



## Evaluating the Influence of Side Stream Cigarette Smoke at an Early Stage of Non-Alcoholic Steatohepatitis Progression in Mice

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Side stream cigarette smoke (SSCS) is known to be as harmful and hazardous to human health as is active smoking. In this study, we investigated the relationship between the exposure to SSCS and its stimulatory and subacute effects on the progression of non-alcoholic steatohepatitis (NASH). A methionine and choline-deficient plus high fat (MCDHF) diet was administered to C57BL/6 mice for 6 weeks. During the first three weeks of MCDHF diet feeding, each diet group was exposed to SSCS (0, 20, 40 µg/L) or fresh air for 2 hrs per day and 5 days per week. Additional experiments were performed by increasing the concentration (0, 30, 60 µg/L) and exposure time (6 hours per day) of SSCS. According to histopathologic analysis and serum levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), there were no differences in hepatic fat deposition, fibrosis, apoptosis or liver damage in MCDHF-fed mice based on SSCS exposure. There were also no differences in the expression of inflammation-, oxidative stress- or fibrosis-related genes between MCDHF-fed mice with or without SSCS exposure. Therefore, it is concluded that SSCS with current exposure amounts does not have additive detrimental effects on the early stage of NASH.

**Key words:** Side stream cigarette smoke, Passive smoking, Liver, Nonalcoholic steatohepatitis

### INTRODUCTION

Over the last several decades, there is increasing concern about the systemic effects of cigarette smoke (CS) on human health. There are two categories of CS: mainstream CS (MSCS), which is inhaled by active smokers, and side stream CS (SSCS), which is inhaled by passive smokers (1). Several

epidemiological trials have clearly demonstrated that CS is a major etiologic factor in the progression of several chronic diseases, especially those involving the lung. Several studies now show that CS exposure is also a risk factor in the development and/or progression of alcoholic-, HBV- and HCV-related cirrhosis and primary biliary cirrhosis (2-5). Furthermore, CS has carcinogenic properties in various organs. In the livers of cirrhotic patients, CS is associated with an increased incidence of hepatocellular carcinoma (6,7).

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of hepatic pathology, ranging from simple steatosis in its mildest form to cirrhosis at its most severe stage (8). Non-alcoholic steatohepatitis (NASH), the intermediate form of NAFLD, is defined by the presence of hepatic steatosis, severe inflammation and ballooning degeneration of hepatocytes. The precise mechanism by which fatty liver progresses into NASH remains unclear. However, several experimental and clinical studies have provided convincing evidence that NASH may develop secondary to the failure of anti-lipotoxic protection systems in the liver, and multiple hits from the gut and/or adipose tissue (9).

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Recent studies suggest that smoking partially enhances the progression of NAFLD through its effect on insulin resistance and/or oxidative stress (10,11). Nicotine, the main constituent of CS, exacerbates metabolic abnormalities that are induced by high-fat intake in mice via increased oxidative stress and inactivation of 5'-AMP-activated protein kinase (AMPK) (12). It is well documented that SSCS contains more oxidants and other harmful compounds than does MSCS. Several experimental studies found that SSCS delays corneal and epidermal wound healing (13,14) and increases oxidative stress in the brainstem, which affects cardiovascular responses (1). Furthermore, consistent with data for active smoking, passive smoking should be regarded as a germ cell mutagen in humans (15). In line with these results, SSCS stimulates fat accumulation in the liver, which is mediated by AMPK and the Sterol regulatory element-binding protein 1 (SREBP-1) (16).

In general, cumulative evidence indicates that passive smoking can be as harmful and hazardous to several organs as is active smoking. However, little is known regarding the effects of SSCS on the development and progression of NASH induced by methionine- and choline-deficient high fat (MCDHF) diet. Recently our unpublished data revealed that exposure of MSCS at an early stage of NASH increased the severity of NASH. Thus, these results implied that SSCS exposure at an early stage of NASH might have similar or intriguing finding. Therefore, this study was designed to evaluate whether SSCS exposure at an early stage of NASH affects its progression.

## MATERIALS AND METHODS

**Animals and experimental design.** Six-week-old male C57BL/6 mice were obtained from Central Lab, Animal Inc., Seoul, Korea. The mice were housed in a standardized ( $24 \pm 2^\circ\text{C}$ ,  $50 \pm 5\%$  humidity), pathogen-free environment and fed a sterile standard control diet or a MCDHF diet (Research diet Inc., New Brunswick, NJ, USA). Mice were provided water *ad libitum*. The experimental and animal management procedures were in accordance with the requirements of the Animal Care and Ethics Committees of Korea Institute of Toxicology. The animal facility at the Korea Institute of Toxicology is fully accredited by the National Association of Laboratory Animal Care. Male mice were either fed a control diet (11% of calories from fat) or a MCDHF diet (L-amino acid rodent diet with 60% of calories from fat, no added choline and 0.1% methionine) for 6 weeks. During the first three weeks of MCDHF diet feeding, each diet group was exposed to SSCS (20, 40  $\mu\text{g}/\text{L}$ ) or fresh air for 2 hrs per day and 5 days per week. Additional experiments were performed by increasing the concentration (30, 60  $\mu\text{g}/\text{L}$ ) and exposure time (6 hrs per day) of SSCS. The mice were divided into control and MCDHF diet groups. Each group was divided into three

groups: CS non-exposed mice (NE), mice exposed to low dose SSCS (CS 20 or 30), and high-dose-exposed mice (CS 40 or 60). There were five mice in each group. Prior to necropsy, animals were fasted overnight. During necropsy, the animals' terminal body weights were measured. Standard necropsy techniques were used (17). Tissues were collected and prepared for future analysis.

