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Kombucha Tea Prevents Obese Mice from Developing Hepatic Steatosis and Liver Damage

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Abstract Nonalcoholic fatty liver disease (NAFLD) is associated with the increased accumulation of hepatocellular lipids. Although Kombucha tea (KT) has emerged as a substance protecting the liver from damage, the effects of KT in NAFLD remain unclear. Hence, we investigated whether KT influenced hepatic steatosis. Db/db mice were fed either control or methionine/choline-deficient (MCD) diets for 4 weeks. The MCD diet group was treated with KT or water for 3 weeks. KT treatment alleviated macrovesicular steatosis compared to the MCD-fed group. The levels of triglyceride, ALT, and AST also decreased in the KT+MCD-treated db/db mice. RNA expression in the MCD+KT group showed reduced triglyceride synthesis and uptake of fatty acids. Immunostaining and western blot assays for active caspase-3 demonstrated a lower level of apoptosis in the MCD+KT than in the MCD group. These results demonstrate that KT attenuated lipid accumulation and protected the liver from damage, promoting liver restoration in mice.

Keywords: Kombucha tea, nonalcoholic fatty liver disease, liver protection, db/db mouse

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a potentially progressive liver disorder ranging from simple steatosis, nonalcoholic fatty liver (NAFL), to severe steatosis, nonalcoholic steatohepatitis (NASH) (1). NASH is characterized by an increased number of apoptotic hepatocytes compared with simple steatosis and progressively causes the inflammation and fibrosis (2). NAFLD is commonly related to obesity, diabetes, insulin resistance, and metabolic syndrome (3). Pharmacological agents have been studied and developed to improve and prevent NAFLD from progressing into chronic stage liver disease (4). Although these medications have been shown to improve liver function and attenuate insulin resistance in patients with NAFLD, they also induce a high incidence of gastrointestinal side effects (4,5). Therefore, dietary supplements with no side effects are needed to prevent NAFLD.

NAFLD is caused by the breakdown of hepatic lipid homeostasis (3). Normally, the liver absorbs the fatty acids (FAs) from dietary foods or lipid-containing particles and stores them as a form of triglyceride (TG). The reserved TGs are enclosed by very low-density lipoproteins (VLDLs) and then released into the blood when energy production is required (3). However, in NAFLD, this lipid metabolism is distorted due to the failure of TG synthesis and VLDL secretion, the excessive uptake of free fatty acids (FFAs), and up-regulated mitochondrial lipid oxidation, leading to an increased accumulation of TGs in hepatocytes (2,3,6). Many lipid metabolism-regulating proteins are abnormally expressed in NAFLD (7). CD36 and PPAR-γ, which are involved in the uptake of FAs, are known to be highly expressed in livers with NAFLD (7,8). In addition, FAS and SREBP-1c, which regulate TG biosynthesis, called de novo lipogenesis, increase in instances of fatty liver, promoting the progression of NAFLD (2,9). Kombucha tea (KT), with a slightly sweet yet acidic flavor, originated in China and has been known for its detoxifying and energizing effects since 220 BC (10). Currently, it is widely consumed in many parts of the world, including Russia, Central Asia, Europe, and USA. KT is a black tea infused with sugar, which is fermented by a symbiotic association of acetic acid bacteria and yeast, forming a pellicle-like cellulose on the surface, called tea fungus (10-12). It has been reported that KT has a higher level of polyphenols and flavonoids than black (13). Antioxidant and antimicrobial properties of KT were also shown to be beneficial to human health (14,15). A recent study has demonstrated that KT has an anti-hyperglycemic effect in streptozotocin-induced diabetic rats (16). In alloxan-induced diabetic rats, KT was shown to attenuate the oxidative damages in liver, kidney, and pancreas (11). In addition, KT was reported to prevent paracetamol-induced hepatotoxicity in rats and protect

hepatocytes from CCl_4 damage or chromate (VI)-induced oxidative stress in albino rats (17-19). However, it remains unclear how KT influences the protective process in a damaged liver. Furthermore, the effects of KT in NAFLD are poorly understood.

