The effect of various high-fat diet on liver histology in the development of NAFLD models in mice

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Abstract

Objectives: Nonalcoholic fatty liver disease (NAFLD) is exceptionally common around the world. The development of NAFLD is increasing rapidly in the world, along with changes in lifestyle. Excess lipid intake is one of the risk factors for NAFLD. The NAFLD model is induced by a high-fat diet contains SFA, MUFA, and ω-6 PUFA. This study aims to assess the effect of high-fat diet variation on liver histology in developing NAFLD models in mice.

Methods: Thirty-six male mice (Balb/c) were divided into six groups fed a high-fat diet containing beef tallow 60%, beef tallow 45%, vegetable ghee, animal ghee + corn oil, vegetable ghee + corn oil for 28 days and compared to a control group fed a chow diet. All of the mice were fed with a high-fat diet in the form of pellets *ad libitum* for 28 days. Bodyweight and food intake were measured every day. At the last day of treatment, animals were sacrificed and the Liver were taken for histological analysis.

Results: This study showed that NAFLD model development was achieved in all group mice fed a high-fat diet with different degrees of NAFLD. Beef tallow 60% had the worst liver histology.

Conclusions: Thus, based on this study, we found that high-fat diet variations influenced the development of NAFLD models in mice, particularly concerning liver histology.

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Keywords: healthy lifestyle; high-fat diet; liver histology; mice; nonalcoholic fatty liver disease.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition caused by anomalies in fat accumulation within the liver, without excessive alcohol intake. Some of the characteristics of NAFLD include steatosis, fibrosis, inflammation, and ballooning [1]. Pathologically, NAFLD is classified into the nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL is a relatively mild liver disorder, characterized by steatosis without ballooning and inflammation. Meanwhile, NASH characterized by steatosis, ballooning, with or without inflammation [1, 2]. NASH is also an essential factor in hepatocellular carcinoma development [3].

Several studies have suggested that an increase in NAFLD prevalence is related to increased mortality of liver disease [4, 5]. In Western countries, the prevalence of NAFLD reaches 15–30% in the population. This prevalence increases to more than 50% in overweight individuals and increases to more than 90% in obese non-diabetic individuals [6]. It is currently known that lifestyle changes have implications for the massive development of NAFLD in the world. Improper dietary habits, especially excessive fat intake, are categorized as an essential risk factor related to the pathogenesis of NAFLD [7]. However, animal and human studies of the effects of fat intake on NAFLD development are limited [8].

Furthermore, the high-fat diet (HFD) is one approach that is often used to develop NAFLD models in experimental animals. HFD contains excess fat composition (varies between 45 and 75%) and is given *ad libitum* to experimental animals for a certain period [9]. The variation in the type of fat used is significant because *in vitro* evidence has found that different fatty acids have different effects on hepatocytes [10]. Most NAFLD model studies with experimental animals focus on HFD lard, while other fats are also widely consumed in daily life [8]. Thus, the

study of animal models of NAFLD-induced HFD using fats from other sources is an interesting thing to explore.

Some of the ingredients developed as HFD include beef tallow, animal ghee, vegetable ghee, and corn oil. Beef tallow and animal ghee are dominant in the content of saturated fatty acids (SFA), with a percentage of 55.4 and 65% [11]. Meanwhile, vegetable ghee is dominant in the content of monounsaturated fatty acids (MUFA). Corn oil is rich in linoleic acid (ω-6 polyunsaturated fatty acids; ω-6 PUFA), with a percentage of 50.4% [12].

In principle, HFD adopts a diet low in carbohydrates but high in fat. When glucose reserves in the body have depleted, the body uses fat as an alternative energy source from breaking down fatty acids in adipose tissue [13]. Furthermore, free fatty acids diffuse into the circulation, which has implications for increasing fatty acid levels in the blood. These things trigger the uptake of fatty acids in the blood by the liver, causing fat accumulation in the liver [14]. The body requires a variety of fatty acids with diverse degrees of saturation. In PUFA deficiency, the body tries to compensate for its needs by synthesizing these fatty acids from SFA and MUFA. This condition causes the fatty liver to worsen due to the high SFA took up by the liver [15].

The saturation and size of the carbon chains of fatty acids also affect their metabolism in the body. SFA and MUFA tend to be difficult to use as energy as soon as they are absorbed. Therefore, these two types of fatty acids are mostly stored in adipose tissue. However, if the body lacks energy, there is a massive breakdown of fatty acids in the adipose tissue. Meanwhile, PUFAs tend to be immediately oxidized to be used as energy in the body or diverted to other functions, for example making cell membranes [16].

