantly reduced after alcohol exposure [34]. Therefore, this study was undertaken to

investigate if there is a mechanistic link between alcohol-induced hepatic zinc deficiency and mitochondrial electron transport chain (ETC) defect and if ETC defect contributes to ROS production.

Materials and Methods

Animal and alcohol feeding experiments

Male Wistar rats were obtained from Harlan (Indianapolis, IN). The animal protocol was approved by the Institutional Animal Care and Use Committee of the North Carolina Research Campus. Eight-week-old male rats were pair-fed a modified Lieber-DeCarli alcohol (Dyets, Bethlehem, PA, #7100027) or isocaloric maltose dextrin control liquid diet (Dyets, #710260) for 5 months (n= 6 for each group) with a stepwise feeding procedure, as described previously [34]. In brief, the ethanol content (%, w/v) in the diet was start with 1.6 and increased with 1 every two days to reach 3.6 at the end of pre-feeding. On the day of feeding, the ethanol content in the diet was 5.0 (36% of total calories) and gradually increased to 6.3 (44% of total calories). At the end of 5-month feeding, rats were anesthetized with inhalational isoflurane. Left lobe of liver was collected for organelle isolation process, and the rest of the liver were fixed for pathology or stored at -80°C.

Staining of frozen tissue section

Liver tissue were frozen in Tissue-Tek OCT (Optimum Cutting Temperature) Compound (VWR, Batavia, IL). Cryostat liver tissue section were cut at 7 μm, fixed and processed with Oil red O solution to stainneutral lipid, or subject to CD68 (AbD Serotec, Atlanta, GA, #MCA341GA, 1:50) immunofluorescent staining to identify Kupffer cells,

or incubated with 5 μM dihydroethidium (DHE, Life technologies, Carlsbad, CA, #D-1168) to detect ROS production. Frozen tissue sections subjected to CD68 immunofluorescent staining were counter stained with DAPI. Quantitative assay of triglycerides, and FFA in liver tissue were measured by Bio Vision assay kits. Quantification of positive cells or staining was performed with Image J (NIH).

Cell culture and treatment

Human HepG2 hepatoma cells obtained from the American Type Culture Collection (Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml) streptomycin sulfate (100 μg/mL) (Invitrogen, Carlsbad, CA). HepG2 hepatoma cells were seeded at 5×10^5 cells per well for 6-well plate, or 1×10^5 cells per well for 8-well chamber slide, or 2×10^4 cells per well for 96-well plate overnight. Then cells were treated with 3 μM N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Sigma-Aldrich) for six hours in absence of FBS.

Determination of ROS

Superoxide levels in the liver of rats or HepG2 cells were detected with DHE After uptake by cells, DHE is oxidized to ethidium, which intercalates into DNA and gives nucleus a bright red fluorescent. The chamber slides were washed twice with PBS. The production of superoxide was detected with fluorescence microscopy. Hydrogen peroxide levels in the cell culture medium were measured by Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen, Waltham, MA, #A22188). Briefly, cells were seeded in 96-well plate overnight. In the next morning, fresh media were changed for

each well. Six hours later, fifty microliter of media was transferred to a clean 96-well plate subject to hydrogen peroxide assay according to instruction provided by manufacture. Then, the production of hydrogen peroxide levels were measured with plate reader at OD560nm.

Immunohistochemical staining

Hepatic 4-hydroxynonenal (4HNE), myeloperoxidase (21), OXPHOS, MTCO1, AMPK, PGC1α, NRF1, and TFAM levels were detected by immunohistochemical staining. Briefly, liver tissue paraffin sections were incubated with 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases. Tissue sections were then incubated with a monoclonal mouse anti-4HNE antibody (Northwest Life Science Specialties, Vancouver, WA, #NWA-HNE020, 1:100), anti-MPO (LSBio, Seattle, WA, #LS-B6699, 1:100), anti-oxphos (Abcam, Cambridge, MA, #ab110413, 1:100), anti-MTCO1 (Abcam, #ab14705, 1:100), anti-p-AMPK (Cell Signaling Technology, Danvers, MA, #2537, 1:100), anti-PGC1α (Calbiochem, #ST1202, 1:100), anti-NRF1 (Boster, Pleasanton, CA, #PA1948, 1:200), or anti-TFAM (Novus biological, Littleton, CO, #NBP1-71648, 1:100). Negative controls were conducted by replacing the primary antibody with PBS, and no positive staining was found. Quantification of positive staining was conducted by Image J.

Immunoblot analysis

Liver tissue or purified hepatic mitochondrial proteins were extracted by T-PER tissue extraction reagent (Thermo scientific) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO). Aliquots containing 80 μg proteins were loaded onto an 8%-15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins

were transferred to a polyvinylidene fluoride membrane. The membrane was probed with polyclonal antibodies against 4HNE (1:500), anti-superoxide dismutase 2 (SOD2, Millipore, Billerica, MA, #06984, 1:1000), anti-heat shock protein 60 (hsp60, BD, Franklin Lake, NJ, #611562, 1:1000), anti-total oxphos cocktail (1:2000), anti-PGC1α (1:1000), anti-NRF1 (1:1000), anti-TFAM (1:1000) respectively. The membrane was then incubated with HRP-conjugated goat anti-rabbit IgG, or goat anti-mouse IgG antibody. The protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ, #34076) and quantified by densitometry analysis.

Measurement of hepatic ATP

Hepatic ATP level was measured by commercial ATP Assay Kit (Abcam, #ab83355). Briefly, liver tissue (approx. 20-30 mg) was freeze-clamped using aluminum block pre-cooled in liquid nitrogen and immersed in liquid nitrogen immediately. Liver samples stored in -80°C. On the day of assay, liver sample was pulverized under liquid nitrogen and homogenized with 6% perchloric acid (PCA). Homogenates were centrifuged at 13,000g for 5 minutes at 4°C, the supernatant were neutralized to pH 7.8 with 2M KOH, placed on ice for 1h and centrifuged at 13,000g for 5 minutes at 4°C. The supernatants were subjected to ATP assay according to manufacturer's instruction. The amount of tissue ATP was determined at OD570nm.

qPCR

The DNA was extracted by QIAamp DNA Mini Kit (Qiagen, Venlo, Limburg, #51306). The forward and reverse primers were purchased from Integrated DNA

Technologies (Coralville, IA). Primer sequences of rat and human NADH dehydrogenase for the qPCRs are described in somewhere else (41). qPCR analysis with SYBR green PCR Master Mix (Qiagen, Valencia, CA, #203445) was performed on the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems).

Silence of mitochondrial respiratory complex in HepG2 cells

HepG2 cells were transfected with human CI-NDUFB8, CIII-UQCRC2 (Santa Cruz Biotechnologies, Santa Cruz, CA), or CIV-MTCO1 shRNA (Applied Biological Materials Inc, Richmond, BC). Stable clones were generated by delivering shRNA lentivirus into cells. The selection of stable silenced clones was started 48 h later with 2μM puromycin.

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential of live cells is assessed by TMRE mitochondrial kit (Abcam, #ab113852), as described previously (34).

Statistics

Results are expressed as mean ± standard deviation. Differences between two groups were analyzed by two-tail Student's *t*-test. Differences among multiple groups were analyzed by analysis of variance followed by Tukey's test. The significance between groups was defined as $P < 0.05$.

Results

Chronic alcohol feeding induces hepatic lipid accumulation and inflammation

The serum ALT, AST, and hepatic and subcellular zinc levels have been reported previously (34).CD68 and MPO staining illustrated that chronic alcohol feeding

significantly increased the number of Kupffer cells and neutrophil infiltration in the liver of alcohol-fed rats (Figure 1A and 1B). Oil red O staining demonstrated that 5 months alcohol feeding increases the number and size of lipid droplets (arrows) in the liver (Fig. 1A). Quantitative measurement of hepatic lipid contents showed that 5 months alcohol feeding significantly increased hepatic triglyceride ($P < 0.05$) and FFA ($P < 0.05$) concentrations (Fig. 1C and 1D).

