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NAC Attenuates Ethanol-Induced Lipid Accumulation Via Scavenging ROS Production in Hepatocytes

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Abstract

Alcoholic liver disease (ALD) is generated from excessive alcohol consumption with characterizing of hepatic steatosis. Reactive oxygen species (ROS) that production from the alcohol metabolism is the main cause of alcoholic fatty lesion in hepatocytes. In this study, we isolated mouse primary hepatocytes by portal vein perfusion technique to investigate the effects of ROS scavenging agent NAC on fatty lesions of hepatocyte caused by alcohol *in vitro*. We found that alcohol treatments resulted in lipid accumulation and high level of ROS production in primary hepatocytes, which were abolished by NAC treatment. Thus, we concluded that controlling the ROS production or its elimination is a promising approach for attenuating ethanol-induced hepatocytes lipid accumulation and ALD treatments.

Keywords

Alcohol; ROS; Hepatocytes; Lipid accumulation.

1. INTRODUCTION

Alcohol is an indispensable consumer product in public life. According to the Global Report on Alcohol and Health published by the World Health Organization, the number of drinkers worldwide is as high as 2.3 billion. However, the harmful use of alcohol is a great threat to health[1]. The liver, the body's main organ of metabolism, can be disorder by prolonged exposure to alcohol and lead to the occurrence of ALD[2]. Alcoholic fatty liver, an early stage in the pathogenesis of ALD, can deteriorate into fibrosis, cirrhosis, alcoholic hepatitis and even hepatocellular carcinoma[3]. In this study, we further aimed to explore the mechanisms of alcoholic fatty lesion in hepatocytes and the treatment of the disease.

Hepatocytes produce a variety of metabolites from alcohol metabolism, and ROS is one of them. ROS from alcohol metabolism can accumulate in cells and have been reported to be closely linked to the development of hepatic steatosis[4][5][6][7][8][9]. N-acetylcysteine (NAC) is a common antioxidant that becomes a potent scavenger of ROS by interacting between its free sulfhydryl side chain and electrophilic groups of free radicals[10]. NAC also plays an indirect antioxidant role by forming glutathione[11][12].

Given the key role of ROS in hepatocellular steatosis[13][14][15], this study will explore whether alcohol-induced ROS production has an eliminative effect on hepatocellular steatosis. It provides a basis for preventing and treating alcoholic fatty liver disease in vivo.

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2. MATERIALS AND METHODS

2.1. Materials and Reagents

Ten male C57BL/6 mice (6-8 weeks old) were purchased from Guangdong Medical Experimental Animal Center; Inverted Microscope was purchased from Guangzhou Liss optical instrument co., Ltd.; CytoFLEX Flow Cytometry was purchased from Beckman; ethanol, isopropanol, N-acetylcysteine, SR9238 and Praeruptorin B were purchased from Sigma-Aldrich; Type II collagenase, Advanced DMEM/F-12 medium and PBS were purchased from Thermo Fisher Scientific; Oil Red O dye solution was purchased from Servicebio; Fetal bovine serum (FBS) was purchased from AusgeneX; 2',7'-dichlorodihydrofluorescein (DCFH-DA) was purchased from GLPBIO; Penicillin-Streptomycin solution was purchased from HyClone; Hank's Balanced Salt Solution (HBSS) was purchased from Procell; 1.25% Avastin solution was purchased from Nanjing Aibei Biotechnology Co., Ltd.

2.2. Methods

2.2.1 Isolation and Culture of Primary Mouse Hepatocytes

The mice were anaesthetized with an intraperitoneal injection of 1.25% Avastin and the abdomens were clipped after dehairing and disinfection. An indwelling needle was inserted into the hepatic portal vein while the inferior vena cava was clipped, perfused and digested with D-Hank's solution and Advanced DMEM/F-12 medium containing type II collagenase. When the surface of the liver was honeycombed, the hepatocytes can be released into the medium and centrifuged several times to obtain viable hepatocytes. The isolated hepatocytes were resuspended in Advanced DMEM/F-12 containing 1% fetal bovine serum and 1% penicillin-streptomycin, then plated and placed in an incubator under controlled conditions of 95% air and 5% CO2 humidified atmosphere at 37℃.

2.2.2 Oil Red O Staining

The treated hepatocytes were rinsed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes. At the end of fixation, the hepatocytes were stained with Oil Red O. After staining, the hepatocytes were rapidly washed with 60% isopropanol and then passed through PBS. The dried hepatocytes were observed and photographed under an inverted microscope.

2.2.3 Determination of Cellular ROS Level with DCFH-DA Probe

Hepatocytes were rinsed with PBS and incubated with 5 μM DCFH-DA in a cell culture incubator protected from light. Single cell suspensions were prepared after PBS washing and detected by flow cytometry.

2.3. Statistical Analysis

All experimental data were assessed for statistical significance by calculating mean ± SD, and differences between groups were compared using the Student t-test. Differences were considered statistically significant at $P < 0.05$ (*), significant at $P < 0.01$ (*), and extremely significant at $P < 0.001$ (*).