Thus, the variation of HFD and the success of creating HFD significantly determine the NAFLD model. This research conducts a study related to the effect of various types of HFD on liver histology to determine and develop NAFLD models.

Materials and methods

Ethical approval

All experiments on animals were carried out at the Animal Research Laboratory of the Faculty of Pharmacy, University of Airlangga, Indonesia, and refer to the Guidelines for the Care and Use of Laboratory Animals released by the Ethical Committee of the Faculty of Veterinary Medicine, University of Airlangga, Indonesia.

Animal and experimental design

Balb/c male mice weighing about 20–30 g were used in this study. They were housed in chip-bedded plastic cages at room temperature (22 ± 2 °C) in a 12 h diffuse light/12 h dark cycle at the Animal Research Laboratory of the Faculty of Pharmacy Universitas Airlangga. Mice were divided into six groups (n=6), fed a high-fat diet containing beef tallow 60%, beef tallow 45%, vegetable ghee, animal ghee + corn oil, vegetable ghee + corn oil for 28 days and compared to a group fed a chow diet (control group). Mice were fed according to the treatment group with 8–12 g of pellets. The remaining pellets were weighed daily for a food intake evaluation. Also, the body weight of the mice was weighed every day during treatment. All treatments lasted 28 days. The HFD was developed in our laboratory. The final composition and caloric value of HFD and chow diet are present in Table 1.

Histological examination

After 28 days of treatment, all mice in each group were sacrificed, and the liver was extracted. Tissue samples were removed directly post-mortem and fixed in 10% formalin, processed, and embedded in paraffin. Then, 3 µm sections were cut and stained with hematoxylin and eosin. The slides were examined under an optical microscope, and the digital images were captured. The liver's histological features

Table 1: The final composition and caloric value of HFD and chow diet.

HFD composition	Composition, %							Caloric value, kcal/100 g
	Chow diet, 306.20 kcal/100 g	Cornstarch, 360 kcal/100 g	Beef tallow, 902 kcal/100 g	Corn oil, 884 kcal/100 g	Milk, 500 kcal/100 g	Vegetable ghee, 834 kcal/100 g	Animal ghee, 894 kcal/100 g	
Chow diet	100%	N/A	N/A	N/A	N/A	N/A	N/A	306.20
HFD beef tallow 60%	15%	25%	60%	N/A	N/A	N/A	N/A	677.13
HFD beef tallow 45%	35%	20%	45%	N/A	N/A	N/A	N/A	585.07
HFD vegetable ghee	30%	20%	N/A	N/A	N/A	50%	N/A	580.86
HFD animal ghee + corn oil	35%	N/A	N/A	10%	25%	N/A	30%	588.77
HFD vegetable ghee + corn oil	35%	N/A	N/A	10%	25%	30%	N/A	570.77

(including steatosis, inflammation, ballooning) were analyzed using a scoring system based on the NAFLD activity score.

Statistical methodology

Bodyweight profile and food intake profile data were statistically analyzed using Two Way ANOVA and followed by post hoc analysis using the Bonferonni test. All calculations were performed using the GraphPad Prism 6 Software (GraphPad, Inc., San Diego, CA, USA). Bodyweight profile and food intake profile data are represented as mean ± SEM.

Results

The effect of HFD variation on body weight profile of mice

The results obtained showed that after 28 days of treatment, there was an increase in body weight of about 5% in the group of mice fed with the chow diet. Further, the group of mice given HFD animal ghee + corn oil and HFD vegetable ghee + corn oil showed an increase in body weight over 20%. Meanwhile, the group of mice that were given HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee showed a weight loss of more than 20% (Figure 1).

The effect of HFD variation on food intake profile of mice

The food intake profile for each mouse was obtained from weighing the remaining feed (pellets) every day for 28 days. Group 1, as a control group, was given 8–12 g chow diet per day containing 306.2 kcal/100 g. Furthermore, the HFD groups were given a variation of HFD 8–12 g per day with almost the same amount of calories for every 100 g of pellets, respectively: group 2 were given HFD beef tallow 60% containing 677.07 kcal/100 g, group 3 were given HFD beef tallow 45% containing 585.07 kcal/100 g, group 4 were given HFD vegetable ghee containing 580.86 kcal/100 g, group 5 were given HFD animal ghee + corn oil containing 588.77 kcal/100 g, and group 6 were given HFD vegetable ghee + corn oil containing 570.77 kcal/100 g. The results obtained showed that the amount of calories intake in the group of mice fed with HFD was higher than the calories intake in the group of mice fed with the chow diet (Figure 2B).