**SSCS exposure.** The 3R4F reference cigarettes were obtained from the University of Kentucky. All cigarettes were conditioned for a minimum of 48 hrs prior to use ( $60 \pm 2\%$  relative humidity,  $22 \pm 1^\circ\text{C}$  according to ISO standard 3402:1999). Smoke was generated on an automatic 30-port carousel smoking machine (JB2080, CH Technologies Inc., Westwood, NJ, USA). The machine operates in basic conformity with ISO 3308 standard smoking protocol (18). Mice were exposed SSCS through their nose using nose-only exposure system (Nose-only Inhalation Chamber NITC-36, HCT, Daejeon, Korea).

**NAFLD activity score (NAS).** H&E stained liver sections were assessed according to the scoring system for NASH proposed by Kleiner *et al.* (19). This system considers the sum of steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning degeneration (0-2) to calculate the NAFLD Activity Score (score 0-2: not NASH, 3-4: borderline, 5-8: NASH). The liver sections were averaged over 5 fields per slide at 200x magnification.

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

**Histopathologic examination.** Livers were fixed in 10% phosphate-buffered formalin, routinely processed, and then embedded in paraffin. A microtome was used to prepare 5  $\mu\text{m}$  tissue sections (HM-340E, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sections were placed on glass slides. H&E staining was performed according to standard techniques. Oil red O staining was performed using standard protocols. The frozen liver sections were air-dried for 30 mins, and then fixed in 4% formaldehyde. In order to evaluate the severity of the fibrosis, liver sections were stained with Direct red 80 and Fast-green FCF (color index 42053) obtained from Sigma-Aldrich Diagnostics (Sigma-Aldrich, St. Louis, MO, USA). After the sections were stained, red-stained collagen fibers were quantified as the percentage of positive area per total liver section. Data were expressed as percentages of the Sirius red-positive area per field. In order to detect apoptotic cells in the liver, the Terminal deoxynucleo-

tidyl 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. Positive reactions were visualized with DAB substrate. Next, nuclear counterstaining was performed using methyl green dye. TUNEL-labeled cells were quantified by the percentage of positive area per high-power field. A total of 10 high-power fields (of liver tissue) were ana-

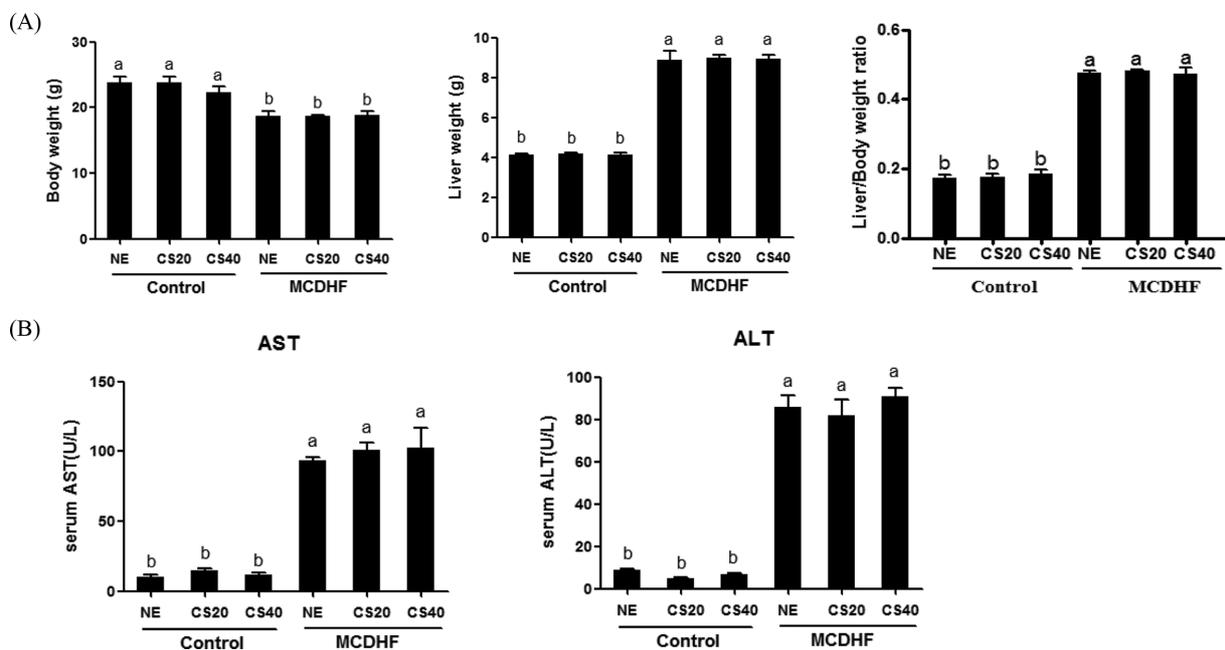
lyzed in each animal. Data were expressed as percentages of TUNEL-positive areas. The total liver section images were analyzed using a light microscope (BX-51, Olympus Corp., Tokyo, Japan) and digital image software (analySIS TS, Olympus Corp.).

