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Original Article

A non-obese, diet-induced animal model of nonalcoholic steatohepatitis in Wistar/ST rats compared to Sprague-Dawley rats

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SUMMARY

Background: Non-alcoholic steatohepatitis (NASH), a subtype of non-alcoholic fatty liver disease (NAFLD), is a potentially progressive liver disease that can lead to cirrhosis. Obesity increases the risk of NAFLD/NASH, but this disease can also be observed in non-obese individuals.

Methods: We investigated the metabolic and histopathological changes in 13 obesity-resistant Slc:Wistar/ST rats fed a high-fat and high-cholesterol (HFC) diet for 9 weeks, and also retrospectively compared the results of 41 Sprague-Dawley (SD) rats that were previously fed with the same protocol to the results of the Slc:Wistar/ST rats.

Results: Of the 13 Slc:Wistar/ST rats fed an HFC diet containing 1.25% or 2.5% cholesterol, 11 (84.6%) developed histologically proven NASH without obesity, an increased visceral fat volume, insulin resistance, histopatological severe lobular inflammation and severe hepatic fibrosis. The HFC diets significantly increased the levels of mRNA encoding collagen type I alpha 1 (COL1A1), transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). The SD rats also developed NASH without obesity, an increased visceral fat volume and insulin resistance, but the metabolic and

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histopathological effects, such as lower serum adiponectin levels, higher serum leptin levels, histopatological severe lobular inflammation and hepatic fibrosis, seemed to be more pronounced in the SD rats than in the Slc:Wistar/ST rats.

Conclusions: These two rat models may reflect the human etiology of NASH that is influenced by dietary factors, and the obesity-resistant Slc:Wistar/ST rat model may be particularly useful for elucidating the pathophysiological mechanism of the so-called "lean NASH".

1. Introduction

Non-alcoholic steatohepatitis (NASH), a subtype of non-alcoholic fatty liver disease (NAFLD), is a potentially progressive liver disease that can lead to cirrhosis, and it has become an important public health issue [1,2]. Understanding the pathogenesis of NASH is important, and adequate animal models are indispensable for the evaluation of pathological mechanism. However, there is currently no animal models that can satisfactorily reflect all of the clinical features of NASH [3]. For the establishment of a diet-induced model of NASH, Splague-Dawley (SD) and Wistar outbred rats were considered to be appropriate standard experimental rodents because they are susceptible to diet-induced obesity and insulin resistance. Moreover, the evaluation of some metabolic parameters, such as by blood sampling, is easier to perform in rats than in mice due to their larger size [4]. However, strain-related differences in physical, biochemical and/or metabolic parameters must be considered. Hayakawa et al. reported that the body weight and food consumption of Wistar Hannover [Crl:WI(Han)] rats were lower than those of SD rats after providing standard diets for 4–26 weeks [5]. Marques et al. compared SD and Wistar rats as models of high-fat diet-induced obesity, and they found that the majority of metabolic changes were more evident or could be detected earlier in Wistar rats than in SD rats [4]. Rosenstengel et al. reported that the development of NAFLD in rat was strain-specific, with different strains revealing different morphological features. In their report, both the SD and Wistar rats fed a high-fat diet developed considerable steatosis, but SD rats showed a higher degree of fibrosis, lobular inflammation and hepatocyte damage (hepatocyte ballooning) as determined using the NAFLD activity score (NAS) [3,6].

We recently reported that the administration of a high-fat and high-cholesterol (HFC) diet to 9-week-old male SD rats induced NASH with hepatic fibrosis within the relatively short period of 9 weeks because SD rats were found to be more sensitive than mice to an HFC diet [7]; however, this rat model did not show obesity with an increased visceral fat volume and insulin resistance [8]. Recently, Slc:Wistar/ST rats were reported to be a model that was resistant to obesity [9]. Therefore, in the present study, we aimed to investigate the metabolic and histopathological changes in Slc:Wistar/ST rats fed an HFC diet for 9 weeks as a potential model for "lean NASH model" [10], and we retrospectively compared the results of 41 SD rats that were previously fed with the same protocol in our laboratory between January 2015 and February 2018 to the results of the Slc:Wistar/ST rats.

2. Materials and methods

2.1. Animals and experimental design

Eight-week-old male Slc:Wistar/ST rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were housed individually in a temperature- and humidity-controlled room (22 °C–24 °C and 50%–

60% relative humidity) with a 12-h light/dark cycle. After 1 week of acclimation with standard rodent chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*, the rats were randomly divided into three groups that were fed for 9 weeks as follows: the W–C group (n = 6) was fed standard rodent chow (MF) as the normal diet; the W–H1.25 group (n = 6) was fed an HFC1.25% diet (69.5% MF, 28.75% palm oil, 1.25% cholesterol and 0.5% sodium cholate); and the W–H2.5 group (n = 7) was fed an HFC2.5% diet (69.5% MF, 27.5% palm oil, 2.5% cholesterol and 0.5% sodium cholate). The proximate dietary compositions of the HFC1.25% and HFC2.5% diets are shown in Table 1. The daily energy intake and body weight were monitored throughout the study. After the 9-week rearing period, the rats were fasted for 8 h and sacrificed under anesthesia with isoflurane and pentobarbital sodium. Blood was collected from the inferior vena cava or heart. The epididymal fat pad and liver were removed, washed in cold saline and weighed. Liver tissues were either placed in 10% neutral buffered formalin or snap frozen in liquid nitrogen and stored at −80 °C. All procedures performed on the animals were approved by the Animal Use Committee of University of Nagasaki (approval numbers were 26-26, 27–14, 28–13, 29–14 and 30–22), and the animals were maintained in accordance with the University of Nagasaki guidelines for the care and use of laboratory animals.

2.2. Serum biochemical and hepatic lipid analyses

Serum triglyceride (TG), total cholesterol (TC) and glucose levels were determined using Triglyceride E test Wako, Cholesterol E test Wako and Glucose C II test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. Serum insulin, leptin and adiponectin levels were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan), a mouse/rat leptin ELISA kit (Morinaga Institute of Biological Science Inc.) and a mouse/rat adiponectin ELISA kit (Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan), respectively. The insulin resistance index was calculated using the homeostasis model of assessment [serum glucose (mg/dL) x serum insulin (ng/mL)/405], and the relative levels were evaluated among groups. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using Transaminase C II test Wako (FUJIFILM Wako Pure Chemical Corporation). Hepatic lipids were extracted from the frozen liver samples using the method of Folch et al. [11]. The extract was dissolved in isopropanol and analyzed for TG and TC with a kit, as described above.