In the present study, we investigated the protective effects of KT on hepatic steatosis in db/db mice, genetically engineered obesity mice without leptin receptor, fed a methionine and choline deficient (MCD) diet. Our results demonstrated that KT reduced hepatic steatosis by inhibiting the uptake of FA and reducing apoptosis in MCD-fed db/db mice, suggesting that KT is a promising dietary supplement for protecting the liver in individuals with NAFLD.

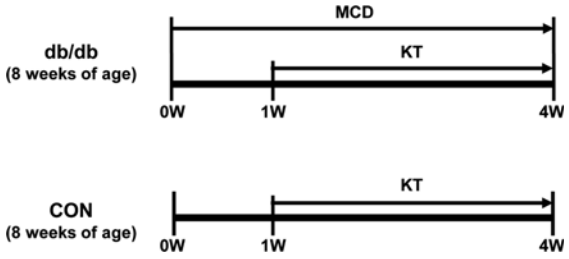

Fig. 1. Schematic diagram of mouse experimental protocol. In the preventive protocol, eight-week-old male db/db mice were treated with normal or MCD diets with KT or water. Normal mice were treated with KT or saline to examine the side-effect of KT.

Materials and Methods

Preparation of KT extract Six gram of black tea (type of tea bag, Lipton, Yellow Label Tea) was added to 600 mL boiling water and was infused for 5 min. Then 10% (w/v) of sucrose was added and stirred to dissolve into the black tea. After cooled to room temperature, the tea was poured into 1 L glass beaker that had been previously sterilized at 121°C for 20 min (11,16,20). Finally, the freshly prepared tea was inoculated with freshly grown KT mat (purchased from Misokombu, Busan, Korea) that had been cultured in the same medium for 14 days. The beaker was covered with clean cheese cloths and fixed with rubber bands. The fermentation was (25±3°C) for 14 days. During fermentation, a daughter mat (new KT mat) was developed over the tea surface. The fermented tea was centrifuged at 7,000× *g* for 20 min and the supernatant was filtered by using 0.45 μm syringe filter (Minisart syringe filters, Sartorius AG, Göttingen, Germany). And then, the filtrate was lyophilized to dry and kept at −80°C for the further experiments (11,16).

Animals and treatments Six-week-old male C57BLKS and C57BLKS db/db mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea) and from Korea Research Institute Bioscience and Biotechnology (Daejeon, Korea), respectively. They were fed with normal diet, watered, and housed with a 12 h light-dark cycle for 2 or 3 weeks for adjustment. Mouse experiments were performed as described Fig. 1. Fifteen db/db mice at 8-week-old (average body weight 38 g) were fed wither the control diet (*n*=4) or the MCD diet (*n*=11; cat no 518810; Central Lab. Animal Inc., Seoul, Korea) for 4 weeks. Six the MCD diet-fed mice were also treated with KT 2 g/kg by oral administration every day for 3 weeks; the remainders were treated with water. KT powder kept at −80°C was completely dissolved in water and used in animal treatment. Liver tissue was collected for histological and biochemical analysis.

To test the toxicity KT, male mice at 9-week-old (average body weight 24 g) were treated with (*n*=5) or without KT (*n*=5; 2 g/kg/day) for 3 weeks. Animal care and surgical procedures were approved by the Pusan National University Institutional Animal Care and Use Committee and carried out in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals.

Liver histology and Immunohistochemistry Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 4 μm section. Specimens were dewaxed, hydrated, and stained usual method with standard hematoxylin and eosin staining (H&E) (21). For immunohistochemistry (IHC), sections were incubated for 10 min in 3% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval was performed by heating in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Sections were treated with Dako protein block (X0909; Dako Envision, Dako Corp., Carpinteria, CA, USA) for 30 min and incubated with casepase-3 (9661s; Cell Signaling Technology, Danvers, MA, USA) or non-immune sera to demonstrate staining specificity at 4°C overnight. Polymer horseradish peroxidase (HRP) anti-rabbit (K 4003; Dako) was used as secondary antibody. 3,3'-Diaminobenzidine (DAB) was employed in the detection procedure.