Chronic alcohol feeding induces oxidative stress in the whole liver and purified hepatic mitochondria

To determine whether liver and mitochondrial oxidative stress is significantly increased by chronic alcohol feeding, ROS in the whole liver and purified hepatic mitochondria was analyzed. DHE fluorescence microscopy showed that alcohol feeding caused ROS accumulation in the liver, as indicated by strong red fluorescence formation in the nuclei (Fig. 2A). A lipid peroxidation product, 4HNE, was detected by immunohistochemistry. As shown in Figure 2A, the intensity of 4HNE staining was increased in the liver of rats after alcohol feeding. Moreover, Western blot analysis showed that alcohol feeding significantly increased 4HNE, SOD2 and hsp60 in the purified hepatic mitochondria compared to the controls (Fig. 2B).

Chronic alcohol feeding impairs mitochondrial oxidative phosphorylation and biogenesis (OXPHOS)

To determine whether alcohol feeding affects mitochondrial OXPHOS, ETC was assessed at protein level. The protein levels of mitochondrial complex I, complex III, complex IV, and complex V were significantly decreased by chronic alcohol feeding (Fig.

3A). Hepatic ATP level was measured to assess the productivity of ETC. Alcohol feeding reduced hepatic ATP level to 87% of that of the controls (Fig. 3B). In addition, immunohistochemical staining of total OXPHOS proteins and complex IV (MTCO1) showed a weaker staining intensity in the liver of alcohol-fed rats compared to the controls (Fig. 3C). Quantification of positive staining further showed significantly decreased expression of total OXPHOS proteins and complex IV protein in the liver of rats after 5 month alcohol exposure (Fig. 3E).

To further explore the possible mechanisms by which chronic alcohol feeding decreases OXPHOS, major mitochondrial biogenesis regulators were evaluated. As shown in Figure 4A, chronic alcohol feeding significantly decreased hepatic p-AMPK, PGC1α, NRF1, and TFAM at protein levels. Meanwhile, immunohistochemical staining demonstrated that the intensities of p-AMPK, nuclear PGC1α and NRF1, and mitochondrial TFAM were reduced in the alcohol-fed rats compared to that of the controls (Fig 4B and 4C). In accordance, hepatic mitochondrial DNA level was significantly decreased in the alcohol fed rats compared to that of the controls (Fig 4C).

Zinc deprivation perturbed mitochondrial biogenesis and oxidative phosphorylation in HepG2 cells

We have previously reported that mitochondrial zinc level was significantly decreased in the liver of rats chronically-fed alcohol; therefore the possible link between zinc deficiency and mitochondrial ETC reduction was evaluated in this study. Zinc deprivation in HepG2 cells was achieved by TPEN treatment. Western blot analysis showed that zinc deprivation did not affect hsp60, but significantly up-regulated SOD2

expression (Fig. 5A). Then the expression of mitochondrial respiratory complexes was assessed. TPEN treatment significantly decreased the expression of complex I, complex III, and complex IV, while increased the expression of complex V at protein level (Fig. 5B). To understand the mechanism by which zinc deprivation perturbs mitochondrial respiratory complexes, mitochondrial biogenesis regulators were evaluated. As shown in Figure 5C, p-AMPK, PGC1α, NRF1, and TFAM were significantly decreased by TPEN treatment. Meanwhile, mitochondrial DNA level in TPEN-treated cells was dramatically decreased to 47% of that in controls (Fig. 5D).

Zinc deficiency-induced defect of mitochondrial ETC led to a decreased mitochondrial membrane potential (MMP) and increased ROS production

We have previously shown that zinc deprivation induced generation of ROS and loss of mitochondria MMP, and thereby caused hepatocyte apoptosis. In the current study, we tested whether ETC protein defect due to zinc deprivation is a cause of increased ROS and decreased MMP in hepatocytes. HepG2 cells were genetically manipulated with scrambled shRNAs, including complex I- NDUFB8 shRNA, complex III-UQCRC2 shRNA, or complex IV-MTCO1 shRNA, respectively. As shown in Figure 6, transfection with all the three mitochondrial complex shRNAs effectively silenced their target genes, respectively.

MMP and ROS were assessed by fluorescence microscopy, and the red fluorescence represents active mitochondria and ROS accumulation, respectively. As shown in Figure 7A, HepG2 cells transfected with NDUFB8 shRNA, UQCRC2 shRNA, or MTCO1 shRNA showed a decreased MMP but an increased ROS production

compared to the HepG2 cells transfected with the scrambled shRNA. The hydrogen peroxide concentration in the cell culture media was measured as well. As shown in Figure 7B, HepG2 cells transfected with NDUFB8 shRNA, UQCRC2 shRNA, or MTCO1 shRNA had a higher hydrogen peroxide concentration in the culture media compared to the HepG2 cells transfected with the scrambled shRNA.

Discussion

Increased generation of ROS is a major cause of alcohol consumption-induced liver damage [23]. Reduced ROS production by genetic manipulation or dietary interventions effectively protected liver injury induced by alcohol consumption. A previous study showed that p47 knockout mice had a decreased ROS, plasma ALT, hepatic lipid accumulation and inflammation compared to wild type mice after alcohol feeding [20]. In addition, our group previously demonstrated that zinc supplementation effectively ameliorated ROS generation and partially reversed plasma ALT, hepatic lipid accumulation and apoptosis after alcohol consumption [18]. In the present study, we found not only an increased ROS level in the whole liver but also accumulated 4HNE in the purified mitochondria. Meanwhile, the increased production of SOD2 was detected. SOD2 is a mitochondrial specific antioxidant that converts superoxide to hydrogen peroxide and the up-regulated expression indicates an increased ROS accumulation in the mitochondria. Therefore, the present study demonstrated that ROS not only accumulated in the whole liver but also in the mitochondria.

Mitochondrion has been referred as a power plant while passing electrons through ETC to produce ATP. Mitochondrial ETC is composed of five multi-subunit complexes.

The subunits of each mitochondrial respiratory complex are encoded by both nuclei DNA (nuDNA) and mitochondrial DNA (mtDNA), except for complex II [29]. Subunits of complex II are completely encoded by nuDNA. Previous studies showed that chronic alcohol exposure repressed OXPHOS by decreased synthesis of mitochondrial respiratory complexes, including complex I, III, IV, and V in rats [7, 8]. Accordingly, mitochondrial respiration (state III) and respiratory control ratio (state III/state IV) were decreased [7], which indicated a decreased ability to convert ADP to ATP. In the current study, we found that chronic alcohol feeding decreased the expression of complex I, III, IV and V, but not complex II, meanwhile the production of ATP was decreased. Interestingly, complex IV was constantly reduced, but complex II was always unchanged in all of the chronically alcohol feeding studies on rats [8, 35, 36]. It seems that complex IV is one of the most sensitive components to oxidative damage in response to alcohol; however, complex II is the most resistant component to alcohol-induced toxicity in rats. Moreover, previous studies suggest that impairment of mtDNA and mitochondrial ribosomes accounts for the decreased synthesis of the subunits encoded by mtDNA [5, 6, 27]. It is still unclear that how alcohol represses the synthesis of respiratory subunits encoded by nuDNA. It is possible that the decreased synthesis of respiratory subunits encoded by mtDNA links with the reduced importation and/or the increased degradation of those subunits encoded by nuDNA in mitochondria. Therefore, the unchanged complex II may be due to the lack of respiratory subunits encode by mtDNA. In addition, we found that chronic alcohol consumption increased hepatic hsp60. It is known that hsp60 is a mitochondrial-specific chaperone protein, and the increased hsp60 level indicates a

folding crisis of mitochondrial proteins [40, 44]. The sustained folding crisis may lead to increased protein degradation [38]. Accordingly, the results suggest that ATP reduction after chronic alcohol exposure may be resulted from the decreased OXPHOS function, as demonstrated by reduced mitochondrial respiratory complex I, III, IV and V proteins.

