

Case Report

Increased hepatic inflammation in a normal-weight mouse after long-term high-fat diet feeding

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Abstract: Among five C57BL/6 mice fed a high-fat diet (HFD) for 12 weeks, one mouse showed a body weight (BW) similar to normal diet (ND)-fed mice. We compared obesity-related parameters of three groups (ND-fed mice, one HFD-fed normal-weight mouse, and HFD-fed overweight mice), including visceral fat weight, serum levels of total cholesterol (TC), glucose, and aminotransferases (AST and ALT), adipocyte size, percentage of crown-like structures, severity of hepatic steatosis, and number of inflammatory foci. Compared to ND-fed mice, the HFD-fed normal-weight mouse exhibited a similar visceral fat weight, similar serum levels of glucose and aminotransferases, and a similar percentage of crown-like structures. On the other hand, the serum TC level, adipocyte size, and hepatic steatosis severity of the HFD-fed normal-weight mouse were intermediate between those of ND-fed mice and HFD-fed overweight mice. Interestingly, the number of hepatic inflammatory foci in the HFD-fed normal-weight mouse was remarkably increased compared with those in HFD-fed overweight mice. These results suggest that having BW or serum ALT levels within normal ranges may not guarantee absence of hepatic inflammation and that the HFD-fed normal-weight mouse can be used as an animal model for the study of liver inflammation, particularly in patients with normal BWs and/or serum ALT values. (DOI: 10.1293/tox.2017-0038; J Toxicol Pathol 2018; 31: 43–47)

Key words: body mass index, calorie dense food, hepatic disease, inflammation, obesity

Obesity is known as a major risk factor for several diseases, including chronic kidney disease¹, type 2 diabetes², and cardiovascular diseases³. Furthermore, it plays an important role in inducing a state of chronic, systemic, low-grade inflammation that increases both circulating and adipose tissue (AT) levels of inflammatory cytokines (e.g., interleukin-6 and tumor necrosis factor- α)^{2, 4}. In addition to genetic factors, environmental factors, such as a sedentary lifestyle and increased intake of calorie-dense foods (e.g., fat-rich foods), are closely associated with obesity. Intake of a high-fat diet (HFD) induces obesity in both humans and animal models. High levels of dietary fat correlate with body weight (BW) and fat mass gain, while reduction of dietary fat results in their loss. Generally, body mass index

is used as a screening tool for identification of overweight individuals^{5, 6}.

The intake of a HFD induces fatty acid uptake by the liver, which leads to liver inflammation and injury. Obesity-induced liver inflammation and injury is closely associated with the development of liver fibrosis and cirrhosis and hepatocellular carcinoma^{7, 8}. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are widely used as a serum enzyme biomarker of liver injury, and their levels have been shown to increase with BW gain in both humans⁹ and rodents¹⁰. Of the two enzymes, ALT is a more specific indicator of liver injury because of its greater concentration in the liver compared with other tissues and is confined to the cytoplasm. On the other hand, the heart has the highest concentration of AST, followed by the liver, skeletal muscles, kidneys, brain, pancreas, and erythrocyte. AST also has two different isoenzyme forms, a mitochondrial and a cytoplasmic form^{11, 12}. Furthermore, ALT has been shown to be the most sensitive biomarker for the presence of hepatic steatosis in both men and women^{13, 14} and is associated with increased abdominal weight and obesity^{9, 15}.

Despite multiple obesity studies, there are limited data available on the obesity-related parameters of HFD-fed normal-weight mice. In the present study, we compared obesi-

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ty-related parameters between a mouse that maintained a normal weight after 12 weeks of HFD feeding and normal diet (ND)-fed mice or HFD-fed overweight mice.

Specific-pathogen-free (SPF) male C57BL/6J mice aged 5 weeks were purchased from Japan SLC (Shizuoka, Japan) and were bred under SPF conditions. Mice were housed with a maximum of 5 mice per cage and were maintained in a temperature-controlled room (22°C) with a 12-h light-dark cycle. They were fed a ND (CE-2, CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum* for a week. The 6-week-old mice were split into two groups, and each group was fed either an ND or HFD (HFD32, CLEA Japan) and tap water *ad libitum* for 12 weeks. The nutritional compositions of the HFD32 and CE-2 are presented in Table 1. HFD32 and CE-2 were stored at -20°C and room temperature, respectively. HFD32 was changed every day, and CE-2 was changed twice a week. All diets were used within the manufacturer's recommended expiry period. Animal studies were performed in accordance with the Guidelines for Animal Experiments established by the Ministry of Health, Labour and Welfare of Japan and by the National Cerebral and Cardiovascular Center Research Institute.