**Quantitative real time polymerase chain reaction (qRT-PCR).** Total RNA was isolated from the tissue using the Easy-Spin Total RNA extraction kit (GeneAll, Seoul,

**Table 1.** Primer sequence of qRT-PCR

Gene	Forward	Reverse
TNF- $\alpha$	5'-GTCTACTCCCAGGTTTCTCTTCAAGG-3'	5'-GCAAATCGGCTGACGGTGTG-3'
IL-1 $\beta$	5'-CTCGCAGCAGCACATCAACA-3'	5'-CCACGGGAAAGACACAGGTA-3'
IL-1ra	5'-TACTCCCAGGTCAAACACCAGGTAA-3'	5'-ATCCTGAGCACACATGGCAAAC-3'
HO-1	5'-TGCAGGTGATGCTGACAGAG-3'	5'-GGGATGAGCTAGTGCTGATC-3'
NOS2	5'-AACGGAGAACGTTGGATTG-3'	5'-CAGCACAAGGGGTTTTCTTC-3'
$\alpha$ SMA	5'-TCAGGGAGTAATGGTTGGAA-3'	5'-CAGTTGGTGATGATGCCGTG-3'
Col1	5'-ACAGGCGAAACCGGTGACAG-3'	5'-GCCAGGAGAACCAGCAGAGC-3'
Col4	5'-AGGAGAGAAGGGTGAACAAG-3'	5'-CCAGGAGTGCCAGGTAAGCC-3'
TGF- $\beta$	5'-TGAACCAAGGAGACGGAATACAGG-3'	5'-GCCATGAGGAGCAGGAAGGG-3'
Timp-1	5'-TCTGGCATCTGGCATCCTCTTG-3'	5'-AACGCTGGTATAAGGTGGTCTCG-3'
GAPDH	5'-ACGGCAAATCAACGGCACAG-3'	5'-AGACTCCACGACATACTCAGCAC-3'

TNF- $\alpha$ : tumor necrosis factor-alpha; IL-1 $\beta$ : interleukin-1 $\beta$ ; IL-1ra: interleukin-1 receptor antagonist;  $\alpha$ SMA: alpha-smooth muscle actin; Col1: type I collagen alpha 1; Col4: type I collagen alpha 4; TGF- $\beta$ : transforming growth factor  $\beta$ ; Timp-1: tissue inhibitor of metalloproteinase-1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



**Fig. 1.** Weight gain and liver injury caused by exposure to SSCS. Six-week-old male mice were divided control and MCDHF diet group. Each group was divided into three groups: non-exposed mice (NE), mice exposed to low dose of SSCS (CS 20), high-dose-exposed mice (CS 40). Five mice were used per group. (A) Changes in body weight, liver weight and liver to body weight ratio for 6 weeks of the experiment. (B) The serum AST and ALT levels were presented among the control and MCDHF diet feeding groups with or without SSCS exposure. Values are expressed as the mean  $\pm$  SEM. Experimental groups marked by different letters represent significant differences between groups at  $p < 0.05$ .

