J Toxicol Pathol 2018; 31: 283–291

Original Article

Hepatic lesions induced by feeding Western diets to Zucker fatty rats, an insulin-resistant model

Tomoyuki Saito[1], Yasufumi Toriniwa[1], Yukihito Ishii[1], Atsuhiro Uemura[1], Katsuhiro Miyajima[1, 2], Kinuko Uno[2], Yuki Shirai[2], Dai Nakae[2], and Takeshi Ohta[1*]

[1] Japan Tobacco Inc., Central Pharmaceutical Research Institute, 1-1 Murasaki-cho, Takatsuki, Osaka 569-1125, Japan
[2] Faculty of Applied Biosciences, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

Abstract: Metabolic diseases including nonalcoholic steatohepatitis develop due to various environmental factors. In particular, the westernization of food is closely related to the development of these diseases. In this study, we investigated pathophysiological changes in the livers of Zucker fatty (ZF) rats induced by feeding Western diets. Male ZF rats were fed a sucrose/fat/cholesterol-enriched diet (Western diet, WD) or standard diet (SD) for 18 weeks, from 7 to 25 weeks of age. Body weight, food intake, and biochemical parameters were periodically measured, histopathological analyses were performed at 25 weeks, and mRNA expression in the liver was determined. ZF rats fed the WD (ZF-WD rats) developed obesity, hyperinsulinemia, hyperglycemia, and hyperlipidemia, and their alanine aminotransferase and aspartate aminotransferase levels increased compared with those of ZF rats fed the SD (ZF-SD rats). Hepatic lesions including fibrosis and necrosis were observed in the ZF-WD rats at 25 weeks; however, fibrosis and necrosis were not observed in the ZF-SD rats. Oxidative stress markers also increased in the livers of ZF-WD rats. Hepatic mRNA expression related to inflammation and fibrosis increased in the ZF-WD rats; however, mRNA expression related to lipid synthesis decreased. Microsomal triglyceride transfer protein mRNA levels in the ZF-WD rats also decreased. In Zucker lean rats fed the WD, similar changes were observed in the liver; however, the hepatic changes were not serious compared with ZF-WD rats. In conclusion, hepatic lesions, such as inflammation, fibrosis, and necrosis, were observed in the ZF-WD rats. The sucrose/fat/cholesterol-enriched diet induced significant lipotoxicity in the livers of animals in this insulin-resistant model. (DOI: 10.1293/tox.2018-0016; J Toxicol Pathol 2018; 31: 283–291)

Key words: hepatic lesion, insulin resistance, nonalcoholic steatohepatitis, Western diet, Zucker fatty rat

Introduction

The prevalence of metabolic diseases including obesity and diabetes is increasing all over the world, and the morbidity and mortality related with secondary complications associated with these diseases, such as cardiovascular disease and diabetic microangiopathy, have similarly increased[1–3]. Obesity is caused by an imbalance between energy intake and energy expenditure, and the energy imbalance is induced by excessive dietary fat[4]. Type 2 diabetes is a metabolic disease characterized by hyperglycemia and dyslipidemia resulting from insulin secretion and insulin resistance[5, 6]. Fat accumulation in peripheral tissues promotes the development of insulin resistance in diabetes, suggesting obesity is closely associated with diabetes[7, 8].

Nonalcoholic steatohepatitis (NASH) is reportedly a warning disease that occasionally leads to cirrhosis and/or hepatocellular carcinoma[9, 10]. Multiple factors, such as adipokines, insulin resistance, oxidative stress, endoplasmic reticulum (ER) stress, genetic differences, and diets, are associated with the development of NASH from nonalcoholic fatty liver disease (NAFLD)[11, 12]. Lipid accumulation in the liver is an important factor in the development of hepatic lesions, and it is closely related to insulin resistance[13]. Both quantitative and qualitative aspects of nutrition have a profound effect on these metabolic diseases.

In this study, we investigated pathophysiological changes in the livers of Zucker fatty (ZF) rats induced by feeding a Western diet. The ZF rats have a missense mutation in the leptin receptor gene that causes hyperphagia and weight gain. Thus, they are used as an insulin-resistant model, showing obesity, hyperinsulinemia, and dyslipidemia. ZF rats fed a standard diet present with hepatocellular steatosis; however, the hepatic lesions do not progress to cirrhosis and/or hepatocellular carcinoma[14, 15]. We examined the pathological changes in the livers of ZF rats by feeding sucrose/fat/cholesterol-enriched diets, so-called Western diets.

Received: 12 March 2018, Accepted: 28 June 2018
Published online in J-STAGE: 2 September 2018
*Corresponding author: T Ohta (e-mail: takeshi.ohta@jt.com)

Materials and Methods

Animals and grouping

This experiment was conducted in compliance with the Guidelines for Animal Experimentation in the Biological/Pharmacological Research Laboratories of the Central Pharmaceutical Research Institute of Japan Tobacco Inc. The animal protocol was designed to minimize pain or discomfort in the animals. Male ZF rats that were 5 weeks of age were purchased from Charles River Laboratories Japan (Yokohama, Japan), and age-matched male Zucker lean (ZL) rats (Charles River Laboratories Japan, Yokohama, Japan) were used as control animals. The rats were housed in suspended bracket cages in a climate-controlled room at a temperature of 23 ± 3°C and humidity of 55 ± 15% with a 12 h dark-light cycle and had free access to standard diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water. After acclimation over one week, the rats were divided into 4 groups (n=5): ZF rats fed a standard diet (ZF-SD rats), ZF rats fed a Western diet (ZF-WD rats), ZL rats fed a standard diet (ZL-SD rats), and ZL rats fed a Western diet (ZL-WD rats). The assignment of rats was performed by matching body weight and blood chemical parameters, such as the plasma glucose, insulin, triglyceride (TG), and total cholesterol (TC) levels. The rats at 7 weeks of age were fed a standard diet or a Western diet (composition of nutrients: sucrose, 25.0%; fat, 15.2%; cholesterol, 2%; CLEA Japan, Tokyo, Japan) for 18 weeks. All animals were sacrificed by exsanguination under light isoflurane anesthesia at 25 weeks of age.

