FORMULATION AND NUTRITIONAL EVALUATION OF HIGH-FAT DIET FEED FOR FATTY LIVER INDUCEMENT IN RODENT MODELS

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ABSTRACT

Non-alcoholic fatty Liver Disease (NAFLD) is one of the most prevalent lipid disorders worldwide. In the pursuit of drug discovery and development, animal modeling is crucial to elucidate the mechanisms underlying a disease. One form of animal model of NAFLD involves the induction of a high-fat diet (HFD). However, most HFDs exhibit complexity in the formulation, and commercial HFDs tend to be costly. This study aimed to formulate an HFD feed with minimal ingredients that are capable of inducing liver steatosis. This study commenced with the development of 4 dietary formulations (F1, F2, F3, and F4) comprising various food components with specific proportions and treatments. A proximate analysis was conducted for each formulation. Subsequently, the most promising formulation was administered to the rats for 14 days. On day 15, liver organs were harvested for histological analysis. The results showed that among the four formulations developed, HFD F4 exhibited the best physical appearance and nutritional proximate components, characterized by its stability in form and high fat (41.8%), high protein (21.5%), and low carbohydrate (23.2%) content. Histology examination revealed that two weeks of administration of F4 administration led to severe degeneration, inflammation, and necrosis in rat hepatocytes compared to the normal group ($p < 0.001$), thus confirming that HFD F4 may be developed as an inducer of steatosis in rats.

Keywords: experimental animal model; high-fat diet; liver steatosis ; NAFLD

INTRODUCTION

Unhealthy lifestyle patterns are among the most influential factors contributing to various health issues worldwide, including diabetes mellitus (DM), cardiovascular diseases (CVD), and obesity. The inclination towards consuming fatty and high-sugar foods is prevalent in different parts of the world, evident from the proliferation of stores offering junk food and beverages with high sweetness levels (Farhud, 2015; Song et al., 2023). Non-alcoholic fatty liver disease (NAFLD) is associated with a high-fat diet. NAFLD is a condition for which treatment is currently undergoing clinical trials. The complexity of identifying NAFLD drugs is attributed to the intricate pathogenesis of NAFLD, which involves various proteins in the metabolic process, including SIRT1 and AMPK (Anggreini et al., 2023a; Yoo et al., 2019).

Animal modeling is a crucial stage that supports the search and development of new drugs (Domínguez-Oliva et al., 2023). Various approaches have been developed in animal models to optimize NAFLD conditions, including methods involving genetic modification, the use of chemicals such as streptozotocin and carbon tetrachloride (CCl_4), and, most commonly, dietary interventions. Diets employed to induce NAFLD exhibit significant

variations, ranging from western diets, atherogenic diets, high-fat diets, high-fructose diets, and others (Van Herck et al., 2017).

The diet consumed encompasses a diverse range of nutrients, and has beneficial effects on the body. However, excessive feed consumption may trigger the onset of a disease. A high-fat diet (HFD) is a hallmark of the development of animal models of NAFLD. Various foods, including nuts, eggs, and oils, can serve as sources of fat (Lian et al., 2020). One fat frequently utilized in cooking is beef tallow. Therefore, the present study involved the formulation of a high-fat diet using beef tallow as the primary fat source, combined with other food ingredients, followed by an assessment of the best feed formula to induce NAFLD or liver steatosis in animal models.

RESEARCH METHODS
Equipment and Materials
The chemicals used in the present study were analytical grade ethanol (Merck), chloroform (Merck), and standard chow BR II (PT. Wonokoyo Jaya Indonesia), homemade beef tallow, palm oil (Sania), egg yolk, egg white, liquid fructose (Rose Brand), powdered fructose (Pudak Scientific), hematoxylin (Merck), and eosin (Merck). The tools used included an oven (Krisbow), Soxhlet (Pyrex), furnace, and Pro-Histo Microscope.