Quantitative real-time PCR Total RNA which had been stored at −80°C was extracted with TRIZOL™ (Ambion® by Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). After assuring RNA quality and concentration, gene expression was evaluated by QRT-PCR analysis. mRNAs were quantified by real-time RT-PCR per the manufacturer's specifications (Mastercycler Real-Time PCR, Eppendorf, Hamburg, Germany). The primer sets used were as follows; 9S forward: 5'-GAC TCC GGA ACA AAC GTG AGG-3', reverse: 5'-CTT CAT CTT GCC CTC GTC CA-3'; CD36 forward: 5'-TCC TCT GAC ATT TGC AGG TCT ATC-3', reverse: 5'-AAA GGC ATT GGC TGG AAG AA-3'; FAS forward: 5'-GCT GCG GAA ACT TCA GGA AAT-3', reverse: 5'-AGA GAC GTG TCA CTC CTG GAC TT-3'; PPAR- forward: 5'-CAG GAG AGC AGG GAT TTG CA-3', reverse: 5'-CCT ACG CTC AGC CCT CTT CAT-3'; SREBP-1c forward: 5'-GGA GCC ATG GAT TGC ACA TT-3', reverse: 5'-GGC CCG GGA AGT CAC TGT-3'; PPAR- forward: 5'-CAC AAT GCC ATC AGG TTT GG-3', reverse: 5'-GCT GGT CGA TAT CAC TGG AGA TC-3'. Samples were analyzed in duplicate according to the ΔΔCt method. All PCR products were directly sequenced for genetic confirmation in Macrogen Inc. (Seoul, Korea).

Western blot assay Total protein was extracted from freeze-

clamped liver tissue sample that had been stored at −80°C. Whole tissues were homogenized in Triton-lysis buffer (TLB) supplemented with protease inhibitors (Complete Mini 11 836 153 001; Roche, Mannheim, Germany). Equal amount of total protein (200 μg) were fractionated by polyacrylamide gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes. Primary antibody against activated caspase-3 (9661s; Cell Signaling Technology) were used in this experiment. Membranes were developed by chemiluminescence (AE-9150 Ez-Capture II; ATTO Corp., Tokyo, Japan). The blots that were obtained from three independent experiments were scanned and an ROI around the band of interest was defined. Band intensities were calculated by using CS analyzer 2.0 program (ATTO Corp.).

Triglyceride measurement Total liver triglycerides were measured by using Triglyceride Colorimetric Assay Kit from Cayman Chemical (Nashville, TN, USA) following the manufacturer's specifications.

Measurement of AST/ALT Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by using a commercial kit (Asan Pharmaceutical, Seoul, Korea) according to the manufacturer's instructions.

Statistical analysis Results are expressed as the mean±SD. Statistical differences were determined by Student's t-test. *p*-values <0.05 were considered to be statistically significant.

Results and Discussion

KT reduced fat accumulation in MCD-fed db/db mice To investigate the effects of KT on histomorphological changes in hepatic steatosis, we performed H&E staining on liver sections from normal mice and chow- or MCD-fed db/db mice with or without KT. There were no visible changes or biochemical differences between KT-treated and non-treated normal mice, indicating that KT rarely caused side effects in the liver (data not shown). In line with other studies, chow-fed db/db mice had the accumulated fatty hepatocytes compared to the control mice. MCD-fed db/db mice showed a dramatically increased accumulation of macro- and microvesicular fats, whereas the MCD-fed db/db mice treated with KT showed decreased macro- and microsteatosis (Fig. 2). Body weight increased in all db/db groups compared to control group, but the degree of body weight increase was lower in KT+MCD-treated than chow- or MCD-fed db/db mice. Increased liver weight/body weight (LW/BW) was observed in chow- and MCD-fed db/db compared to control mice, which indicates that fatty hepatocytes were massively accumulated in chow- and MCD-fed db/db mice (1.33-fold increase in chow-fed mice, 1.65-fold increase in MCD-fed db/db mice, *p<0.05, **p<0.005, compared to control). KT treatment strikingly reduced the LW/BW in MCD-fed db/db mice, which was almost

