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Mainstream cigarette smoke accelerates the progression of nonalcoholic steatohepatitis by modulating Kupffer cell-mediated hepatocellular apoptosis in adolescent mice

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HIGHLIGHTS

- Mainstream cigarette smoke accelerates the progression of nonalcoholic steatohepatitis.
- Mainstream cigarette smoke activates Kupffer cells to release inflammatory cytokines and oxidative stress.
- Mainstream cigarette smoke induces hepatocellular apoptosis.

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ABSTRACT

Cigarette smoking in adolescents is considered to be a major cause of preventable morbidity and mortality. The purpose of this study is to investigate the role of mainstream cigarette smoke (MSCS) on the progression of nonalcoholic steatohepatitis in adolescents. Three-week-old C57BL/6 mice were fed either a methionine and choline-deficient plus high fat (MCDHF) diet for 6 weeks. Each group was exposed to MSCS (300, 600 ug/L) or fresh air for 2 h per day during the first 3 weeks of MCDHF diet feeding. MSCS increased MCDHF diet-induced NASH by increasing serum ALT/AST levels, steatosis, inflammation, and fibrosis. Furthermore, MSCS was associated with the degree of oxidative stress and hepatocellular apoptosis in NASH mice, but not prominent in controls. *In vitro*, cigarette smoke extract (CSE) activated Kupffer cells (KCs) to release inflammatory cytokines and oxidative stress, which induced hepatocellular apoptosis. In conclusion, MSCS exposure accelerates the progression and severity of NASH by modulating KC-mediated hepatocellular apoptosis. Our results support the regulation of CS in adolescents with steatohepatitis.

1. Introduction

Cigarette smoking is considered to be a major cause of potentially preventable morbidity and mortality, and preventing adolescents and adults from smoking is a public health priority for comprehensive cigarette smoking control (He et al., 2005; Viswanath et al., 2010). Although the adult smoking rate has declined over the past few years, this trend is far less evident among adolescents (Johnston et al., 2005). Recent data suggest that approximately 90,000 adolescents around the world start smoking every day, and many become regular smokers (Prokhorov et al., 2006); if this pattern persists, an estimated one-third of adolescent

Abbreviations: NAFLD, non-alcoholic fatty liver disease; MSCS, mainstream cigarette smoke; NASH, nonalcoholic steatohepatitis; MCDHF, methionine- and choline-deficient plus high fat; CSE, cigarette smoke extract; KCs, Kupffer cells; SSCS, side stream CS; NAS, NAFLD activity score; ALT, alanine aminotransferase; AST, aminotransferase; TG, triglyceride; TC, total cholesterol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; MDA, malondialdehyde; GSH, glutathione.

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smokers will die from smoking-related diseases during their lifetime (Pbert et al., 2003).

Non-alcoholic fatty liver disease (NAFLD) is currently the most common form of chronic metabolic liver disorder affecting both adults and children (Wieckowska and Feldstein, 2005). One in three adults and one in 10 adolescents in the United States have hepatic steatosis along the spectrum of NAFLD, which includes hepatic pathologies ranging from simple, non-inflammatory triglyceride accumulation in hepatocytes ("simple steatosis or fatty liver") to steatosis with inflammation and fibrosis (steatohepatitis) (Browning et al., 2004; Schwimmer et al., 2006). Possible mechanisms include pro-inflammatory cytokine production, oxidative stress, and mitochondrial dysfunctions, which are known to be associated with progression from NAFLD to nonalcoholic steatohepatitis (NASH), can progress to cirrhosis with complications such as portal hypertension and hepatocellular carcinoma (Sanyal, 2005; Marra et al., 2008).

Cigarette smoke (CS) is generally divided into two categories. Mainstream CS (MSCS) refers to the smoke inhaled by the smoker, while side stream CS (SSCS) is the smoke emanating from the cigarette between puffs (Smith and Fischer, 2001). A previous study demonstrated that prolonged exposure to MSCS increases the risk of cardiovascular and pulmonary disease and lung cancer in human (Pham et al., 2006). In recent years, there has been increasing evidence that MSCS negatively influences the incidence, severity, and clinical course of various types of chronic liver diseases including hepatitis C, primary biliary cirrhosis, and hepatocellular carcinoma in patients (Pessione et al., 2001; Gershwin et al., 2005; Marrero et al., 2005). Furthermore, MSCS was also shown to exacerbate the severity of NAFLD in obese rats and to be associated with liver fibrosis in a large multi-center cohort of NAFLD patients (Azzalini et al., 2010; Zein et al., 2011).