Mitochondrial biogenesis is a process by which cells replenish the damaged and degraded mitochondria to maintain their mitochondrial number and function. The regulation of mitochondrial biogenesis involves more than 1000 genes encoded by both mtDNA and nuDNA. The master regulator of mitochondrial biogenesis is PGC1α [37]. The downstream factors of PGC1α include NRF1, peroxisome proliferator receptors (PPARs) and estrogen related receptors (ERRs); they all can activate TFAM to promote mitochondrial gene expression [3]. It has been reported that PGC1α and TFAM positively correlate with mitochondrial respiratory complexes [16, 43]. In consistent with previous findings, we demonstrated that alcohol consumption decreases both PGC1α and mitochondrial respiratory complexes in the liver. In addition to PGC1α and TFAM, the current study demonstrated, for the first time, that chronic alcohol feeding also reduces hepatic NRF1, a key nuclear transcription factor for mitochondrial respiration. It has been reported that NRF1 binding sites are found in a numerous genes involved in respiratory chain [10]. Most of these genes were involved in mitochondrial respiratory complexes, but others related to expression and assembly of the respiratory apparatus. NRF1 plays an important role in nucleo-mitochondrial communication by which the proteins involved in mitochondrial respiration can be produced accordingly in both nuclear and mitochondria. Moreover, it has been reported that homozygous NRF1 null blastocysts have impaired

mitochondrial membrane potential and severely reduced mtDNA level [17]. Thus, the decreased NRF1 level may play an important role in alcohol-induced decrease in mitochondrial respiration and function in addition to regulation of mitochondrial biogenesis. Thus, the current study suggests that inactivation of PGC1α, NRF1 and TFAM represent the key mechanism underlying chronic alcohol consumption-induced reduction of mitochondrial biogenesis and respiration.

Zinc deficiency has been reported constantly in human and animals with ALD, and zinc supplement to alcohol fed animals showed beneficial effect [18, 26]. A previous study demonstrated that zinc supplementation could partially rescue alcohol consumption-induced morphological change of mitochondria [47]. In addition, it has been shown that zinc is required to maintain mitochondrial complex IV content and ATP production in mammary gland [32]. In the current study, we found that zinc deficiency reduced the expression of mitochondrial respiratory complex I, III, IV, and V. Moreover, zinc deficiency also decreased the expression of PGC1α, NRF1 and TFAM and reduced the level of mtDNA. It has been reported that zinc plays an important role in cell proliferation via zinc proteins [30, 39]; thus it is not surprising that zinc regulates mitochondrial biogenesis. However, the exact molecular mechanisms need to be further explored. It is noteworthy to mention that experimental zinc deprivation induced complex V expression instead of a decreased expression as found in the *in vivo* study. This controversial result indicates that other factors in combination with zinc deficiency may contribute to the reduction of mitochondrial respiratory complexes in rats chronically fed alcohol. In addition, we did not find an altered hsp60 in the *in vitro* study, which further

suggests that other factors in addition to zinc must play a role in mitochondrial dysfunction after alcohol exposure. Further investigations are required to define the mechanisms by which alcohol reduces the mitochondrial complex V. Furthermore, oxidative stress, in addition to PGC1α inactivation, may also contribute to the reduction of mtDNA level by experimental zinc deficiency based on the observation from our group that experimental zinc deficiency by TPEN treatment increased MDA level in HepG2 cells [18]. As a whole, we demonstratedthat chronic alcohol consumption downregulates hepatic PGC1α, NRF1 and TFAM and mitochondrial respiratory complex I, III, and IV, and zinc deficiency is likely a mediator of the alcohol effects.

Mitochondrion is the major site of ROS production. Normally, about 1-2% of the oxygen consumed in mitochondria is converted into ROS [12, 21]. It has been shown that mitochondrial respiratory complex I and III are the sites leaking electrons, which, in turn, react with oxygen to generate ROS in the mitochondrial matrix [29]. However, under disease conditions, other mitochondrial respiratory complexes in addition to complex I and III may also contribute to mitochondrial origin of ROS generation. A previous study showed that defective complex II was mainly responsible for the overproduction of mitochondria-oriented ROS in mice with Rett syndrome [9]. Furthermore, although it is unlikely that ROS can be generated during enzyme turnover of complex IV, one study demonstrated that cytochrome oxidase contributed to the production of ROS after myocardial ischemia/perfusion [28]. In addition, Galati, D *et al.* further showed that knockdown of cytochrome c oxidase subunit Vb increased mitochondrial ROS level [14].The exact site where does defective cytochrome oxidase

generate ROS is still unknown. We reported coexistence of ROS overproduction and MMP impairment in the ALD model, which seems contradictory to the existing theory that increased MMP is necessary for ROS production via enhancing proton backpressure and inducing electron leakage. Interestingly, previous studies also reported the same finding as ours in their disease models [4, 9]. It is likely that, under disease condition, the more ROS is generated through defected ETC, the more mitochondria are damaged. As a result, the total number of mitochondria is decreased and leads to decreased MMP. Meanwhile, the remaining mitochondria with impaired ETC generates more than usual ROS.

It is noteworthy to mention that NDUFB8 and UQCRC2 are accessory subunit of the complex I and complex III, respectively [25], and they are required to anchor the corresponding complexes. Previous studies have shown that selective inhibition of electron transfer from iron-sulfur center of complex I or cytochrome b_H of complex III to ubiquinone with rotenone or antimycin induced ROS overproduction [1]. In the current study, we selectively knocked-down accessory subunit of complex I and III to understand whether non-catalytic subunit deficiency also contribute to dysfunction of complexes and subsequent ROS overproduction. In addition, MTCO1 is one of the core subunit of complex IV, and plays catalytic role for the function of complex IV. It has been shown that increased ROS cause reduction of complex IV [2], meanwhile a few study also showed dysfunction of complex IV, under disease conditions, may contribute to ROS production [28].Therefore, the role of MTCO1 was assessed. In the present study, selective knockdown of either NDUFB8 (subunit of complex I), UQCRC2 (subunit of

complex III), or MTCO1 (subunit of complex IV) increased ROS production and decreased MMP. The results demonstrated, for the first time, that defective complex IV, as the defective complex I and III, could cause overproduction of ROS *in vitro*. In accordance, the data suggests that alcohol-induced defect of ETC could be an important factor in the generation of oxidative stress after chronic alcohol feeding.

In summary, the present study demonstrated that chronic alcohol consumption impairs mitochondrial biogenesis and perturbs ETC, leading to generation of ROS. As illustrated in Figure 8, experimental zinc deprivation reduced the expression of mitochondrial biogenesis regulators, PGC1α, NRF1, and TFAM as well as mtDNA, which accounts for the reduction of mitochondrial respiratory complex proteins. Another important finding in the present study is that the reduction of mitochondrial respiratory complex IV may also contributes to ROS generation. However, it is still unclear whether increased production of ROS is directly through defected complex IV or other mechanisms are also involved. These results provide a novel insight into the role of zinc in mitochondrial biogenesis and function, and a new evidence of zinc therapy for treating ALD.

Acknowledgement

Qian Sun conducted subcellular compartments isolation, all of the assays, western blot, cell culture and fluorescence staining studies. Qian Sun, Dr. Wei Zhong, and Dr. Wenliang Zhang performed animal study. Dr.Zhanxiang Zhou performed immunohistochemical staining. Dr. Zhanxiang Zhou and Qian Sun designed the studies; Qian Sun analyzed the data and draft the manuscript. All authors read and approval the

final manuscript. This study was supported by a grant from the NIH National Institute on Alcohol Abuse and Alcoholism (R01AA1020212 and 2R01AA1018844 to Z.Z).

References

1. Bailey SM, and Cunningham CC. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radic Biol Med* 32: 11-16, 2002.

2. Bansal S, Srinivasan S, Anandasadagopan S, Chowdhury AR, Selvaraj V, Kalyanaraman B, Joseph J, and Avadhani NG. Additive effects of mitochondrion-targeted cytochrome CYP2E1 and alcohol toxicity on cytochrome c oxidase function and stability of respirosome complexes. *J Biol Chem* 287: 15284-15297, 2012.

3. Bayeva M, Gheorghiade M, and Ardehali H. Mitochondria as a therapeutic target in heart failure. *J Am Coll Cardiol* 61: 599-610, 2013.

4. Bobba A, Amadoro G, Valenti D, Corsetti V, Lassandro R, and Atlante A. Mitochondrial respiratory chain Complexes I and IV are impaired by beta-amyloid via direct interaction and through Complex I-dependent ROS production, respectively. *Mitochondrion* 13: 298-311, 2013.

5. Cahill A, Baio DL, Ivester P, and Cunningham CC. Differential effects of chronic ethanol consumption on hepatic mitochondrial and cytoplasmic ribosomes. *Alcohol Clin Exp Res* 20: 1362-1367, 1996.