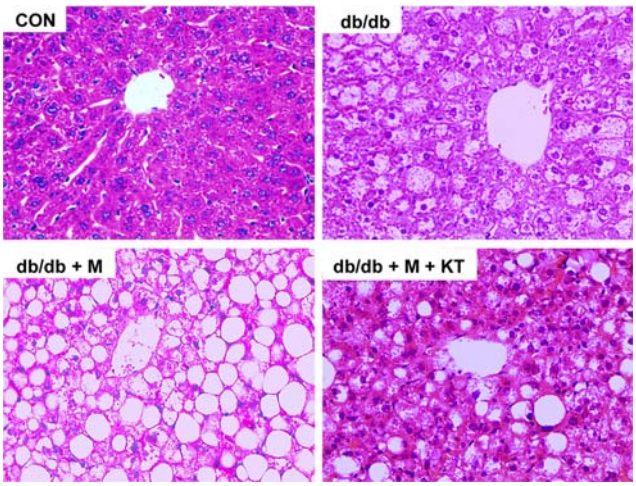

Fig. 2. KT improves hepatic histomorphology in MCD-fed db/db mice. H&E stained liver sections from representative mice from each treatment group (CON: normal-fed wild-type; db/db: normal-fed db/db mice; db/db+M: MCD-fed db/db mice; db/db+M+KT: MCD and KT-treated db/db mice).

equivalent to that of the control (Fig. 3A). To confirm the decreased fatty hepatocytes, we assessed the level of TG. The level of hepatic TG was significantly lower in KT-treated mice than in non-treated db/db mice fed an MCD diet, supporting the greatly reduced fat levels in the MCD-fed fatty liver mice treated with KT (Fig. 3B). In addition, MCD-treated db/db mice had elevated serum ALT and AST, whereas the KT supplement attenuated ALT (control-10.70±0.98, db/db-52.65±12.51, db/db+M-97.74±9.87, and db/db+M+KT-59.31±4.22) and AST (control-71.55±8.67, db/db-118.93±35.49, db/db+M-127.67 ±31.37, and db/db+M+KT-74.69±8.56) in MCD-treated db/db mice (Fig. 3B). These results indicate that KT protected the livers from severe steatosis caused by MCD diet.

KT improved lipid metabolism in MCD-fed db/db mice NAFLD is the most common chronic liver disease and is associated with dysregulated lipid metabolism (7). Lipid metabolism in the liver is regulated by a balance among multiple pathways including de novo lipogenesis, fatty acid uptake, fatty acid oxidation, and very low-density lipoprotein (VLDL) secretion (2,3,7). In db/db mice, high TG levels are caused by upregulated lipogenesis and delivery of FFA to the liver, resulting in the development of mild steatosis (22,23). MCD diets result in the reduced secretion of VLDL and mitochondrial beta-oxidation of fatty acids to the livers of db/db mice, eventually promoting severe steatosis and apoptosis of hepatocytes, as in NASH (24). Thus, db/db mice fed an MCD diet are known to develop NASH with fibrosis within 4 to 8 weeks (22), providing a well-established small animal model for progressive NAFLD (1,22,25). Recent studies have demonstrated that increased uptake of FFA into the liver, with an impaired hepatic SREBP-1c-mediated lipogenic pathway, mitochondrial beta-oxidation of FAs, and VLDL secretion, contributes to TG accumulation, promoting the progression of NASH (2,26,27). Hence,