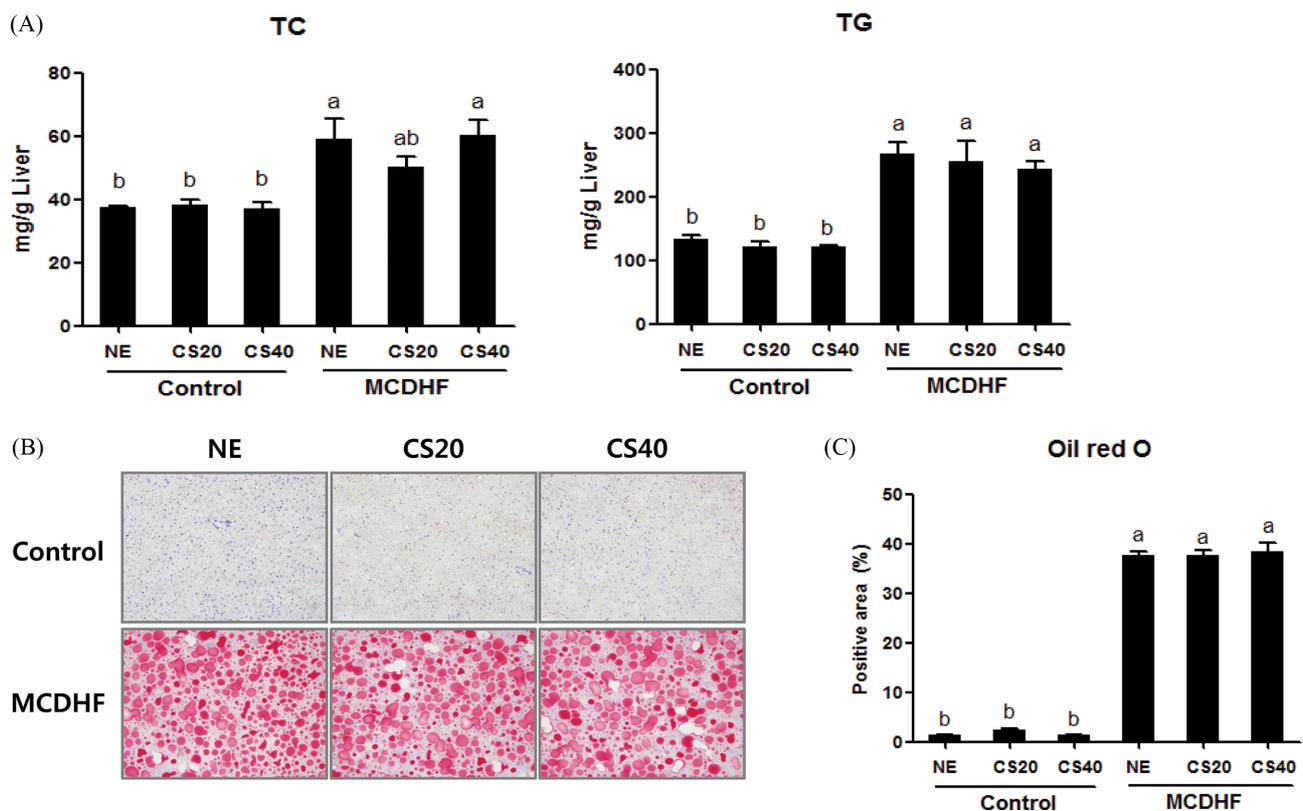


reduced TBW, because of poor body condition. However, SSCS did not affect TBW or mortality in either the MCDHF or control group. Although the MCDHF-fed mice displayed significantly increased liver weights compared to those of the control-fed mice, the liver weight was not affected by SSCS exposure. A similar pattern was observed in the liver to body weight ratio (Fig. 1A). The MCDHF-fed groups had higher levels of AST and ALT, regardless of SSCS exposure, than did the control-fed groups; however, there was no significant difference between the SSCS exposed and non-exposed mice (Fig. 1B). Collectively, our results suggest that SSCS exposure does not affect the liver or systemic condition in general.

**Histopathological evaluation of SSCS exposure.** After H&E staining, the histopathological severity of NASH was also analyzed. The MCDHF-fed groups had significantly increased hepatic inflammatory immune cell infiltration and lipid accumulation compared to those of the control diet groups. However, there were no such microscopic changes between the SSCS exposed and non-exposed mice independent of the diet type (Fig. 2A). According to a well-documented

grading system (19), the MCDHF-fed groups demonstrated more severe inflammation, hepatic steatosis, ballooning degeneration, and a significantly higher NAFLD activity score than did the control diet groups. Although SSCS exposure induced a mild increase in ballooning degeneration in some control mice, it had no effect on ballooning degeneration in the MCDHF-fed groups. Moreover, the degree of inflammation, hepatic steatosis and total activity did not differ in mice with regard to SSCS exposure (Fig. 2B). These findings indicate that SSCS exposure does not increase histopathological changes in the liver.

**Effects of SSCS exposure on hepatic lipid accumulation.** Previous work has suggested that SSCS exposure may influence lipid metabolism (11). Therefore, the effects of SSCS on lipid accumulation in hepatocytes, and lipid levels in liver tissue extracts were quantified. In addition, the cellular lipid content was evaluated in liver tissue sections stained with Oil Red O. The degree of hepatic steatosis, determined by measuring the concentrations of TC and TG in liver extracts, was higher in the MCDHF-fed groups than it was in the control groups (Fig. 3A). In addition,