After 12 weeks of HFD feeding, blood samples from all mice were collected from the caudal vena cava. Blood was collected in BD Microtainer® Blood Collection tubes (BD, Franklin Lakes, NJ, USA), and serum samples were obtained by centrifuging the tubes for five minutes at 10,000 g. Total cholesterol (TC) in the serum samples was measured using the Cholesterol E-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The level of serum glucose

was measured using a glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA). AST and ALT were measured using a transaminase assay kit (Transaminase C II-test, Wako) according to the manufacturer's instructions.

Visceral epididymal adipose and liver tissues were removed and fixed in 10% buffered formalin. After routine processing by paraffin embedding, tissue sectioning, and slide mounting, sections were stained with hematoxylin and eosin (HE) or immunostained with anti-F4/80 antibody (monoclonal antibody; Abcam, Cambridge, UK). Adipocyte diameters were determined using the ImageJ software¹⁶. Percentages of crown-like structures were measured as previously described¹⁷. Micro- and macrovesicular steatosis severity were determined at 50–100× magnification and expressed as percentages of the total surface area. Micro- and macrovesicular steatosis were differentiated from each other by whether centrally placed nuclei were visible in the foamy hepatocytes (micro) or not (macro). In addition, the number of inflammatory foci was counted in five fields viewed for each sample and expressed as the average number of inflammatory foci per field (view size 2.2 mm²).

Intake of a HFD induces obesity and BW gain in humans^{5, 6} and animals^{10, 18, 19}. However, among the five mice fed a HFD for 12 weeks, one mouse showed a BW similar to the ND-fed mice (Table 2). We compared obesity-related parameters of all three groups (ND-fed mice, the one HFD-fed normal-weight mouse, and HFD-fed overweight mice), including visceral fat weight, serum levels of TC, glucose, and aminotransferases (AST and ALT), adipocyte size, percentage of crown-like structures, severity of hepatic steatosis, and number of inflammatory foci. Visceral fat weight, serum levels of glucose and aminotransferases, and the percentage of crown-like structures of the normal-weight mouse after long-term HFD feeding were similar to those of the ND-fed mice. Serum TC level, adipocyte size, and hepatic steatosis severity of the HFD-fed normal-weight mouse were higher than those of ND-fed mice but lower than those of HFD-fed overweight mice (Table 2). A significant relationship between dietary fat intake and serum TC levels has been observed in humans^{5, 6} and rodents^{18, 19}, but patients with severe cirrhosis have shown decreased serum levels of TC²¹.

Several studies have suggested a high positive cor-

Table 1. Nutritional Compositions of HFD32 and CE-2

Nutritional composition (per 100 g diet)	Diet	
	HFD32	CE-2
Moisture (%)	6.2	8.9
Crude protein (%)	25.5	24.9
Crude fat (%)	32.0	4.6
Crude fiber (%)	2.9	4.1
Crude ash (%)	4.0	6.6
Nitrogen free extract (%)	29.4	51.0
Energy (kcal)	507.6	344.9
Fat kcal (%)	56.7	11.6

Table 2. Obesity-related Parameters in the ND-fed and HFD-fed Mice

Parameters	BW (g)	Fat weight (g)	TC (mg/mL)	AST (U/L)	ALT (U/L)	Glucose (mg/dL)	Adipocyte size (µm)	Crown-like structures (%)	Steatosis (%)	No. of inflamma- tion foci
ND-fed mice (n=5)	28.2 ± 1.0	0.56 ± 0.12	124.5 ± 9.8	32.4 ± 2.2	23.7 ± 2.3	143.1 ± 18.8	49.3 ± 3.2	0.2 ± 0.1	2.8 ± 0.5	0.2 ± 0.14
HFD-fed normal-weight mouse	28.6	0.61	149.9	35	21	146.2	65.3	0.8	33.5	12.8
HFD-fed overweight mice (n=4)	41.6 ± 4.3	2.63 ± 0.19	241.5 ± 16.6	45.0 ± 8.2	29.6 ± 2.2	170.3 ± 13.5	99.0 ± 10.1	9.6 ± 5.2	61.8 ± 9.9	1.25 ± 0.75

Data for ND-fed and HFD-fed overweight mice except glucose and AST values were obtained from our previous study²⁰. Data are presented as the mean ± SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; HFD, high-fat diet; ND, normal diet; TC, total cholesterol.

relation among adipocyte size, adipose tissue macrophage (ATM) infiltration, and adipocyte death in obese mice and humans^{17, 22–24}. Adipocyte death and ATM infiltration increase the occurrence of crown-like structures. Furthermore, infiltrating ATMs in crown-like structures stimulate the secretion of inflammatory-related cytokines such as tumor necrosis factor α and interleukin-6^{17, 22, 24, 25} and the development of hepatic steatosis^{22, 23}. In the present study, the percentage of crown-like structures decreased among groups in the following order: HFD-fed overweight mice > HFD-fed normal-weight mouse > ND-fed mice (Table 2 and Fig. 1a). This supports the previous findings that HFD feeding leads to increased damage or apoptosis in adipose tissues.