6. Cahill A, Wang X, and Hoek JB. Increased oxidative damage to mitochondrial DNA following chronic ethanol consumption. *Biochem Biophys Res Commun* 235: 286-290, 1997.

7. Coleman WB, and Cunningham CC. Effect of chronic ethanol consumption on hepatic mitochondrial transcription and translation. *Biochim Biophys Acta* 1058: 178-186, 1991.

8. Coleman WB, and Cunningham CC. Effects of chronic ethanol consumption on the synthesis of polypeptides encoded by the hepatic mitochondrial genome. *Biochim Biophys Acta* 1019: 142-150, 1990.

9. De Filippis B, Valenti D, de Bari L, De Rasmo D, Musto M, Fabbri A, Ricceri L, Fiorentini C, Laviola G, and Vacca RA. Mitochondrial free radical overproduction due to respiratory chain impairment in the brain of a mouse model of Rett syndrome: protective effect of CNF1. *Free Radic Biol Med* 83: 167-177, 2015.

10. Evans MJ, and Scarpulla RC. NRF-1: a trans-activator of nuclear-encoded respiratory genes in animal cells. *Genes Dev* 4: 1023-1034, 1990.

11. Feldmann G, Maurice M, Husson JM, Fiessinger JN, Camilleri JP, Benhamou JP, and Housset E. Hepatocyte giant mitochondria: an almost constant lesion in systemic scleroderma. *Virchows Arch A Pathol Anat Histol* 374: 215-227, 1977.

12. Forman HJ, and Kennedy JA. Role of superoxide radical in mitochondrial dehydrogenase reactions. *Biochem Biophys Res Commun* 60: 1044-1050, 1974.

13. Fukada T, Yamasaki S, Nishida K, Murakami M, and Hirano T. Zinc homeostasis and signaling in health and diseases: Zinc signaling. *J Biol Inorg Chem* 16: 1123-1134, 2011.

14. Galati D, Srinivasan S, Raza H, Prabu SK, Hardy M, Chandran K, Lopez M, Kalyanaraman B, and Avadhani NG. Role of nuclear-encoded subunit Vb in the assembly and stability of cytochrome c oxidase complex: implications in mitochondrial dysfunction and ROS production. *Biochem J* 420: 439-449, 2009.

15. Gao B, and Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology* 141: 1572-1585, 2011.

16. Han D, Ybanez MD, Johnson HS, McDonald JN, Mesropyan L, Sancheti H, Martin G, Martin A, Lim AM, Dara L, Cadenas E, Tsukamoto H, and Kaplowitz N. Dynamic adaptation of liver mitochondria to chronic alcohol feeding in mice: biogenesis, remodeling, and functional alterations. *J Biol Chem* 287: 42165-42179, 2012.

17. Huo L, and Scarpulla RC. Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice. *Mol Cell Biol* 21: 644-654, 2001.

18. Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, and Zhou Z. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. *Hepatology* 50: 1241-1250, 2009.

19. Koch OR, Fusco S, Ranieri SC, Maulucci G, Palozza P, Larocca LM, Cravero AA, Farre SM, De Spirito M, Galeotti T, and Pani G. Role of the life span determinant P66(shcA) in ethanol-induced liver damage. *Lab Invest* 88: 750-760, 2008.

20. Kono H, Rusyn I, Yin M, Gabele E, Yamashina S, Dikalova A, Kadiiska MB, Connor HD, Mason RP, Segal BH, Bradford BU, Holland SM, and Thurman RG. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J Clin Invest* 106: 867-872, 2000.

21. Kudin AP, Bimpong-Buta NY, Vielhaber S, Elger CE, and Kunz WS. Characterization of superoxide-producing sites in isolated brain mitochondria. *J Biol Chem* 279: 4127-4135, 2004.

22. Levene AP, and Goldin RD. The epidemiology, pathogenesis and histopathology of fatty liver disease. *Histopathology* 61: 141-152, 2012.

23. Lu Y, and Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free Radic Biol Med* 44: 723-738, 2008.

24. Mann RE, Smart RG, and Govoni R. The epidemiology of alcoholic liver disease. *Alcohol Res Health* 27: 209-219, 2003.

25. Mimaki M, Wang X, McKenzie M, Thorburn DR, and Ryan MT. Understanding mitochondrial complex I assembly in health and disease. *Biochim Biophys Acta* 1817: 851-862, 2012.

26. Mohammad MK, Zhou Z, Cave M, Barve A, and McClain CJ. Zinc and liver disease. *Nutr Clin Pract* 27: 8-20, 2012.

27. Patel VB, and Cunningham CC. Altered hepatic mitochondrial ribosome structure following chronic ethanol consumption. *Arch Biochem Biophys* 398: 41-50, 2002.

28. Prabu SK, Anandatheerthavarada HK, Raza H, Srinivasan S, Spear JF, and Avadhani NG. Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury. *J Biol Chem* 281: 2061-2070, 2006.

29. Raimundo N. Mitochondrial pathology: stress signals from the energy factory. *Trends Mol Med* 20: 282-292, 2014.

30. Sandstead HH, Lanier VC, Jr., Shephard GH, and Gillespie DD. Zinc and wound healing. Effects of zinc deficiency and zinc supplementation. *Am J Clin Nutr* 23: 514-519, 1970.

31. Schiavi A, and Ventura N. The interplay between mitochondria and autophagy and its role in the aging process. *Exp Gerontol* 56: 147-153, 2014.

32. Seo YA, Lopez V, and Kelleher SL. A histidine-rich motif mediates mitochondrial localization of ZnT2 to modulate mitochondrial function. *Am J Physiol Cell Physiol* 300: C1479-1489, 2011.

33. Sun Q, Li Q, Zhong W, Zhang J, Sun X, Tan X, Yin X, Sun X, Zhang X, and Zhou Z. Dysregulation of hepatic zinc transporters in a mouse model of alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* 307: G313-322, 2014.

34. Sun Q, Zhong W, Zhang W, Li Q, Sun X, Tan X, Sun X, Dong D, and Zhou Z. Zinc deficiency mediates alcohol-induced apoptotic cell death in the liver of rats through activating ER and mitochondrial cell death pathways. *Am J Physiol Gastrointest Liver Physiol* 308: G757-766, 2015.

35. Thayer WS, and Rubin E. Molecular alterations in the respiratory chain of rat liver after chronic ethanol consumption. *J Biol Chem* 256: 6090-6097, 1981.

36. Venkatraman A, Landar A, Davis AJ, Chamlee L, Sanderson T, Kim H, Page G, Pompilius M, Ballinger S, Darley-Usmar V, and Bailey SM. Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. *J Biol Chem* 279: 22092-22101, 2004.

37. Ventura-Clapier R, Garnier A, and Veksler V. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. *Cardiovasc Res* 79: 208-217, 2008.

38. Voos W. Chaperone-protease networks in mitochondrial protein homeostasis. *Biochim Biophys Acta* 1833: 388-399, 2013.

39. Watanabe T, Arakawa T, Fukuda T, Higuchi K, and Kobayashi K. Zinc deficiency delays gastric ulcer healing in rats. *Dig Dis Sci* 40: 1340-1344, 1995.

40. Yoneda T, Benedetti C, Urano F, Clark SG, Harding HP, and Ron D. Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J Cell Sci* 117: 4055-4066, 2004.

41. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, and Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 464: 104-107, 2010.

42. Zhang W, Zhong W, Sun X, Sun Q, Tan X, Li Q, Sun X, and Zhou Z. Visceral white adipose tissue is susceptible to alcohol-induced lipodystrophy in rats: role of acetaldehyde. *Alcohol Clin Exp Res* 39: 416-423, 2015.

43. Zhao L, Zou X, Feng Z, Luo C, Liu J, Li H, Chang L, Wang H, Li Y, Long J, Gao F, and Liu J. Evidence for association of mitochondrial metabolism alteration with lipid accumulation in aging rats. *Exp Gerontol* 56: 3-12, 2014.

44. Zhao Q, Wang J, Levichkin IV, Stasinopoulos S, Ryan MT, and Hoogenraad NJ. A mitochondrial specific stress response in mammalian cells. *EMBO J* 21: 4411-4419, 2002.

45. Zhao Y, Zhong W, Sun X, Song Z, Clemens DL, Kang YJ, McClain CJ, and Zhou Z. Zinc deprivation mediates alcohol-induced hepatocyte IL-8 analog expression in rodents via an epigenetic mechanism. *Am J Pathol* 179: 693-702, 2011.

