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Dietary fat, cholesterol, and cholic acid affect the histopathologic severity of nonalcoholic steatohepatitis in Sprague-Dawley rats

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ABSTRACT

Understanding of the pathogenesis of nonalcoholic steatohepatitis (NASH)-associated fibrosis has been hampered by the lack of a comprehensive and physiological small animal model of NASH with fibrosis. Feeding a high-fat and high-cholesterol (HFC) diet supplemented with cholic acid to rats is known to replicate human NASH pathology, and it induces fibrosis earlier than with an HFC diet alone. In the present study, physiological and histopathological observations from 65 Sprague-Dawley (SD) rats fed an HFC diet with or without cholic acid for 9 or 18 weeks in our laboratory between January 2013 and February 2018 were retrospectively reviewed. The liver weight/body weight ratio at the end of the rearing period was higher in rats fed an HFC diet than in rats fed a normal diet in a cholesterol dose-, cholic acid dose-, or rearing period dependent manner. Dietary fat, cholesterol and/or cholic acid and rearing period affected the histopathologic severity of NASH. Overall, 56 (86.2%) of 65 SD rats fed an HFC diet for 9 or 18 weeks developed histopathologically proven NASH. It is noted that the SD rats fed an HFC diet supplemented with 2% (w/w) cholic acid for 18 weeks frequently developed advanced fibrosis, including cirrhosis. Thus, this diet-induced NASH rat model is likely to be a highly reproducible.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), which is increasing in prevalence worldwide, is strongly associated with metabolic syndrome, including obesity and type 2 diabetes. NAFLD is a spectrum of liver disorders that includes nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NASH is a potentially progressive liver disease that can lead to cirrhosis [1], and liver fibrosis is the most important predictor of mortality due to NAFLD [2]. However, the mechanisms involved in the progression to fibrosis remain largely unknown, but there is growing evidence that dietary cholesterol intake and the consequent increase in hepatic cholesterol are critical factors in the development of hepatic steatosis and fibrosis [3–7].

Understanding of the pathogenesis of NASH-associated fibrosis has been hampered by the lack of a comprehensive and physiological small animal model of NASH with fibrosis [8,9]. We recently reported that the administration of a high-fat and high-cholesterol (HFC) diet to Sprague-Dawley (SD) rats induced NASH with advanced fibrosis within the relatively short period of 9 or 18 weeks [10,11]. In our model, cholic acid, one of the primary bile acids, was supplemented to the HFC diet in order to promote the absorption of excessive amounts of cholesterol because rats do not have a gallbladder and therefore lack the ability to concentrate bile. Although the mechanism of the development of NASH in our model has not been fully elucidated, this HFC diet increased the total cholesterol (TC) levels in liver, and suppressed the mRNA expressions of microsomal triglyceride (TG) transfer protein which is a rate-limiting lipid transfer protein involved in the synthesis and excretion of very-low-density lipoprotein from the liver, adenosine triphosphate binding cassette transporter G5 which facilitates cholesterol efflux across the bile canalicular membrane, bile acid CoA: amino acid *N*-acyltransferase which conjugates bile acid to taurine or glycine, and bile salt export pump; it also suppressed the enzymatic activity of carnitine palmitoyltransferase which is a rate-limiting fatty acid transporter involved in β-oxidation [10,11].

In the present study, to confirm the overall incidence of NASH and the influence of dietary fat, cholesterol, cholic acid and rearing period on the histopathological findings of NASH, we conducted a retrospective review of the experimental results of 65 SD rats fed an HFC diet (1.25% or 2.5% cholesterol, w/w) with or without (0%, 0.1%, 0.5% or 2%, w/w) cholic acid in our laboratory between January 2013 and February 2018.

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2. Methods

2.1. Animals and experimental design

We retrospectively reviewed the experimental results of 97 SD rats fed a normal rodent diet (MF; Oriental Yeast, Tokyo, Japan) or HFC diet in our laboratory between January 2013 and February 2018. All rats (8-week-old male SD rats) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats were housed individually and were maintained at 22–24 °C with 50–60% relative humidity and a 12-h dark/light cycle. After one week of acclimation with a normal rodent diet (MF; Oriental Yeast) and water ad libitum, the rats were fed one of the following six diets: MF diet (formulated to provide adequate growth of rats), high-fat and high-cholesterol (1.25%, w/w) diet without cholic acid (HFC1.25-0 diet), high-fat and high-cholesterol (1.25%, w/w) diet supplemented with 0.1% (w/w) cholic acid (HFC1.25-0.1 diet), high-fat and high-cholesterol (1.25%, w/w) diet supplemented with 0.5% (w/w) cholic acid (HFC1.25-0.5 diet), high-fat and high-cholesterol (1.25%, w/w) diet supplemented with 2% (w/w) cholic acid (HFC1.25-2 diet), or high-fat and high-cholesterol (2.5%, w/w) diet supplemented with 2% (w/w) cholic acid (HFC2.5-2 diet). Proximate dietary composition of each diet is shown in Table 1.

Allocation of the 97 SD rats into the 9 diet and feeding period groups was as follows in the present study: Control-9 group (n = 27), fed a MF diet for 9 weeks; A group (n = 5), fed an HFC1.25-0 diet for 9 weeks; B group (n = 5), fed an HFC1.25-0.1 diet for 9 weeks; C group (n = 24), fed an HFC1.25-0.5 diet for 9 weeks; D group (n = 10), fed an HFC1.25-2 diet for 9 weeks; E group (n = 9), fed an HFC2.5-2 diet for 9 weeks; Control-18 group (n = 5), fed a MF diet for 18 weeks; F group (n = 6), fed an HFC1.25-2 diet for 18 weeks; and G group (n = 6), fed an HFC2.5-2 diet for 18 weeks.

The rats in all groups had free access to food and water, and daily energy intake and body weight were monitored throughout the study. At 18 or 27 weeks of age, the rats were fasted for 8 h and killed under anesthesia with pentobarbital sodium. The epididymal fat pad and liver were removed, washed in cold saline, and weighed. Liver tissue portions were fixed in 10% neutral buffered formalin for histopathological examination.

All procedures performed on the animals were approved by the Animal Use Committee of University of Nagasaki, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals, University of Nagasaki.

2.2. Serum biochemical analysis

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using Transaminase C II test Wako (Wako Pure Chemical Industries, Osaka, Japan).