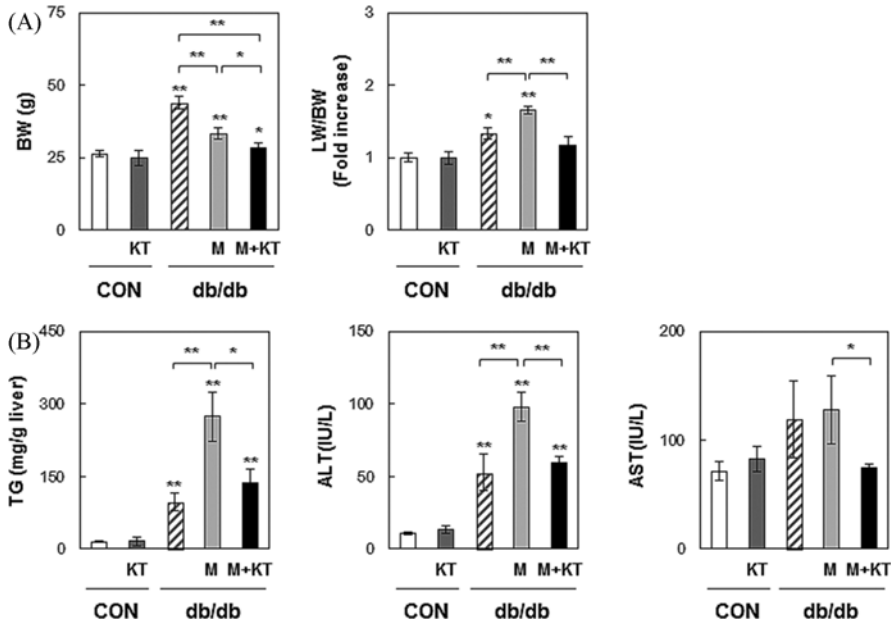

Fig. 3. KT improves liver function in MCD-fed db/db mice. (A) Body weight (BW) and relative liver weight/body weight (LW/BW) of mice. (B) Hepatic content of triglycerides, serum ALT and AST levels. Data represent the Mean±SD results are graphed. (*p<0.05, **p<0.005 vs control)

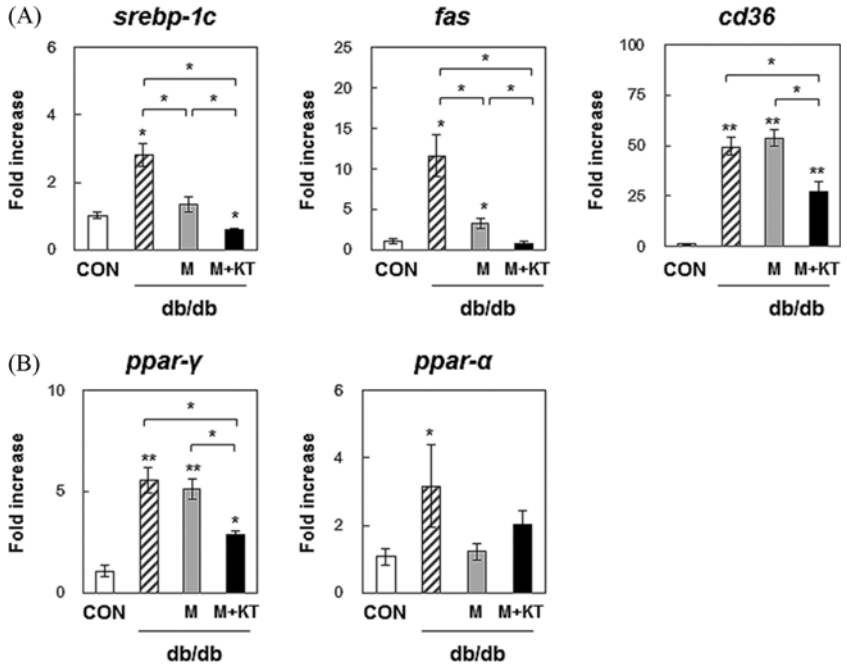

Fig. 4. KT influences expression of markers related with lipid metabolism in damaged livers of MCD diet-treated db/db mice. QRT-PCR analysis for fas, srebp-1c, cd36, ppar-γ, and ppar-α of liver from each treatment group (n≥4 representative mice/group). Mean±SD results are graphed (*p<0.05, **p<0.005 vs control).

