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Effect of High-Fat Diet Variations on Cholesterol Levels and Hepatic Histopathology in Mouse Models of Type 2 Diabetes Mellitus

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Abstract
Excessive consumption of fatty foods causes lipid accumulation, leading to hypercholesterolemia and type 2 diabetes mellitus. This study aims to determine the effect of a high-fat diet with various fat types together with alloxan induction on cholesterol levels and hepatic histopathology. This study used a completely randomized design (CRD); twenty male mice were divided into five treatment groups, namely KK (standard feed), KP (placebo), P1 (vegetable oil), P2 (beef tallow), and P3 (goat tallow). The feed, fat, and duck egg yolk ratio was 80%:15%:5% given ad libitum at a dose of 5 gram/day for eight weeks, followed by alloxan injection to induce diabetes mellitus in mice. Liver histopathology were prepared using paraffin method and Hematoxylin-eosin (HE) staining. Hepatocytes were assessed based on inflammatory damage, swelling, and steatosis. Total cholesterol levels were analyzed by ANOVA, followed by Duncan's test. Kruskal-Wallis test followed by Mann-Whitney was used to analyzes liver histopapathology. Total cholesterol levels of P2 had drastic increased every week. Hepatic histopathology showed significant differences between groups. It can be concluded that a high-fat diet using beef tallow had the highest effect to type 2 diabetes mellitus in regards to total cholesterol levels at 132±4.24 mg/dL and hepatic histopathology, resulting in steatosis.

Keywords: beef tallow; goat tallow; vegetable oil; steatosis; diabetes.

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterised by impaired insulin secretion, leading to elevated blood sugar levels (hyperglycaemia) (Budianto *et al.*, 2022). Lifestyle is one of the primary risk factors for type 2 diabetes mellitus (T2DM), with most patients having an unhealthy lifestyle. A diet high in fat and calories, often resulting from fast food consumption, can lead to energy imbalance, thereby triggering overweight and obesity (Evan *et al.*, 2017; Moreno-Fernandez *et al.*, 2018).

Obesity is a health condition caused by excessive fat accumulation that can disrupt bodily functions and lead to health issues (Aswad *et al.*, 2022). Excess fat storage in the body, particularly in adipose tissue, triggers adipocyte hypertrophy, systemic inflammation, cellular dysfunction, and reduced pancreatic β-cell response and insulin sensitivity. (Handari *et al.*, 2023; Ruze *et al.*, 2023). Disrupted insulin pathway and elevated cholesterol levels in the body potentially lead to hepatic steatosis or fatty liver and liver damage (Delfina *et al.*, 2021).

Lipid accumulation in the liver can originate from de novo lipogenesis, esterification of free fatty acids (FFA), or increased dietary fat intake (Softic *et al.*, 2016). Triglyceride accumulation in the blood is closely associated with lipoproteins, as lipid transport in the blood by proteins that bind to fats is referred to as lipoproteins (Laila *et al.*, 2022). If the level of lipoproteins in the blood is high, it will cause high cholesterol levels. An increase in LDL levels by HDL can lead to atherosclerosis, which is the blockage of arterial walls, increasing the risk of heart attacks and strokes (Anggraeni *et al.*, 2021). Lipid accumulation in the liver can be caused by high triglyceride levels with low HDL levels, leading to non-alcoholic fatty liver disease (NAFLD) (Deprince *et al.*, 2020). Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of fat in liver cells in patients without excessive alcohol consumption.

Efforts to develop effective treatment strategies for type 2 diabetes mellitus require animal models that mimic key aspects of human disease. One of the most commonly used animal models for this research is the mouse (*Mus musculus*). Mice are mammals with physiological and biochemical characteristics similar to humans, particularly in the reproductive, respiratory, and circulatory systems

(Fadilah *et al*., 2022). The selection of animal models is done to align with the pathology of the disease being studied, as animal models play a role in understanding the pathogenesis of diabetes through the combination of these characteristics and functionalities (Al-Awar *et al.*, 2016).