2.3. Hepatic lipid analysis

Hepatic lipids were extracted from frozen liver by the method of Folch et al.[12] . The extracts were dissolved in isopropanol and analyzed for TG and TC using Triglyceride E test Wako and Cholesterol E test Wako (Wako Pure Chemical Industries), respectively.

2.4. 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. Tsuneyama) who was blinded to the experimental design and sample identity. Histological findings from the liver were scored using the NASH Clinical Research Network Scoring System based on the following four semi-quantitative factors: steatosis (0–3), lobular inflammation (0–3), hepatocyte ballooning (0–2), and fibrosis (0–4). The NAFLD activity score (NAS) was taken as the unweighted sum of the scores for steatosis, lobular inflammation, and hepatocyte ballooning. For the diagnosis of steatohepatitis, NAS scores ≥ 5 and ≤ 2 were considered to be diagnostic and not diagnostic, respectively [13]. Liver fibrosis (0–4) was also assessed according to this system. Fibrosis scores were further classified as follows: score 0.5 was indicated a scores between 0 and 1; score 1.5 indicated a scores between 1 and 2; score 2.5 indicated a scores between 2 and 3, and score 3.5 indicated a scores between 3 and 4.

2.5. 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 Scheffe's post hoc test, chi-square test, or Fisher's exact probability test. All analyses were performed using IBM SPSS statistics software, version 24 (IBM, Chicago, IL, USA) on a computer with a Windows operating system. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Body weight gain and cumulative energy intake during the rearing period

Compared to the Control-9 group, the body weight gain in the 9-week study was significantly higher in the A and B groups ($p = 0.005$ and $p = 0.003$, respectively) and significantly lower in the E group ($p < 0.001$). The body weight gain was also significantly higher in the

Table 1
Proximate dietary compositions.

Ingredient/Diet	Control (MF*)	HFC1.25-0	HFC1.25-0.1	HFC1.25-0.5	HFC1.25-2	HFC2.5-2
Water (g)	79.0	55.3	55.2	54.9	53.7	53.7
Crude protein (g)	231.0	161.7	161.5	160.5	157.1	157.1
Crude lipid (g)	51.0	35.7	35.6	35.4	34.7	34.7
Crude ash (g)	58.0	40.6	40.5	40.3	39.4	39.4
Crude fiber (g)	28.0	19.6	19.6	19.5	19.0	19.0
Nitrogen free extract (g)	553.0	387.1	386.5	384.3	376.0	376.0
Palm oil (g)	0.0	287.5	287.5	287.5	287.5	275.0
Cholesterol (g)	0.0	12.5	12.5	12.5	12.5	25.0
Sodium cholate (g)	0.0	0.0	1.0	5.0	20.0	20.0
Total (g)	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Protein energy ratio (%)	25.70	12.67	12.66	12.63	12.49	12.77
Lipid energy ratio (%)	12.77	56.99	57.02	57.15	57.62	56.65
Carbohydrate energy ratio (%)	61.53	30.34	30.32	30.23	29.89	30.57
Energy (kcal/1000g)	3595.0	5104.0	5100.4	5086.0	5032.1	4919.6

HFC, high-fat and high-cholesterol diet.