3. RESULTS AND DISCUSSION

3.1. Establishment of Alcoholic Fatty Liver Cell Model *in vitro*

Previous studies have shown that alcohol is closely related to the occurrence of fatty liver, but no studies have been conducted directly at the hepatocyte level. We isolated primary mouse hepatocytes and cultured them for 24 hours in vitro with different concentrations of alcohol. The results of Oil Red O staining (Figure 1A, B) showed that lipid droplets became larger (Figure 1D) and increased (Figure 1C) in alcohol-treated hepatocytes. These results indicated that we had successfully established an alcoholic fatty liver cell model in vitro.

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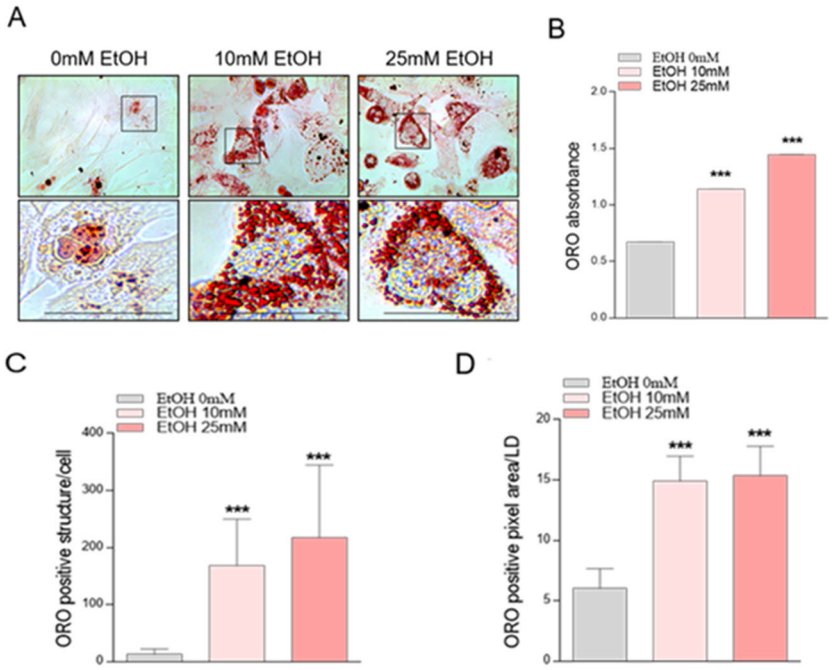

Figure 1. Establishment of alcoholic fatty liver cell model. (A) Representative images of the results of oil red O staining, Bar = 50μm. (B) Absorbance values of Oil Red O Staining for the same number of hepatocytes. Statistical results of the number of lipids(C) and lipid size(D) in hepatocytes that were treated with different concentrations of alcohol.

3.2. Alcohol Promotes High Levels of ROS Production in Hepatocytes

Primary mouse hepatocytes were cultured in 24-well plates. DCFH-DA was used to detect changes in ROS levels in hepatocytes treated with different concentrations (0, 10, 25 mM) of ethanol content. The results showed that the fluorescence intensity of the alcohol-treated group was significantly higher than that of the control group. This indicates that alcohol treatment induced an increase in the level of ROS in hepatocytes, positively correlating with the alcohol concentration.

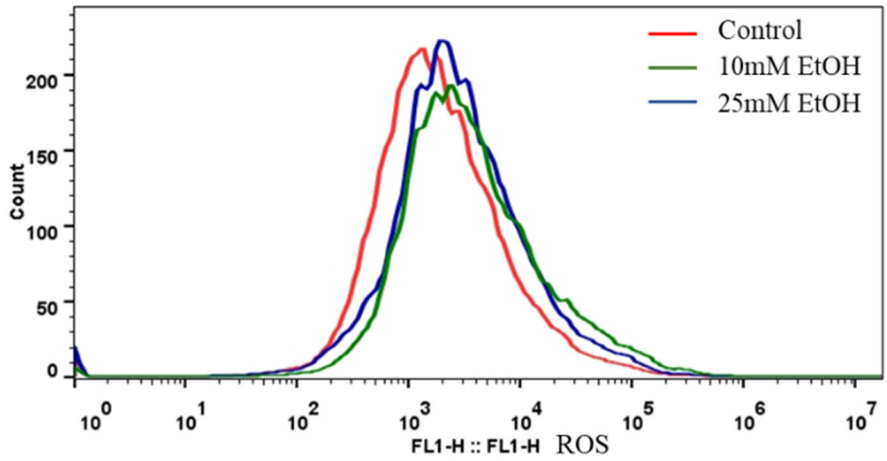

Figure 2. Alcohol treatment induces ROS production in hepatocytes. Primary mouse hepatocytes were treated with alcohol for 24 hours before analysis.