2.3. Histopathological assessment of the liver

After fixation in neutral buffered formalin, the liver tissues were embedded in paraffin, sectioned and stained with Azan as well as hematoxylin and eosin. All histopathological examinations were performed by a pathologist (K.T.) who was blinded to the experimental data. Histological findings from the liver were scored using the NASH Clinical Research Network Scoring System based on the following

Table 1
Proximate dietary composition.

Constituents	Control	HFC1.25%	HFC2.5%
Water (g)	7.90	5.49	5.49
Crude protein (g)	23.10	16.05	16.05
Crude lipid (g)	5.10	3.54	3.54
Crude ash (g)	5.80	4.03	4.03
Crude fiber (g)	2.80	1.95	1.95
Nitrogen-free extract (g)	55.30	38.43	38.43
Palm oil (g)	0.00	28.75	27.50
Cholesterol (g)	0.00	1.25	2.50
Sodium cholate (g)	0.00	0.50	0.50
Total (g)	100.00	100.00	100.00
Protein-energy ratio (%)	25.70	12.63	12.91
Lipid-energy ratio (%)	12.77	57.15	56.18
Carbohydrate-energy ratio (%)	61.53	30.23	30.91
Energy (kcal/100 g)	359.50	508.60	497.35

four semi-quantitative factors: steatosis (0–3), lobular inflammation (0–3), hepatocyte ballooning (0–2) and fibrosis (0–4). The NAS was defined as the unweighted sum of the scores for steatosis, lobular inflammation and hepatocyte ballooning. A NAS $\geq$5 and a NAS $\leq$2 were considered to be diagnostic and not diagnostic, respectively, for steatohepatitis. Liver fibrosis (0–4) was also assessed according to this system [6]. The scores for fibrosis were further classified as follows: a score of 0.5 represented scores between 0 and 1; a score of 1.5 represented scores between 1 and 2; and a score of 2.5 represented scores between 2 and 3.

2.4. Quantification of mRNA using real-time RT-PCR

Total RNA from the liver was extracted using RNAiso Plus (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA templates using a commercial kit (PrimeScript RT Master Mix, Takara Bio Inc.). Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) analysis was performed as described previously [8]. Specific primers were designed using the primer-designing tool Primer-BLAST (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) and were synthesized by Greiner Bio-One Japan (Tokyo, Japan; Table

Table 2
Primer sequences for real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

Primer	Sequence (5′ to 3′)
Hmgcr	Forward: GAAACCCTCATGGAGACGCA
	Reverse: AGCAAGCTCCCATCACCAAG
Ldlr	Forward: AGGAGTGCAAGACCAACGAG
	Reverse: TATCTTCACACTGGTGGCCG
Abcg5	Forward: ACGCTGTGAACCTCTTTCCC
	Reverse: GCTGCTGAAAATCACCGTGG
Cyp7a1	Forward: TGCCGGTACTAGACAGCATC
	Reverse: CCGTCCTCAAGATGGAGAGTG
Abcb11	Forward: TGAAGGCATGGTGACTCTGG
	Reverse: AGTGGTGGAGAACAGAACGG
Fasn	Forward: CAACATTGACGCCAGTTCCG
	Reverse: TTCGAGCCAGTGTCTTCCAC
Slc27a5	Forward: CTTCTACTTCCGAGACCGCC
	Reverse: ACCTTACCCTCACACCCTGG
Mttp	Forward: CAAGCTCAAGGCAGTGGTTG
	Reverse: AGCAGGTACATCGTGGTGTC
Nr1h4	Forward: TGGGAATGTTGGCTGAATGTTTG
	Reverse: TGCATAGCTTGGTCGTGGAG
Nr1h3	Forward: CAGGACCAGCTCCAAGTAGA
	Reverse: GAACATCAGTCGGTCGTGG
Srebf1	Forward: CATGGACGAGCTACCCTTCG
	Reverse: GAAGCATGTCTTCGATGTCGG
Col1a1	Forward: GCGTAGCCTACATGGACCAA
	Reverse: AAGTTCCGGTGTGACTCGTG
Tgfb1	Forward: CTTTGTACAACAGCACCCGC
	Reverse: TAGATTGCGTTGTTGCGGTC
Tnf	Forward: CGTCGTAGCAAACCACCAAG
	Reverse: CCACCAGTTGGTTGTCTTTGAG
Ccl2	Forward: TCTGTCACGCTTCTGGGCCTGT
	Reverse: GGGGCATTAACTGCATCTGGCTGAG
Cyp2e1	Forward: CCCATCCTTGGGAACATTTTT
	Reverse: GCCAAGGTGCAGTGTGAACA
Hmox1	Forward: CACAGGGTGACAGAAGAGGCTAA
	Reverse: GGGACTCTGGTCTTTGTGTTCCT
Sod2	Forward: GACCTGCCTTACGACTATG
	Reverse: TACTTCTCCTCGGTGACG
Rplp0	Forward: ATTGCGGACACCCTCTAGGA
	Reverse: GGTGTTTGACAATGGCAGCAT