we investigated whether KT influenced lipid metabolism in MCD-treated db/db mice. QRT-PCR analysis showed that fas and srebp-1c, well-known markers for de novo lipogenesis, and cd36 and ppar-γ, well-known markers for FFA uptake into the liver, were up-regulated in the livers of chow-fed db/db mice and compared to control mice, indicated increases in both TG synthesis and FA uptake in db/db mice (Fig. 4). In the MCD-fed db/db mice, the expression of fas and srebp-1c decreased compared to db/db mice, but the expression of cd36 and ppar-γ increased, which was almost equivalent to that of the chow-fed db/db mice, indicating impaired TG synthesis with the up-regulated uptake of fats in the MCD-fed db/db mice. In addition, the expression of PPAR-α, a well-known factor for FA oxidation (2,7), was higher in chow-fed than MCD-fed mice, indicating that the fat removal process in the liver was impaired in MCD-fed db/db mice

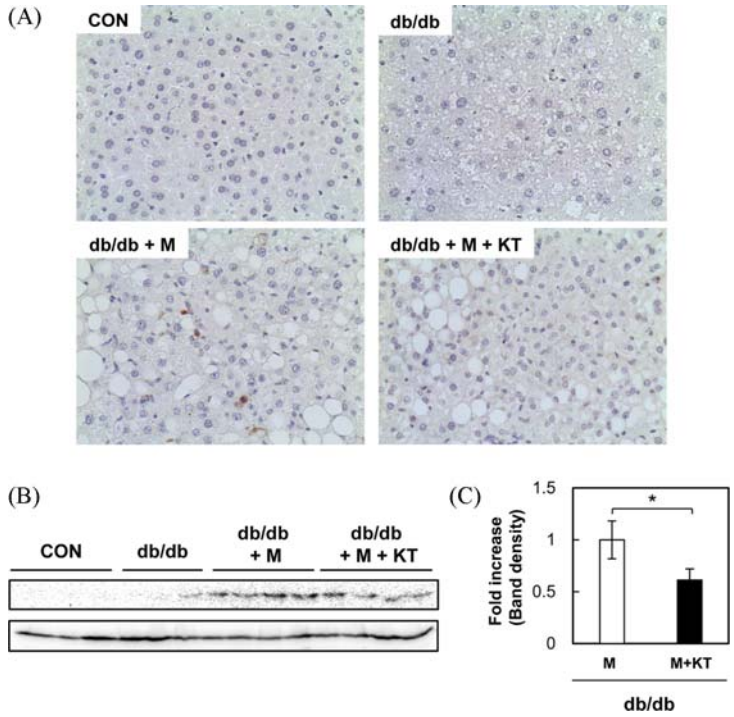

Fig. 5. KT treatment reduces the level of active-caspase 3 in MCD-fed db/db mice. (A) Immunohistochemical staining for active caspase-3 in liver sections from representative mice from each treatment group (CON: normal-fed wild-type; db/db: normal-fed db/db mice; db/db+M: MCD-fed db/db mice; db/db+M+KT: MCD and KT-treated db/db mice). (B) Representative western blot for active caspase-3 (GAPDH was used as an internal control) (C) Band density of MCD-fed db/db mice versus MCD+KT-treated db/db mice. Mean±SD results are graphed (*p<0.05).