Biological parameters

Food intake, body weight, and biochemical parameters, such as the plasma glucose, insulin, TG, TC, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels, were measured every 4 weeks from 9 to 25 weeks of age. The energy contents of the standard and Western diets were 3.57 and 4.04 kcal/g, respectively. Because there were multiple rats in each cage, the calorie intake was calculated by dividing the total cage calorie intake by the number of animals per cage. Blood samples were collected from the tail veins of rats. Plasma glucose, TG, TC, ALT, and AST levels were measured as biochemical parameters using commercial kits (Roche Diagnostics, Basel, Switzerland) in an automatic analyzer (Hitachi 7170S, Hitachi, Tokyo, Japan). Plasma insulin was measured using a commercial ELISA kit (Rat Insulin ELISA Kit, Morinaga Institute of Biological Science, Yokohama, Japan).

Tissue sampling and histopathology

Necropsy was performed at 25 weeks of age as mentioned in the *Animals and grouping* section. The livers were immediately sampled and fixed in 10% neutral-buffered formalin. After resection, the tissue was embedded in paraffin using standard techniques and sliced into thin sections (3 to 5 μm). The sections were stained with hematoxylin and eosin (HE) and Sirius Red. These samples were all examined histopathologically in a blinded manner, and findings were graded from normal (−) to severe (3+). Immunohistochemical analysis of 4-hydroxynonenal (HNE), which is one of the major end products of lipid peroxidation and has been used as an indicator of oxidative stress, was performed on the liver sections. Staining was visualized using a DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA) to produce a brown reaction product indicating antigen localization. Anti 4-HNE monoclonal antibody (1:100, clone HNE-J2, JaICA, Shizuoka, Japan) and N-Histofine Simple Stain Rat MAX-PO (MULTI) (Nichirei, Tokyo, Japan) were used for immunohistochemical detection of hepatocytes in the liver.

mRNA quantification with real-time quantitative PCR

Total RNA was extracted from the liver at 25 weeks of age using an miRNeasy Mini Kit (Qiagen, Hilden, Germany) that included QIAzol Lysis Reagent to facilitate lysis of fatty liver and was used according to the manufacturer's protocols. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). The reaction mixture was incubated for 10 min at 25°C, 2 h at 37°C, and 5 min at 85°C. Real-time PCR quantification was performed in a 20 μL reaction mixture on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). The reaction mixture contained 1× TaqMan Universal PCR Master Mix II (Applied Biosystems), 50 ng of synthesized cDNA, and 0.9 μmol/L primers/0.25 μmol/L probes or TaqMan primers/probe mix (TaqMan Gene Expression Assays, Applied Biosystems). The cycle parameters were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The following primer and FAM-conjugated probe were designed using the Primer Express software (Applied Biosystems): fatty acid synthase (FAS) (forward, ACTGAACGGCATTACTCGGTCC; reverse, GTGTCCCATGTTGGATTTGGTG; probe, TTCCGCCAGAGCCCTTTGTTAATTGG), acetyl-CoA carboxylase (ACC) (forward, GCAGCTATGTTCAGAGAGTTCACC; reverse, CCACCTCACAGTTGACTTGTTTTC; probe, CGGCGACTTACGTTCCTAGTTGCACAAAA). The following gene expression was confirmed using TaqMan Gene Expression Assays: beta-actin (Rn00667869_m1), tumor necrosis factor-alpha (TNF-α) (Rn99999017_m1), monocyte chemoattractant protein-1 (MCP-1) (Rn00580555_m1), transforming growth factor-beta (TGF-β) (Rn99999016_m1), collagen type I alpha-1 (COL1A1) (Rn01463848_m1), alpha-smooth muscle actin (α-SMA) (Rn01759928_g1), microsomal triglyceride transfer protein (MTP) (Rn01522970_m1), and sterol regulatory binding protein-1 (SREBP-1) (Rn01495769_m1).

Statistical analysis

Biological parameters, with the exception of food intake, are expressed as mean values ± standard deviations. Food intake is expressed in mean values. Statistical analyses of differences between mean values in the standard diet group and Western diet group were performed using an F-test, followed by Student's *t*-test or Aspin-Welch's *t*-test. Differences were considered significant at $p<0.05$.

Results

Food intake, body weight and biochemical parameters

Both ZF-SD rats and ZF-WD rats presented with hyperphagia and obesity, and there were no significant differences between the two groups. Obesity was sustained in both groups until 25 weeks of age (Fig. 1). Both ZF-SD rats and ZF-WD rats presented with hyperinsulinemia, suggesting insulin resistance was maintained (Fig. 2B). Plasma glucose, insulin, and TG levels were lower in ZF-WD rats than in ZF-SD rats before 17 weeks of age (Fig. 2A–C). Plasma TC levels were higher in ZF-WD rats than in ZF-SD rats at 9, 13, and 17 weeks of age (Fig. 2D). Plasma ALT and AST levels in ZF-WD rats increased throughout the observation period (Fig. 2E and F). In ZL rats, plasma TC, ALT, and AST levels increased or tended to increase in ZL-WD rats compared with ZL-SD rats throughout the observation period.

Histopathological analyses

Liver histopathology was examined by HE staining and Sirius Red staining (Table 1). In ZF-SD rats, moderate fatty changes (2+) and hypertrophy of hepatocytes were observed at 25 weeks of age, with very slight (±) changes in infiltration of inflammatory cells including neutrophils, lymphocytes, and macrophages (Fig. 3A). In ZF-WD rats, severe fatty changes (3+) and hypertrophy of hepatocytes were observed in 3 rats, and moderate (2+) or severe (3+) changes in infiltration of inflammatory cells were also observed (Fig. 3B). In ZL-WD rats, slight (+) or moderate (2+) fatty changes and moderate (2+) or severe (3+) changes in infiltration of inflammatory cells were observed; however, hypertrophy of hepatocytes was not observed (Fig. 3D). Fibrosis and necrosis were not observed in ZF-SD rats; however, these changes were significant in ZF-WD rats (Fig. 3B, 4A, 4B). Focal necrosis of hepatocytes was accompanied by infiltration of inflammatory cells, and slight (+) to moderate (2+) perisinusoidal fibrosis was sporadically noted; however, there was no correlation of the location between these two findings. Some ZL-WD rats presented with fibrosis and necrosis (Fig. 3D, 4D); however, the pathological changes in ZL-WD rats were not serious, as compared with those in ZF-WD rats (Table 1). Focal necrosis in ZL-WD rats was located in the area of lobules with and/or without fatty change of hepatocytes. No histopathological changes were observed in ZL-SD rats (Fig. 3C, 4C).