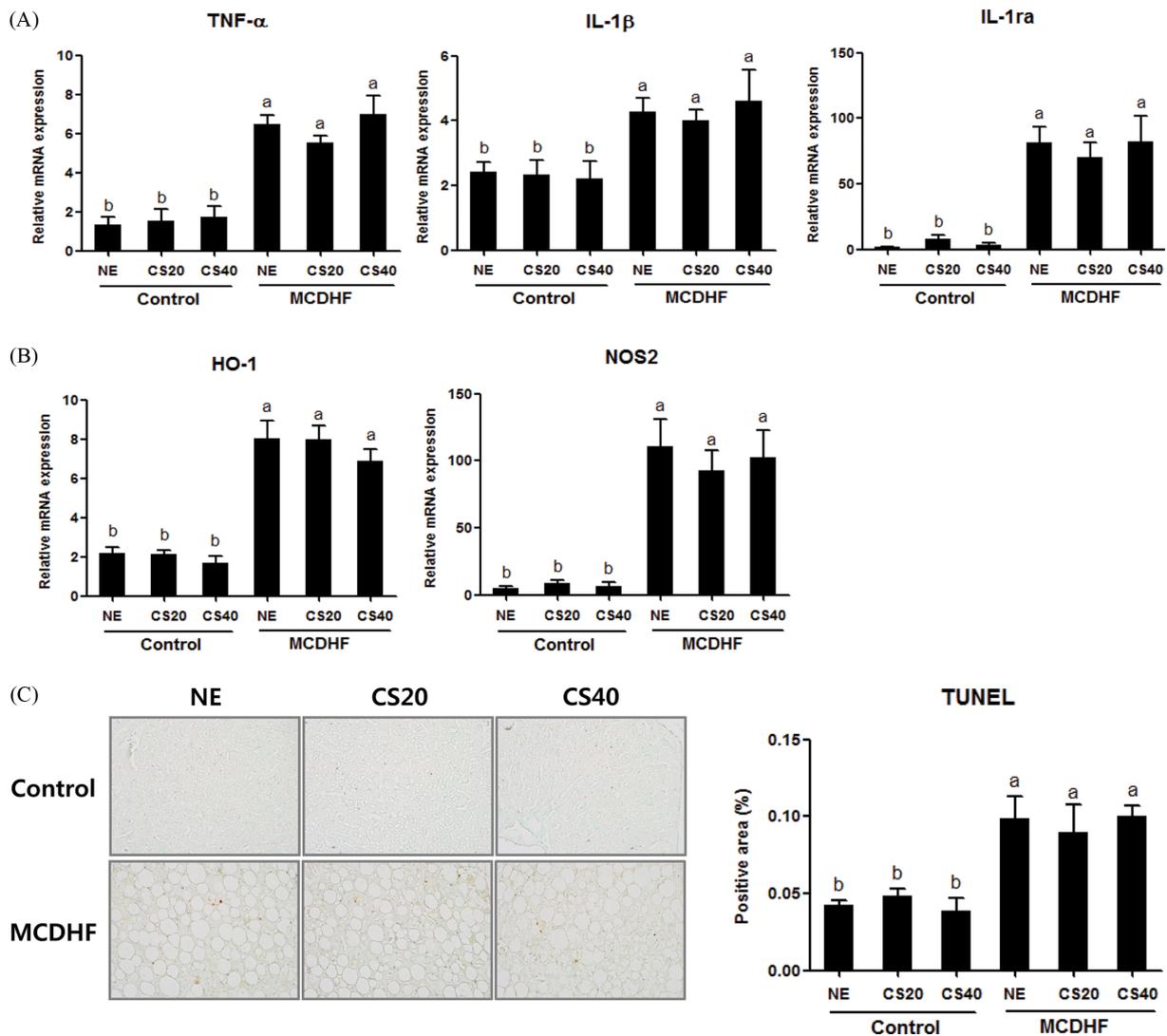


**Fig. 3.** Evaluation of lipid accumulation in liver. (A) Hepatic TC and TG content were measured using commercial kit. (B) Sections of liver tissue from control and MCDHF diet group with or without exposure to SSCS. Tissues were fixed and sections were stained with Oil Red O to observe the hepatic steatosis. Pictures are shown at 200 $\times$  magnification. (C) Lipid accumulation was evaluated by using an image analysis system. Values are expressed as the mean  $\pm$  SEM. Experimental groups marked by different letters represent significant differences between groups at  $p < 0.05$ .

there was increased cellular lipid accumulation in liver sections stained with Oil red O in the MCDHF diet groups than in the control groups (Fig. 3B). However, there were no significant differences between the groups with regard to the levels of hepatic TC, TG and fat accumulation according to SSCS exposure (Fig. 3A-3C). Overall, these results indicate that SSCS exposure (at the indicated concentration and exposure period) does not induce lipid accumulation in the liver.

**Effects of SSCS exposure on inflammation, oxidative stress and apoptosis in the liver.** In order to determine the effects of SSCS exposure on inflammation, we first measured pro-inflammatory gene expression using qRT-PCR.

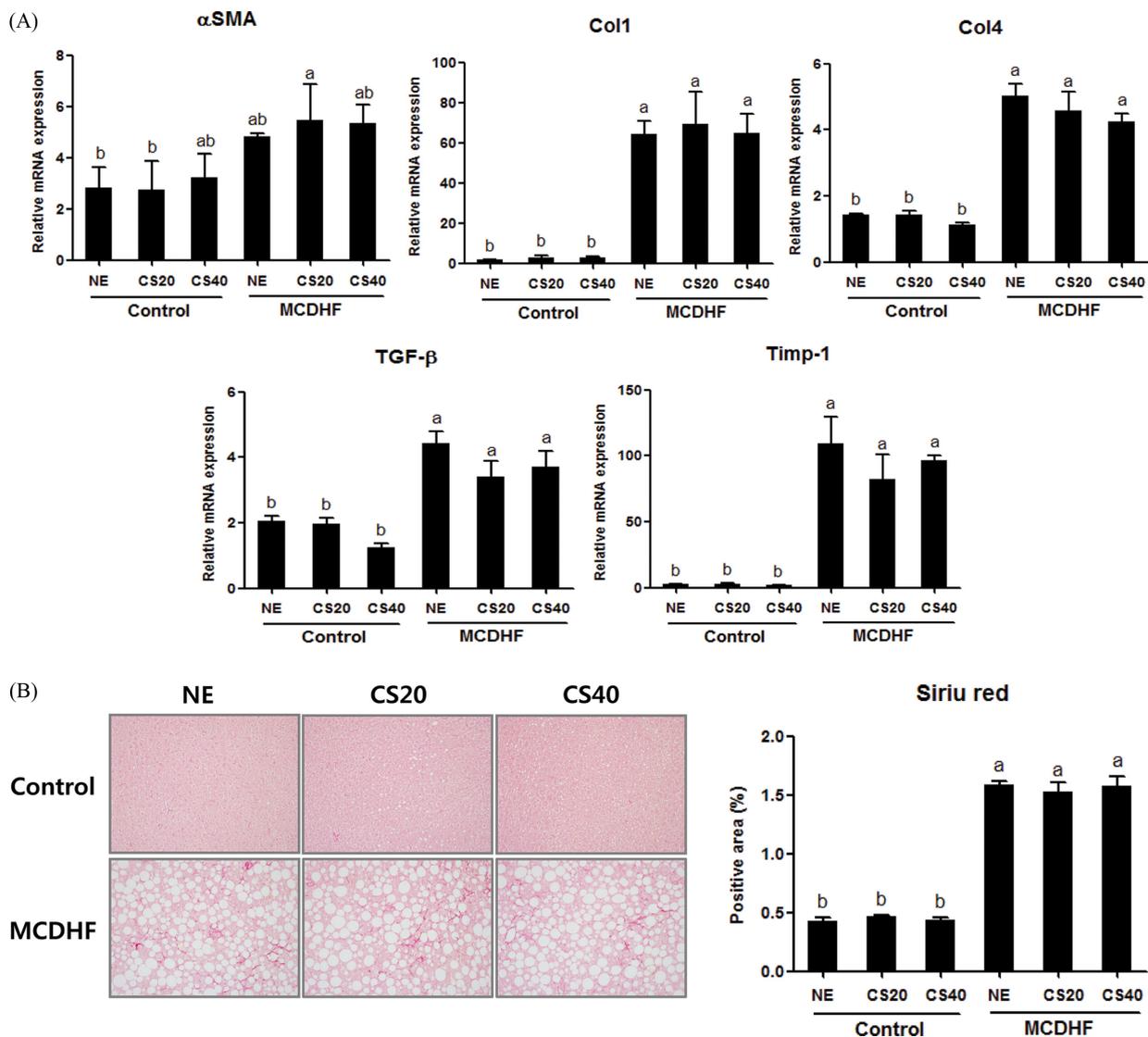
Hepatic mRNA levels of tissue tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-1 beta (IL-1 $\beta$ ) were markedly upregulated in the MCDHF-fed groups compared to those of the control diet groups (Fig. 4A). In addition, the mRNA expression of IL-1 receptor antagonist (IL-1ra) was examined, which is known as a non-invasive inflammatory marker for NASH (20). In accordance with the results of pro-inflammatory gene expression, IL-1ra expression was significantly increased in the MCDHF-fed groups. However, such-inflammatory parameters were not changed, regardless of SSCS exposure, in the control and MCDHF-fed groups. In order to analyze NASH severity, we evaluated whether oxidative stress, an important pathogenic event in NASH progression



