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Experimental Research Article

Cholic acid enhanced hypercholesterol parameters in high cholesterol diet fed Sprague-Dawley rats

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ABSTRACT

Introduction: Hypercholesterolemia is a condition in which the blood contains elevated levels of low-density lipoprotein (LDL) and non-high-density lipoprotein (HDL). There are varieties of different diets used by different laboratories as a recipe for induction with varying levels of hypercholesterolemia. This study aims to investigate the role of cholic acids in enhancing hypercholesterolemia parameters in Sprague Dawley rats.

Methods: Nine Sprague Dawley rats (250 g ± 50 g BW) were used to investigate the most effective diet that is cost-effective for inducing hypercholesterolemia. The rats were randomly divided into 3 groups: normal diet (ND) (n=3), high cholesterol diet (HCD 1), a combination of 2% cholesterol and 0.5% cholic acid (n=3), and high HCD 2, a combination of 2% cholesterol and 30% ghee (n=3). After 4 weeks of feeding, blood samples were collected for lipid profiling, which included total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). The liver, kidney, and brain were removed for histopathological examination using hematoxylin and eosin (H&E) staining.

Results: The lipid profile measurements show significant differences between the HCD 1 group for total cholesterol, LDL cholesterol, non-HDL cholesterol, and total cholesterol/HDL ratio compared to the normal group. HCD 2 shows no significant changes in lipid profiles compared to the normal group.

Conclusion: Cholic acid helps in the absorption of cholesterol and enhances the hypercholesterol parameters in diet-induced SD rats based on lipid profile analysis and histology of the liver and kidney.

Keywords:
Dietetical recipe
Hypercholesterolemia
Low-density lipoprotein
SD rats

Introduction

Hyperlipidemia is a condition in which the concentration of cholesterol or triglyceride-carrying lipoproteins in the blood exceeds a predetermined normal limit. Increased lipoprotein deposition blocks blood flow to the heart, resulting in myocardial infarction (Bentzon et al., 2014). Ischemic stroke due to cholesterol accumulation blocking the blood flow to the brain will be triggered (Hackam and Hegele 2019). The term hypercholesterolemia is used when blood contains high non-HDL

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cholesterol and LDL cholesterol. One way to test for hypercholesterolemia is by performing the lipid profile test. The markers for this test are total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL). Low-density lipoprotein (LDL) is referred ~~to~~ as "bad cholesterol" as it can cause fatty accumulation in the vessel and lead to atherosclerosis. VLDL is also known as bad lipoprotein because it carries triglycerides that contribute to arterial plaque formation. Meanwhile, HDL (high-density lipoprotein) is considered "good cholesterol" as it transports cholesterol to the liver to be removed from the body (Águila et al., 2002; Yin et al., 2012). The liver is the primary organ involved in cholesterol metabolism, including cholesterol synthesis and clearance, and can be affected by high cholesterol levels. High cholesterol can lead to fatty liver disease (steatosis) and impairment of the liver's ability to break down and eliminate excess cholesterol, leading to an increase in cholesterol levels in the blood (Corso et al., 2017). High cholesterol can also indirectly affect the kidneys by contributing to the buildup of cholesterol in the blood vessels supplying the kidneys, making it harder for the kidneys to work properly (Pan 2022). High cholesterol can also increase the risk of kidney disease, particularly when combined with high triglycerides (Pandya et al., 2015).

Animal models have been widely used to understand the effectiveness of any drugs before clinical trials and translation into clinical practice. Therefore, the choice of animal model is crucial for accurate results. Generally, rodents are the preferred choice due to their anatomical, physiological, and genetic similarities to humans (Gajda et al., 2007; Wong et al., 2016). Sprague Dawley is one of the animal models that are more suitable compared to Wistar rats to induce dyslipidemia by diet (Udomkasemsab and Prangthip 2019; Wu et al., 2021). In addition to animal models, the induction method with either chemicals or diet is important to ensure similarity with human diseases. For hypercholesterolemia, there are many types of diets created by researchers to induce hypercholesterolemia in animal models, such as a combination of cholesterol, cholic acid and many bad cholesterol agents, such as maize oil (Coelho et al., 2018), peanut oil (Wu et al., 2021), coconut oil (Romain et al., 2018), egg yolk powder (Aminlari et al., 2019), lard (Romain et al., 2018) and butter (El-Sayyad et al., 2018)

These agents not only increase blood cholesterol levels but also significantly elevate the body weight of the animal.