In immunohistochemical examinations, immunostaining reactions for 4-HNE, which is an indicator of lipid peroxidation, were apparently detected in hepatocytes in tissues of ZF-WD rats and ZL-WD rats but were very weakly detected in tissue in the standard diet group (Fig. 5). As a positive control in immunohistochemistry for 4-HNE, specimens of liver in a rat fed a choline-deficient diet for six months were used, and these sections were prepared from a formalin-fixed, paraffin-embedded block in the same manner as in the main experiment. Positive cells were detected in the specimen in the positive control, while there was no positive reaction in any negative control slide in which normal mouse serum was mounted instead of the primary antibody.

Liver mRNA expression

The mRNA levels of inflammatory genes (TNF-α and MCP-1), fibrotic genes (TGF-β, COL1A1, and α-SMA), and lipogenic genes (MTP, SREBP-1, ACC, and FAS) in the liver were measured using real-time PCR at 25 weeks of age. TNF-α mRNA levels were significantly higher in both ZF-WD rats and ZL-WD rats, and MCP-1 mRNA levels were also higher in ZL-WD rats than in ZL-SD rats (Fig. 6A and B). TGF-β mRNA levels were significantly higher in both ZF-WD rats and ZL-WD rats; however, COL1A1 and α-SMA mRNA levels in both rats were comparable to corresponding control rats (Fig. 6C and D). MTP mRNA levels in both ZF-WD and ZL-WD rats were significantly reduced, as compared with those in corresponding control rats (Fig. 6F). The mRNA levels of lipogenic genes, such as SREBP-1, ACC, and FAS, were significantly lower in ZF-WD rats than in ZF-SD rats, and ACC mRNA levels in ZL-WD rats were significantly reduced, as compared with those in ZL-SD rats (Fig. 6G–I).

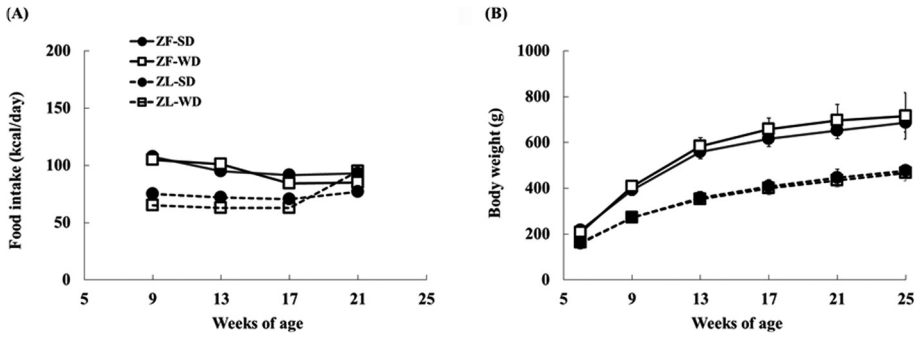

Fig. 1. Changes in food intake (A) and body weight (B) in Zucker fatty rats fed a standard diet (ZF-SD) or Western diet (ZF-WD) and Zucker lean rats fed a standard diet (ZL-SD) or Western diet (ZL-WD). Data are shown as means for food intake and are shown as means ± standard deviations for body weight (n=5).

Discussion

NASH is one of the notable hepatic lesions in which many factors, such as lipid accumulation, insulin resistance, and inflammation, are intricately related and may lead to liver cirrhosis and hepatocellular carcinoma[9, 10]. The pathogenesis of NASH remains poorly understood, and effective pharmacological therapies for NASH have not been