2). The levels of mRNA relative to those of the internal control acidic ribosomal phosphoprotein (36B4) mRNA (*Rplp0*) were determined using the 2-ΔΔCt method. For studies in rats, the hepatic expression of genes involved in cholesterol uptake, biosynthesis and excretion [*Hmgcr* encoding 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), *Ldlr* encoding low density lipoprotein receptor (LDLR) and *Abcg5* encoding ATP-binding cassette, subfamily G, member 5 (ABCG-5)], bile acid synthesis, conjugation and excretion [*Cyp7a1* encoding cytochrome P450 family 7 subfamily A polypeptide 1 (CYP7A1) and *Abcb11* encoding bile salt export pump (BSEP)], fatty acid synthesis, uptake and very-low-density lipoprotein (VLDL) synthesis [*Fasn* encoding fatty acid synthase (FAS), *Slc27a5* encoding fatty acid transport protein 5 (FATP-5) and *Mttp* encoding microsomal triglyceride transfer protein (MTP)], lipid homeostasis regulated by nuclear transcription factors [*Nr1h4* encoding farnesoid X receptor (FXR), *Nr1h3* encoding liver X receptor-α (LXR-α) and *Srebf1* encoding sterol regulatory element-binding protein-1c (SREBP-1c)], fibrosis [*Col1a1* encoding collagen type I alpha 1 (COL1A1) and *Tgfb1* encoding transforming growth factor-β (TGF-β1)], inflammation [*Tnf* encoding tumor necrosis factor-α (TNF-α) and *Ccl2* encoding monocyte chemoattractant protein-1 (MCP-1)] and oxidative stress [*Cyp2e1* encoding cytochrome P450 family 2 subfamily E polypeptide 1 (CYP2E1), *Hmox1* encoding heme oxygenase-1 (HO-1) and *Sod2* encoding manganese superoxide dismutase (MnSOD)] were quantified. All data were expressed as the fold-change relative to the W–C group.

2.5. Comparison of the experimental results between Slc:Wistar/ST and SD rats

We retrospectively compared our previous experimental results from 41 SD rats fed a MF diet (S–C group, n = 17) or HFC1.25% diet (S–H1.25 group, n = 24) for 9 weeks in our laboratory between January 2015 and February 2018 to the results of the above 12 Slc:Wistar/ST rats (6 W–C group rats and 6 W–H1.25 group rats). All SD rats (8-week-old males) were purchased from Japan SLC Inc. The rearing environment, rearing methods and sample collection methods used for the SD rats were the same as those used for the Slc:Wistar/ST rats.

2.6. Statistical analysis

All values were expressed as mean ± standard error (SE). Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, chi-square test or Fisher's exact probability test. All analyses were performed using IBM SPSS statistics software, version 25 (IBM, Chicago, IL, USA) on a computer with a Windows operating system. A p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Cumulative energy intake, body weights and relative organ weights

The cumulative energy intake during the 9-week rearing period was significantly higher in the W–H1.25 and W–H2.5 groups than in the W–C group ($p = 0.002$ and 0.006, respectively). The final body weight at 18 weeks of age, body weight gain during the 9-week rearing period (9–18 weeks of age) and food efficacy, calculated as the body weight gain (g)/cumulative energy intake (kcal), did not differ significantly among the three groups. The liver weight/body weight ratio at 18 weeks of age was significantly higher in the W–H1.25 and W–H2.5 groups than in the W–C group ($p < 0.001$ for all), whereas the epididymal fat pad weight/body weight ratio did not differ significantly among the three groups (Table 3).

3.2. Serum biochemical parameters and hepatic lipid concentrations

The serum TG levels at 18 weeks of age were not significantly different among the three groups, but the serum TC levels were significantly higher in the W–H1.25 and W–H2.5 groups than in the W–C

Table 3
Cumulative energy intake, body weights, relative organ weights, serum parameters and hepatic lipid concentrations in Slc:Wistar/ST rats after the 9-week rearing period.

Item/Group	W–C (n = 6)	W–H1.25 (n = 6)	W–H2.5 (n = 7)
Cumulative energy intake (kcal)	5528 ± 167[a]	6337 ± 131[b]	6200 ± 93[b]
Final body weight (g)	479 ± 16	509 ± 7	492 ± 11
Body weight gain (g)	185 ± 13	214 ± 6	200 ± 9
Food efficacy (g/kcal)	0.0332 ± 0.0014	0.0338 ± 0.0004	0.0323 ± 0.0012
Liver weight/body weight (%)	2.89 ± 0.08[a]	4.85 ± 0.11[b]	4.97 ± 0.07[b]
Epididymal fat pad weight/body weight (%)	1.93 ± 0.11	2.06 ± 0.17	1.82 ± 0.12
Serum triglyceride (mg/dL)	74.4 ± 10.6	69.4 ± 14.8	54.7 ± 5.2
Serum total cholesterol (mg/dL)	67.3 ± 5.4[a]	89.5 ± 6.5[b]	89.7 ± 4.9[b]
Serum glucose (mg/dL)	207 ± 18[a]	184 ± 8[ab]	156 ± 9[b]
Serum insulin (ng/mL)	1.39 ± 0.29	1.93 ± 0.51	1.21 ± 0.28
Insulin resistance index	0.73 ± 0.19	0.90 ± 0.27	0.46 ± 0.10
Serum leptin (ng/mL)	4.47 ± 0.60[ab]	5.05 ± 0.72[a]	2.92 ± 0.28[b]
Serum adiponectin (μg/mL)	5.08 ± 0.15[a]	4.44 ± 0.22[a]	3.63 ± 0.17[b]
Serum aspartate aminotransferase (IU/L)	46.6 ± 5.2[a]	59.7 ± 5.4[ab]	76.9 ± 6.6[b]
Serum alanine aminotransferase (IU/L)	9.7 ± 1.0[a]	18.6 ± 1.1[ab]	24.2 ± 3.0[b]
Hepatic triglyceride (mg/g tissue)	38.8 ± 8.4[a]	65.7 ± 2.9[b]	39.8 ± 2.5[a]
Hepatic total cholesterol (mg/g tissue)	4.8 ± 0.7[a]	70.1 ± 3.7[b]	79.1 ± 4.9[b]

Values are expressed as means ± SE.
[ab] Values not sharing the same lowercase letter within a row are significantly different at $p < 0.05$ among groups.
Food efficacy was calculated as the body weight gain (g)/cumulative energy intake (kcal).
W–C, Slc:Wistar/ST rats fed a control diet (MF); W–H1.25, Slc:Wistar/ST rats fed a HFC1.25% diet; W–H2.5, Slc:Wistar/ST rats fed a HFC2.5% diet.

group ($p = 0.044$ and 0.034, respectively). The serum glucose levels were significantly higher in the W–C group than in the W–H2.5 group ($p = 0.030$), whereas the serum insulin levels and the insulin resistance indices did not differ significantly among the three groups. The serum leptin levels were significantly higher in the W–H1.25 group than in the W–H2.5 group ($p = 0.037$), and the serum adiponectin levels were significantly higher in the W–C and W–H1.25 groups than in the W–H2.5 group ($p < 0.001$ and 0.004, respectively). The serum AST and ALT levels were significantly higher in the W–H2.5 group than in the W–C group ($p = 0.006$ and < 0.001, respectively).