46. Zhong W, Zhang W, Li Q, Xie G, Sun Q, Sun X, Tan X, Sun X, Jia W, and Zhou Z. Pharmacological activation of aldehyde dehydrogenase 2 by Alda-1 reverses alcohol-induced hepatic steatosis and cell death in mice. *J Hepatol* 62: 1375-1381, 2015.

47. Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, and Kang YJ. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. *Am J Pathol* 166: 1681-1690, 2005.

48. Zhu H, and Li YR. Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Exp Biol Med (Maywood)* 237: 474-480, 2012.

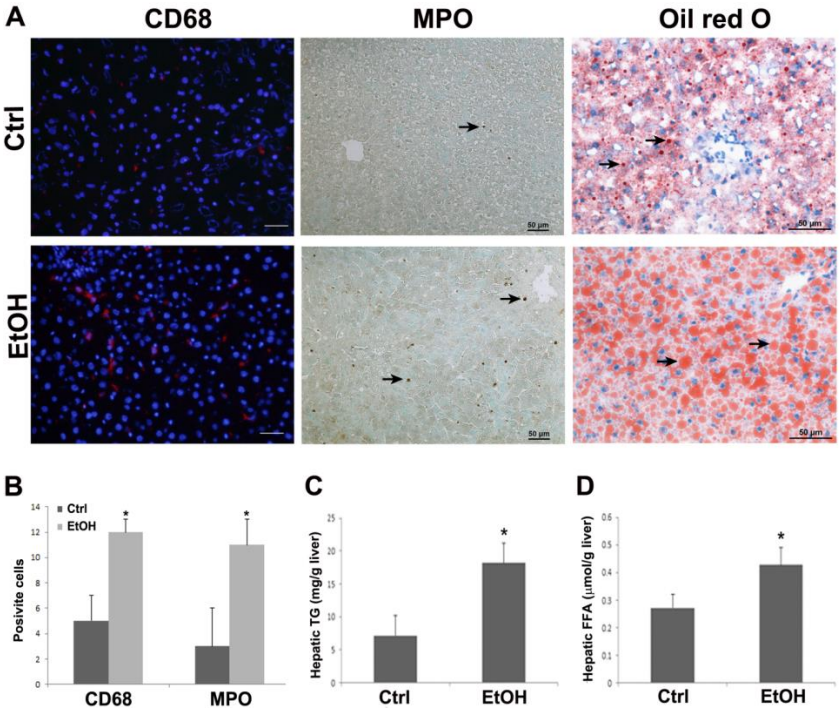


Figure 5.1. Inflammatory Cells Infiltration and Lipid Accumulation in Rats Chronically Fed Alcohol for 5 Months. (A) Immunofluorescent staining of CD68 in the liver, which was counter stained with DAPI; immunohistochemical staining of MPO in the liver; Oil red O staining of the neutral lipids in the liver. CD68 positive cells are showed as red staining in the cytosol. The brownish staining are MPO positive cells, as indicated by arrows. Lipid droplets are stained with red color, as indicated by arrows. (B) Quantitative measurements of positive cells of CD68 and MPO. (C) Quantitative measurements of hepatic concentration of triglycerides and (D) free fatty acids. Data are expressed as mean ± SD from 6 rats. Statistical difference ($P < 0.05$) was analyzed by Student's *t*-test. Ctrl: control; EtOH: ethanol.

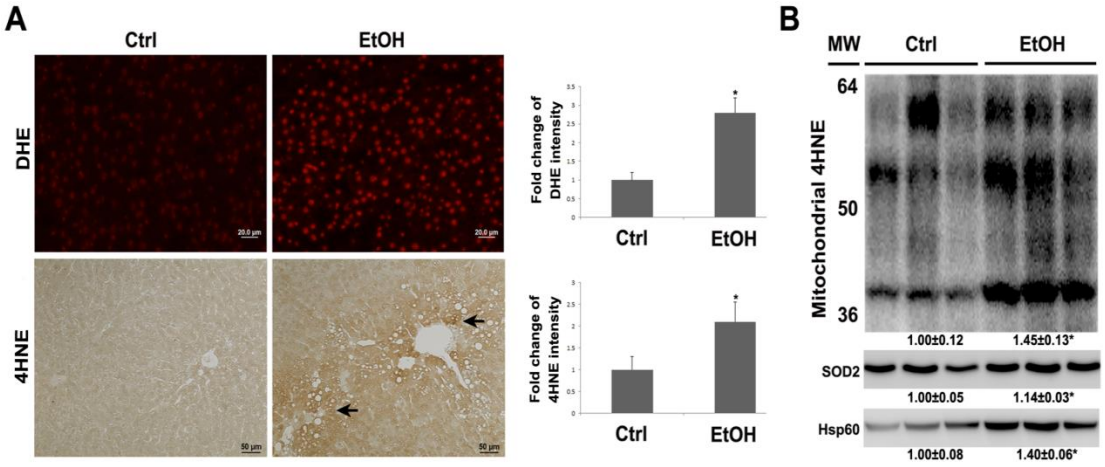


Figure 5.2. Oxidative Stress in The Whole Liver and Purified Mitochondria in Rats Chronically Fed Alcohol for 5 Months. (A) ROS accumulation in the liver was detected by dihydroethidium, as indicated by red fluorescence in the nuclei (upper panel). Scale bar: 20μm.The formation of 4HNE adduct (arrows) was detected by immunohistochemistry (lower panel). Scale bar: 50μm. (B) The immunoblot bands of 4HNE, SOD2, and hsp60 in purified mitochondria. Quantification of DHE and 4HNE staining intensity was conducted by Image J. Data are expressed as mean ± SD from 6 rats. Statistical difference ($P < 0.05$) was analyzed by Student's *t*-test. Ctrl: control; EtOH: ethanol.

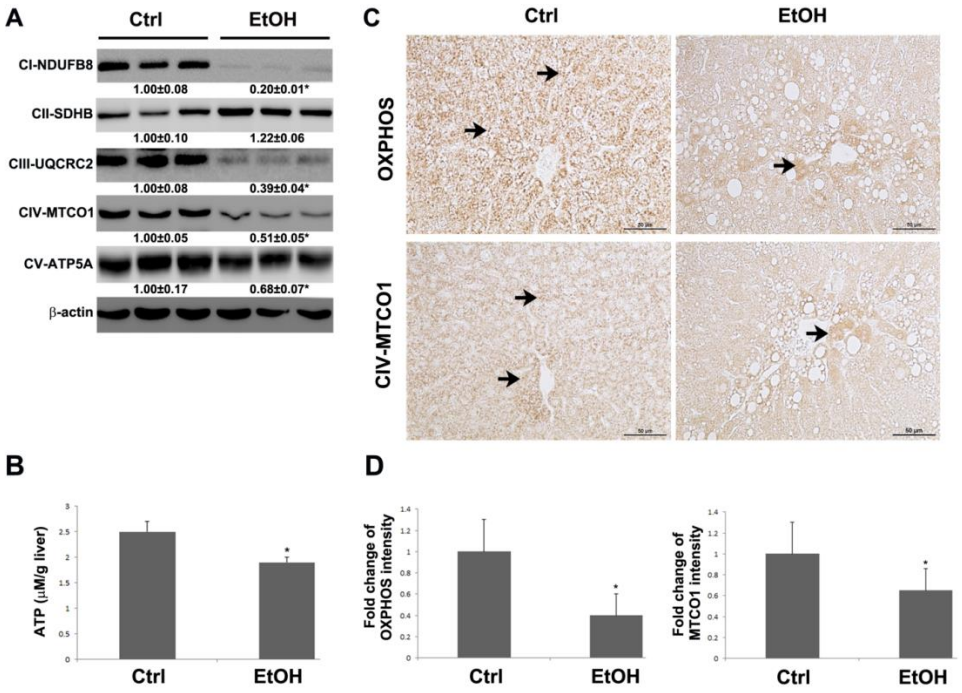


Figure 5.3. Protein Levels of Mitochondrial Respiratory Complexes and ATP Production in The Liver of Rats Chronically Fed Alcohol for 5 Months. (A) The immunoblot bands of OXPHOS proteins, subunit of complex I (CI-NDUFB8), subunit of complex II (CII-SDHB), subunit of complex III (UQCRC2), subunit of complex IV (MTCO1), and subunit of complex V (ATP5A) (B) ATP level in the liver of rat was detected by commercial ATP assay kit. (C) The immunohistochemical staining of total mitochondrial respiratory complex proteins (upper panel) and complex IV MTCO-1 proteins (lower panel). Arrows indicate positive staining. (D) The fold change of immunohistochemical staining intensity. The quantification was conducted by Image J.Data are expressed as mean ± SD from 6 rats. Statistical difference (*$P < 0.05$) was analyzed by Student's *t*-test. Ctrl: control; EtOH: ethanol.