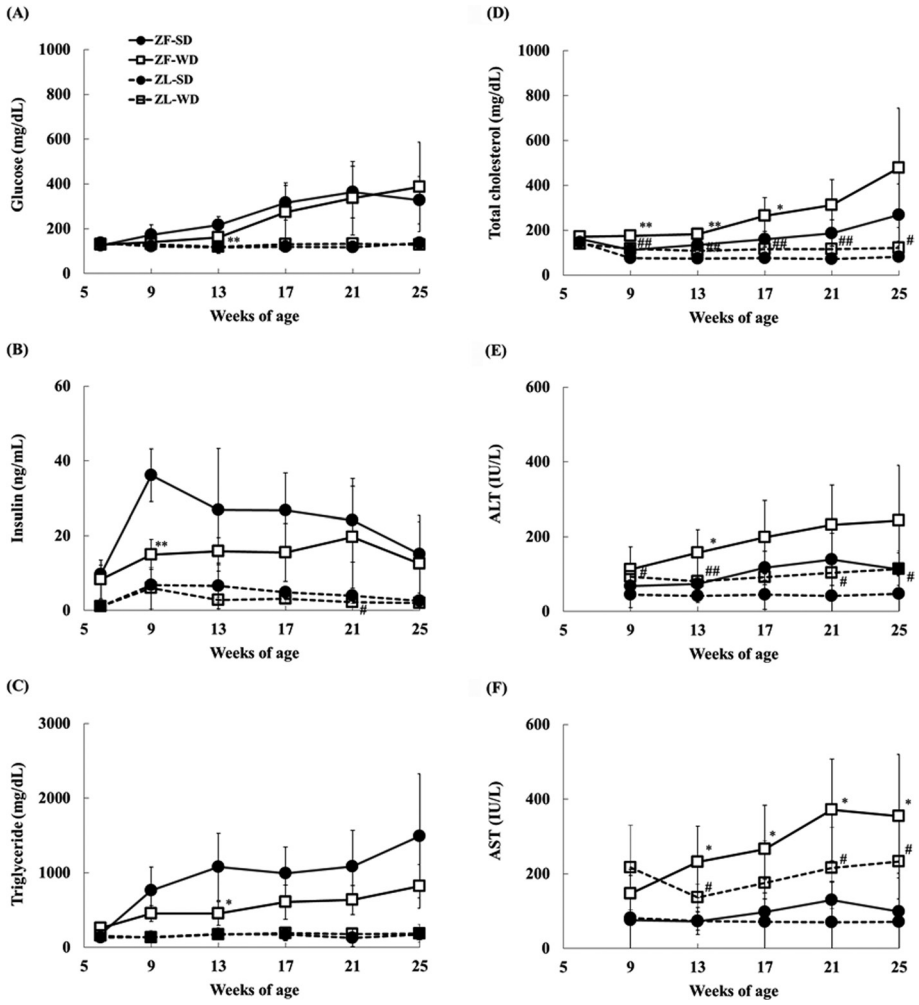

Fig. 2. Changes in blood chemical parameters in Zucker fatty rats fed a standard diet (ZF-SD) or Western diet (ZF-WD) and Zucker lean rats fed a standard diet (ZL-SD) or Western diet (ZL-WD). (A) Glucose. (B) Insulin. (C) Triglyceride. (D) Total cholesterol. (E) Alanine aminotransferase (ALT). (F) Aspartate aminotransferase (AST). Data are shown as means ± standard deviations (n=5). *Significant difference between ZF-SD and ZF-WD ($p<0.05$). **Significant difference between ZF-SD and ZF-WD ($p<0.01$). #Significant difference between ZL-SD and ZL-WD ($p<0.05$). ##Significant difference between ZL-SD and ZL-WD ($p<0.01$).

Table 1. Microscopic Findings of the Liver in Male Zucker Fatty Rats and Zucker Lean Rats

Liver	25 weeks of age																			
	ZF-SD					ZF-WD					ZL-SD					ZL-WD				
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Findings																				
Fatty change	2+	2+	2+	2+	2+	3+	2+	3+	3+	+	−	−	−	−	−	+	+	2+	+	+
Hypertrophy, hepatocyte	2+	2+	2+	2+	2+	3+	2+	3+	3+	+	−	−	−	−	−	−	−	−	−	−
Infiltration, inflammatory cell, focal	±	±	±	±	±	2+	3+	3+	2+	3+	−	−	−	−	−	2+	3+	2+	2+	2+
Fibrosis, focal	−	−	−	−	−	+	2+	2+	2+	2+	−	−	−	−	−	−	±	−	+	±
Necrosis, focal	−	−	−	−	−	+	3+	3+	+	2+	−	−	−	−	−	±	2+	+	+	+

Symbols: −, negative; ± , very slight; +, slight; 2+, moderate; 3+, severe. ZF-SD, Zucker fatty rats fed a standard diet; ZF-WD, Zucker fatty rats fed a Western diet; ZL-SD, Zucker lean rats fed a standard diet; ZL-WD, Zucker lean rats fed a Western diet.

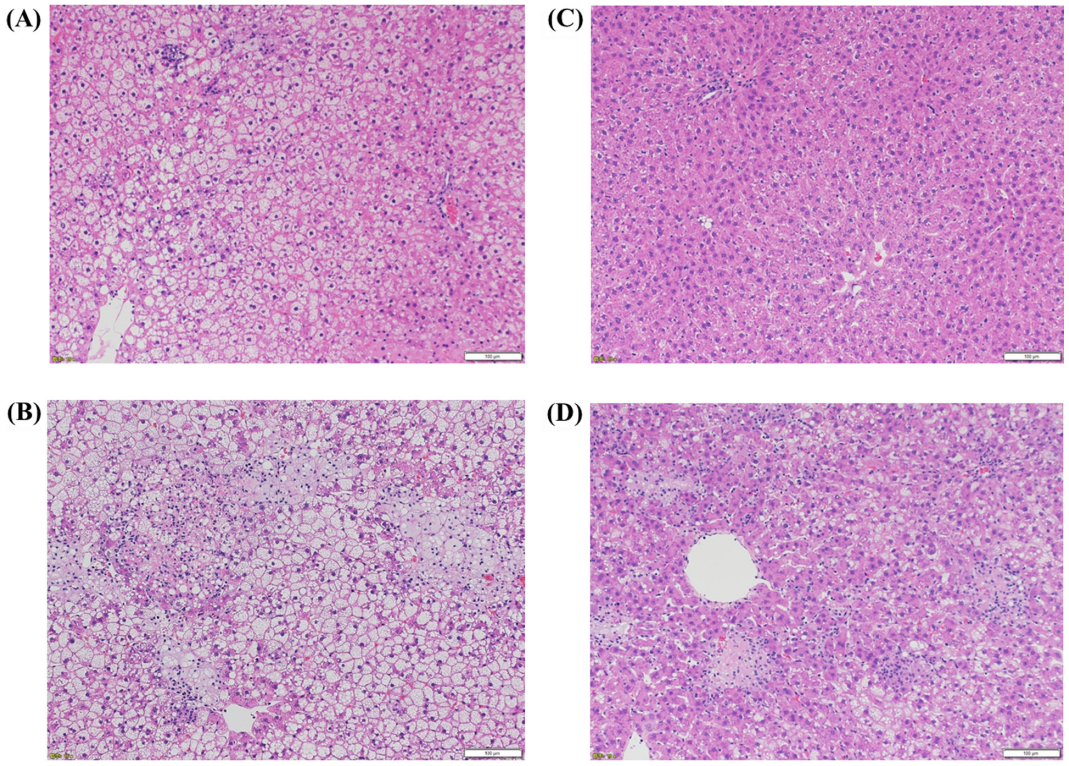

Fig. 3. Liver histopathology at 25 weeks of age. (A) Zucker fatty rats fed a standard diet. (B) Zucker fatty rats fed a Western diet. (C) Zucker lean rats fed a standard diet. (D) Zucker lean rats fed a Western diet. Hematoxylin and eosin (HE) staining. The scale bar represents 100 µm.