The hepatic TG concentrations were significantly higher in the W–H1.25 group than in the W–C and W–H2.5 groups ($p = 0.007$ for all), and the hepatic TC concentrations were significantly higher in the W–H1.25 and W–H2.5 groups than in the W–C group ($p < 0.001$ for all; Table 3).

3.3. Histopathological findings of the liver

Figure 1 shows representative morphological and histopathological findings of the liver according to the NASH Clinical Research Network Scoring System [6] in W–C, W–H1.25% and W–H2.5% groups. The histopathological assessments of the liver in Slc:Wistar/ST rats after the 9-week rearing period according to the above scoring system are shown in Table 4. Severe hepatic steatosis (score 3) was observed in all 13 rats in the W–H1.25 and W–H2.5 groups, but it was not observed in any of the 6 rats in the W–C group ($p < 0.001$). Mild to severe lobular inflammation (score 1 to 3) was also observed in all 13 rats in the W–H1.25 and W–H2.5 groups, but it was not observed in any of the 6 rats in the W–C group ($p = 0.002$). Hepatocyte ballooning was not observed in most of the 19 rats; it was seen only in 2 rats in the W–H1.25 group and 1 rat in the W–H2.5 group (score 1). Five (83.3%) of 6 rats in the W–H1.25 group and 6 (85.7%) of 7 rats in the W–H2.5 group had a NAS of 5 or 6, which was considered to be diagnostic for NASH, whereas all 6 rats in the W–C group had a NAS of 0, which was considered not to be diagnostic for NASH ($p = 0.004$). Hepatic fibrosis was not observed in any of the 12 rats in the W–C and W–H1.25 groups, but a score of 1 or 1.5 fibrosis was observed in 4 (57.1%) of 7 rats in the W–H2.5 group ($p = 0.069$; Table 4).

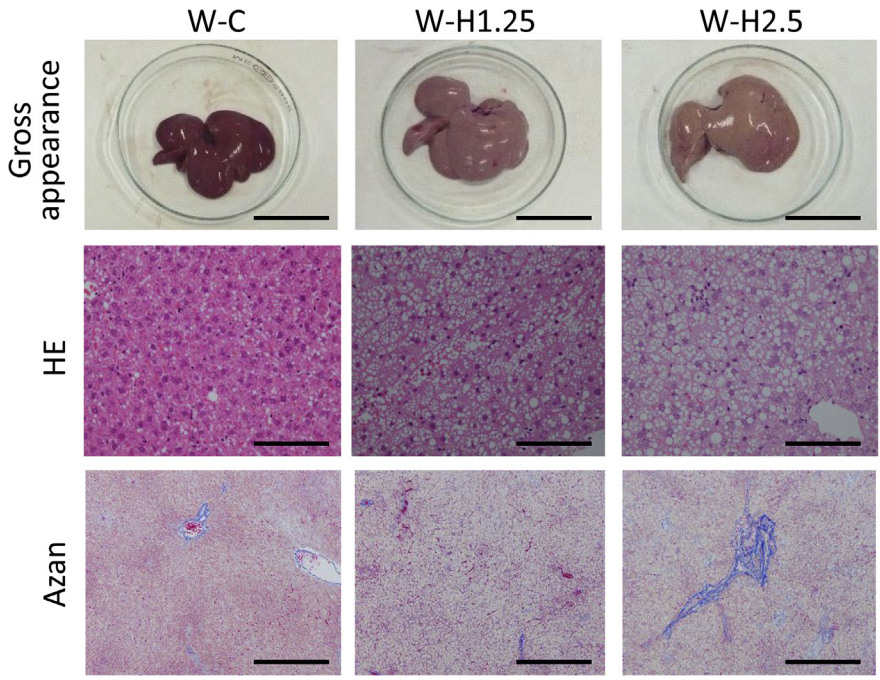

Fig. 1. Representative morphological and histopathological findings of the liver in Slc:Wistar/ST rats fed a control (MF), HFC1.25% or HFC2.5% diet for 9 weeks. The livers in the W–H1.25 and W–H2.5 groups were grossly enlarged and pale in color. In a representative rat of the W–C group, steatosis, lobular inflammation, hepatocyte ballooning and fibrosis were not observed. In a representative rat of the W–H1.25 group, severe steatosis (score 3), moderate lobular inflammation (score 2) and a few hepatocyte ballooning were observed. The NAS was 6 and fibrosis was not observed. In a representative rat of the W–C2.5 group, severe steatosis (score 3), moderate lobular inflammation (score 2) and no hepatocyte ballooning were observed. The NAS was 5 and mild fibrosis (score 1) was also observed. Gross appearance of the liver surface: scale bars = 4 cm. Hematoxylin and eosin (HE)-stained sections: original magnification, 200×; scale bars = 200 μm. Azan-stained sections: original magnification, 100×; scale bars = 400 μm. W–C, Slc:Wistar/ST rats fed a control diet (MF); W–H1.25, Slc:Wistar/ST rats fed an HFC1.25% diet; W–H2.5, Slc:Wistar/ST rats fed an HFC2.5% diet.

3.4. Hepatic mRNA expression

The levels of mRNA encoding FAS (*Fasn*) which catalyzes fatty acid synthesis were significantly lower in the W–H1.25 and W–H2.5 groups than in the W–C group ($p = 0.006$ and 0.001, respectively). The levels of mRNA encoding LDLR (*Ldlr*) which plays a critical role in regulating the amount of cholesterol in blood, FATP-5 (*Slc27a5*) which is a long-chain fatty acid transporter, LXR-α (*Nr1h3*) which is a key nuclear transcription factor involved in hepatic lipid homeostasis, and SREBP-1c (*Srebf1*) which regulates genes required for fatty acid and lipid production were significantly lower in the W–H2.5 group than in the W–C group ($p = 0.032$, 0.049, 0.016 and 0.002, respectively). The levels of mRNA encoding BSEP (*Abcb11*) which is responsible for the transport of taurocholate and other cholate conjugates from hepatocytes to bile, MTP (*Mttp*) which is the rate-limiting lipid transfer protein in the synthesis and excretion of VLDL from the liver, FXR (*Nr1h4*) which plays an important role in bile acid and lipid metabolism and CYP2E1 (*Cyp2e1*) which is a member of the cytochrome P450 mixed-function oxidase system also tended to be lower in the W–H1.25 and W–H2.5 groups than in the W–C group in a cholesterol dose-dependent manner, although the difference was not statistically significant.