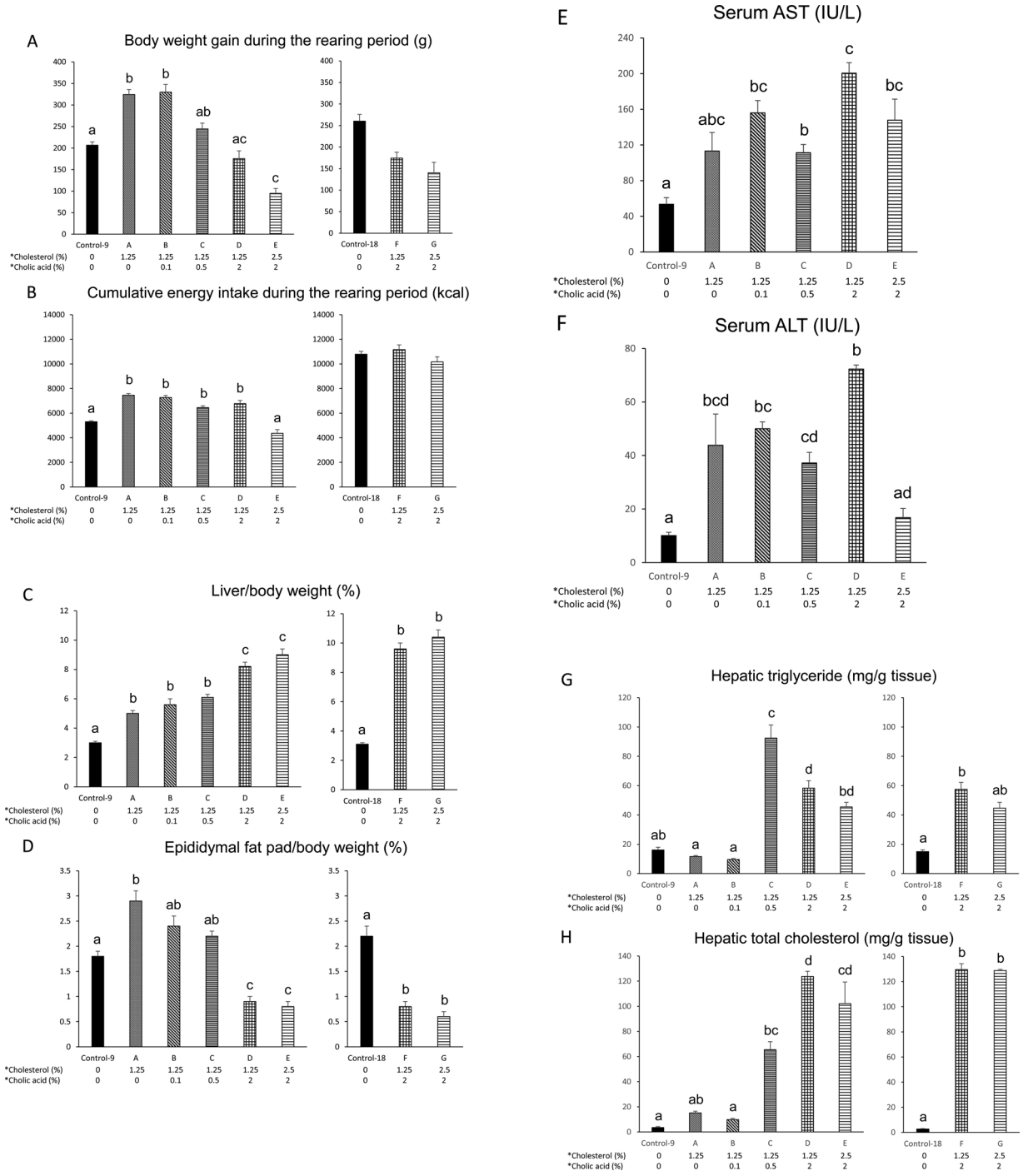

Fig. 1. The body weight gain during the rearing period (A), cumulative energy intake during the rearing period (B), liver/body weight (C), epididymal fat pad/body weight (D), serum aspartate aminotransferase (AST) level (E), serum alanine aminotransferase (ALT) level (F), hepatic triglyceride (TG) concentration (G), and hepatic total cholesterol (TC) concentration (H) in each group. Serum AST and ALT in Control-18, F and G groups were not tested. [abcd] Values not sharing the same lowercase letter are significantly different among groups ($P < 0.05$). *Dietary cholesterol or cholic acid concentration.

A and B group than the D and E group, respectively ($p < 0.001$ each) and higher in the C group than the E group ($p < 0.001$). The body weight gain during the 18-week study was not significantly different among the Control-18, F and G groups (Fig. 1A). The cumulative energy intake in the 9-week study was significantly higher in the A, B, C and D groups than the Control-9 group, respectively ($p < 0.001$, $p = 0.001$, $p = 0.001$, and $p = 0.001$). This energy intake was also significantly higher in the A, B, C and D than the E group, respectively ($p < 0.001$ each). The cumulative energy intake in the 18-week study was not significantly different among the Control-18, F and G groups (Fig. 1B).

3.2. Liver weight/body weight ratio and epididymal fat pad weight/body weight ratio

Compared to the Control-9 group, the liver weight/body weight ratio at 18 weeks of age was significantly higher in the A, B, C, D and E group, respectively ($p = 0.020$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.001$). This ratio was also significantly higher in the D group than the A, B and C group, respectively ($p < 0.001$, $p = 0.002$ and $p < 0.001$), and was significantly higher in the E group than the A, B and C group, respectively ($p < 0.001$ each). The liver weight/body

weight ratio at 27 weeks of age was significantly higher in the F and G group than the Control-18 group, respectively ($p < 0.001$ each, Fig. 1C). The epididymal fat pad weight/body weight ratio at 18 weeks of age was significantly higher in the A group ($p < 0.001$), and lower in the D and E group, respectively ($p < 0.001$ each) than the Control-9 group. This ratio was also significantly higher in the A, B and C group than the D and E group, respectively ($p < 0.001$ each). The epididymal fat pad weight/body weight ratio at 27 weeks of age was significantly higher in the Control-18 group than the F and G group, respectively ($p < 0.001$ each, Fig. 1D).

3.3. Serum levels of AST and ALT

Compared to the Control-9 group, the serum AST level at 18 weeks of age was significantly higher in the B, C, D and E group, respectively ($p = < 0.001$, $p = 0.002$, $p < 0.001$, $p < 0.001$ and $p = 0.002$). This AST level was also significantly higher in the D group than the C group ($p = 0.032$, Fig. 1E). The serum ALT level at 18 weeks of age was significantly higher in the A, B, C and D group than the Control-9 group, respectively ($p = 0.002$, $p < 0.001$, $p < 0.001$ and $p < 0.001$). This ALT level was also significantly higher in the B and D group than the E group, respectively ($p = 0.035$ and $p < 0.001$), and in the D group than the C group ($p = 0.016$, Fig. 1F). The serum AST and ALT levels were not tested in the Control-18, F and G groups.

3.4. Hepatic TG and TC concentrations

The hepatic TG concentration at 18 weeks of age was significantly higher in the C group than the Control-9, A, B, D and E group, respectively ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p = 0.032$ and $p < 0.001$). This TG concentration was also significantly higher in the D group than the Control-9, A and B group, respectively ($p = 0.001$, $p = 0.006$ and $p = 0.003$), and in the E group than the A and B group, respectively ($p = 0.047$ and $p = 0.026$). The hepatic TG concentration at 27 weeks of age was significantly higher in the F group than the Control-18 group ($p = 0.012$, Fig. 1G). The hepatic TC concentration at 18 weeks of age was significantly higher in the D group than the Control-9, A, B and C group, respectively ($p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.012$). This TC concentration was also significantly higher in the E group than the Control-9, A and B group, respectively ($p < 0.001$ each), and in the C group than the Control-9 and B group, respectively ($p < 0.001$ and $p = 0.020$). The hepatic TC concentration at 27 weeks of age was significantly higher in the F and G group than the Control-18 group, respectively ($p < 0.001$ each, Fig. 1H).