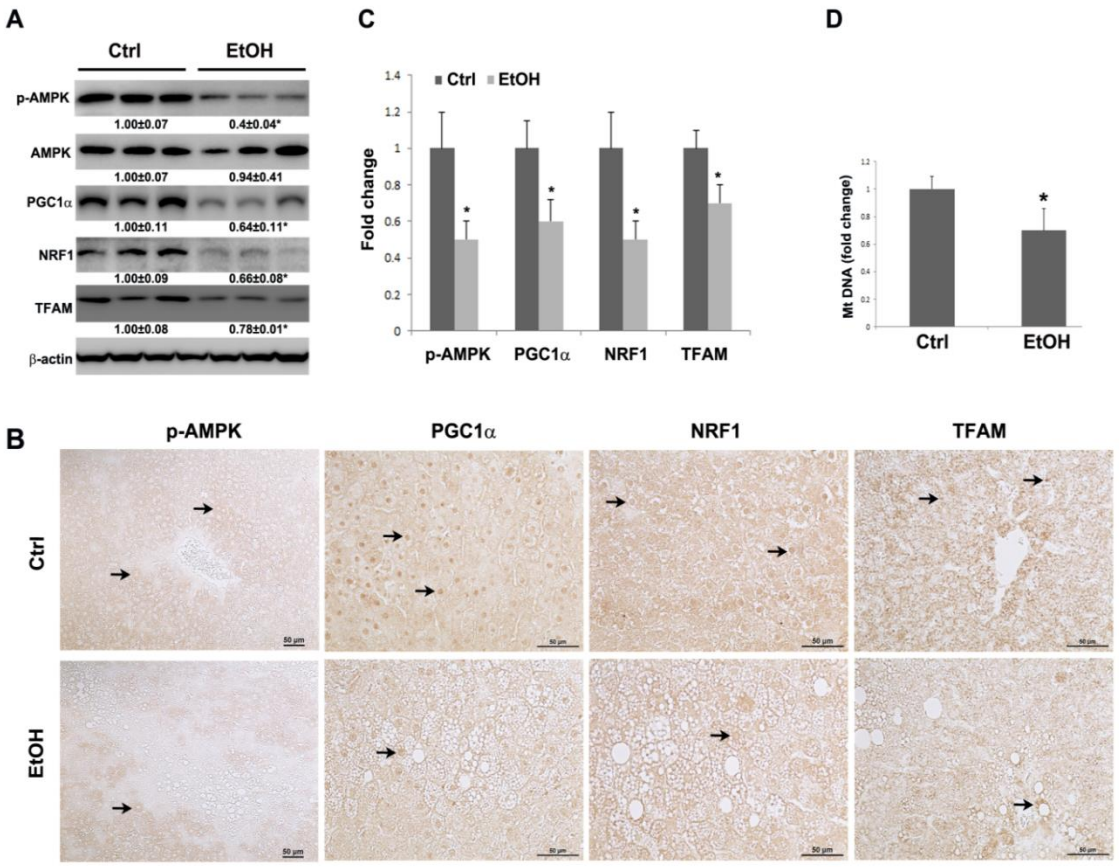


Figure 5.4. Protein Levels of Mitochondrial Biogenesis Regulators and mtDNA Level in The Liver of Rats Chronically Fed Alcohol for 5 Months. (A) The immunoblot bands of p-AMPK, AMPK, PGC1α, NRF1, and TFAM. (B) The immunohistochemical staining of p-AMPK, PGC1α, NRF1 and TFAM proteins. Arrows indicate positive staining. (C) The fold change of immunohistochemical staining intensity. The quantification was conducted by Image J. (D) The mtDNA level was measured with NADH dehydrogenase subunit 6 by qPCR. Data are expressed as mean ± SD from 6 rats. Statistical difference (*$P < 0.05$) was analyzed by Student's *t*-test. Ctrl: control; EtOH: ethanol.

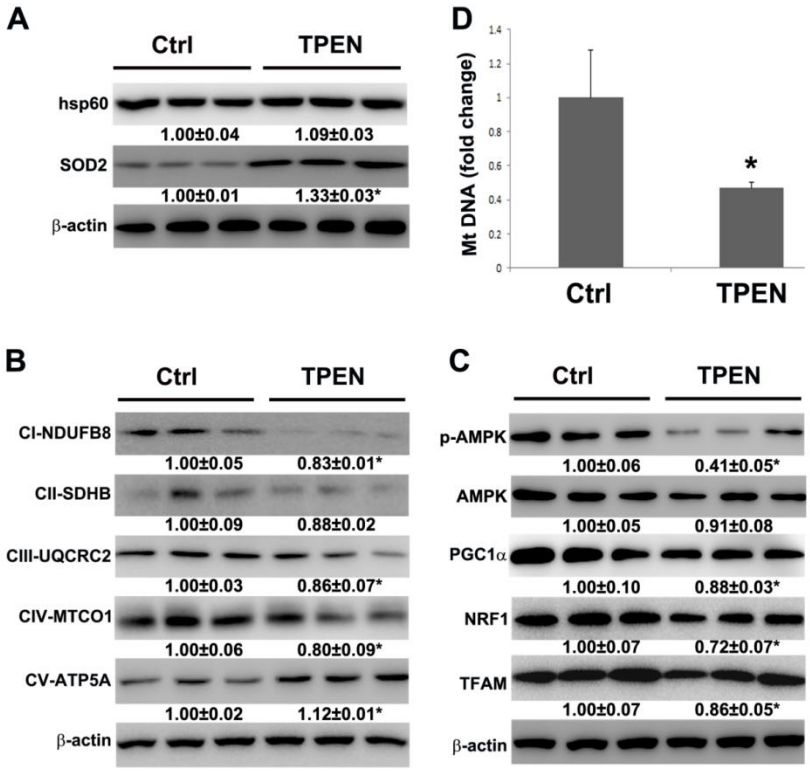


Figure 5.5. Effect of TPEN on hsp60, SOD2, OXPHOS Proteins, Mitochondrial Biogenesis Regulators, and mtDNA Level in Human HepG2 Cells. Human HepG2 cells were treated with 3 μM TPEN without FBS for 6h. (A) The immunoblot bands of hsp60 and SOD2. (B) The immunoblot bands of OXPHOS proteins, subunit of complex I (CI-NDUFB8), subunit of complex II (CII-SDHB), subunit of complex III (CIII-UQCRC2), subunit of complex IV (CIV-MTCO1), and subunit of complex V (CV-ATP5A). (C) The immunoblot bands of p-AMPK, AMPK, PGC1α, NRF1, and TFAM. (D) The mtDNA level was measured with NADH dehydrogenase subunit 6 by qPCR. Data are expressed as mean ± SD (n=3). Statistical difference (*P< 0.05) was analyzed by Student's t-test. Ctrl: control.