In the present study, we measured the effects of MSCS exposure on the progression of NASH in an adolescent NASH mouse model. To the best of our knowledge, our results are the first to demonstrate that the detrimental effects of MSCS occur *via* KC-mediated hepatocellular apoptosis.

2. Materials and methods

2.1. Animals and experimental protocol

Three-week-old male C57BL/6 mice were used for this experiment. Mice were obtained from Central Lab. Animal Inc., Seoul, Korea. Mice were maintained under standard conditions (24 ± 2 °C, 50 ± 5% humidity) in a pathogen-free environment and fed either a sterile standard control diet or a methionine- and choline-deficient high-fat (MCDHF) diet and provided water *ad libitum*. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of the Korea Institute of Toxicology. The animal facility of the Korea Institute of Toxicology is fully accredited by the National Association of Laboratory Animal Care. Male mice were fed either a control diet or an MCDHF diet (L-amino acid rodent diet with 60 Kcal% fat, no added choline, and 0.1% methionine, Research Diets, USA) for 6 weeks. Each diet group was exposed to MSCS (300, 600 ug/L) or fresh air for 2 h per day, 5 days per week during the first 3 weeks of the MCDHF diet. Mice were divided into six groups: control nonsmoker (Negative control, NC), control smoker (CS 300), control heavy smoker (CS 600), NASH nonsmoker (MCDHF), NASH smoker (MCDHF + CS 300), and NASH heavy smoker (MCDHF + CS 600). Prior to necropsy, animals were fasted overnight. Eight mice were used in each group. At necropsy, terminal body and liver weights were measured, and standard necropsy techniques were used. Tissues were collected and prepared for future analysis.

2.2. Exposure to MSCS

The 3R4F reference cigarettes were obtained from the University of Kentucky. All cigarettes were conditioned for a minimum of 48 h prior to use (60 ± 2% relative humidity, 22 ± 1 °C according to ISO standard 3402:1999). Smoke was generated on an automatic 30-port carrousel smoking machine (JB2080, CH Technology, USA). The machine operates in basic conformity with the ISO 3308 standard smoking protocol (Carmines and Gaworski, 2003). Mice were exposed to MSCS using nose-only exposure system (Nose-only Inhalation Chamber NITC-36, HCT, Korea).

2.3. CSE extraction

CSE was extracted from 3R4F reference cigarettes into 30 ml of PBS for 5 min using impingers, according to the ISO3308 standard method. This solution was considered to be 100% CSE.

2.4. NAFLD Activity Score (NAS)

H&E-stained liver sections were assessed according to the scoring system for NASH proposed by Kleiner et al., which considers the sum of steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning degeneration (0–2) scores to be the NAFLD Activity Score (score of 0–2: not NASH; 3–4: borderline; 5–8: NASH) (Miura et al., 2012).

2.5. Biochemical measurements

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using AM101-K spectrophotometric assay kits (ASAN Pharmaceutical, Hwasung, Korea). The triglyceride (TG) and total cholesterol (TC) contents in the liver were determined by an AM202-K spectrophotometric assay kit (ASAN Pharmaceutical, Hwasung, Korea).

2.6. Histopathologic examination

Livers were fixed in 10% phosphate-buffered formalin, routinely processed, and then embedded in paraffin. Tissue sections (5 μm) were prepared using a microtome (HM-340E, Thermo Fisher Scientific Inc., MA, USA) and placed on glass slides. H&E staining was performed according to standard techniques. For oil red O staining, 5 μm frozen liver sections were air-dried for 30 min, followed by fixation in 4% formaldehyde. Oil red O staining was performed using standard protocols. To evaluate the severity of fibrosis, liver sections were stained with Direct red 80 and Fast-green FCF (color index 42053) obtained from Sigma-Aldrich Diagnostics. After the liver sections were stained, red-stained collagen fibers were quantified by the percentage of positive area per total liver section. Data were expressed as the percentage of Sirius red-positive area per field. For detection of apoptotic cells in the liver, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed on paraffin-embedded sections using an ApopTaq Peroxidase *in situ* apoptosis detection kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. The positive reaction was visualized with DAB substrate, and then nuclear counterstaining was performed using methyl green dye. TUNEL-labeled cells were quantified as the percentage of positive area per high-power field. A total of 10 high-power fields were analyzed from the liver tissue of individual animals. Data were expressed as the percentage of TUNEL-positive area. Total liver section images were analyzed for each animal using light microscopy (BX-51, Olympus Corp., Tokyo, Japan) and digital imaging software (analySIS TS, Olympus Corp., Tokyo, Japan).