3.5. Histopathological findings of the liver

Fig. 2 shows representative histopathological findings in the liver according to the NASH Clinical Research Network Scoring System [13], and histopathological assessment of the liver in each group according to the above scoring system is shown in Table 2. Hepatic steatosis was not observed in 31 (96.9%) of 32 rats in the Control-9 and Control-18 groups, but it was observed in all 65 rats of the A, B, C, D, E, F and G groups. In the A group, all 5 rats had moderate steatosis (score 2). In the B, C and D group, severe steatosis (score 3) was seen in 3 (60%) of 5 rats, 23 (95.8%) of 24 rats, and 9 (90%) of 10 rats, respectively. In the E, F and G groups, all 21 rats had severe steatosis (score 3). Lobular inflammation was not observed in any of the 32 rats in the Control-9 and Control-18 groups, but it was observed in all 65 rats in the A, B, C, D, E, F and G groups. In the A group, mild (score 1) and moderate (score 2) lobular inflammation was seen in 3 (60%) and 2 (40%) of 5 rats, respectively. In the B, C, D and E group, severe lobular inflammation (score 3) was seen in 2 (40%) of 5 rats, 5 (20.8%) of 24 rats, 4 (40%) of 10 rats, and 3 (33.3%) of 9 rats, respectively. In the F and G groups, severe lobular inflammation (score 3) was seen in all 12 rats. Hepatocyte ballooning was not observed in any of the 10 rats in the A and B groups, nor in any of the 32 rats in the Control-9 and Control-18 groups. In the C group, 5 (20.8%) of 24 rats had a few ballooning hepatocytes (score 1). In the D, E, F and G group, many ballooning hepatocytes (score 2) were seen in 4 (40%) of 10 rats, 5 (55.6%) of 9 rats, 6 (100%) of 6 rats, and 6 (100%) of 6 rats, respectively (Fig. 3). According to the NASH Clinical Research Network Scoring System [13], all 32 rats in the Control-9 and Control-18 groups had a NAS of 0 or 1, indicating “not diagnostic of NASH”. In the A group, all 5 rats had a NAS of 3 or 4, representing a “borderline” diagnosis. In the B and C group, 3 (60%) of 5 rats and 22 (91.7%) of 24 rats had a NAS of ≥ 5, representing “NASH”, respectively. In the D, E, F and G groups, all 31 rats had a NAS of ≥ 5, representing “NASH”. Overall, 56 (86.2%) of 65 rats fed an HFC diet developed NASH. Hepatic fibrosis was not observed in all 5 rats in the A group and 4 (80%) of 5 rats in the B group, as well as in all 32 rats in the Control-9 and Control-18 groups. In the C, D, E, F and G group, score 3.5 or 4 fibrosis was seen in none of 24 rats, 3 (30%) of 10 rats, 3 (33.3%) of 9 rats, 5 (83.3%) of 6 rats, and 6 (100%) of 6 rats, respectively. It is noted that 2 (33.3%) of 6 rats in the F group and 5 (83.3%) of 6 rats in the G group showed score 4 fibrosis, indicating cirrhosis [11].

3.6. Effects of dietary cholesterol consumption

To assess the effects of dietary cholesterol consumption, data were compared between the D group (fed an HFC1.25-2 diet for 9 weeks) and the E group (fed an HFC2.5-2 diet for 9 weeks), and between the F group (fed an HFC1.25-2 diet for 18 weeks) and the G group (fed an HFC2.5-2 diet for 18 weeks). The high cholesterol diet significantly reduced the cumulative energy intake of rats during the rearing period and the serum ALT level at 18 weeks of age in the 9-week study; however, the body weight gain during the rearing period in both the 9- and 18-week studies, the liver/body weight and epididymal fat pad/body weight ratios, and the serum AST, serum ALT, hepatic TG, and hepatic TC levels at both 18 and 27 weeks of age were not significantly affected by the dietary cholesterol consumption (Fig. 1). Histopathologically, hepatic steatosis, lobular inflammation, hepatocyte ballooning, NAS score and hepatic fibrosis were not significantly affected by the dietary cholesterol consumption (Table 2).

3.7. Effects of dietary cholic acid consumption

To assess the effects of dietary cholic acid consumption, data among the A group (fed an HFC1.25-0 diet for 9 weeks), B group (fed an HFC1.25-0.1 diet for 9 weeks), C group (fed an HFC1.25-0.5 diet for 9 weeks) and D group (fed an HFC1.25-2 diet for 9 weeks) were compared. The high cholic acid diet significantly reduced the body weight gain of rats during the rearing period and the epididymal fat pad/body weight ratio at 18 weeks of age; it also significantly increased the liver/body weight ratio and the hepatic TG and TC concentrations at 18 weeks of age (Fig. 1). Histopathologically, the scores of hepatic steatosis ($p < 0.001$), lobular inflammation ($p = 0.043$), hepatocyte ballooning ($p = 0.003$), NAS ($p < 0.001$) and hepatic fibrosis ($p < 0.001$) were significantly increased by the dietary cholic acid consumption (Table 2).

4. Discussion

A diet enriched in fat and cholesterol is well known to cause hepatic steatosis and inflammation in animal models. The development of fibrosis, the hallmark that differentiates progressive NASH from NAFL, is probably caused not only by dietary cholesterol, but also by the interaction between dietary cholesterol and dietary fat [5,6,7,9]. A cholic acid-supplemented HFC diet, or atherogenic diet, is also known to replicate human NASH pathology and to induce early fibrosis when compared to a diet supplemented with only high fat and cholesterol [8,14,15]. In the present study, the liver weight/body weight ratio was higher in rats fed an HFC diet than in rats fed a normal diet in a cholesterol dose-, cholic acid dose-, or rearing period-dependent manner (Fig. 1C).