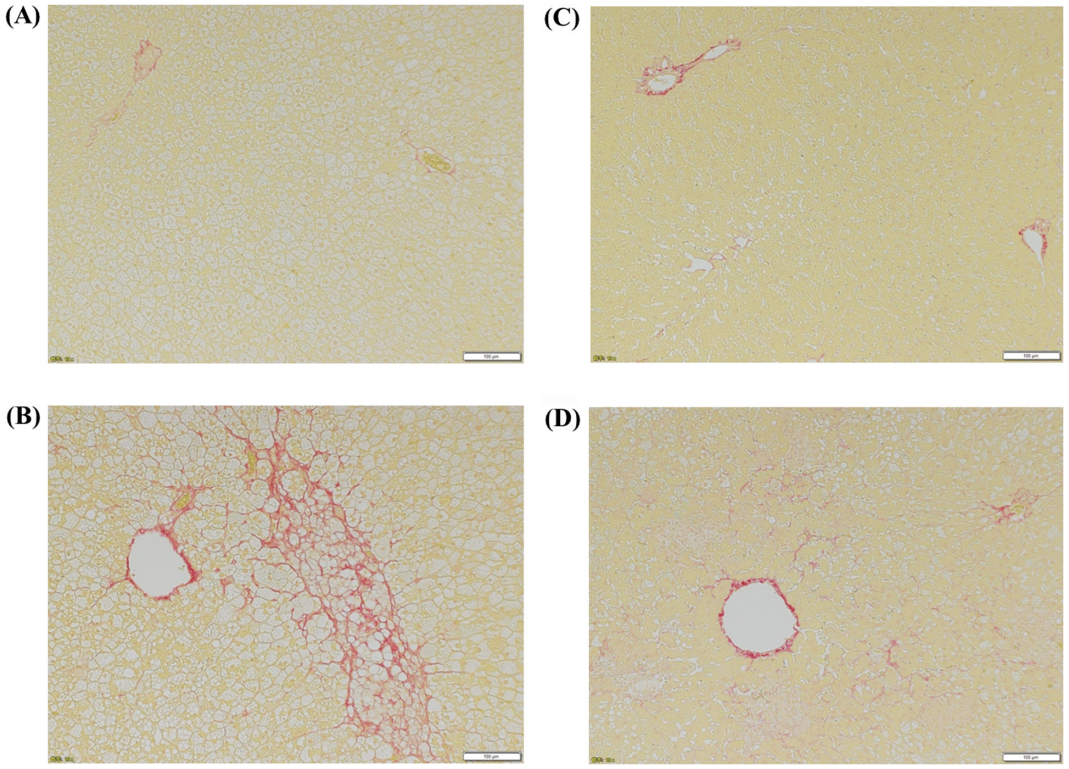

Fig. 4. Liver histopathology at 25 weeks of age. (A) Zucker fatty rats fed a standard diet. (B) Zucker fatty rats fed a Western diet. (C) Zucker lean rats fed a standard diet. (D) Zucker lean rats fed a Western diet. Sirius Red staining. The scale bar represents 100 µm.

approved. To understand the pathophysiological features of NASH or develop new therapies for the disease, animal models offer a source of important information. Rodents fed a methionine and choline-deficient (MCD) diet are frequently used as a NASH model. However, this model does not exhibit any other metabolic features that are seen in human NASH, such as obesity and insulin resistance[16]. A Western diet containing high sucrose/fat/cholesterol not only leads to obesity and metabolic syndrome in mice or rats but also induces NAFLD-like changes including hepatosteatosis; however, in this model, NASH-like lesions and fibrosis were not observed[17, 18] or the progression of fibrosis was slow[18, 19]. Rodent models with increased appetite including ZF rats, ob/ob mice, and db/db mice are used as NAFLD models and spontaneously develop hepatosteatosis based on insulin resistance and obesity, but these rodents do not exhibit liver fibrosis when fed a normal chow[14, 15, 20]. In a previous study, ZF rats fed a high-fat diet presented with pathological NAFLD-like hepatic changes; however, fibrosis and/or necrosis were not observed[21]. In this study, we investigated pathophysiological changes in the livers of ZF rats, an insulin-resistant model, induced by feeding a Western diet. The results showed that ZF-WD rats exhibited NASH-like lesions including hepatic fibrosis with lipid accumulation and inflammation and also exhibited hepatic necrosis. Therefore, the ZF-WD rats may be used as a novel obesity-associated NASH model.

Hyperinsulinemia and obesity were maintained in the ZF-WD rats, demonstrating that insulin resistance was maintained. The ZF-WD rats showed lipid accumulation in the liver; however, mRNA expression related to lipid synthesis in the liver was reduced, which is considered to be