High-fat diet induction commonly uses various lipids from animal or plant for example vegetable oil, beef tallow, and goat tallow. According to Husnah *et al.* (2020), the unsaturated fatty acid content of liquid palm oil consists of 5–14% linoleic acid, 38–50% oleic acid, and 1% linolenic acid, while the saturated fatty acids are composed of approximately 1% lauric acid, 1–2% myristic acid, and 4–10% stearic acid. In a study by Ginting *et al.* (2022), 100 g of goat tallow was found to contain 320 mg of cholesterol. Davis *et al.* (2022) explained that cattle have several types of fat contents in raw intramuscular fat, composed of around 46% saturated fatty acids (SFA), 46%monounsaturated fatty acids (MUFA), and 7% polyunsaturated fatty acids (PUFA). Research on high-fat diet variations has been done in animal model studies of non-alcoholic fatty liver disease (NAFLD), with results showing that beef tallow caused hepatic steatosis and high triglyceride levels (Rahmadi *et al.*, 2021).

However, different lipids with different characteristics resulted in different effects on the body. Research on high-fat diet variations has been used in animal model studies of NAFLD, with results showing that beef oil can cause liver steatosis and high triglyceride levels (Rahmadi *et al.*, 2021). According to research by Aminah and Qomariyah (2023), goat tallow mixed with 20% egg yolk in the diet, induced by alloxan, was able to induce hyperglycaemia and hypercholesterolemia in mice.

This study was designed to evaluate the effects of different types of fat in a high-fat diet for T2DM induction on total cholesterol levels and histopathological conditions of liver in mice. By using an animal model, it is expected that clearer information can be obtained regarding the impact of each fat source on the development of dyslipidaemia and histopathological changes in the liver. The primary objective of this study is to determine the differences in the effects of vegetable oil, beef tallow, and goat tallow in a high-fat diet on total blood cholesterol levels and liver histopathology, particularly steatosis, inflammation, and ballooning hepatosis in T2DM mice models.

MATERIALS AND METHODS

This study is an experimental study with a completely randomised design. Mice were divided into five treatment groups; the sandard feed group (KK) as normal control, the placebo group (KP) which was given 95% feed + 5% duck egg yolk, the vegetable oil group (P1) given 80% standard feed + 15% vegetable oil + 5% duck egg yolk, the beef tallow group (P2) given 80% standard feed + 15% beef tallow + 5% duck egg yolk, and the goat tallow group (P3) given 80% standard feed + 15% goat tallow + 5% duck egg yolk. The preparation of the high-fat diet (HFD), animal acclimatisation, HFD and alloxan induction, data collection, and animal care were carried out at the Animal Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Surabaya State University. Processing and evaluation of liver histopathology were conducted in the Microtechnique Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Surabaya State University.

The vegetable oil used in the study was palm oil (Frybest), commonly used for frying food. Goat and beef tallow purchased from the market and heated separately until melted. The oil was then stored in a refrigerator and heated beforehand before use. Induction of HFD was performed for 8 weeks until mice developed hypercholesterolemia.

The mice *(Mus musculus)* used were male from Deutsch Denken Yoken (DDY) strain, aged 6–8 weeks, weighed 25–30 grams each. Mice was obtained from the Veterinary Pharmaceutical Centre (PUSVETMA) in Surabaya. Mice were checked first of its health, indicatted by normal fur, clear eyes, expected behaviour, and no physical defects. Before treatment, the mice were acclimatised for 7 days in plastic cages measuring 46x30x12 cm with wire mesh lids, lined with rice husks, and containing 4 mice. All mice in each group were fed 20 grams of feed per cage in the morning, and drinking water was provided ad libitum until the study was completed.

After acclimatization, all mice were weighed, and their total cholesterol and fasting blood sugar levels were measured. Feed was first blended and oven-dried at 60^{o} for 3 days. Feed for HFD consisted of a mixture of 800 grams of ground BR 1 COMFEED feed, 15 grams of oil or fat (vegetable oil, beef tallow, goat tallow), and 5 grams of egg yolk, with 20 grams administered daily over eight weeks. The standard group was only given ground BR 1 COMFEED feed, and the placebo group was given a mixture of 995 grams of ground BR 1 COMFEED feed with 5 grams of duck egg yolk. Every week, all treatment groups were weighed and their total cholesterol levels were measured. In groups other than the normal control group, alloxan dissolved in 0.1 M sodium citrate buffer was administered intraperitoneally at a dose of 100 mg/kgBW (Zuhriyah *et al.*, 2021). Total cholesterol were measured

using cholesterolmeter (EasyTouch GCU) from peripheral blood. Mice are considered hypercholesterolemic if they have a cholesterol level of ≥ 130 mg/dL (Saputri *et al.*, 2017).