In contrast, the levels of mRNA encoding TGF-β1 (*Tgfb1*) which is a key inducer of fibrogenesis were significantly higher in the W–H1.25 group than in the W–C group ($p = 0.019$), and in the W–H2.5 group than in the W–C and W–H1.25 groups ($p < 0.001$ and 0.028, respectively). The levels of mRNA encoding COL1A1 (*Col1a1*) which produces a component of type I collagen, TNF-α (*Tnf*) and MCP-1

Table 4
Histopathological assessment of the liver in Slc:Wistar/ST rats after the 9-week rearing period according to the NASH Clinical Research Network Scoring System [6].

Item/Group	Score	W–C	W–H1.25	W–H2.5
Steatosis	0	6	0	0
	1	0	0	0
	2	0	0	0
	3	0	6	7
Lobular inflammation	0	6	0	0
	1	0	1	1
	2	0	5	5
	3	0	0	1
Hepatocyte ballooning	0	6	4	6
	1	0	2	1
	2	0	0	0
NAFLD activity score (NAS)*	0–2	6	0	0
	3–4	0	1	1
	5–8	0	5	6
Fibrosis	0	6	6	3
	0.5	0	0	0
	1	0	0	3
	1.5	0	0	1
	2–4	0	0	0

Values indicate the number of rats.
*A NAS of 5–8 was considered to be diagnostic for NASH, and a NAS of 0–2 was considered not to be diagnostic for NASH.
W–C, Slc:Wistar/ST rats fed a control diet (MF); W–H1.25, Slc:Wistar/ST rats fed a HFC1.25% diet; W–H2.5, Slc:Wistar/ST rats fed a HFC2.5% diet.

(*Ccl2*) which are both involved in inflammation were significantly higher in the W–H2.5 group than in the W–C group ($p = 0.002$, 0.034 and 0.007, respectively). The levels of mRNA encoding ABCG-5 (*Abcg5*) which facilitates cholesterol efflux across the bile canalicular membrane, CYP7A1 (*Cyp7a1*) which is the rate-limiting enzyme for bile acid synthesis, HO-1 (*Hmox1*) which is an inducible enzyme that is activated in response to oxidative stress and MnSOD (*Sod2*) which is a member of the iron/manganese superoxide dismutase family also tended to be higher in the W–H1.25 and W–H2.5 groups than in the W–C group, although the difference was not statistically significant.

The levels of mRNA encoding HMGCR (*Hmgcr*) which is the rate-limiting enzyme for cholesterol biosynthesis did not differ significantly among the three groups (Fig. 2).

3.5. Comparison of the experimental results between Slc:Wistar/ST and SD rats

The cumulative energy intake and body weight gain during the 9-week rearing period did not differ significantly between the W–C and W–H1.25 groups, but they were significantly higher in the S–H1.25 group than in the S–C group ($p < 0.001$ and 0.033, respectively). The final body weight at 18 weeks of age did not differ significantly between the W–C and W–H1.25 groups or between the S–C and S–H1.25 groups, but it was highest in the S–H1.25 group. Food efficacy was also similar between the W–C and W–H1.25 groups as well as between the S–C and S–H1.25 groups, but it was significantly higher in the S–C group than in the W–C group ($p = 0.010$). The liver weight/body weight ratio at 18 weeks of age was significantly higher in the W–H1.25 group than in the W–C group, and also in the S–H1.25 group than in the S–C group ($p < 0.001$ for all). Moreover, this ratio was significantly higher in the S–H1.25 group than in the W–H1.25 group ($p = 0.005$). In contrast, the epididymal fat pad weight/body weight ratio did not differ significantly among the four groups (Fig. 3).

The serum TG levels at 18 weeks of age were not significantly different among the four groups, but the serum TC levels were significantly higher in the W–H1.25 group than in the W–C group, and also in the S–H1.25 group than in the S–C group ($p = 0.041$ and < 0.001, respectively). The serum glucose and insulin levels did not differ significantly between the W–C and W–H1.25 groups, or between the S–C and S–H1.25 groups, but the serum glucose levels were significantly higher in the W–C group than in