Cholic acid serves as an agent to induce hypercholesterolemia as it facilitates cholesterol absorption in the small intestine (Bethesda 2012; Woollett et al., 2004). Cholic acid is a primary bile acid that is used to treat patients with genetic deficiencies in the synthesis of bile acids (Gonzales et al., 2018). It is synthesized from cholesterol in the liver and is conjugated to either glycine or taurine. Cholic acid facilitates fat absorption and cholesterol excretion and acts as a signaling molecule, affecting bile acid synthesis. Cholic acid is also used to help treat patients with symptoms of liver disease, steatorrhea, or difficulty in absorbing fat-soluble vitamins (Hofmann 1990). Since cholic acid will add to the cost of the study, it is crucial to examine the effectiveness of adding cholic acid to the diet to induce hypercholesterolemia.

Knowledge about how cholic acid affects cholesterol metabolism can help with risk assessment and personalized preventative care plans that include dietary and lifestyle adjustments. This information could be useful in the creation of novel treatment modalities, such as pharmaceutical interventions and prophylactic measures. Hence, this research aims to investigate the role of cholic acids in enhancing hypercholesterolemia parameters in Sprague Dawley rats.

Material and methods

Animals, Diet, and Experimental Protocol

The protocols of this study were designed to minimize animal suffering has been approved by USM Institutional Animal Care and Use Committee, Universiti Sains Malaysia, Malaysia (USM IACUC) [USM/IACUC/2021/ (131) (1165)].

Nine Sprague Dawley rats weighing 250 g ± 50 g were used in this study. Rats were housed in the animal house at 26–28 °C temperature under dark (12-h) and light (12-h) cycles with free access to standard animal chow/high cholesterol diet and water *ad libitum*. After 7 days of adaptation to the environment, the Sprague Dawley rats were randomly divided into 3 groups: normal diet (ND) (n = 3), high cholesterol diet type 1 (HCD 1) (n = 3), and high cholesterol diet type 2 (HCD 2) (n = 3). Table 1 shows the composition of the diet used in this study. Both diet compositions were optimized based

TABLE 1: Composition of the diet

GENERAL			
	NORMAL	HCD 1	HCD 2
Normal pellet	100 g	97.5 g	68 g
Cholesterol	-	2 g	2 g
Cholic acid	-	0.5 g	-
Ghee	-	-	30 g
SPECIFIC			
Metabolic energy	3188 kcal	3108.3 kcal	2394.66 kcal
fat	12 g	11.7 g	37.86 g
protein	24 g	23.4 g	-
carbohydrates	64 g	62.4 g	-
Cholesterol	-	2 g	2 g
Cholic acid	-	g	-

on the previous study with a slight modification (Antona et al., 2020; Yu et al., 2021). The normal group received normal pellets from Altromin Spezialfutter GmbH&Co. KG (Code 1324); the HCD 1 group received a combination of a normal diet, cholesterol, and cholic acid, while the HCD 2 group received a combination of a normal diet, ghee, and cholic acid. Cholesterol and cholic acid are from Nacalai Tesque and ghee is from Crispo.

The body weight of each group was recorded weekly. After 4 weeks of diet induction, blood samples were collected and were outsourced to a clinical laboratory for blood analysis that included a lipid profile test using blood serum. The Sprague Dawley rats were euthanized, and the organs were removed for histopathological examination.

Change in body weight

The rats' body weight was measured weekly on days 0, 7, 14, 21, and 28 and expressed as the percentage change in body weight relative to the baseline weight (day 0), as shown in the formula:

$$\%\ \text{Change of body weight} = \frac{\text{BW on Day N-BW on Day 0}}{\text{BW on Day 0}}\ \text{x } 100\%$$

In which 'N' refers to the specific day when the body weight was recorded.