Research Procedure
1. Formulation of high-fat diet (HFD)

In the present study, four different formulas (F1-F4) presented in Table I, were prepared. The first step in preparing a high-fat diet was the melting process of the beef tallow by heating it in an oven at 200 °C for 10 – 20 minutes, and adding powdered standard chow, followed by the addition of other ingredients such as eggs, wheat bran, wheat flour, fructose, and palm oil, to complement the nutritional composition of the feed formula. The feed was prepared by blending these ingredients, and the finalized feed underwent the last stage of solidification and was compressed to make pellets (weighing 2-3 g per pellet) and further processed differently for each formula using two methods: heating and freezing (Table I).

Table I. Formula of High Fat Diet

Ingredients	F1	F2	F3	F4
Standard chow	-	100 g	100 g	100 g
Wheat bran	100 g	-	-	-
Wheat flour	70 g	q.s	q.s	q.s
Bean flour	57 g	-	-	-
Beef tallow	70 g	100 g	100 g	100 g
Egg yolk	70 g	25 g	25 g	25 g
Egg white	-	8 g	8 g	8 g
Palm oil	40 mL	7 mL	7 mL	7 mL
Liquid fructose	-	25 g	25 g	25 g
Powdered sucrose	40 g	-	-	-
Final Process	Roasted at 150°C for 10 minutes	Roasted at 150°C for 10 minutes	Roasted at 100°C for 10 minutes	Frozen and stored at 4 – 8°C

2. Proximate analysis
   a. Fat content

   The fat content was determined using the Soxhlet method (Ni'Mah & Ameswari, 2019). Feed samples (5 g) were extracted using petroleum benzene (150 mL) in Soxhlet flasks. The filtrate was then evaporated to separate the solvent. The fat was heated in an oven at 85 °C for 15 minutes, cooled, and weighed.

b. Protein content

Proteins were analyzed using the Kjeldahl method (Ni'Mah & Ameswari, 2019). Feed samples (0.1 g), sulfuric acid (2.5 mL), and Kjeldahl catalyst (1 tablet) were placed in a Kjeldahl flask, destroyed for 2 hours at 85 °C, and then cooled. Distilled water (50 mL), sodium hydroxide (50 %; 10 mL), and 5 pieces of boiling chips were then added, and the mixture was further distilled. The distillate was placed in an Erlenmeyer flask containing 10 mL of boric acid and the indicator solution (methyl red and methyl blue) and was titrated using 0.02 N a standard hydrochloric acid solution. The volume of the hydrochloric acid solution at the end of titration was recorded, and the protein content was calculated.

c. Water content

Water content was measured using the gravimetric method (Ni'Mah & Ameswari, 2019). The porcelain cup was prepared by heating it in an oven at 105 °C for 30 minutes, cooling it in a desiccator, and weighing it. A feed sample (10 g) was placed in a porcelain cup and heated in an oven at 105 °C for 5 hours. The difference between the initial and final weights was calculated as water content.

d. Ash content

The ash content was measured by the gravimetric method (Ni'Mah & Ameswari, 2019), similar to that of the water content analysis, with a difference in the heating temperature at 450 °C for 1 hour (first step) and 600 °C (second step) until the feed ash and the result were weighed.

e. Carbohydrate content

Carbohydrate content was analyzed using the by-difference method with the following formula:

% carbohydrate = 100 - % content of (water + ash + fat + protein).

3. Modelling for NAFLD

This animal study was conducted according to the 3Rs Principles of the ARRIVE Guidelines (https://arriveguidelines.org/) and approved by the Ethical Committee of the Faculty of Pharmacy, Mulawarman University (No. 062/KEPK-FFUNMUL/EC/EXE/05/2023). The procedures were carried out at the Laboratory of Pharmaceutical Research & Development for Pharmaka Tropis of Mulawarman University, Samarinda, Indonesia. Male (6-8 weeks old) DDY mice (n = 6) were randomly divided into 2 groups: (1) normal group and (2) high-fat diet (HFD) group. All mice were maintained in a controlled environment at 25 °C, following a 12:12 hour light/dark cycle, with unrestricted access to food and water.