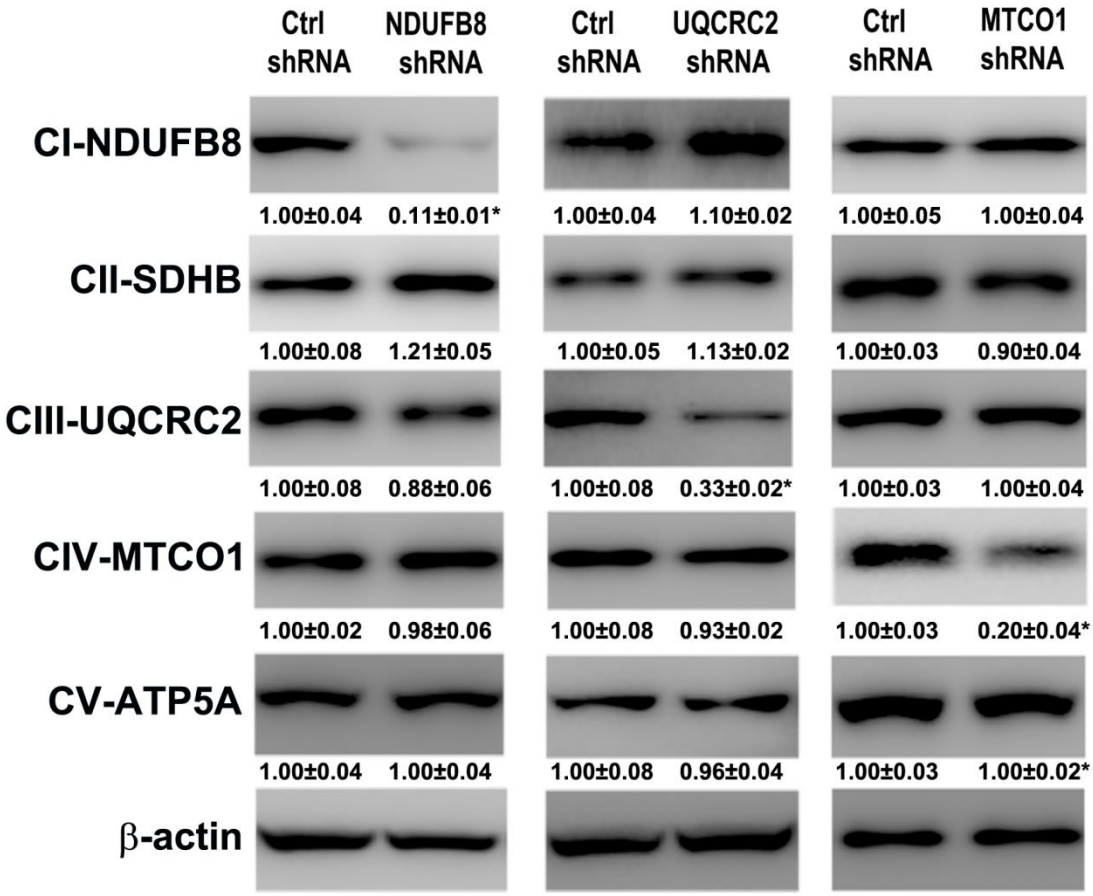


Figure 5.6. The Efficiency of Transfection with shRNAs. The human HepG2 cells were transfected with scrambled, CI-NDUFB8, CIII-UQCRC2, CIV-MTCO1 shRNA lentivirus for 48h and the stable clone was selected with 2 μM puromycin. The immunoblot bands of OXPHOS proteins in stable transfected cells. Data are expressed as mean ± SD (n=3). Statistical difference (*P< 0.05) was analyzed by Student's *t*-test. Ctrl: control.

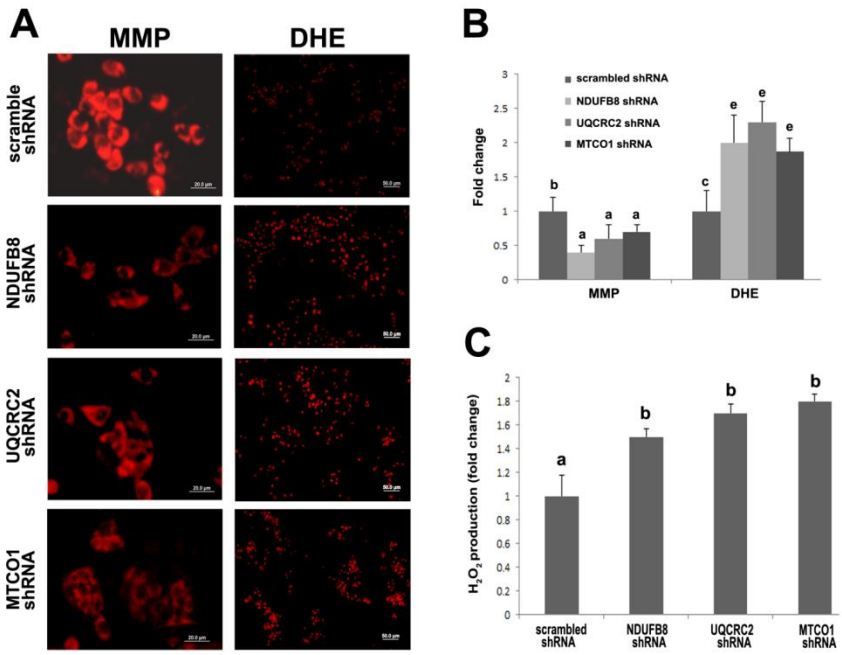


Figure 5.7. Effect of Defective Mitochondrial Respiratory Complex on Mitochondrial Membrane Potential (MMP) and ROS Generation. HepG2 cells transfected with scrambled shRNA, NDUFB8 shRNA, UQCRC2 shRNA, or MTCO shRNA were stained with TMRE or DHE for assessing MMP and ROS, respectively. (A) Red fluorescence represents TMRE labeled active mitochondria (left panel, Scale bar: 20 μm) or cells with ROS production (Right panel, scale bar: 50 μm). (B) The fold change of fluorescent intensity. The quantification was conducted by Image J. (C) Hydrogen peroxide secreted into cell cultural media was detected by Hydrogen Peroxide/Peroxidase assay kit. Data are expressed as mean ± SD (n=3). Results for bars that do not share a letter differ significantly (P <0.05). Significant differences among groups were determined by ANOVA followed by Tukey's test.

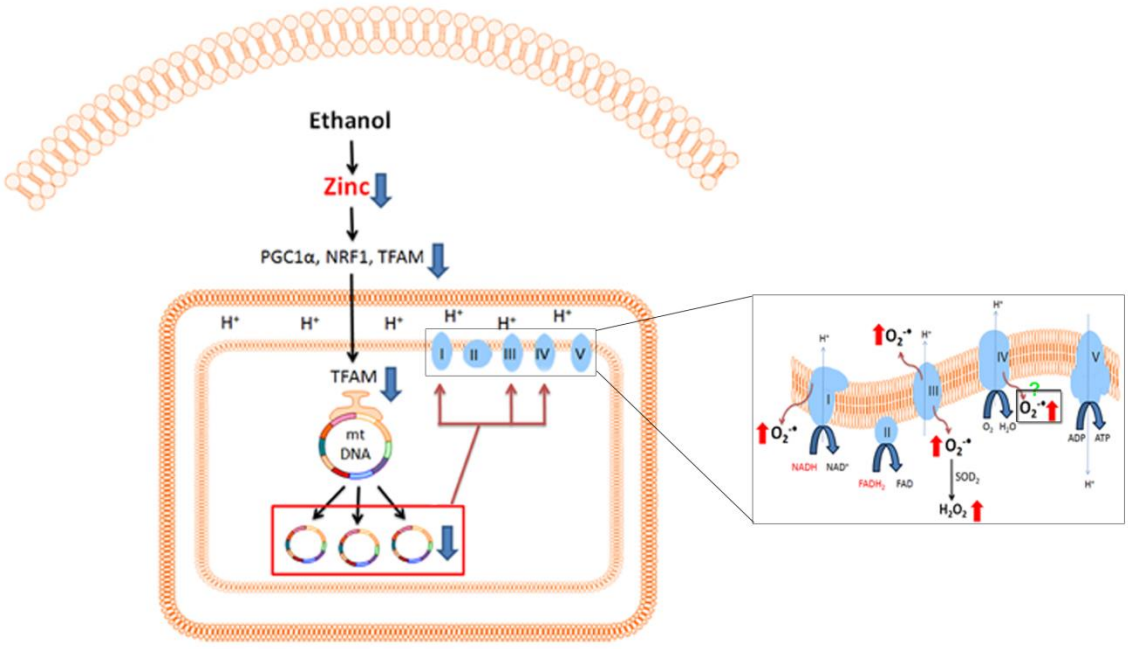


Figure 5.8. Schematic Hypothesis on Zinc Deprivation Induced Accumulation of ROS Based on The Results of The Present Study. Alcohol consumption-induces a decrease in zinc level in the liver. Zinc deficiency down-regulates expression of PGC1□, NRF1, and TFAM, which results in reduced mitochondrial biogenesis and mtDNA replication. Impairment of mtDNA leads to defect of mitochondrial respiratory complexes, namely complex I, III, and IV. As a result, defective complex I, III, and IV causes ROS overproduction in ALD.