Atherogenic Index and Coronary Risk Index

The atherogenic index (AI = LDL-C/HDL-C) and CRI (CRI = TC/HDL-C) were calculated to assess the correlation between the risk factors for CVD (Kazemi et al., 2018).

Biochemical measurement

At the end of the experiment, animals were anesthetized by intraperitoneal sodium pentobarbital from Alfasan International BV (Netherlands) and blood samples were taken into yellow top vacutainer to collect serum and were outsourced for lipid profile test using an automated blood analyzer. The parameters of the lipid profile were TC, TG, HDL, and LDL.

$$\text{Dosage of pentobarbital sodium} = \frac{\text{Body weight (kg)x 100 mg/kg}}{\text{200 mg/ml}}$$

Histology

The liver, kidney, and brain were stored in 10% formalin upon harvesting from each rat. After 24 hours, tissues were cleared in xylene and descending concentrations of ethanol (100%, 75%, and 50% alcohol in distilled water) for 1 hour each. The tissues were processed into blocks of paraffin. A microtome was used to cut the tissue block into 4 µm sections. The tissues were stained with hematoxylin and eosin (H&E) to check for organ changes in rats from different groups. The histological scoring was calculated based on the steatosis level and damage of the cells (Veteläinen et al., 2006). The liver and kidney were examined since they are the crucial organ affected by hypercholesterolemia. The brain was also analyzed whether the diet composition crosses the blood-brain barrier.

Statistical analysis

Data are presented as the mean ± standard error of the mean. One-way ANOVA was used for data analy-

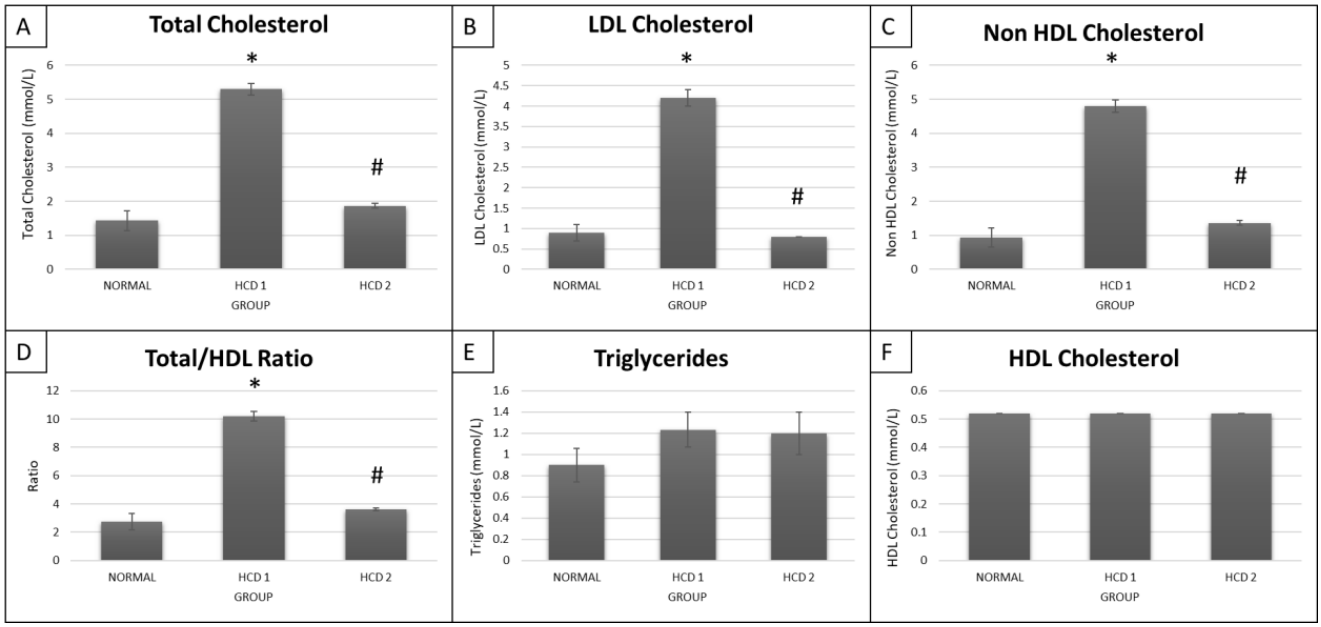

FIGURE 1. Lipid profile (A) total cholesterol, (B) LDL cholesterol, (C) non-HDL cholesterol, (D) total/HDL ratio, (E) triglycerides, (F) HDL cholesterol in the normal, high cholesterol diet 1 (HCD 1) and HCD 2 groups. * shows a significant difference between the normal and HCD groups. # shows a significant difference between the HCD1 and HCD2 groups. The data are presented as the mean ± standard error of the mean (SEM) (n=3), *$P < 0.05$.