Intake of a HFD induces structural alterations of the liver, including micro- and macrovesicular steatosis. Hepatic steatosis is associated with severe liver diseases (e.g., non-alcoholic steatohepatitis [NASH] and fibrosis)^{26–28}. Steatosis and lobular and portal inflammation have been found to be significantly higher in patients with definite NASH compared with those without NASH²⁶. Significant correlation has been reported between microvesicular steatosis and NASH and fibrosis in human patients and between macrovesicular steatosis and NASH and fibrosis in HFD-fed mice. In our study, the severity of hepatic steatosis decreased in the following order: HFD-fed overweight mice > HFD-fed normal-weight mouse > ND-fed mice (Table 2 and Fig. 1b). These results show that intake of a HFD is associated with an increased risk of hepatic steatosis.

On the other hand, the number of hepatic inflammatory foci in the HFD-fed normal-weight mouse was remark-

ably increased compared with the numbers in the ND-fed mice and HFD-fed overweight mice (Table 2 and Fig. 1b). Serum ALT levels are broadly used as a sensitive biomarker of liver damage or injury^{9, 11, 12–14}. However, several studies have suggested that ALT levels may lack correlation with the severity of a patient's liver disease (advanced fibrosis and NASH)^{10, 29–31}. Bridging fibrosis and cirrhosis have been identified in patients with normal ALT values³¹, and NASH has been found in more than 50% of patients with normal ALT values³⁰. However, another study demonstrated that ALT levels are higher in patients with definite NASH than in those without and higher in patients with mild/moderate and bridging fibrosis than in those with cirrhosis²⁶.

In our study, the serum ALT levels were similar between the one HFD-fed normal-weight mouse and ND-fed mice, but the hepatic inflammatory foci in the HFD-fed normal-weight mouse were remarkably increased compared with those in the ND-fed mice and HFD-fed overweight mice (Table 2 and Fig. 1b). Although it is not clear why the number of inflammation foci was increased in the HFD-fed normal-weight mouse, several studies have reported that mice showing acute or severe hepatic inflammation either do not become obese or show a small weight loss^{32, 33}. Therefore, increased hepatic inflammation in the HFD-fed normal-weight mouse may be associated with the reduction in BW gain. These results indicate that a normal BW or serum ALT value may not guarantee absence of hepatic inflammation and that the HFD-fed normal-weight mouse may potentially be used as an animal model for the study of liver inflammation in patients with normal BWs and/or serum ALT values.

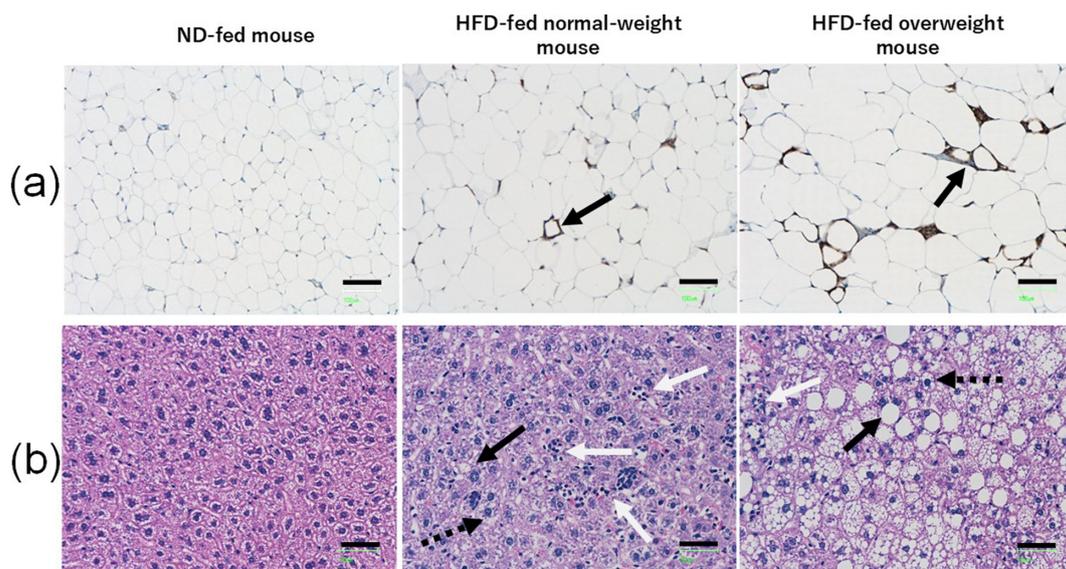


Fig. 1. (a) Representative photomicrographs of F4/80-stained adipose tissue cross sections. The black arrows show typical crown-like structures. Scale bar, 100 μ m. (b) Representative pictures of HE-stained liver cross sections. The black arrows show typical macrovesicular steatosis, the dotted black arrows show typical microvesicular steatosis, and the white arrows show inflammatory foci. Scale bar, 50 μ m. ND, normal diet; HFD, high-fat diet.

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