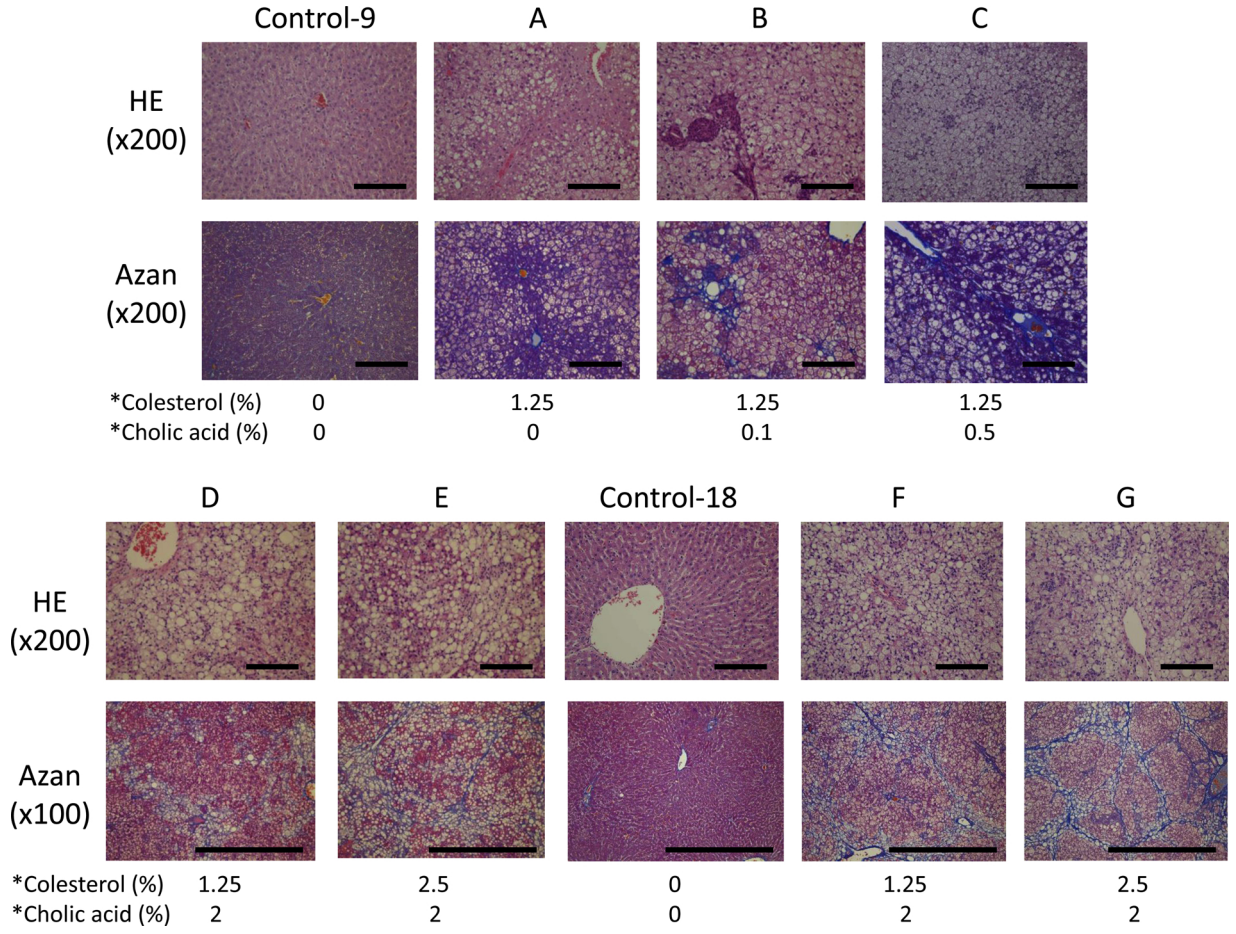

Fig. 2. Representative histopathology of the rat liver in each group. HE: Hematoxylin and eosin-stained section; original magnification, x200; scale bars = 200 μm. Azan: Azan-stained section; original magnification, x100 or x200; scale bars = 200 μm. *Dietary cholesterol orcholic acid concentration.

SD rats were found to be more sensitive than mice to a high-fat and high-cholesterol diet [8]. Previously, we reported that SD rats fed an HFC diet supplemented with cholic acid developed hepatic features of NASH and progressed to cirrhosis within 9–18 weeks, but they lacked obesity and insulin resistance [9–11]. In the present study, the body weight gain during the rearing period and the epididymal fat pad weight/body weight ratio at 18 weeks of age were decreased in the C, D and E groups in a cholic acid dose-dependent manner. At 27 weeks of age, these two parameters were also lower in the F and G groups than the Control-18 group, whereas the cumulative energy intake during the rearing period did not differ among the three groups (Fig. 1A, B and D). The mechanism underlying these results has not been elucidated, but one possibility is that cholic acid acts as a signaling molecule for the G protein-coupled receptor TGR5, which is an up-regulator of fatty acid

Table 2
Histopathological assessment of the liver in each group according to the NASH Clinical Research Network Scoring System [13].

Item/Group*	Score	Control-9	A	B	C	D	E	Control-18	F	G	Total
Steatosis	0	26	0	0	0	0	0	5	0	0	31
	1	1	0	1	0	0	0	0	0	0	2
	2	0	5	1	1	1	0	0	0	0	8
	3	0	0	3	23	9	9	0	6	6	56
Lobular inflammation	0	27	0	0	0	0	0	5	0	0	32
	1	0	3	2	3	0	0	0	0	0	8
	2	0	2	1	16	6	6	0	0	0	31
	3	0	0	2	5	4	3	0	6	6	26
Hepatocyte ballooning	0	27	5	5	19	3	0	5	0	0	64
	1	0	0	0	5	3	4	0	0	0	12
	2	0	0	0	0	4	5	0	6	6	21
NAFLD activity score (NAS)**	0–2	27	0	1	0	0	0	5	0	0	33
	3–4	0	5	1	2	0	0	0	0	0	8
	5–8	0	0	3	22	10	9	0	6	6	56
Fibrosis	0	27	5	4	0	0	0	5	0	0	41
	0.5–1	0	0	1	16	3	2	0	0	0	22
	1.5–2	0	0	0	4	4	2	0	0	0	10
	2.5–3	0	0	0	4	0	2	0	1	0	7
	3.5–4	0	0	0	0	3	3	0	5	6	17

Values indicate number of rats.

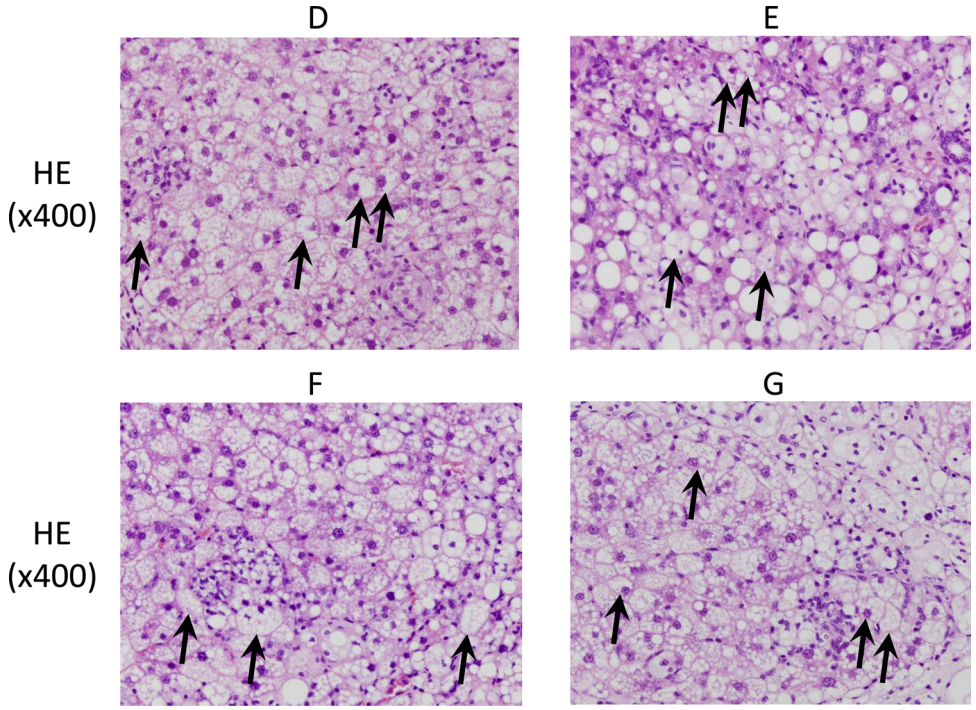

Fig. 3. Hepatocyte ballooning (score 2) according to the NASH Clinical Research Network Scoring System [13] was observed in the D, E, F and G groups (arrows). HE: Hematoxylin and eosin-stained section; original magnification, x400).

oxidation and induces elevated energy expenditure, resulting in decreased body weight or adipose tissue [16].