**Fig. 4.** Assessment of inflammation, oxidative stress and apoptosis in liver. (A) Results of qRT-PCR to detect TNF- $\alpha$ , IL-1 $\beta$  and IL-1ra mRNA levels are shown. (B) The mRNA levels of oxidative stress-related gene HO-1 and NOS2 were analyzed. (C) Representative TUNEL staining for detection of apoptosis in liver tissue was examined. Pictures are shown at 200x magnification. The proportion of TUNEL-positive area was calculated using an image analysis system. Values are expressed as the mean  $\pm$  SEM. Experimental groups marked by different letters represent significant differences between groups at  $p < 0.05$ .

(21), was affected by SSCS exposure. Although MCDHF feeding markedly increased the mRNA expression of heme oxygenase-1 (HO-1) and nitric oxide synthase 2 (NOS2), SSCS exposure did not affect the expression of such oxidative stress-related genes in either group (Fig. 4B). A similar pattern was observed with regard to cellular apoptosis. The apoptotic phenotypes in SSCS-exposed and unexposed mice were very similar, as evidenced by TUNEL staining and quantitative assessment (Fig. 4C). Collectively, these data suggest that SSCS exposure (of the stated concentration and exposure time) does not induce inflammation, oxidative stress or apoptosis in the liver.

**Effects of SSCS exposure on NASH-associated liver fibrosis.** In order to analyze NASH progression, we used qRT-PCR to measure the mRNA levels of hepatic fibrosis-related genes such as alpha smooth muscle actin ( $\alpha$ SMA), type I collagen alpha 1 (col1), alpha 4 (col4), transforming growth factor-beta (TGF- $\beta$ ) and tissue inhibitor of metalloproteinase-1 (Timp-1). After Sirius red staining, histological assessment of liver fibrosis was also performed using an image analysis system. The mRNA levels of the hepatic fibrosis-related gene were significantly higher in the MCDHF-fed groups compared to those in the control diet groups (Fig. 5A). In addition, there was increased liver fibrosis with Sir-