After eight weeks of HFD induction and 21 days after alloxan injection, mice were anesthetized and dissected to harvest the liver. Before removal, the organ was washed with NaCl and placed in 10% NBF for fixation. After fixation, the liver was washed with running water overnight. Dehydration was performed by placing the organs in 70% alcohol (4x), 80% alcohol (2x), 90% alcohol, 96% alcohol, and 100% alcohol for 30 minutes in each concentration. The clearing process is performed by immersing the organ in xylol I for 15 minutes and xylol II for overnight. Infiltration begun with Paraffin: xylol (1:1) for 30 minutes, followed by three phases ofparaffins, each for 1 hour. Then, for the embedding process, the organ was placed in a mould filled with paraffin liquid and left until the paraffin hardened. After that, sectioning was performed using a microtome with a thickness of 4–5 µm. The organ sections were put on bject glass coated with Mayer's albumin. The sections were placed in an oven (50°C) for at least 2 hours. After that, staining was performed using Hematoxylin-Eosin (HE) stain (Khaleyla *et al.*, 2021). Slides were observed under a light microscope. Liver damage was assessed using the scoring system based on Fitmawati *et al.* (2018) and Liem *et al.* (2023). Data were analysed using statistical tests by using SPSS softwaree.

RESULTS

The high-fat diet (HFD) was administered for eight weeks, and the increase in total cholesterol levels for each treatment during the eight weeks are presented in Figure 1.

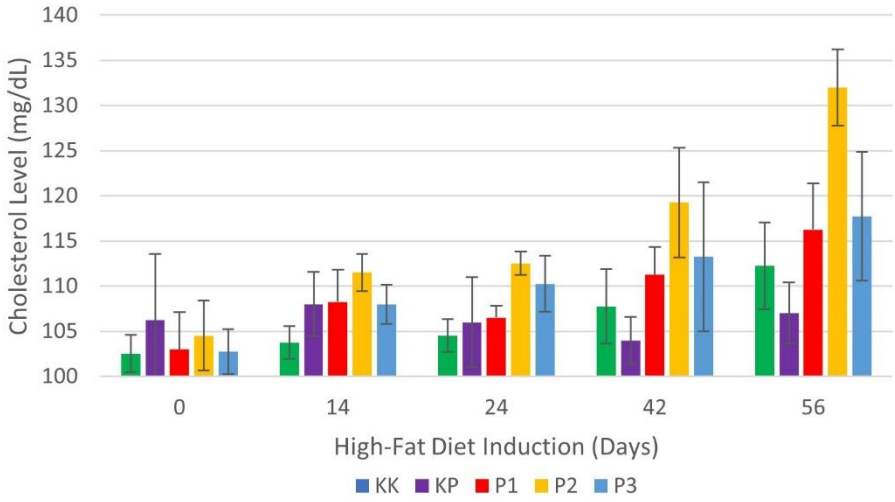

Figure 1. Total cholesterol level increase of each group. Description: KK = normal group, KP= placebo group, P1 = vegetable oil group, P2 = beef tallow group, P3 = goat tallow group.

Based on Figure 1, during the eight-week high-fat diet induction period, each treatment group showed different trends in cholesterol level increases. The KK group showed a gradual increase with a slight but stable rise. The KP group showed an unstable increase in total cholesterol levels and exhibited fluctuations in total cholesterol levels. The P1 group showed gradual increase, where total cholesterol level continued to rise until day 56, at 116.25±5.12 mg/dL which was less than hypercholesterolemic range. Group P2 experienced a sharp and consistent increase in cholesterol levels, with a surge occurring gradually with total cholesterol levels reached to 132±4.24 mg/dL, the highest cholesterol level among all treatments and reached hypercholesterolemic range. The increase pattern in P3 was similar to that in P1 with cholesterol level just slightly higher from P2 (Figure 1).

The KK group was not induced with alloxan and served as the normal control group. The KP, P1, P2, and P3 groups were induced with alloxan at 100 mg/kgBW after HFD. Cholesterol levels on day 7 were normally distributed ($p>0.05$). There was a significant difference between KP and P2 and P3, while P1 differed significantly from P2 and P3 (Table 2). Cholesterol levels on day 14 after alloxan induction ranged from 118±7.37 mg/dL to 133.5±6.45 mg/dL and on day 21 after alloxan induction ranged from 115 ± 10.42 mg/dL to 130.25 ± 4.50 mg/dL (Table 2). Duncan test showed a significant difference between KP and P3, while P3 differed significantly from KP, P1, and P2 on day 14 and day 21 there were significant differences between KP and P1, P2, and P3 (Table 2).