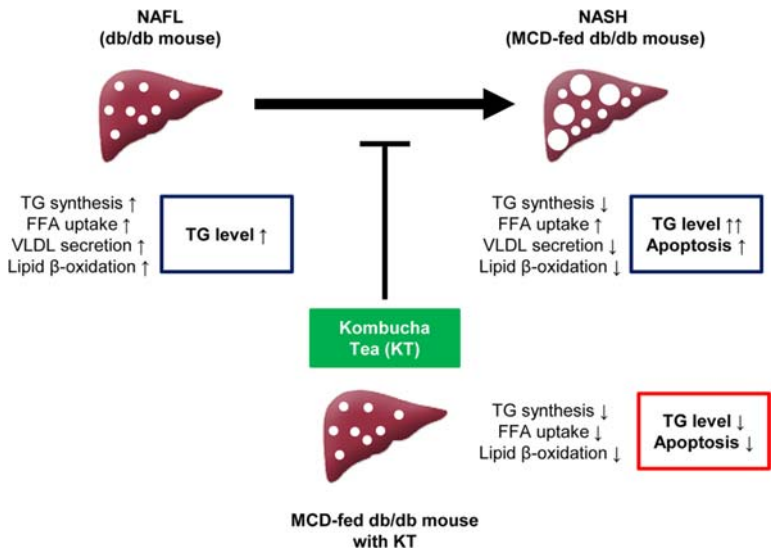

Fig. 6. Schematic representation of the proposed events occurring in the MCD-fed db/db mice with KT treatment. KT exerts preventive effect on the progression of hepatic steatosis by suppressing the accumulation of hepatic TG and the apoptosis of damaged liver cells in MCD-fed db/db mice.

with NASH (Fig. 4). However, KT-treated db/db mice fed MCD diets had a significantly lower expression of fas, srebp-1c, cd36, and ppar-γ than chow-fed db/db mice with or without MCD. PPAR-α was not significantly affected by the 3-week KT treatment (Fig. 4). These results suggest that KT contributed to reduced hepatic fat accumulation by decreasing TG synthesis and FFA delivery to the liver.

Although the mRNA level of fas and srebp-1c decreased in both the KT-treated and non-treated db/db mice with MCD diets, these results suggest different meanings. In NASH, de novo lipogenesis is known to decline due to impaired TG synthesis (27,28) and the current results also showed decreased expression of lipogenesis-related genes in the db/db mice fed MCD diets. In the KT-treated db/db group fed MCD diets, both genes were also down-regulated, almost equivalent to that of the control mice, suggesting lower, not

impaired, synthesis of TG, because this group had a reduced level of hepatic TG due to decreased delivery of FFA to the liver. In addition, the expression of ppar-α increased in db/db mice compared to healthy mice, whereas its expression did not significantly change in either MCD-treated db/db group. In line with our findings, it is known that hepatic lipid removal through mitochondrial β-oxidation remains intact in obesity and insulin resistance, but is inhibited in individuals with MCD diets (7,24). Thus, the mitochondrial β oxidation-related gene, ppar-α, was up-regulated to remove the lipid in db/db mice, while it was inactivated in MCD-fed db/db mice despite a significant accumulation of TG. The expression of ppar-α did not change in the KT-treated group. It is possible that decreased TG synthesis and uptake of FFA into the liver induced the lower fat accumulation, which was, in turn, not sufficient to activate mitochondrial beta oxidation. However, further study is required to demonstrate this possibility.

KT prevented hepatic apoptosis in MCD-fed db/db mice Although steatosis is a typical feature of NAFL and NASH, there is a disparity in the severity of hepatocyte injury because there are more apoptotic hepatocytes in NASH than in NAFL (2,25). Hence, we investigated the effect of KT on hepatic apoptosis induced by an MCD diet. Immunohistochemistry for active caspase-3, an apoptosis marker (29), showed that more positive cells for this marker were observed in MCD-fed db/db mice, whereas those cells were rarely detected in the livers of both normal- and KT+MCD-treated db/db mice (Fig. 5A). Western blot analysis also confirmed a greater increase in apoptosis in MCD-fed db/db mice than in KT treated db/db mice fed MCD diets (0.6-fold decrease compared to MCD-treated db/db; $p<0.05$) (Fig. 5B and 5C). These data suggest that KT exerted an anti-apoptotic effect on the damaged liver cells.

In conclusion, these results demonstrate that KT suppresses the accumulation of hepatic lipids and prevents progression to NASH, contributing to liver restoration (Fig. 6). Our findings suggest KT as a potential dietary strategy for preventing the progression of NAFLD.

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Disclosure The authors declare no conflict of interest.

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