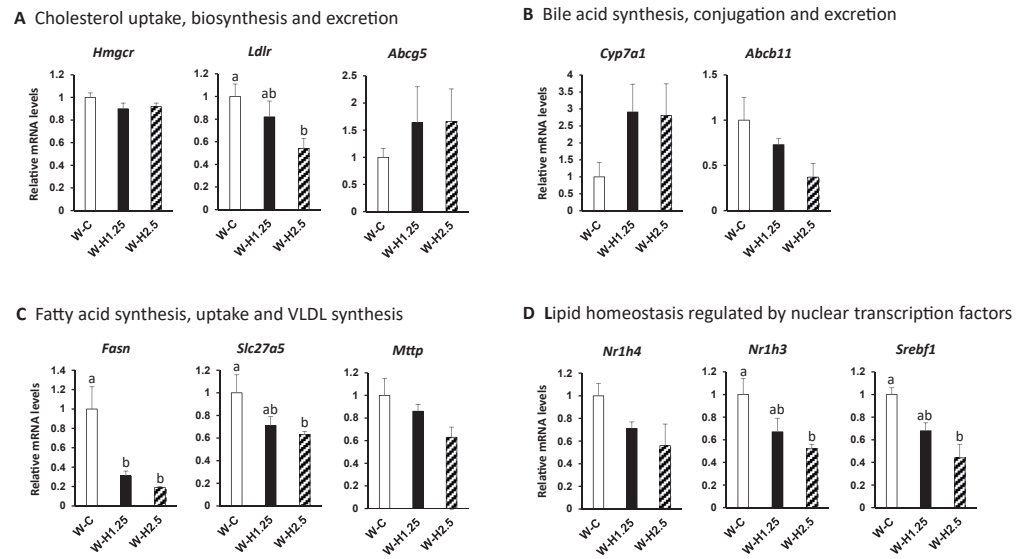

Fig. 2. Hepatic expression of genes involved in cholesterol, lipid and bile acid metabolism, fibrogenesis, inflammation and oxidative stress in Slc:Wistar/ST rats fed a control (MF), HFC1.25 or HFC2.5 diet for 9 weeks. The mRNA levels were determined by real-time RT-PCR, and data are expressed relative to the levels of the W−C group (mean ± SE), which was set to 1. [abc] Values not sharing the same lowercase letter are significantly different at $p < 0.05$ among groups. W−C, Slc:Wistar/ST rats fed a control diet (MF); W−H1.25, Slc:Wistar/ST rats fed an HFC1.25% diet; W−H2.5, Slc:Wistar/ST rats fed an HFC2.5% diet.

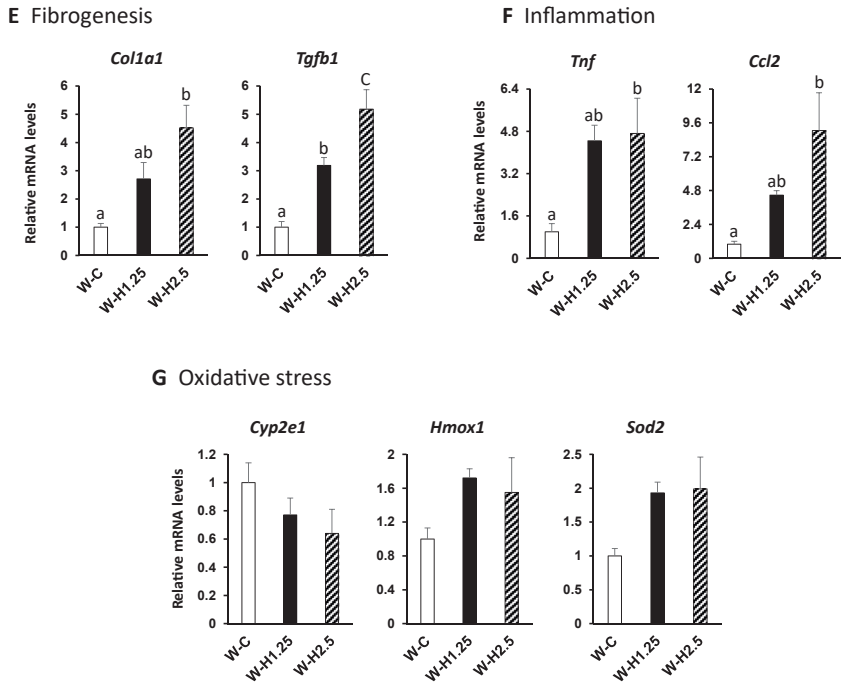

Fig. 2. (*continued*).

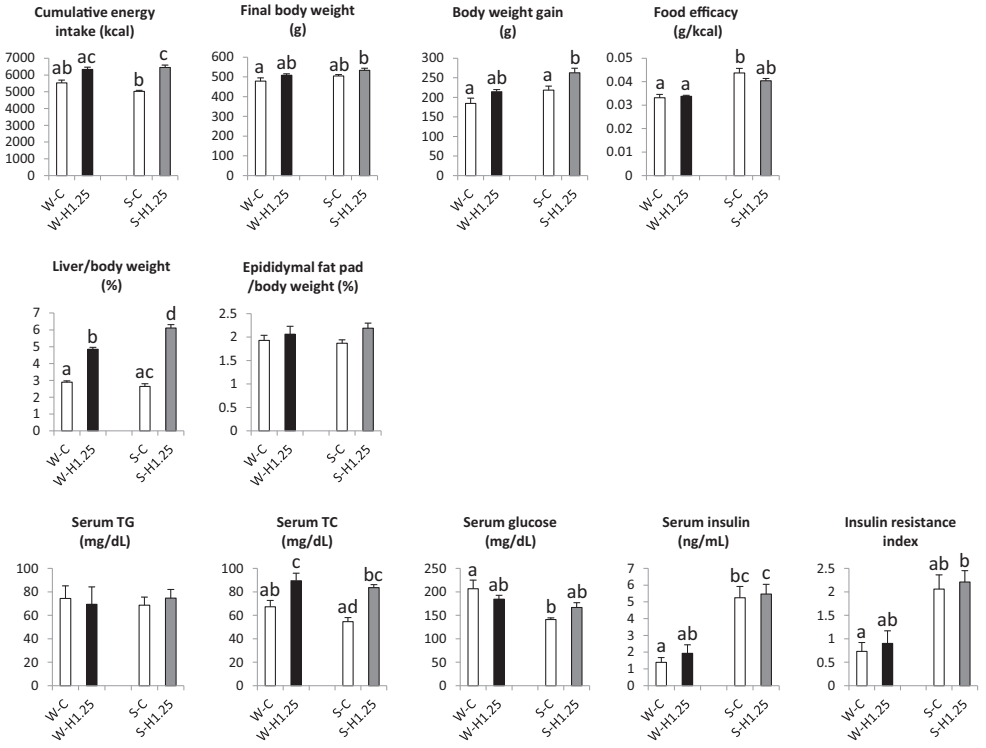

Fig. 3. Cumulative energy intake, body weights, relative organ weights, serum parameters and hepatic lipid concentrations in Slc:Wistar/ST and SD rats fed a control or HFC1.25% diet for 9 weeks. [abcd] Values not sharing the same lowercase letter are significantly different at $p < 0.05$ among groups. W–C, Slc:Wistar/ST rats fed a control diet (MF); W–H1.25, Slc:Wistar/ST rats fed an HFC1.25% diet; S–C, SD rats fed a control diet (MF); S–H1.25, SD rats fed an HFC1.25% diet; TG, triglyceride; TC, total cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

the S–C group ($p = 0.005$), and the serum insulin levels were significantly higher in the S–C group than in the W–C group ($p = 0.015$) and in the S–H1.25 group than in the W–H1.25 group ($p = 0.023$). The insulin resistance indices did not differ significantly between the W–C and W–H1.25 groups, or between the S–C and S–H1.25 groups, but they tended to be higher in the S–C group than in the W–C group as well as in the S–H1.25 group than in the W–H1.25 group, although the difference was not statistically significant. The serum leptin and adiponectin levels did not differ significantly between the W–C and W–H1.25 groups, or between the S–C and S–H1.25 groups, but the serum leptin levels were highest in the S–H1.25 group and the serum adiponectin levels were highest in the W–C group. The serum ALT levels were significantly higher in the S–H1.25 group than in the S–C group ($p = 0.002$; Fig. 3).