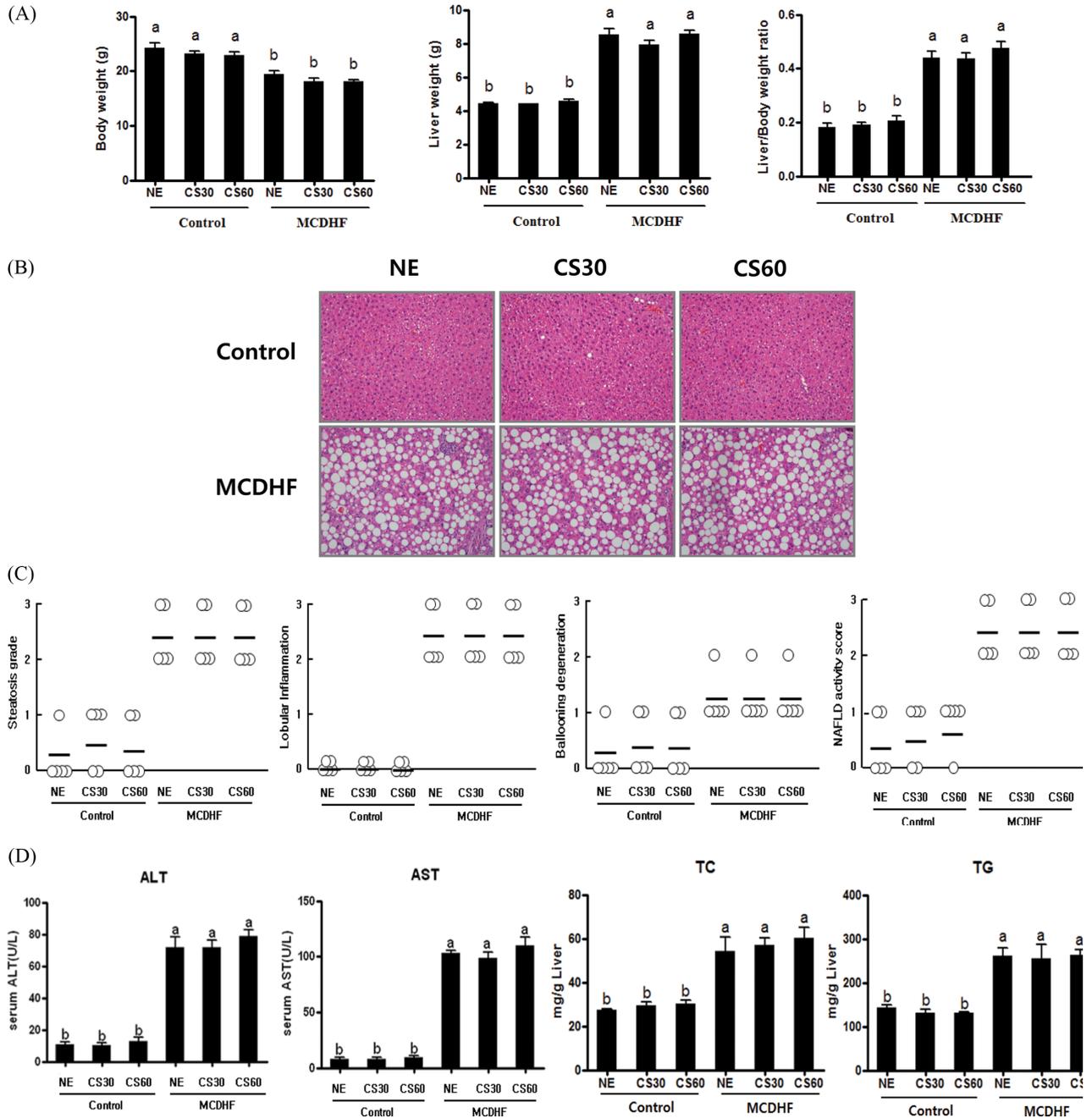


**Fig. 5.** Measurement of liver fibrosis. (A) Results of qRT-PCR to evaluate the mRNA levels of hepatic fibrosis-related genes are shown. (B) Histological analysis of the liver to assess hepatic fibrosis was performed after Sirius red staining. Pictures are shown at 200 $\times$  magnification. Furthermore, liver fibrosis was evaluated by calculating Sirius red-positive area using an image analysis system. Values are expressed as the mean  $\pm$  SEM. Experimental groups marked by different letters represent significant differences between groups at  $p < 0.05$ .

ius red staining in the MCDHF-fed groups than there was in the control diet groups (Fig. 5B). However, the mRNA levels and histological evaluation of liver fibrosis did not differ between the groups with regard to SSCS exposure. Therefore, these findings reveal that SSCS exposure does not

affect NASH-related fibrogenesis in the liver.

**Effects of SSCS concentration and time exposure on NASH development.** In order to clarify the role of SSCS in NASH, additional experiments were conducted by increas-



**Fig. 6.** Additional experiments with increasing SSCS concentration and exposure time. Mice were divided into three groups: non-exposed mice (NE), mice exposed to low dose of SSCS (CS 30), high-dose-exposed mice (CS 60). (A) Effects of SSCS exposure on body weight, liver weight and liver to body weight ratio for 6 weeks of the experiment. (B) Representative H&E-stained liver sections are shown. Pictures are shown at 200x magnification. (C) Histological analysis of H&E-stained liver sections using NAS. (D) The serum AST, ALT, hepatic TC and TG levels were presented among the control and MCDHF diet feeding groups with or without SSCS exposure. Values are expressed as the mean  $\pm$  SEM. Experimental groups marked by different letters represent significant differences between groups at  $p < 0.05$ .

ing the SSCS concentration (30, 60  $\mu\text{g/L}$ ) and exposure time (6 hrs per day). Even with increased concentration and exposure time, however, SSCS still did not affect TBW, liver weight or mortality in either the MCDHF or control-feeding groups. A similar pattern was shown in the liver to body weight ratio (Fig. 6A). Histopathologic analyses revealed that hepatic fat deposition, inflammation and ballooning degeneration were similar in the MCDHF-fed mice regardless of SSCS exposure (Fig. 6B and 6C). Similar to previous results, there were no significant differences in the levels of serum ALT, AST, hepatic TC and TG according to SSCS exposure (Fig. 6D). These consistent findings, despite increased SSCS concentration and exposure time, provide compelling evidence that second-hand smoke did not affect NASH development or severity.