Tabel 2. Average cholesterol levels in post-aloxan mice on days 7, 14, and 21.

Group	Cholesterol Level Post-Alloxan (mg/dL)			
	H-0	H-7	H-14	H-21
KK	112.25 ± 5.05[a]	114 ± 5.05[a]	111.25 ± 5.05[a]	114.25 ± 4.21[a]
KP	107 ± 3.37[ab]	116 ± 709[a]	118 ± 7.37[a]	115 ± 10.42[a]
P1	116.25 ± 5.12[b]	119 ± 829[a]	126 ± 13.39[ab]	125.5 ±6.95[b]
P2	132 ± 4.24[c]	131.25 ± 3.95[b]	128 ± 3.56[ab]	132.75 ± 4.11[b]
P3	111.75 ± 7.14[b]	131 ± 8.76[b]	134.25 ± 5.19[c]	124.5 ±4.80[ab]

Description: KK = Normal group, KP = Placebo Group, P1 = Vegetable Oil Group, P2 = Beef Tallow Group, P3 = Goat Tallow Group. Different notations indicate significant differences between groups ($p<0.05$).

Based on Table 3, steatosis was only found in P2 and P1, at 0.8±1.15 and 0.1±0.37. Steatosis observed in tissue was not stained by HE because part of the cytoplasm was replaced by fat vacuoles. Scoring of inflammatory in liver shows that the range of cell damage due to inflammation is from 0.15 ± 0.37 to 1.4 ± 0.75 (Table 3.). Inflammation itself occurs because the body is in the inflammatory stage. On the other hand, the lowest ballooning was observed in KK and the highest in P2, with average score of 0.00 ± 0.00 to 1.1 ± 0.79. Based on the results of the one-way ANOVA test, significant effect of the administration of kedondong leaf extract on the reduction of total cholesterol levels was found ($p<0.05$).

Figure 2 shows, necrosis and cellular degeneration from all treatment groups. The extent of damage was less in the KK and KP groups compared to P1, P2, and P3. However, inflammation was more pronounced in the KP group compared to the KK group. In the P1, P2, and P3 groups, ballooning cells characterized by enlarged hepatocytes with pale cytoplasm due to the continuous accumulation of fat were also found. On the other hand, steatosis only occurred in the P2 and P1 groups, indicating severe damage caused by the lysis of hepatocytes due to massive fat accumulation, resulting in the formation of vacuoles. Binucleated cells were also observed, where a single cell contained two nuclei.

Tabel 3. Liver histological score from each treatment group

Group	Histological score		
	Steatosis	Inflamation	Ballooning
KK	0.00 ± 0.00[a]	0.15 ± 0.37[a]	0.00 ± 0.00[a]
KP	0.00 ± 0.00[a]	0.60 ± 0.75[a]	0.45 ± 0.51[b]
P1	0.1 ± 0.37[a]	1.4 ± 0.75[b]	0.75 ± 0.72[b]
P2	0.8 ± 1.15[b]	1.05 ± 0.89[ab]	1.1 ± 0.79[b]
P3	0.00 ± 0.00[a]	0.9 ± 0.83[ab]	0.50 ± 0.51[b]

Description: KK = normal control, KP = Placebo Group, P1 = Vegetable Oil Group, P2 = Beef tallow Group, P3 = Goat tallow Group. Different notations indicate significant differences between groups based on statictical test ($p<0.05$).