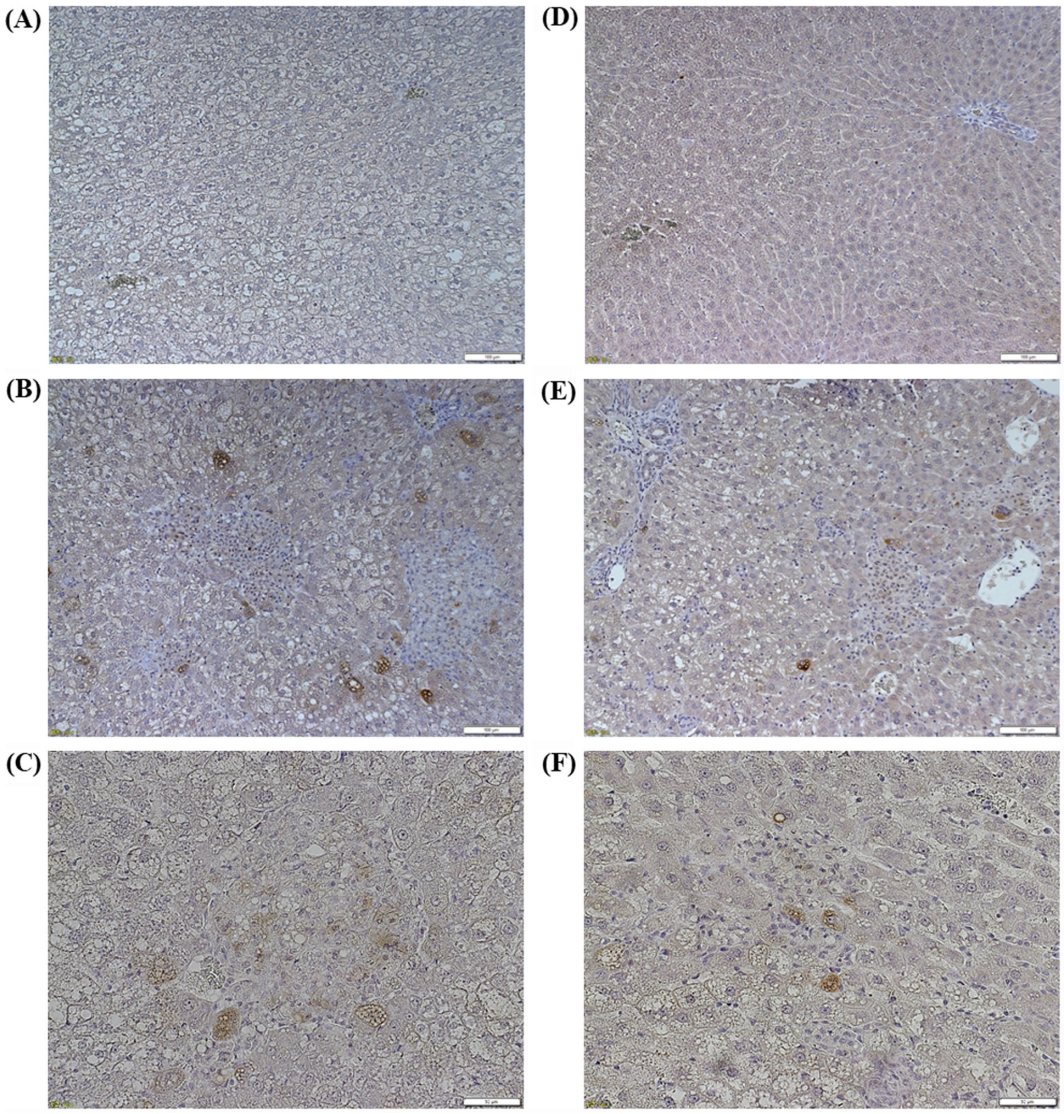

Fig. 5. Liver histopathology at 25 weeks of age. (A) Zucker fatty rats fed a standard diet. (B, C) Zucker fatty rats fed a Western diet. (D) Zucker lean rats fed a standard diet. (E, F) Zucker lean rats fed a Western diet. Immunohistochemistry for 4-hydroxynonenal (HNE). The scale bar represents 100 µm (A, B, D, E) or 50 µm (C, F).