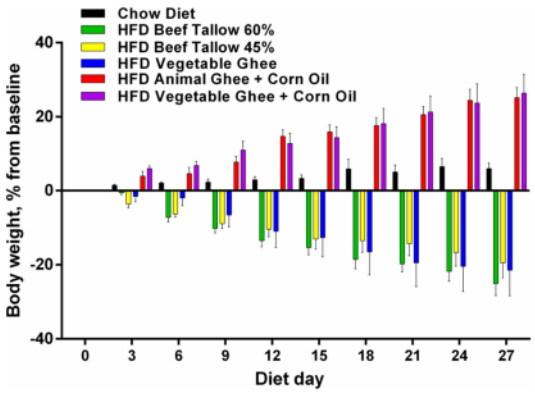

Figure 1: Bodyweight profile of each group of tested animals.

The effect of HFD variation liver histology of mice

Histological examination of the liver was observed under an optical microscope at 400× magnification. Based on microscopic observations, the liver histology of mice fed a chow diet showed nuclei of cells with cytoplasm and sinusoid in a regular pattern around the central vein (Figure 3A). The histology of mice given HFD beef tallow 60% showed macrovesicular steatosis and ballooning accompanied by inflammation that resembled the NASH pattern (Figure 3B). Meanwhile, the histology of mice given HFD beef tallow 45% and HFD vegetable ghee showed an abnormal hepatocyte structure, characterized by microvesicular steatosis, ballooning, and inflammation around the central vein. In both experimental groups, there was also a borderline/probable NASH which is the boundary for NAFL and NASH (Figure 3C, B). Further, in the group of mice given HFD animal ghee + corn oil and HFD vegetable ghee + corn oil showed hepatocytes with mostly normal cell structures, but some cells showed ballooning. Liver histology in these two groups showed a more NAFL-oriented pattern which is the initial stage of NAFLD development (Figure 3E, F).

Discussion

HFD-induced NAFLD is the preferred method in animal studies for reproducing the conditions observed in humans, including the biochemical and histopathological aspects of developing NAFLD [9]. This study evaluates the effects of various types of HFD, including; HFD beef tallow 60%, HFD beef tallow 45%, HFD vegetable ghee, HFD animal ghee + corn oil, and HFD vegetable ghee + corn oil

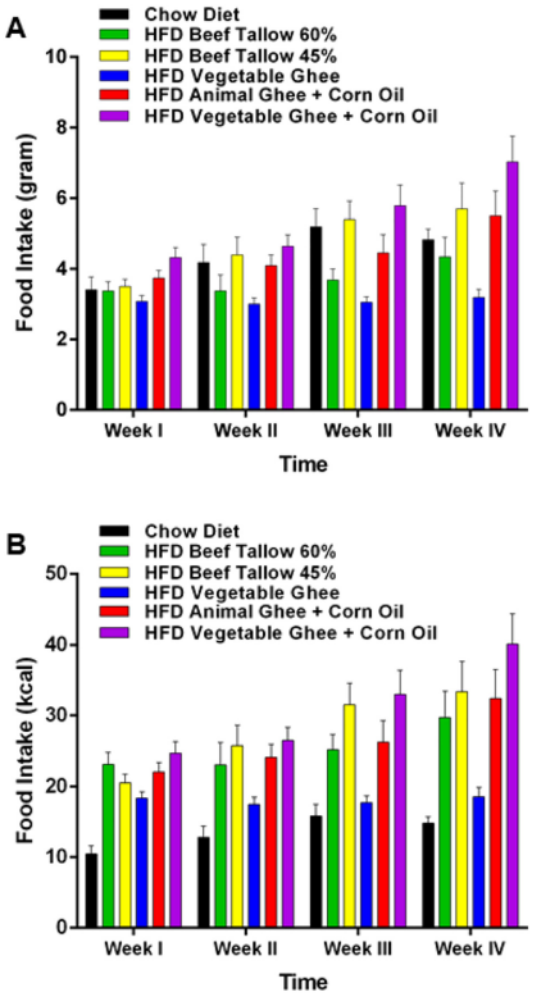

Figure 2: Food intake profile of each group of tested animals for 28 days {units of grams (A), units of calories (B)}.

on the development of NAFLD models in mice. The parameters observed in this study include; body weight profile, food intake profile, and liver histology in mice.