Histopathologic steatosis was obvious in rats fed an HFC diet, and among rats in the A, B and C groups in the present study, severe steatosis (score 3) was seen more frequently in a cholic acid dose-dependent manner. Therefore, it is conceivable that fat, cholesterol and cholic acid affected the histopathologic severity of steatosis. Histopathologic lobular inflammation was also obvious in rats fed an HFC diet, and among rats in the A, B, C and D groups, severe lobular inflammation (score 3) was seen more frequently in a cholic acid dose-dependent manner. Moreover, rats with severe lobular inflammation (score 3) were seen more frequently in the F group than the D group and in the G group than the E group; in other words, even with the same diet, a longer feeding duration resulted in a higher frequency of severe lobular inflammation. These results suggest that fat, cholesterol, cholic acid and rearing period affect the histopathologic severity of lobular inflammation. Histopathologic hepatocyte ballooning was not observed in rats fed an HFC diet without or with a low dose of cholic acid supplementation (0.1%). Rats showing a higher hepatocyte ballooning score were seen more frequently in the D group (fed an HFC1.25-2 diet) than the C group (fed an HFC1.25-0.5 diet), and also in the E group (fed an HFC2.5-2 diet) than the D group (fed an HFC1.25-2 diet). Moreover, in comparisons among groups with matched diets (the F group versus the D group, and the G group versus the E group), rats with hepatocyte ballooning were seen more frequently in the longer feeding duration groups. These results suggest that cholesterol, cholic acid and rearing period affect the histopathologic severity of hepatocyte ballooning. Consequently, the NAS score, defined as the unweighted sum of the scores for steatosis, lobular inflammation, and hepatocyte ballooning [13], was obviously higher in rats fed an HFC diet than the Control groups. Among the A, B, C and D groups, rats diagnosed with NASH (NAS of ≥ 5) were seen more frequently in a cholic acid dose-dependent manner. Moreover, a longer duration of feeding these diets resulted in a more frequent occurrence of high scores of NAS; the frequency was higher in the F group (score 8 in 6 rats) than the D group (score 5 in 2 rats, score 6 in 3 rats, score 7 in 4 rats, and score 8 in 1 rat), and also in the G group (score 8 in 6 rats) than the E group (score 6 in 1 rat, and score 7 in 8 rats). These results suggest that fat, cholesterol, cholic acid and rearing period affect NAS score. Histopathologic hepatic fibrosis was seldom seen in rats fed an HFC diet without or with a low dose of cholic acid supplementation (0.1%). Among the B, C and D groups, rats with advanced fibrosis were seen more frequently in a cholic acid dose-dependent manner. Moreover, in comparisons among groups with matched diets (the F group versus the D group, and the G group versus the E group), rats with advanced fibrosis were seen more frequently in the longer feeding duration groups. These results suggest that cholic acid and rearing period affect the histopathologic severity of hepatic fibrosis.

Bile acids are synthesized from cholesterol in the liver and stored in the gall bladder. They are secreted into the intestine as a constituent of bile upon ingestion of a fatty meal, and they facilitate digestion and absorption of dietary fats and lipid-soluble vitamins [17]. In addition, bile acids regulate lipid, glucose and energy metabolism as signaling molecules [18]. Despite its physiological importance, cholic acid is known to show toxicity to membrane components of cells, such as liver injury, inflammation and fibrosis above certain concentrations [19]. Indeed, cholic acid specifically enhances the expression of hepatic fibrosis-related genes, such as those of the collagen family [20]. In the present study, the effects of dietary cholic acid consumption on the severity of NASH were more evident than those of dietary cholesterol consumption. Feeding of an HFC diet was reported to increase the expression of hepatic *Cyp7a1*mRNA, which catalyzes the rate-limiting step in bile acid biosynthesis from cholesterol [21] . Although the hepatic *Cyp7a1*mRNA levels were not systematically assessed in our rat model, they tended to be higher in the D ($n = 5$) and E ($n = 5$) groups than the Control-9 ($n = 5$) group, and in the F ($n = 6$) and G ($n = 6$) groups than the Control-18 ($n = 5$) group, although the differences were not statistically significant (data not shown) [10,11]. Overexpression of this gene may lead to the excessive accumulation of toxic bile acids [22]. Thus, the presence of cholic acid in an HFC diet complements the hepatotoxicity of cholesterol not only by enhancing lipid absorption in the intestine, but also by enhancing inflammation in the liver when in excess, and as a result, contributes to the severity of NASH [23]. Results of the present study corroborated previously reported findings.

The liver pathogenesis leading to NASH due to cholic acid-supplemented HFC diet is also facilitated by oxidative stress [23,24]. In our rat model, oxidative stress was not systematically assessed, but the mRNA

levels of *Hmox1*encoding heme oxygenase-1 (HO-1) and *Sod2*encoding manganese superoxide dismutase (MnSOD), both oxidative stress markers, tended to be higher in the C group (n = 6) than the Control-9 group (n = 6) [25,26]. Moreover, immunohistochemical analysis of 4-hydroxynonenal (4-HNE) as a marker of oxidative stress revealed that its expression in the liver was higher in the C group (n = 6) than the Control-9 group (n = 6) (data not shown) [26].