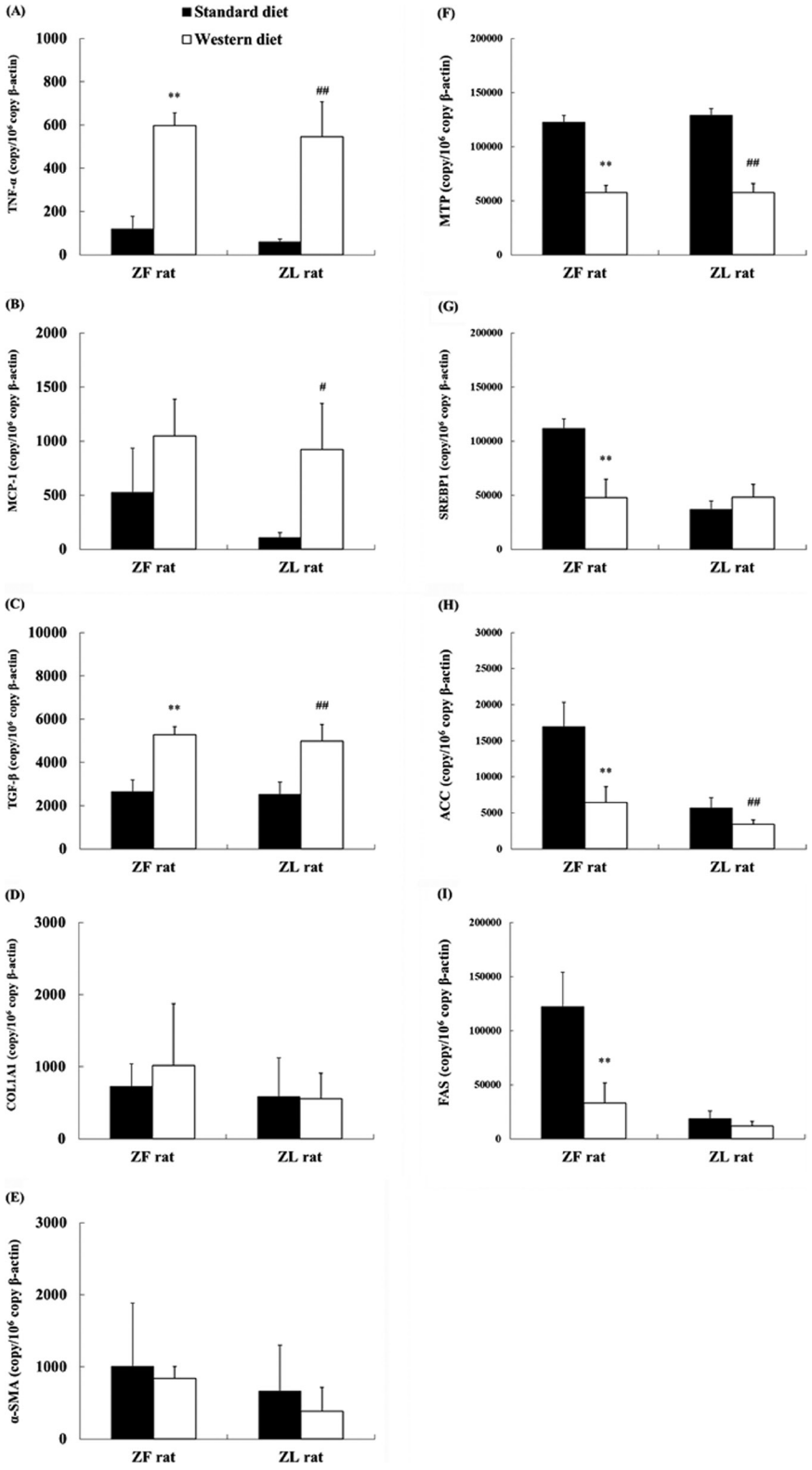

Fig. 6. Expression of hepatic genes related to inflammation, fibrosis, and triglyceride secretion and synthesis in Zucker fatty (ZF) rats and Zucker lean (ZL) rats at 25 weeks of age. (A) Tumor necrosis factor (TNF)-α. (B) Monocyte chemotactic protein (MCP)-1. (C) Transforming growth factor (TGF)-β. (D) Collagen type I α-1 (COL1A1). (E) α-Smooth muscle actin (SMA). (F) Microsomal triglyceride transfer protein (MTP). (G) Sterol regulatory binding protein (SREBP)-1. (H) Acetyl-CoA carboxylase (ACC). (I) Fatty acid synthase (FAS). Data are shown as means ± standard deviations (n=5). **Significant difference between ZF rats fed a standard diet and ZF rats fed a Western diet ($p<0.01$). #Significant difference between ZL rats fed a standard diet and ZL rats fed a Western diet ($p<0.05$). ##Significant difference between ZL rats fed a standard diet and ZL rats fed a Western diet ($p<0.01$).

due to feedback suppression of SREBP processing by dietary cholesterol[22]. Hepatic fatty changes are considered to be closely related to decreases in mRNA levels of MTP, which plays an important role in the assembly and secretion of apolipoprotein B-containing lipoproteins in the liver. The reduction of MTP levels in the liver reportedly induces hepatic fatty changes[23, 24]. The ZF-WD rats showed significant increases in plasma ALT and AST activities, upregulation of TNF-α mRNA in the liver, and infiltration of inflammatory cells into the liver. These results indicate that ZF-WD rats had liver injuries and inflammation. We found upregulation of TGF-β mRNA in the liver of ZF-WD rats. It has been reported that TGF-β activates fibroblasts including hepatic stellate cells and contributes to extracellular matrix remodeling[25], suggesting that the cytokine microenvironment in the liver is profibrotic in ZF-WD rats. As mentioned above, ZF-WD rats showed NASH-like hepatic lesions, including fibrosis, lipid accumulation, and inflammation, based on insulin resistance. Furthermore, in ZF-WD rats, hepatic necrosis was observed. Development of hepatic necrosis is considered to be caused by an increase in oxidative stress-associated events, similar to free radical production, lipid peroxidation, and participation of cytokines, in the liver[26, 27]. Actually, oxidative stress marker 4-HNE, as the major end product of lipid peroxidation, was detected in the hepatocytes of ZF-WD rats.

Unexpectedly, the ZL-WD rats, nonobese rats, in this study also showed hepatic changes, such as fibrosis and necrosis. The reason for this observation is unknown; however, it is interesting to observe NASH-like lesions in nonobese rats that do not show metabolic abnormalities, such as insulin resistance, hyperglycemia, and dyslipidemia. One noticeable finding was the observation of hepatic fibrosis/necrosis in ZF-WD and ZL-WD rats. Since the other animal models fed Western diets did not develop a hepatic necrosis[28], species/strain differences may be the reason. The degrees of pathological changes, including fibrosis and necrosis, were serious in ZF-WD rats compared with ZL-WD rats. This suggests that insulin resistance and/or obesity closely contribute to the onset of hepatic lesions due to WD feeding.

In conclusion, male ZF rats fed sucrose/fat/cholesterol-enriched diets developed NASH-like lesions including hepatic fibrosis with lipid accumulation and inflammation and also exhibited hepatic necrosis, suggesting that the Western diets induced significant lipotoxicity in the livers of animals in this insulin-resistant model.

Disclosure of Potential Conflicts of Interest: Tomoyuki Saito, Yasufumi Toriniwa, Yukihito Ishii, Atsuhiro Uemura, and Takeshi Ohta are employees of Japan Tobacco Inc.