The hepatic TG concentrations did not differ significantly between the W–C and W–H1.25 groups, but they were significantly higher in the S–H1.25 group than in the S–C group ($p < 0.001$); among the four groups, they were highest in the S–H1.25 group. The hepatic TC concentrations were significantly higher in the W–H1.25 group than in the W–C group, and also in the S–H1.25 group than in the S–C group ($p < 0.001$ for all); among the four groups, they were highest in the W–H1.25 group (Fig. 3).

The histopathological assessments of the liver in W–C, W–H1.25, S–C and S–H1.25 groups are shown in Table 5. Hepatic steatosis, lobular inflammation, hepatocyte ballooning and hepatic fibrosis were not observed in any of the W–C and S–C group rats, except for one S–C rat with mild steatosis. These rats had a NAS of 0, which is considered not to be diagnostic for NASH. In contrast, severe hepatic

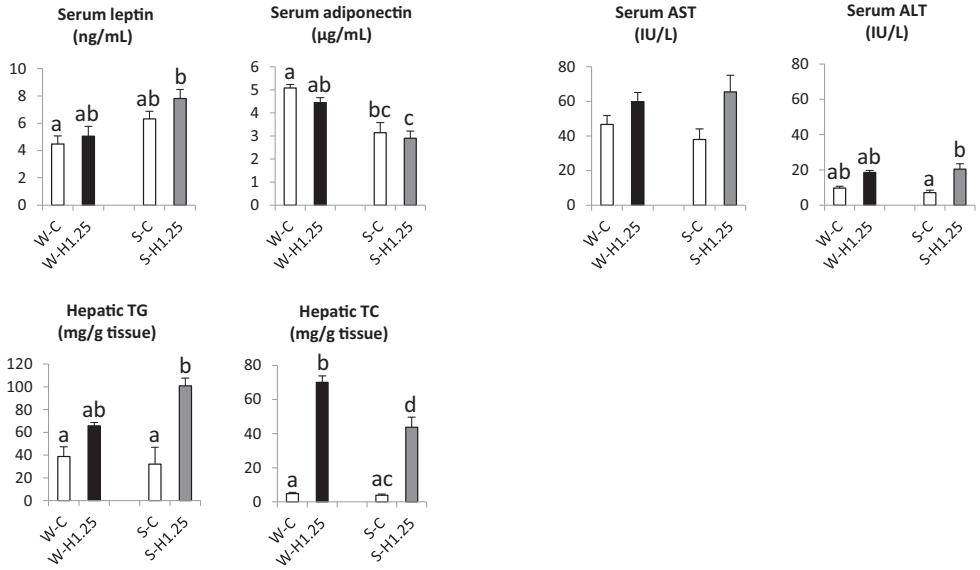

Fig. 3. (*continued*).

steatosis (score 3) was observed in 6 (100%) of 6 rats in the W–H1.25 group and in 23 (95.8%) of 24 rats in the S–H1.25 group ($p < 0.001$ vs W–C or S–C group, respectively). Lobular inflammation was observed (score 1 to 3) in all 30 rats in the W–H1.25 and S–C1.25 groups ($p < 0.001$ vs W–C or S–C group, respectively). Severe lobular inflammation (score 3) was observed only in 5 (20.8%) of 24 rats in the S–H1.25 group. A few ballooning hepatocytes (score 1) were observed in 2 (33.3%) of 6 rats in the W–H1.25 group and in 5 (20.8%) of 24 rats in the S–H1.25 group. Five (83.3%) of 6 rats in the W–H1.25 group had a NAS of 5 or 6, and 22 (91.7%) of 24 rats in the S–H1.25 group had a NAS of 5, 6, or 7, which are considered to be diagnostic for NASH ($p < 0.001$). Hepatic fibrosis was not observed in any of the 6 rats in W–H1.25 group, but a fibrosis score of 0.5–3 was observed in all (100%) of the 24 rats in the S–H1.25 group ($p < 0.001$; Table 5).

4. Discussion

NAFLD is considered to be a hepatic manifestation of metabolic syndrome, and patients with NAFLD are more likely to have obesity, insulin resistance, abnormal glucose metabolism and a higher risk for the development of diabetes [12]. However, NAFLD can also be observed in non-obese individuals [13]. Recently, NAFLD in the absence of overweight and/or obesity has been designated as “lean NAFLD”. Lean NAFLD is generally considered a less severe form of liver disease than NAFLD in obese patients, but recent data suggest that patients with lean NAFLD have higher mortality rates and more morbidities [1]. Feng et al. reported that lean NAFLD patients had lower levels of blood glucose, hyperlipidemia and insulin resistance than overweight-obese NAFLD patients, but normal-weight individuals were more likely to have diabetes and metabolic syndrome if they had NAFLD [12].

Wistar rats have breed-specific characteristics [14]. Hashimoto et al. reported Slc:Wistar/ST rats as a model that was resistant to obesity because the rats did not become obese when fed high-fat diets due to accelerated lipolysis, suppressed levels of VLDL secretion from the liver, and/or altered lipid and glucose metabolism [9]. In the present study, 11 (84.6%) of 13 Slc:Wistar/ST rats fed an HFC1.25 or HFC2.5 diet for 9 weeks developed histologically proven NASH. The HFC diets increased the liver weight, the serum TC, AST and ALT levels and the hepatic TC concentration, but they did not increase, and rather decreased, the final body weight, epididymal fat pad weight, the serum TG, glucose, insulin

Table 5
Histopathological assessment of the liver in Slc:Wistar/ST and SD rats fed a control or HFC1.25% diet for 9 weeks according to the NASH Clinical Research Network Scoring System [6].