The majority of high fat diet-induced animal NAFLD models use fat with high amounts of corn oil or lard [27], but in our rat NASH model, palm oil was used as the source of fat. Sales et al. recently reported that palm oil induced NASH in mice via disturbed hepatocyte transcription [28]. Palm oil is the most consumed oil in the world, and it is rich in saturated fatty acids, especially palmitic acid. NAFLD patients with advanced liver fibrosis had higher levels of palmitic acid (C16:0) and stearic acid (C18:0) in their erythrocytes [29], and C18:0/C16:0 ratio was lower in the NASH group than the NAFL group [30]. Palmitic acid induces proinflammatory cytokines such as interleukin (IL)-1β and IL-18, and stimulates hepatic stellate cells, which play a pivotal role in NASH development [31,32]. Therefore, the palm oil used in the present study can affect NASH development.

There were several limitations to the present study. First, despite the fact that all rats were obtained from the same supplier and were reared under the same breeding environment, i.e., they were housed individually and were maintained at 22–24 °C with 50–60% relative humidity and a 12-h dark/light cycle, and all histopathological assessments were made by one pathologist (K. Tsuneyama) who was blinded to the experimental design and sample identity, the rearing year and breeders were not the same throughout the entire experiment. Animal experiments are complicated, and the results and their interpretation are not always straightforward, as no single experiment can be executed perfectly. Although there are several possible sources of bias in animal studies, reliable conclusions can be obtained to some extent from the analysis of combined results from other studies; this circumvents unnecessary duplication of animal studies. In the present study, all animal experiments were deemed to be sufficiently homogeneous, in terms of the animal species, interventions, designs and outcomes, for reliable conclusions [33]. Second, the MF diet is formulated to provide adequate growth for rats, but the exact ingredient composition of this diet is not disclosed by the manufacturer, and there may be inconsistencies in the composition from year to year; such variations in the ingredient composition of the MF diet may have affected the results of this study. Third, there was considerable variation in the number of rats in each group because the present study was a retrospective historical investigation. Thus, future investigations should employ a systematic study.

In conclusion, 56 (86.2%) of 65 SD rats fed an HFC diet for 9 or 18 weeks developed histopathologically proven NASH according to the NASH Clinical Research Network Scoring System [13], although this model lacked visceral obesity [10,11]. Notably, SD rats fed an HFC diet supplemented with 2% (w/w) cholic acid for 18 weeks frequently developed advanced fibrosis including cirrhosis. The amounts of dietary fat, cholesterol and/or cholic acid, and the rearing period affected the histopathologic severity of NASH. Further studies are needed to determine whether comparable results can be obtained using rats of other ages or strains.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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References