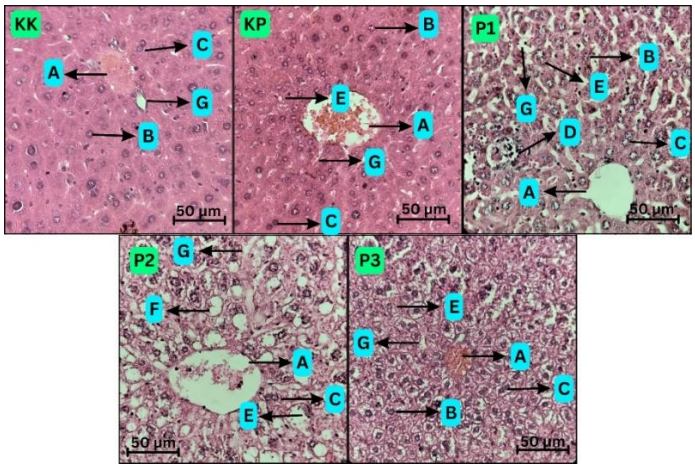

Figure 2. Histopathological findings of the liver in the treatment groups. KK= normal group, KP= placebo group, P1 = vegetable oil group, P2 = beef tallow group, P3 = goat tallow group. A: central vein; B: normal hepatovytes; C: binucleated cells; D: inflammatory cells; E: ballooning cells; F: steatosis; G: Sinusoid

DISCUSSION

Induction of HFD for 56 days and injection of alloxan at 100 mg/kg bw increased total cholesterol levels in the P1, P2, and P3 treatment groups. The increase occurred because the administration of HFD for 56 days caused fat accumulation in the blood, resulting in hypercholesterolemia. According to Hutapea *et al.* (2021), free fatty acids in oil are saturated fatty acids that contain cholesterol and contribute to increased cholesterol levels in the body. Consuming foods high in free fatty acids increases the levels of Low-Density Lipoprotein (LDL) in the blood. Yuningrum *et al.* (2022) stated that one of the contributing factors to elevated acetyl-CoA levels is the intake of saturated fatty acids, which stimulate HMG-CoA reductase enzyme production.

On day 56 of treatment P2, the beef tallow group increased cholesterol levels to 132 ± 4.24 mg/dL, categorized as hypercholesterolemia. Saputri *et al.* (2017) defined hypercholesterolemia as ≥130 mg/dL of total cholesterol. Davis *et al.* (2022) reported that beef tallow contains approximately 46% saturated fatty acids (SFA), 46% monounsaturated fatty acids (MUFA), and 7% polyunsaturated fatty acids (PUFA). The high content of SFA is known to increase LDL levels, leading to cardiovascular disease and hypercholesterolemia. In line with Perna and Hewling (2022), high SFA content can cause hypercholesterolemia because the consumption of saturated fat in food increases low-density lipoprotein (LDL), which leads to cardiovascular disease. This mechanism further supports the observed increase in total cholesterol levels, especially in group P2 (beef tallow),

Group P3 (goat tallow) did not experience hypercholesterolemia on day 56, but after alloxan injection, there was an increase in total cholesterol levels reaching 131 ± 8.76 mg/dL. Alloxan injection causes Type 2 diabetes and increases cholesterol levels in the body. This occurs because insulin typically inhibits cholesterol synthesis, but in Type 2 diabetes, cholesterol synthesis continues unabated, leading to hypercholesterolemia and atherosclerosis. According to Klein *et al.* (2022), insulin inhibits cholesterol synthesis, and its absence or resistance in type 2 diabetes mellitus causes continuous cholesterol production. Ruze *et al.* (2023) reported that elevated plasma free fatty acid (FFA) levels result in enlarged adipose tissue, which is closely associated with obesity.

Sopianti *et al.* (2017) showed that vegetable oil has only 1% FFA content, which can be metabolized efficiently by the body into energy. This supports the finding that group P1 (vegetable oil) did not experience hypercholesterolemia. Xiao *et al.* (2020) added that saturated fatty acids in egg yolk help increase antioxidant activity, maintain lipid membrane integrity, and support development. Saturated fatty acids in egg yolk function to increase boost antioxidants, form lipid membranes, and regulate their development (Xiao *et al.*, 2020). In the placebo group, which was only given 5% duck egg yolk, total cholesterol levels also did not increase. The combination of MUFA, PUFA, and antioxidants in egg yolk has a neutral or even protective effect on lipid metabolism when not excessive. Meanwhile, in the standard diet group, the fat content in the diet was only around 5% of the total diet per bag, so the likelihood of an increase in total cholesterol levels was low.

The KK (standard) and KP (placebo) groups had low to moderate damage, while the P1 (vegetable oil), P2 (beef tallow), and P3 (goat tallow) groups had severe damage. Liver cell damage is caused by compounds accumulated in the liver, including alloxan and fats from the high-fat diet (HFD) induction. In the KK group, liver cells appear normal with no damage, characterized by normal hepatocyte shape, no swelling or shrinkage of the cell nucleus, and the nucleus and cytoplasm are evenly distributed. This is consistent with Dhanti and Mulyanto (2021), who stated that normal hepatocytes have a round nucleus located in the center of the polygonal (homogeneous) cytoplasm with sinusoids spreading around the central vein arranged radially.