The body weight profile of mice related to the lipolysis and esterification processes in adipose tissue. Triglycerides (TG) in adipose tissue are stored statically but may always undergo two-way reactions, namely lipolysis, and esterification. In this study, mice were given HFD animal ghee + corn oil, and HFD vegetable ghee + corn oil showed an increase in body weight. This finding may be due to the esterification process to form TG beyond lipolysis so that TG accumulates in adipose tissue and causes obesity. The presence of PUFA contained in HFD animal ghee + corn oil, and HFD vegetable ghee + corn oil may also inhibit the breakdown of fatty acids in adipose tissue [14].

In contrast, mice fed with HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee showed weight loss (Figure 1). This finding may be due to the breakdown of fatty acids in adipose tissue and lipolysis that is greater than esterification. Fatty acids formed as a result of partial lipolysis impossible to be re-esterified because they were not kept up with the rate of lipolysis. As a result, free fatty acids that accumulate in adipose tissue immediately diffuse into the circulation, leading to weight loss and increased fatty acids in the blood [14, 17]. The high lipolysis caused by HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee may be due to the dominant SFA and MUFA content so that the body compensates for PUFA deficiency by synthesizing these fatty acids from SFA and MUFA. This circumstance results in a greater breakdown of SFA/MUFA in adipose tissue. Besides, HFD, which is low in carbohydrates, causes reduced levels of glucose and insulin in the blood resulting in a decrease in the amount of glucose that enters the adipocytes. This condition results in reduced esterification and increased lipolysis [17].

Furthermore, the results of liver histology in this study showed that all mice fed HFD develop a NAFLD but to varying degrees. The group of mice that were fed with HFD vegetable ghee + corn oil and HFD animal ghee + corn oil, which were rich in SFA/MUFA + PUFA content, experienced NAFLD with a relatively lower degree than the group of mice fed other types of HFD. These findings were in line with the postulates put forward by several previous studies, where PUFA was shown to induce NAFLD because it causes the accumulation of arachidonic acid in the membrane [18]. However, when PUFA is combined with SFA/MUFA, it was known to prevent insulin resistance and avoid SFA/MUFA-induced endoplasmic reticulum stress and prevent inflammation in the liver [19]. These are probably why the development of NAFLD in these two groups had a lower degree than the group that was fed other types of HFD in this study.

Whereas in mice fed HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee were found to develop a more severe degree of NAFLD. This condition may be caused by the buildup of excess free fatty acids in the adipose tissue and diffuse into the circulation which causes an increase in fatty acids in the blood and is taken up by the liver in large amounts, exacerbating the condition of NAFLD [14, 17]. Furthermore, several studies suggest that the dominant HFD beef tallow contains SFA injure hepatocyte directly through several mechanisms, including; cell death induced stress of endoplasmic reticulum, causing intrinsic mitochondrial apoptosis, as well as triggering inflammatory