Mice in the normal group received a standard diet composed of ground wheat, corn, rice, and soybean, whereas the HFD group received the best HFD feed formula. Feeding was performed daily for 14 days. On the final day, the mice were sacrificed, and the livers were collected for histology analysis.

4. Histological examination

To assess the success of the feed in inducing NAFLD, an analysis was conducted on the liver organs of the mice. The liver harvested on day 15 was immersed in 10% neutral-buffered formalin. Subsequently, they underwent paraffin embedding and were thinly sliced to a thickness of approximately 4 μm using a microtome. The liver was stained with hematoxylin-eosin (H&E), and further morphological analysis, as well as the determination of NAFLD activity score (NAS) using a Pro-Histo Microscope at 5 fields of view at 400x magnification. Ballooning degeneration, inflammation, and necrosis were scored as follows: 0: if absent, 1: if <25%, 2: if 25-50%, 3: if 50-75%, and 4: if >75%.

Data Analysis

Statistical analysis was performed using a T-test. Statistical analyses and graph creation were performed using GraphPad Prism (version 7).

RESULTS AND DISCUSSION

The proximate analysis and texture of the HFD

The primary approach to animal models of NAFLD involves utilizing diet as an inducing agent. Beef tallow was used as the predominant fat source in this study. Proximate analysis revealed a reduction in fat content in chow subjected to heating (F1-F3) compared to those not subjected to the heating process (F4). The proximate analysis of the four formulas of the HFD is presented in Table II. HFD F4 was selected as the best HFD formula, as it revealed the highest content of fat (41.8%) and protein (21.5%), and the lowest carbohydrate content (23.2%).

Table II. The proximate analysis of different formulas of HFD

Formula	Proximate analysis (%)				
	Fat Content	Protein Content	Carbohydrate Content	Ash Content	Water Content
F1	29.2	20.3	37.7	0.5	12.0
F2	20.4	20.4	39.63	0.5	9.2
F3	29.3	20.4	39.1	0.5	10.4
F4	41.8	21.5	23.2	0.5	12.8

Differences were observed in the appearance of the formulated chows. The pellets were dark brown with a mushy texture prior to the final process. The heating process (150 °C and 100 °C) of F1 – F3 changed the consistency to harder, crunchy, and sticky because of the melting of the fat. Conversely, HFD F4, which was cold-prepared by freezing at 4 – 8 °C, showed a hard and stable texture (Figure 1).

Figure 1. The texture of HFD F4

Beef tallow is a solid fat at room temperature owing to its high content of saturated fatty acids, namely stearic acid and palmitic acid (Limmatvapirat et al., 2021). Beef tallow is a popular animal fat source used in cooking, and stearate and palmitate fatty acids dominate its composition. Compared with other animal fats, beef tallow has a higher saturated fatty acid content, approximately 55-68%, compared than chicken fat or lard (32-67%) (Naquiah et al., 2013).

Saturated fatty acids have a higher melting point than unsaturated fats. Upon heating, beef tallow becomes liquid, causing leakage of its fat components in formulas F1-F3. However, no significant differences were observed in other macronutrients, such as proteins and carbohydrates, among the four formulas. Based on these findings, we propose that HFD F4 is the best considering its structural characteristics, protein, and fat content.