[1] Z.M. Younossi, Non-alcoholic fatty liver disease – a global public health perspective, J. Hepatol. 70 (2019) 531–544.
[2] P.S. Dulai, S. Singh, J. Patel, M. Soni, L.J. Prokop, Z. Younossi, G. Sebastiani, M. Ekstedt, H. Hagstrom, P. Nasr, P. Stal, V.W. Wong, S. Kechagias, R. Hultcrantz, R. Loomba, Increased risk of mortality by fibrosis stage in non- alcoholic fatty liver disease: Systematic review and meta-analysis, Hepatology 65 (2017) 1557–1565.
[3] K. Wouters, P.J. van Gorp, V. Bieghs, M.J. Gijbels, H. Duimel, D. Lutjohann, A. Kerksiek, R. van Kruchten, N. Maeda, B. Staels, M. van Bilsen, R. Shiri- Sverdlov, M.H. Hofker, Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis, Hepatology 48 (2008) 474–486.
[4] D.M. Van Rooyen, C.Z. Larter, W.G. Haigh, M.M. Yeh, G. Ioannou, R. Kuver, S.P. Lee, N.C. Teoh, G.C. Farrell, Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis, Gastroenterology 141 (2011) 1393–1403.
[5] T. Teratani, K. Tomita, T. Suzuki, T. Oshikawa, H. Yokoyama, K. Shimamura, S. Tominaga, S. Hiroi, R. Irie, Y. Okada, C. Kurihara, H. Ebinuma, H. Saito, R. Hokari, K. Sugiyama, T. Kanai, S. Miura, T. Hibi, A high-cholesterol diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate cells, Gastroenterology 142 (2012) 152–162.
[6] C. Savard, E.V. Tartaglione, R. Kuver, W.G. Haigh, G.C. Farrell, S. Subramanian, A. Chait, M.M. Yeh, L.S. Quinn, G.N. Ioannou, Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis, Hepatology 57 (2013) 81–92.
[7] Y. Miyaoka, D. Jin, K. Tashiro, S. Masubuchi, M. Ozeki, F. Hirokawa, M. Hayashi, S. Takai, K. Uchiyama, A novel hamster nonalcoholic steatohepatitis model induced by a high-fat and high-cholesterol diet, Exp. Anim. 67 (2018) 239–247.
[8] S.H. Ibrahim, P. Hirsova, H. Malhi, G.J. Gores, Animal models of nonalcoholic steatohepatitis: eat, delete, and inflame, Dig. Dis. Sci. 61 (2016) 1325–1336.
[9] S.C. Yanguas, B. Cogliati, J. Willebrords, M. Maes, I. Colle, B. van den Bossche, C.P.M.S. de Oliveira, W. Andraus, V.A. Alves, I. Leclercq, M. Vinken, Experimental models of liver fibrosis, Arch. Toxicol. 90 (2016) 1025–1048.
[10] M. Ichimura, M. Kawase, M. Masuzumi, M. Sakaki, Y. Nagata, K. Tanaka, K. Suruga, S. Tamaru, S. Kato, K. Tsuneyama, K. Omagari, High-fat and high- cholesterol diet rapidly induces nonalcoholic steatohepatitis with advanced fibrosis in Sprague-Dawley rats, Hepatol. Res. 45 (2015) 458–469.
[11] M. Ichimura, M. Masuzumi, M. Kawase, M. Sakaki, S. Tamaru, Y. Nagata, K. Tanaka, K. Suruga, K. Tsuneyama, S. Matsuda, K. Omagari, A diet induced Sprague-Dawley rat model of nonalcoholic steatohepatitis-related cirrhosis, J. Nutr. Biochem. 40 (2017) 62–69.
[12] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[13] D.E. Kleiner, E.M. Brunt, M. Van Natta, C. Behling, M.J. Contos, O.W. Cummings, L.D. Ferrell, Y.-C. Liu, M.S. Torbenson, A. Unalp-Arida, M. Yeh, A.J. McCullough, A.J. Sanyal, Design and validation of a histological scoring system for nonalcoholic fatty liver disease, Hepatology 41 (2005) 1313–1321.
[14] N. Matsuzawa, T. Takamura, S. Kurita, H. Misu, T. Ota, H. Ando, M. Yokoyama, M. Honda, Y. Zen, Y. Nakanuma, K. Miyamoto, S. Kaneko, Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet, Hepatology 46 (2007) 1392–1403.
[15] J.K.C. Lau, X. Zhang, J. Yu, Animal models of non-alcoholic fatty liver disease: current perspectives and recent advance, J. Pathol. 241 (2017) 36–44.
[16] M. Watanabe, S.M. Houten, C. Mataki, M.A. Christoffolete, B.W. Kim, H. Sato, N. Messaddeq, J.W. Harney, O. Ezaki, T. Kodama, K. Schoonjans, A.C. Bianco, J. Auwerx, Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation, Nature 439 (2006) 484–489.
[17] L.B. Agellon, Metabolism and function of bile acids, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of Lipids, Lipoproteins and Membranes. 4th ed, Elsevier Science B.V., Amsterdam, 2002, pp. 433–448.
[18] T. Li, J.Y.L. Chiang, Bile acid signaling in liver metabolism and diseases, J. Lipids 2012 (2012) 754067.
[19] S. Watanabe, K. Tsuneyama, Eicosapentaenoic acid attenuates hepatic accumulation of cholesterol esters but aggravates liver injury and inflammation in mice fed a cholate-supplemented high-fat diet, J. Toxicol. Sci. 38 (2013) 379–390.
[20] L. Vergnes, J. Phan, M. Strauss, S. Tafuri, K. Reue, Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression, J. Biol. Chem. 278 (2003) 42774–42784.
[21] H. Yetti, H. Naito, Y. Yuan, X. Jia, Y. Hayashi, H. Tamada, K. Kitamori, K. Ikeda, Y. Yamori, T. Nakajima, Bile acid detoxifying enzymes limit susceptibility to liver fibrosis in female SHRSP5/Dmcr rats fed with a high-fat-cholesterol diet, PLoS One 13 (2018) e0192863.
[22] Y. Yuan, H. Naito, X. Jia, K. Kitamori, T. Nakajima, Combination of hypertension along with a high fat and cholesterol diet induces severe hepatic inflammation in rats via a signaling network comprising NF-kB, MARK, and Nrf2 pathways, Nutrients 9 (2017) 1018.
[23] L.N. Tu, M.R. Showalter, T. Cajka, S. Fan, V.V. Pillai, O. Fiehn, V. Selvaraj, Metabolomic characteristics of cholesterol-induced non-obese nonalcoholic fatty liver disease in mice, Sci. Rep. 7 (2017) 6120.
[24] T. Moriya, K. Kitamori, H. Naito, Y. Yanagiba, Y. Ito, N. Yamagishi, H. Tamada, X. Jia, S. Tsuchikura, K. Ikeda, Y. Yamori, T. Nakajima, Simultaneous changes in high-fat and high-cholesterol diet-induced steatohepatitis and severe fibrosis and those underlying molecular mechanisms in novel SHRSP5/Dmcr rat, Environ. Health Prev. Med. 17 (2012) 444–456.
[25] K. Omagari, K. Suruga, A. Kyogoku, S. Nakamura, A. Sakamoto, S. Nishioka, M. Ichimura, Y. Miyata, K. Tajima, K. Tsuneyama, K. Tanaka, A fermented mixed tea made with camellia (Camellia japonica) and third-crop green tea leaves prevents nonalcoholic steatohepatitis in Sprague-Dawley rats fed a high-fat and high-cholesterol diet, Hepatobiliary Surg. Nutr. 7 (2018) 175–184.
[26] K. Omagari, A. Fukuda, M. Suga, A. Ogata, S. Nishioka, K. Suruga, M. Ichimura,

K. Tsuneyama, Peptides obtained by enzymatic decomposition of mackerel prevent nonalcoholic steatohepatitis in Sprague-Dawley rats fed a high- fat and high-cholesterol diet, OBM Hepatol. Gastroenterol. 2 (2018) 8.
[27] O. Kucera, Z. Cervinkova, Experimental models of non-alcoholic fatty liver disease in rats, World J. Gastroenterol. 20 (2014) 8364–8376.
[28] R.C. Sales, P.C. Medeiros, F. Spreafico, P.C. de Velasco, F.K.A. Goncalves, R. Martin-Hernandez, D.C. Mantilla-Escalante, J. Gil-Zamorano, W.A.F. Peres, S.A.L. de Souza, A. Davalos, M.G. Tavares do Carmo, Olive oil, palm oil, and hybrid palm oil distinctly modulate liver transcriptome and induce NAFLD in mice fed a high-fat diet, Int. J. Mol. Sci. 20 (2019) 8.
[29] K. Cansancao, L. Silva Monteiro, N. Carvalho Leite, A. Davalos, M.D.G. Tavares do Carmo, W. Arantes Ferreira Peres, Advanced liver fibrosis is independently associated with palmitic acid and insulin levels in patients with non-alcoholic fatty liver disease, Nutrients 10 (2018) 1586.
[30] K. Yamada, E. Mizukoshi, H. Sunagozaka, K. Arai, T. Yamashita, Y. Takeshita, H. Mizu, T. Takamura, S. Kitamura, Y. Zen, Y. Nakanuma, M. Honda, S. Kaneko, Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis, Liver Int. 35 (2015) 582–590.
[31] C. Cai, X. Zhu, P. Li, J. Li, J. Gong, W. Shen, K. He, NLRP3 deletion inhibits the non-alcoholic steatohepatitis development and inflammation in Kupffer cells induced by palmitic acid, Inflammation 40 (2017) 1875–1883.
[32] N.N. Duan, X.J. Liu, J. Wu, Palmitic acid elicits hepatic stellate cell activation through inflammasomes and hedgehog signaling, Life Sci. 176 (2017) 42–53.
[33] C.R. Hooijmans, J. IntHout, M. Ritskes-Hoitinga, M.M. Rovers, Meta-analyses of animal studies: an introduction of a valuable instrument to further improve healthcare, ILAR J. 55 (2014) 418–426.