References

1. Wild S, Roglic G, Green A, Sicree R, and King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. **27**: 1047–1053. 2004. [Medline] [CrossRef]
2. Stein CJ, and Colditz GA. The epidemic of obesity. J Clin Endocrinol Metab. **89**: 2522–2525. 2004. [Medline] [CrossRef]
3. Halford JC, Boyland EJ, Blundell JE, Kirkham TC, and Harrold JA. Pharmacological management of appetite expression in obesity. Nat Rev Endocrinol. **6**: 255–269. 2010. [Medline] [CrossRef]
4. Prentice AM, and Poppitt SD. Importance of energy density and macronutrients in the regulation of energy intake. Int J Obes Relat Metab Disord. **20**(Suppl 2): S18–S23. 1996. [Medline]
5. Saltiel AR. New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. Cell. **104**: 517–529. 2001. [Medline] [CrossRef]
6. Zimmet P, Alberti KG, and Shaw J. Global and societal implications of the diabetes epidemic. Nature. **414**: 782–787. 2001. [Medline] [CrossRef]
7. Asrih M, and Jornayvaz FR. Metabolic syndrome and nonalcoholic fatty liver disease: Is insulin resistance the link? Mol Cell Endocrinol. **418**: 55–65. 2015. [Medline] [CrossRef]
8. Caprio S, Perry R, and Kursawe R. Adolescent Obesity and Insulin Resistance: Roles of Ectopic Fat Accumulation and Adipose Inflammation. Gastroenterology. **152**: 1638–1646. 2017. [Medline] [CrossRef]
9. Malik SM, Gupte PA, de Vera ME, and Ahmad J. Liver transplantation in patients with nonalcoholic steatohepatitis-related hepatocellular carcinoma. Clin Gastroenterol Hepatol. **7**: 800–806. 2009. [Medline] [CrossRef]
10. Ertle J, Dechêne A, Sowa JP, Penndorf V, Herzer K, Kaiser G, Schlaak JF, Gerken G, Syn WK, and Canbay A. Nonalcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis. Int J Cancer. **128**: 2436–2443. 2011. [Medline] [CrossRef]
11. Takaki A, Kawai D, and Yamamoto K. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). Int J Mol Sci. **14**: 20704–20728. 2013. [Medline] [CrossRef]
12. Altinbas A, Sowa JP, Hasenberg T, and Canbay A. The diagnosis and treatment of non-alcoholic fatty liver disease. Minerva Gastroenterol Dietol. **61**: 159–169. 2015. [Medline]
13. de Alwis NM, and Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatol. **48**(Suppl 1): S104–S112. 2008. [Medline] [CrossRef]
14. Hockings PD, Changani KK, Saeed N, Reid DG, Birmingham J, O'Brien P, Osborne J, Toseland CN, and Buckingham RE. Rapid reversal of hepatic steatosis, and reduction of muscle triglyceride, by rosiglitazone: MRI/S studies in Zucker fatty rats. Diabetes Obes Metab. **5**: 234–243. 2003. [Medline] [CrossRef]
15. Ran J, Hirano T, and Adachi M. Angiotensin II type 1 receptor blocker ameliorates overproduction and accumulation of triglyceride in the liver of Zucker fatty rats. Am J

Physiol Endocrinol Metab. **287**: E227–E232. 2004. [Medline] [CrossRef]
16. Rinella ME, Elias MS, Smolak RR, Fu T, Borensztajn J, and Green RM. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. J Lipid Res. **49**: 1068–1076. 2008. [Medline] [CrossRef]
17. Schierwagen R, Maybüchen L, Zimmer S, Hittatiya K, Bäck C, Klein S, Uschner FE, Reul W, Boor P, Nickenig G, Strassburg CP, Trautwein C, Plat J, Lütjohann D, Sauerbruch T, Tacke F, and Trebicka J. Seven weeks of Western diet in apolipoprotein-E-deficient mice induce metabolic syndrome and non-alcoholic steatohepatitis with liver fibrosis. Sci Rep. **5**: 12931. 2015. [Medline] [CrossRef]
18. Asgharpour A, Cazanave SC, Pacana T, Seneshaw M, Vincent R, Banini BA, Kumar DP, Daita K, Min HK, Mirshahi F, Bedossa P, Sun X, Hoshida Y, Koduru SV, Contaifer D Jr, Warncke UO, Wijesinghe DS, and Sanyal AJ. A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. J Hepatol. **65**: 579–588. 2016. [Medline] [CrossRef]
19. Savard C, Tartaglione EV, Kuver R, Haigh WG, Farrell GC, Subramanian S, Chait A, Yeh MM, Quinn LS, and Ioannou GN. Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis. Hepatology. **57**: 81–92. 2013. [Medline] [CrossRef]
20. Ge F, Zhou S, Hu C, Lobdell H 4th, and Berk PD. Insulin- and leptin-regulated fatty acid uptake plays a key causal role in hepatic steatosis in mice with intact leptin signaling but not in ob/ob or db/db mice. Am J Physiol Gastrointest Liver Physiol. **299**: G855–G866. 2010. [Medline] [CrossRef]
21. Tan Y, Kim J, Cheng J, Ong M, Lao WG, Jin XL, Lin YG, Xiao L, Zhu XQ, and Qu XQ. Green tea polyphenols ameliorate non-alcoholic fatty liver disease through upregulating AMPK activation in high fat fed Zucker fatty rats. World J Gastroenterol. **23**: 3805–3814. 2017. [Medline] [CrossRef]
22. Horton JD, Goldstein JL, and Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest. **109**: 1125–1131. 2002. [Medline] [CrossRef]
23. Higuchi N, Kato M, Tanaka M, Miyazaki M, Takao S, Kohjima M, Kotoh K, Enjoji M, Nakamuta M, and Takayanagi R. Effects of insulin resistance and hepatic lipid accumulation on hepatic mRNA expression levels of apoB, MTP and L-FABP in non-alcoholic fatty liver disease. Exp Ther Med. **2**: 1077–1081. 2011. [Medline] [CrossRef]
24. Lin M, Zhao S, Shen L, and Xu D. Potential approaches to ameliorate hepatic fat accumulation seen with MTP inhibition. Drug Saf. **37**: 213–224. 2014. [Medline] [CrossRef]
25. Border WA, and Noble NA. Transforming growth factor beta in tissue fibrosis. N Engl J Med. **331**: 1286–1292. 1994. [Medline] [CrossRef]
26. Ingawale DK, Mandlik SK, and Naik SR. Models of hepatotoxicity and the underlying cellular, biochemical and immunological mechanism(s): a critical discussion. Environ Toxicol Pharmacol. **37**: 118–133. 2014. [Medline] [CrossRef]
27. Contreras-Zentella ML, and Hernández-Muñoz R. Is liver enzyme release really associated with cell necrosis induced by oxidant stress? Oxid Med Cell Longev. **2016**: 3529149. 2016. [Medline] [CrossRef]
28. Han H, Qiu F, Zhao H, Tang H, Li X, and Shi D. Dietary flaxseed oil prevents western-type diet-induced nonalcoholic fatty liver disease in apolipoprotein-E knockout mice. Oxid Med Cell Longev. **2017**: 3256241. 2017. [Medline] [CrossRef]