Item/Group	Score	W–C (n = 6)	W–H1.25 (n = 6)	S–C (n = 17)	S–H1.25 (n = 24)
Steatosis	0	6	0	16	0
	1	0	0	1	0
	2	0	0	0	1
	3	0	6	0	23
Lobular inflammation	0	6	0	17	0
	1	0	1	0	3
	2	0	5	0	16
	3	0	0	0	5
Hepatocyte ballooning	0	6	4	17	19
	1	0	2	0	5
	2	0	0	0	0
NAFLD activity score (NAS)*	0–2	6	0	17	0
	3–4	0	1	0	2
	5–8	0	5	0	22
Fibrosis	0	6	6	17	0
	0.5	0	0	0	9
	1	0	0	0	7
	1.5	0	0	0	0
	2	0	0	0	4
	2.5	0	0	0	1
	3	0	0	0	3

Values indicate the number of rats.
*A NAS of 5–8 was considered to be diagnostic for NASH, and a NAS of 0–2 was considered not to be diagnostic for NASH.
W–C, Slc:Wistar/ST rats fed a control diet (MF); W–H1.25, Slc:Wistar/ST rats fed a HFC1.25% diet; S–C, SD rats fed a control diet (MF); S–H1.25, SD rats fed a HFC1.25% diet.

and adiponectin levels, and the insulin resistance index. These results indicate that our Slc:Wistar/ST rat model of NASH did not exhibit obesity with an increased visceral fat volume and insulin resistance as did our SD rat model of NASH fed an HFC diet for 9 or 18 weeks [8,15]. Moreover, severe fibrosis does not develop as readily in our Slc:Wistar/ST rat model when compared to our SD rat model [8] since only mild (score 1 or 1.5) fibrosis was observed in 4 (57.1%) of 7 rats in the W–H2.5 group.

The so-called “lean NAFLD” is not a benign disease, and it can develop into the full spectrum of liver damage that characterize “obese NAFLD” [13]. The “lean NAFLD” can result from a heterogeneous spectrum of causes, including environmental factors, e.g., high-fructose or high-fat intake, dysfunctional adipose tissues that lead to a decrease in adiponectin levels, an altered body composition, e.g., lipodystrophy or sarcopenia, malnutrition, e.g., Kwashiorkor, endocrine disorders as well as drug-related disorders; however, the pathophysiological mechanisms are not yet completely understood [4]. In the present study, the HFC diets resulted in increased hepatic cholesterol accumulation, downregulated cholesterol and bile acid metabolism with the inactivation of LDLR and BSEP, and downregulated lipid synthesis with the inactivation of FAS, FATP-5, LXR-α and SREBP-1c (Fig. 2). These results suggest that the HFC diets did not induce lipogenesis. FXR plays an important role in bile acid, glucose and lipid metabolism. FXR regulates the synthesis and enterohepatic circulation of bile acids. Bile flow is prompted through FXR activation in the liver, and FXR blocks bile acid synthesis through CYP7A1 [16]. In our Slc:Wistar/ST rat model, the HFC diets tended to reduce the mRNA expression of FXR, although the difference was not statistically significant; this may induce the mRNA expression of CYP7A1.

An altered lipid metabolism eventually leads to an increase in fat accumulation by hepatocytes, causing oxidative stress and cellular damage [16]. The export of hepatic TG in the form of VLDL particles from the liver is mediated by plasma apoB-lipoprotein and MTP. Lowered hepatic expression of MTP plays a crucial role in NAFLD development [17], and the decrease may be induced by enhanced oxidative stress [18]. In our Slc:Wistar/ST rat model, the HFC diets tended to reduce MTP expression,

and induced the mRNA expressions of genes related to fibrogenesis, inflammation and oxidative stress (Fig. 2).

When the data from the Slc:Wistar/ST rats were compared to those from our previously tested SD rats, the serum adiponectin levels and hepatic TC concentrations were highest in the W–H1.25 and W–C groups, respectively. In contrast, the cumulative energy intake, final body weight, body weight gain, liver weight, serum insulin levels, insulin resistance indices, serum leptin levels and hepatic TG concentrations were highest in the S–H1.25 group, regardless of statistical significance. Moreover, histopathological severe lobular inflammation and fibrosis were only observed in SD rats fed an HFC1.25% diet (S–H1.25 group). These results indicate that the metabolic and histopathological effects caused by the HFC diets appeared more pronounced in the SD rats than in the Slc:Wistar/ST rats. In other words, our Slc:Wistar/ST rat model likely constitutes a "lean NASH model" [10].

There were several limitations to our comparative study between the Slc:Wistar/ST and SD rat models. First, despite the fact that all rats were obtained from the same supplier and were reared under the same breeding environment, and all histopathological assessments were made by one pathologist (K.T.) who was blinded to the experimental design and sample identity, the rearing year and breeders were not the same. Second, there was considerable variation in the number of rats in each group because our comparative study was a retrospective investigation. Third, the data from the comparisons of body weight, liver weight and the serum biochemical parameters between the Slc:Wistar/ST and SD rats should be assessed with caution because the normal range of these parameters in these two rat strains are not completely identical [19]. Thus, a systematic study is needed for the next investigation.

In conclusion, Slc:Wistar/ST rats fed the HFC diets (HFC1.25% or HFC2.5% diet) for 9 weeks developed histologically proven NASH without obesity, an increased visceral fat volume and insulin resistance. SD rats fed the HFC diets for 9 weeks also developed NASH with the same characteristics, but metabolic and histopathological effects caused by the HFC diets seemed to be more pronounced in the SD rats than in the Slc:Wistar/ST rats. These two rat models can reflect the human etiology of NASH that is influenced by dietary factors, and the obesity-resistant Slc:Wistar/ST rat model may be particularly useful for elucidating the pathophysiological mechanism of the so-called "lean NASH".

Conflict of interest

The authors of this manuscript state that there are no conflicts of interest to disclose.

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