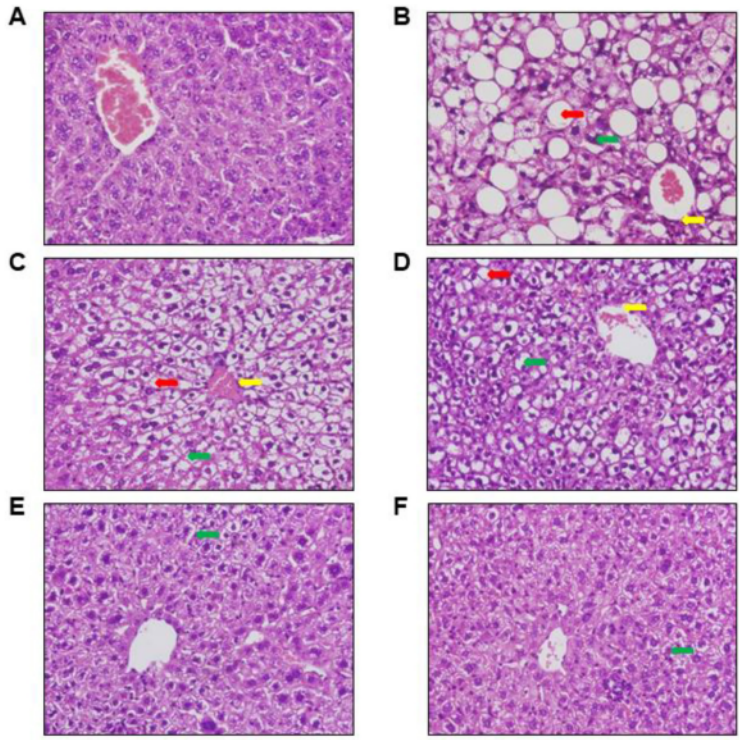

Figure 3: Histological analysis with hematoxylin-eosin staining from the liver revealed damage caused by HFD, according to the type of fat used; (A) control group, (B) beef tallow 60% group, (C) beef tallow 45% group, (D) vegetable ghee group, (E) anima ghee + corn oil group, (F) vegetable ghee + corn oil group. Red arrows indicate steatosis, green arrows indicate ballooning, and yellow arrows indicate inflammation.

mediators (e.g., cytokines) [20]. Besides, SFA also causes an increase in adipocyte oxygen consumption and leads to hypoxia of adipose tissue *in vivo* [21]. Meanwhile, MUFA-enriched HFD vegetable ghee was associated with *in vivo* adipocyte hyperplasia. One study has also reviewed that administration of MUFA causes liver steatosis and inflammation of adipose tissue [22].

Studies conducted in humans have shown that SFA tends to induce steatosis, insulin resistance, and proinflammatory cytokines (such as TNF-α) compared to PUFA [23]. These findings also are attributed to this study, where the histological results of mice fed a combination of HFD (HFD vegetable ghee + corn oil, and HFD animal ghee + corn oil) showed less degeneration of ballooning than the mice fed with vegetable ghee or HFD beef tallow only (Figure 3).

A study examining the SFA and MUFA fats found that these two types of fat cause NASH, which is an advanced stage of NAFLD disease. In addition, it was also found that different compositions of the same fat type influence the development of different NAFLD [18]. These findings are in line with the results of this study, where the experimental group fed with HFD beef tallow 45% (predominantly containing SFA) and HFD vegetable ghee (predominantly containing MUFA) showed a more probable NASH pattern (Figure 3C, D). In comparison, the experimental group fed with HFD beef tallow 60% showed a definite NASH pattern (Figure 3B).

In terms of food intake, this study found differences in the food intake profile between the experimental groups (Figure 2). Several studies have tried to examine the factors that may affect the profile of food intake in mice, including mouse strains, genetic background (transgenic and knockout mice), age, gender, stress and habituation, body composition and body fat distribution, diet composition, and appetite [24]. Among these, factors that may have played a significant role in our study were dietary composition and appetite. Our finding is in line with several previous studies, which revealed that diet composition affects appetite, which results in a change in the food intake profile of experimental animals. On the other hand, the variations in the types and characteristics of protein in the diet also produce different metabolic and physiological responses. This condition affects the digestion kinetics and digestibility where it is strongly associated with the resulting satiety [25]. Interestingly, in our study, we also found that each group's food intake profile tended to increase with each week. This condition may be due to

ad libitum feeding, which stimulates caloric intake in rodents over time [26].

In particular, several studies reported that MUFA caused more severe NAFLD compared to SFA at the same calorie amount [22]. Interestingly, in our research, we found that the SFA-enriched HFD beef tallow 45% tended to have the same severity as MUFA-enriched HFD vegetable ghee. This finding may be attributed to the total calories intake of the group of mice fed with HFD beef tallow 45%, which tended to be grated than the total calories intake of the group of mice fed with HFD vegetable ghee (Figure 2B), although the HFD of beef tallow 45% and HFD vegetable ghee had the same total calories per 100 g. Thus, apart from HFD composition, the proportion of HFD intake may also influence the severity of NAFLD development in mice.

Conclusions

The development of the NAFLD model could be achieved in all groups of mice, where the liver histology level was the most severe, respectively; HFD beef tallow 60%, HFD beef tallow 45% and HFD vegetable ghee, and HFD animal ghee + corn oil and HFD vegetable ghee + corn oil. The results of this study may contribute to the development of the NAFLD model as an essential approach to studying the pathological state of disease. Apart from that, it may also be useful to discover and develop relevant therapeutic agents for this disease.

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