## DISCUSSION

NASH, a more severe form of NAFLD, is characterized by liver steatosis, which progresses to an inflammatory state, fibrosis and eventual loss of organ function. Triglycerides are the main lipids stored in the hepatocytes of NAFLD patients. In a previous study, long term exposure of SSCS markedly increased hepatic lipid accumulation and TG levels via AMPK and SREBP-1 in high fat diet-fed mice (16). However, our results was not consistent with previous study and it is presumably because of different NAFLD models and also exposure concentration since MCDHF model mainly induces steatohepatitis via blocking of lipid metabolism in hepatocyte. Originally, we questioned whether SSCS exposure at an early stage of NASH increases the severity of NASH since our unpublished data revealed that exposure of MSCS at an early stage of NASH increased the severity of NASH.

Recent evidence indicates that TG accumulation in hepatocytes is actually just an “innocent bystander.” Triglyceride accumulation in macrophages actually exerts protective functions with regard to cellular injury and inflammation (22,23). Free fatty acids (FFA) in non-adipose cells, and especially in hepatocytes, may exert harmful effects by increasing insulin resistance, lipotoxicity and apoptosis, leading to cell dysfunction (24,25). Exposure to SSCS increases oxidative stress and lipid anomalies in other organs; therefore, further investigation is necessary to investigate the effects of SSCS on FFA-mediated oxidative stress (1,26). One recent study found that free cholesterol accumulation sensitizes hepatocytes to TNF- $\alpha$  and Fas-induced apoptosis, facilitating the progression from steatosis to NASH (27). However, our results showed that SSCS exposure did not affect the level of hepatic TC or apoptotic cell death.

A previous study from our group found that SSCS exposure at a later stage of NASH (during the last 3 weeks of MCDHF feeding) did not affect NASH development and progression. Furthermore, another unpublished data of our

experiment found that subacute exposure of MSCS at an early stage of NASH increased the severity of NASH via Kupffer cell activation, but decreased the severity of NASH if MSCS at a later stage of NASH is exposed although exposure concentration and smoke constitution are different between MSCS and SSCS. Therefore, we assumed that SSCS exposure may act as a stimulant of NASH early on. However, results from the current study reveal that SSCS exposure in the first 3 weeks of MCDHF feeding did not affect NASH severity. A recent clinical study found an association between SSCS exposure and NAFLD in children (28). The experimental exposure period used in our study was shorter than that of human or other experimental studies, which employed 12~19 weeks of SSCS exposure (16,29). Akhter et al. exposed mice to SSCS in a whole-body chamber, set at  $30 \pm 2 \text{ mg/m}^3$  for 4 hrs/day and 5 days/week for 12 consecutive weeks. The group found that the urinary cotinine levels in SSCS-exposed mice ranged 2.46~3.54  $\mu\text{g}/\text{mg}$  creatinine, which is similar to those of humans who smoke approximately 10~15 cigarettes per day (29). The SSCS exposure concentrations used in our study (20  $\mu\text{g/L}$  or 40  $\mu\text{g/L}$ ) are similar or slightly higher than those of other studies (13,16,29). Therefore, we performed additional experiments with increased exposure time and SSCS concentration during the first 3 weeks of MCDHF feeding. The longer exposure time and higher concentration (30, 60  $\mu\text{g/L}$ ), however, did not affect the histopathologic changes, inflammation or fibrogenesis in either group.

Recent clinical survey study concerning association between SSCS exposure and NAFLD indicated that the incidence of NAFLD development in child was increased 1.8 times for every extra pack per day smoked at home (28). Since the age is the one of the major risk factors for advanced NAFLD/NASH (30), the possibility of development and progression of these liver diseases might be increased by SSCS exposure in old population. However, we herein found that subacute exposure of SSCS at an early stage of NASH did not affect the disease severity in mice. Also, similar results were observed in adolescent mice exposed to SSCS under the same condition (data not shown). Since there is difference between our finding and clinical survey study regarding the effect of SSCS exposure on NASH progression, we speculated that such discrepancy might be caused by difference of SSCS exposure period. Recent study provided that the maximum  $\text{PM}_{2.5}$  concentration of environmental tobacco smoke (ETS), composed both from MSCS and SSCS, was measured as  $1560 \pm 500 \text{ }\mu\text{g}/\text{m}^3$  by using commercial cigarettes and  $1050 \pm 230 \text{ }\mu\text{g}/\text{m}^3$  by using 3R4F reference cigarettes in closed door condition with inner volume of  $1.75 \text{ m}^3$  (31). Another study showed that the mean concentration of respirable particles in ETS ranged from 32.4 to 76.9  $\mu\text{g}/\text{m}^3$  in house (32). Also, mean  $\text{PM}_{2.5}$  concentrations reported for outdoor condition when smokers were present ranged from 8.32 to 124  $\mu\text{g}/\text{m}^3$  at hospitality venues (33). Based on such

information, the concentration of SSCS exposure in this study is much higher than those of SSCS or ETS exposure in human. It is difficult to assume how SSCS with above mentioned concentrations epidemiologically affect a NAFLD incidence, but it is presumed that lifetime (chronic) exposure of SSCS or species specificity may have different outcome of the NAFLD progression compared to our study.

In conclusion, our study found that currently used SSCS exposure concentration and time at an early stage of NASH did not affect the NASH-related inflammatory and fibrogenic response.

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