Histological examination

Based on the proximate analysis and texture of the HFD, formula 4 (F4) was selected for further analysis to evaluate the ability to induce hepatic steatosis. Mice (n = 3) were fed HFD F4 for 2 weeks, and their livers were examined. Our research shows that the administration of HFD F4 for 2 weeks can trigger hepatic steatosis in experimental mice (Figure 2). HFD F4 induces changes in liver morphology, characterized by inflammation, ballooning degeneration, and necrosis. The results indicated that hepatocyte cells in the normal group exhibited no abnormalities, with inflammation occurring in less than 25% of cases. In contrast, the High-Fat Diet (HFD) showed evidence of ballooning degeneration (red arrow), necrosis (yellow arrow), and inflammation (blue arrow) in the liver cross-section at 400x magnification (Figure 2).
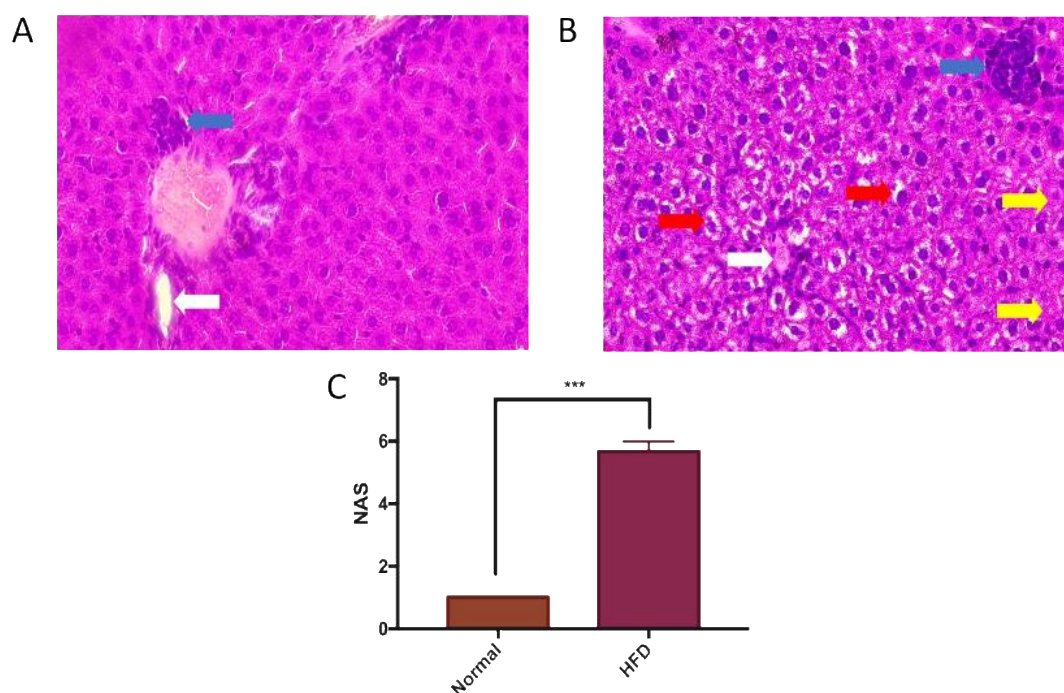

Figure 2. *The liver morphology of (A) the normal group and (B) the HFD group and (C) the NAFLD activity score (NAS) revealed a significant difference between the two groups.* **The NAS was analyzed using unpaired t-tests. Each bar represents the mean ± SEM of 3 mice. ***p < 0.001. White arrow = central vein. The blue arrow indicates the inflammation. Red arrow = ballooning degeneration. Yellow arrow = necrosis**

We conducted NAS analysis to obtain insights into the likelihood of NAFLD occurrence in animals fed a HFD. These results indicate that the HFD group had a significantly higher NAS score than the normal group, based on unpaired t-test analysis. According to NAS, the HFD group was categorized as non-alcoholic hepato-steatosis (NASH). NASH is a severe condition within NAFLD that is characterized by inflammation, fibrosis, and necrosis in hepatocytes (Pouwels et al., 2022). NASH is distinguished by the presence of lipid droplets in hepatocytes and inflammation (Figure 2b). A previous study showed that the administration of a HFD with 60% fat content induced NASH conditions in mice fed this diet for 12 weeks (Sabir et al., 2022). Other studies combining a HFD with fructose and glucose diets for 8 to 16 weeks showed an increase in BW, changes in lipid profiles, and the occurrence of NASH in experimental mice (Liu et al., 2018).