CHAPTER VI

EPILOGUE

Conclusion and Future Study

Prolonged and excessive alcohol consumption exerts health concerns, and there is no effective treatment for ALD. Thus, ALD study is still ongoing, with the objective of elucidating the underlying mechanisms and discovering potential therapeutic target. In my dissertation research, I studied the mechanism underlies chronic alcohol consumption-induced hepatic zinc deficiency and dissect the effect of zinc deficiency on mitochondrial apoptosis. My study provided evidence that zinc deficiency could damage ER and mitochondrial function and lead to apoptosis.

In chapter III, we demonstrated that prolonged alcohol consumption, 2-week, 4-week, and 8-week, continuously decrease hepatic zinc level, in line with increased urinary zinc excretion. However, plasma zinc level showed an increase after alcohol feeding at all three time points. Zinc finger transcription factors, PPARα and HNF4α, were decrease at protein levels as early as 2-week of alcohol feeding. Another zinc protein, SOD1, showed reduced activity at week-2 of alcohol feeding. Hepatic zinc transporters were analyzed at both mRNA and protein levels. Consistent alterations were observed at protein levels after 2-week, 4-week, and 8-week of alcohol feeding, with reduction of ZIP5 and ZIP14, and elevation of ZIP7 and ZnT7. Chronic alcohol consumption also induced the expression of CYP 2E1 and formation of 4-HNE adduct,

and the amount of CYP 2E1 and 4-HNE was dependent on the duration of alcohol-feeding. Mechanistic study elucidated that 4-HNE increased the expression of ZIP5 and ZIP7, and hydrogen peroxide increased the expression of ZIP7 and ZnT7 but decreased the expression of ZIP14 at protein levels.

As a whole, chapter III demonstrated that chronic alcohol consumption altered the expression of hepatic zinc transporters, by which the uptake of zinc by liver was reduced. Since only ZIP14 has been reported located on the plasma membrane of hepatocyte, we predicted that decreased ZIP14 responsible for decreased zinc levels in the whole liver. Moreover, increased oxidative stress by alcohol consumption may be the cause of the alteration of the zinc transporters. Thus, chapter III fulfill the objective in Aim 1

In chapter IV, zinc levels were evaluated at subcellular compartments, including nuclei, ER, mitochondria, and cytosol, meanwhile zinc transporters were measured in ER and mitochondria. Results showed that five months of alcohol exposure significantly decreased hepatic zinc levels, along with decreased zinc levels in ER and mitochondria. Mitochondria had the highest zinc level among all the tested subcellular compartments. The expression of zinc transporters in the organelles is limited. ZIP 7, ZIP8, ZIP13 and ZnT7 were detected in ER, while ZIP8, ZnT1 and ZnT4 were detected in mitochondria. ZIP13 was increased in ER and ZIP8 and ZnT4 was increased in mitochondria after chronic alcohol administration. The expression of ER stress makers, p-eIF-2α, ATF4, and CHOP, were upregulated and mitochondrial cytochrome C release was increased by alcohol exposure. TUNEL assay showed increased number of apoptotic cells after five months of alcohol feeding. Mechanistic study showed that zinc deprivation increased

reactive oxygen species (ROS), ER stress, as well as impaired mitochondrial membrane potential (MMP). However antioxidant, N-acetyl cysteine (NAC), treatment only partially reversed zinc deprivation induced apoptosis.

As a whole, chapter IV demonstrated that reduced zinc levels in ER and mitochondria may, at least partially, contribute to chronic alcohol consumption induced hepatic zinc deficiency. In addition, increased ZIP13 and ZIP8 may responsible for the zinc reduction in ER and mitochondria, respectively. Zinc deficiency triggers ER stress-induced apoptosis via impair MMP, and this process is partially dependent on ROS. Chapter IV accomplishes the objective in Aim 2.

In chapter V, the mechanism underlies zinc deficiency induces mitochondrial stress induced apoptosis was carefully examined. The results elucidated that chronic alcohol consumption increased the ROS in whole liver and mitochondria. In addition, the level of ATP and the expression of mitochondrial ETC complexes were impaired by alcohol exposure. Mitochondrial biogenesis markers were significantly decreased by 5 months of alcohol exposure, in line with decreased mitochondria DNA (mtDNA) levels. Zinc deprivation study showed that zinc deficiency induced reduction of mitochondrial biogenesis markers, decreased mtDNA levels, as well as decreased ETC complex I, III and IV. Selective knockdown of ETC complex I, or III, or IV, induced the production of ROS and decreased the MMP.

As a whole, chapter V demonstrated that zinc deficiency lead to defect of ETC, which promotes ROS overproduction and loss of MMP.

Collectively, my dissertation research suggests that chronic alcohol consumption-induced hepatic zinc deficiency, mainly from ER and mitochondrial, may be resulted from altered zinc transporters by increased oxidative stress. In addition, decreased hepatic zinc level lead to activation of mitochondria mediated apoptotic cell death pathway by increased ER and mitochondrial stress.

While working on my dissertation research, I encountered some problems. Since increased acetaldehyde is always a concern for alcohol-consumption induced liver injury, it is of importance to evaluate the effect of acetaldehyde on the expression of zinc transporters. However, it is hard to determine the exact concentration of acetaldehyde in the cell culture media due to low boiling point, which is 68.4^{0}F. The effect of acetaldehyde with a reasonable dose on the expression of zinc transporters may be achieved through *in vivo* study. We have demonstrated that several compounds, for instance alda-1 could accelerate acetaldehyde clearance and decrease hepatic acetaldehyde level [3]; therefore the distribution and expression of hepatic zinc transporters and zinc level can be assessed in mice fed with alcohol diet with or without alda-1 treatment to determine whether acetaldehyde plays a role in the expression of zinc transporters. In addition, fluorescence microscope was used to evaluate the MMP. Although the fluorescent intensity among treatment groups was measured with Image J (NIH), the morphology of mitochondria could not be well-recognized from the fluorescence microscope. Therefore, confocal microscope should be used in the future study to give a better sight that the TMRE dye stained the active mitochondria instead of the cytosol. Moreover, the production of superoxide was evaluated with DHE staining by

fluorescence microscope. However, it has been found that there is a spectral overlapping between superoxide derived maker product and other non-specific oxidized fluorescent product [1]. Therefore, HPLC method should be utilized to measure the production of superoxide in the future study.

Apart from the conclusions that have been made in the dissertation research, studies are still need to investigate i) the effect of subcellular organelle zinc deficiency on the organelle functions; and ii) the effect of zinc supplementation on subcellular zinc levels and the subcellular organelle functions. Therefore the overall objective of my future project is to determine the effectiveness of zinc in treating ALD. The central hypothesis is that zinc supplementation protect against ALD by improving organelle functions.

To test the hypothesis and attain the objective of the project, the following two aims will be accomplished:

Aim 1: Define the link between organelle zinc levels and organelle function in alcohol-fed mice with or without zinc supplementation.

Aim 2: Determine the mechanism underlies subcellular organelle zinc levels and subcellular organelle dysfunctions.

Collectively, my dissertation research studied the mechanism underlies alcohol induces hepatic zinc deficiency and zinc deficiency induces hepatocyte apoptosis. In my future study, I will focus on how does zinc deficiency affect liver function at subcellular level.

References

1. Kalyanaraman B, Dranka BP, Hardy M, Michalski R, and Zielonka J. HPLC-based monitoring of products formed from hydroethidine-based fluorogenic probes--the ultimate approach for intra- and extracellular superoxide detection. *Biochim Biophys Acta* 1840: 739-744, 2014.

2. Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, and Zhou Z. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. *Hepatology* 50: 1241-1250, 2009.

3. Zhong W, Zhang W, Li Q, Xie G, Sun Q, Sun X, Tan X, Sun X, Jia W, and Zhou Z. Pharmacological activation of aldehyde dehydrogenase 2 by Alda-1 reverses alcohol-induced hepatic steatosis and cell death in mice. *J Hepatol* 62: 1375-1381, 2015.