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3.3. NAC Attenuates Lipid Accumulation in Hepatocytes by Eliminating ROS

To clarify the correlation between alcohol-induced lipid accumulation and increased ROS levels, hepatocytes were treated with NAC, a ROS scavenging agent, in addition to alcohol treatment. Lipid accumulation and intracellular ROS changes were observed by Oil Red O staining and DCFH-DA, respectively. Figure 3A shows an increase in ROS production after alcohol treatment and a relative decrease in ROS production after co-treatment of alcohol and NAC. The results of Oil Red O staining (Figure 3B, C, D) showed that lipid droplets in the hepatocytes of the alcohol-treated group increased in number and volume compared to the control group, whereas, the phenotypes were abolished by the treatment of NAC. Based on this, we found that reducing ROS levels inhibited alcohol-induced accumulation of lipids in hepatocytes.

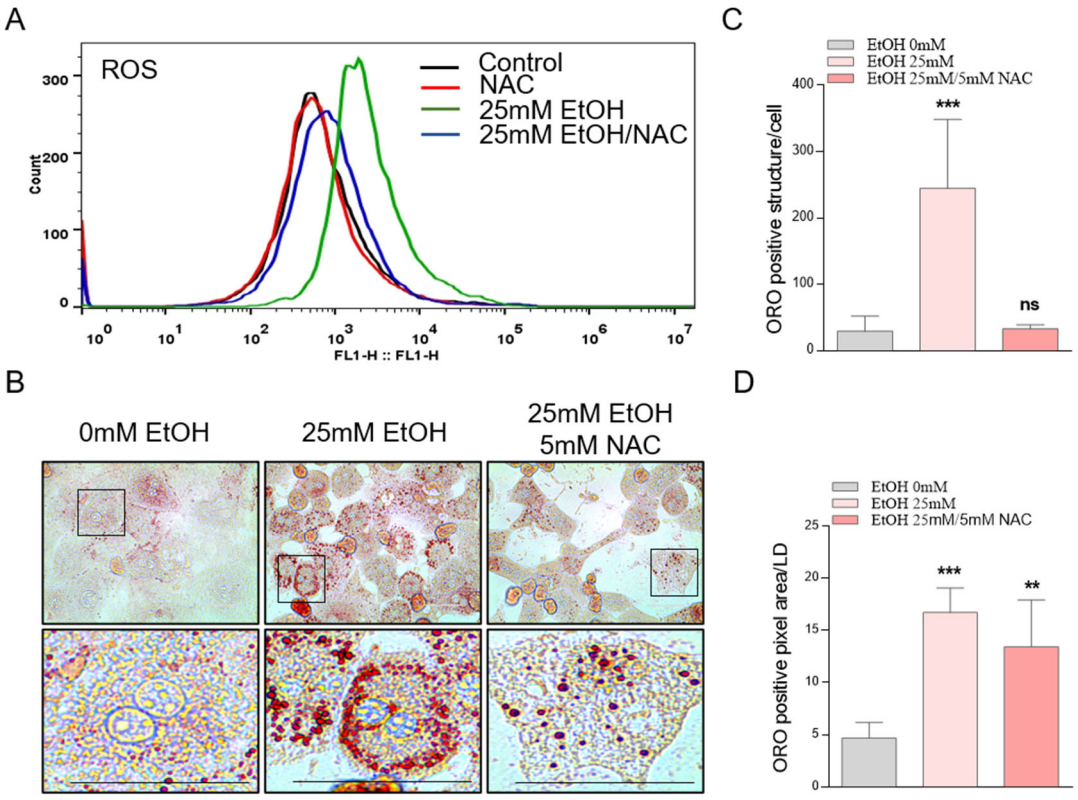

Figure 3. Reducing ROS levels improved alcohol-induced accumulation of lipids. (A)Changes of ROS in hepatocytes after alcohol, alcohol and NAC combined treatments. (B) Representative pictures of ORO staining results of hepatocytes treated with alcohol, alcohol and NAC, Bar=50μm. Statistical results of the number of lipids(C) and lipid size(D) in hepatocytes that were treated with specific condition.

4. CONCLUSION

Hepatic steatosis, the most distinguished pathological feature of ALD, is characterized by elevated intrahepatic lipid accumulation. The production of ROS from alcohol metabolism is the key causation of lipid disorders. In the present study, we established primary mouse hepatocytes models of alcoholic fatty hepatocyte to investigate the role of ROS scavenging agent in alcoholic steatosis. Our recent study revealed that ROS inhibition reduced hepatic lipid accumulation by controlling the production of alcohol related ROS. However, the central issue of ROS action on ethanol-induced hepatic steatosis is not completely understood. Further study needed to investigate how ROS effects lipid metabolism in hepatocytes and test in alcohol-fed mouse.

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In conclusion, our work demonstrated that the NAC, a scavenging agent of ROS, plays a critical role in attenuating ethanol-induced hepatic steatosis. It exhibited its preventive and therapeutic effects on ALD by ameliorating alcohol-induced intracellular lipid deposition in hepatocytes though inhibited ROS production from alcohol metabolism. Our study provides a potential drug target and approach for ALD treatment.

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