Fat entering the body undergoes a metabolic process, with a portion converted into energy or utilized for other physiological functions, while the rest is stored as triglycerides in organs, such as the liver and muscles (Chandel, 2021). However, continuous and excessive fat intake can lead to disturbances in lipid metabolism, resulting in various conditions, such as dyslipidemia, obesity, DM, CVD, and liver damage (Natesan & Kim, 2021).

Saturated fatty acids play a crucial role in the development of hepatic steatosis through lipotoxic processes that cause cell dysfunction, activation of the endoplasmic reticulum (ER) stress pathway, reactive oxygen species (ROS), oxidative stress accumulation, and dysregulation of mitochondrial metabolism (Leamy et al., 2013). ER stress activates the unfolded protein response (UPR): (1) inositol-requiring enzyme 1 (IRE1), (2) PKP-like endoplasmic reticulum kinase (PERK), or (3) activating transcription factor 6 (ATF6). In association with NAFLD development due to high-fat and high-carbohydrate intake, both IRE1 and PERK branches are involved in different patterns (Zhou et al., 2020).

Several studies have indicated that using beef tallow as a fat source in diets can trigger dyslipidemia, inflammation, and steatosis (Sabir et al., 2022; Yustisia et al., 2022). Furthermore, the fructose content of HFD F4 expedited hepatic steatosis. In addition to fatty foods, high-sugar diets also lead to fat accumulation in the liver through de novo lipogenesis, where sugars, such as fructose and sucrose, are converted into fatty acids and stored as triglycerides in hepatocytes (Anggreini et al., 2023b). Recent studies have suggested that high intake of fat and carbohydrates induces dynamic changes in autophagy and hepatic ER stress (Chan et al., 2013; Nakamura et al., 2012; Ren et al., 2012; Wang et al., 2015; Zhang et al., 2020).

Eggs were also used as a source of fat and protein in the formula. Clinical research using cohort methods showed that there is a debated association between egg consumption and the incidence of CVD and changes in lipid profiles (Mohseni et al., 2023; Sugano & Matsuoka, 2021). Palm oil containing palmitic fatty acids is another fat source in our formulation. Previous studies have shown that the consumption of palmitic fatty acids for 6 weeks leads to weight gain, hyperglycemia, steatosis, and hepatic injury in zebrafish (Park et al., 2019). Cholesterol-enriched HFD administered to mice for 6 months showed a notable increase in hepatic steatosis owing to its effect in inducing inflammation and fibrogenesis (Ioannou et al., 2017). Another study showed that long-term HFD loading in C57Bl/6J mice resulted in obesity and insulin resistance and induced NASH and hepatic tumorigenesis (Nakamura et al., 2012).

Furthermore, human studies have revealed that a high intake of fructose is positively correlated with the prevalence of insulin resistance and DM (Aeberli et al., 2013; Tappy & Le, 2010). Healthy, normal-weight male adult participants were given four different drinks for 3 weeks, which contained medium fructose at 40 g/day and high fructose, high glucose, and high sucrose each at 80 g/day. It has been reported that moderate amounts of fructose and sucrose significantly alter hepatic insulin sensitivity and lipid metabolism compared to similar amounts of glucose (Aeberli et al., 2013).

Taken together, the combination of several ingredients applied in HFD F4 offers the potential to serve as an easily accessible and rapid inducer of hepatic steatosis in experimental animals.

CONCLUSION

The importance of modeling in animal experiments must be supported by methods that closely resemble real-life situations. Our research aimed to formulate a high-fat diet capable of creating an animal model of lipid disorders, particularly non-alcoholic fatty liver disease (NAFLD). Our study indicated that the use of a high-fat diet with the proposed formula (HFD F4) induced steatosis in mice exposed to HFD for 14 days. However, we are still unaware of the impact of HFD on blood lipid levels, which constitutes a limitation of this study. Further research is required to support the development of this high-fat formula.

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