Groups KP, P1, P2, and P3 show inflammatory cells around hepatocytes. Inflammation is an immune response in the body when there is a harmful stimulus, such as the entry of pathogens or damaged cells. Listina *et al.* (2024) explained that inflammation arises when the immune system responds to harmful stimuli such as toxins or damaged cells, and may worsen under weakened immune conditions. Hendrawan *et al.* (2023) added that hepatocyte necrosis releases mediators that initiate inflammation and attract immune cells, supporting the observed inflammation in KP, P2, and P3 groups. The necrosis and degeneration of hepatocytes lead to the accumulation of inflammatory cells, indicating inflammation within the liver.

In ballooning cell damage, swelling of the cytoplasm can be observed. Ballooning damage is equivalent to hydrophilic degeneration. Fitriani *et al.* (2020) described ballooning damage (hydrophilic degeneration) is characterized by larger hepatocytes, with the cell nucleus and cytoplasm appearing more swollen than in the control group, and the cytoplasm also appears wider. Degeneration is an early sign of cell damage caused by toxic compounds, indicating cellular abnormalities resulting from minor

structural damage that disrupts metabolic processes. Putri *et al.* (2021) explained that this is caused by disruption in fluid regulation within the cell, leading to vesicle formation in mitochondria and the endoplasmic reticulum. Kakisaka *et al.* (2021) noted that ballooning is also associated with insulin resistance and obesity, which were observed in the beef tallow (P2) group.

Hepatocyte steatosis is severe damage induced by a high-fat diet (HFD). HFD induction causes lipid accumulation in the liver, leading to hepatic steatosis. In Figure 2, P2 (beef tallow) exhibits macrovesicles within cytoplasmic vacuoles, marked by white areas in the cytoplasm Sedeman *et al.* (2022) stated that microvesicular steatosis occurs when hepatocytes are filled with lipids that push the nucleus to the periphery and cause it to fade. Goh *et al.* (2019) supported this by stating that steatotic vacuoles displace the nucleus laterally. Inaba *et al.* (2023) further explained that hepatic steatosis hampers regeneration, causing liver dysfunction and progression to Non-Alcoholic Steatohepatitis (NASH), as observed in P2. Hepatic steatosis increases hepatocyte cell death during the regeneration period, leading to chronic liver damage.

High blood sugar and cholesterol levels in treatment P2 (beef tallow) caused lipid accumulation in the liver. High blood sugar levels were caused by insulin resistance due to damage to insulin signalling in the liver. This is in line with the statement by Jung and Choi (2014) that insulin resistance occurs because GLUT4-mediated glucose uptake decreases, particularly in skeletal muscle and adipocytes. During fasting, the liver maintains glucose levels through glycogenolysis and gluconeogenesis. When glycogen reserves are depleted, gluconeogenesis becomes dominant, utilising non-carbohydrate substrates. An increase in hepatic de novo lipogenesis is observed in the post-absorptive state, associated with the expression of key genes for glycolysis and de novo lipogenesis. According to Vázquez-Borrego et al (2023), steatosis is associated with increased de novo lipogenesis, measured through the uptake of deuterium-labelled water into fatty acids associated with triglycerides.

The inflammatory aspect of NASH is characterized by the infiltration of immune cells into the liver, causing local inflammation within the hepatic lobules. Singla *et al.* (2023) stated that lobular inflammation in NASH involves immune cell infiltration around swollen hepatocytes, which exacerbates tissue injury. The presence of oxidative stress, as an imbalance between reactive oxygen species (ROS) and antioxidant defenses, plays a central role in the pathogenesis of NASH. The histological damage in P2 supports its classification under early-stage Non-Alcoholic Fatty Liver Disease (NAFLD) or NASH.

CONCLUSION

Mice given beef tallow experienced an increase in cholesterol levels after treatment and liver damage to the extent of steatosis. The P2 group (beef tallow) was found to be the most suitable for NAFLD animal models. Based on these findings, it is recommended that future studies use individual caging systems to monitor feed intake and animal behaviour more accurately.

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CONFLICT OF INTEREST

There is no conflict of interest

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