TABLE 2: Percentage change in body weight and relative organ weight

	NORMAL	HCD 1	HCD 2
Body Weight Change (g)	27.8186± 6.0784	19.3451± 0.5164	42.6720± 4.1015$^{\#}$
BMI	0.6647 ± 0.02193	0.7001 ± .71346	0.7631 ± 0.1075
Relative Organ Weight (g)			
Liver	0.0349 ± 0.0034	0.0474 ± 0.0008	0.0330 ±0.0033$^{\#}$
Kidney	0.0061 ± 0.0002	0.0063 ± 0.0002	0.0055 ± 0.0003
Heart	0.0033 ± 0.0002	0.0322 ± 0.0002	0.0030 ± 0.0001
Lung	0.0043 ± 0.0001	0.0035 ± 0.0006	0.0034 ± 0.0001
Brain	0.0055 ± 0.0004	0.0051 ± 0.0001	0.0049 ± 0.0005
Pancreas	0.0026 ± 0.0005	0.0027 ± 0.0005	0.0024 ± 0.0003
Spleen	0.0022 ± 0.0004	0.0199 ± 0.0003	0.0016 ± 0.0001

The data are presented as the mean ± standard error of the mean (SEM). $^{*}P<0.05$ shows the significance compared to the normal group. $^{\#}P<0.05$ shows the significance compared to HCD 1 group (n=3).

sis within different groups using IBM SPSS Statistics Version 27. Post hoc tests were used for comparisons between groups. All values are expressed as the means followed by the standard error of the mean (SEM). Differences were considered significant at $P< 0.05$.

Results

Percentage change in body weight and relative organ weight

Table 2 shows the percentage change in body weight and relative organ weight for each group.

The HCD 2 group showed a significant change in

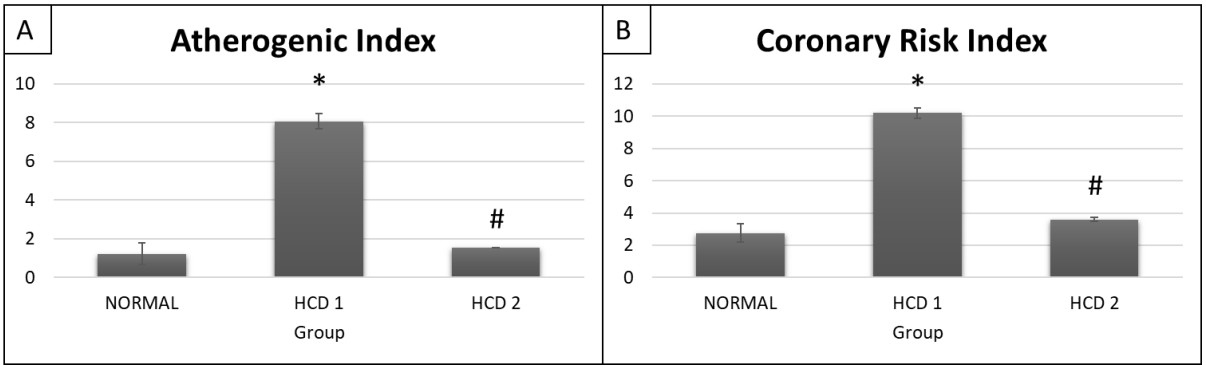

FIGURE 2. (A) atherogenic index, (B) coronary risk index in the normal, high cholesterol diet 1 (HCD 1) and high cholesterol diet 2 (HCD 2) groups. * shows a significant difference between the normal and HCD1 groups. # shows a significant difference between the HCD1 and HCD2 groups.

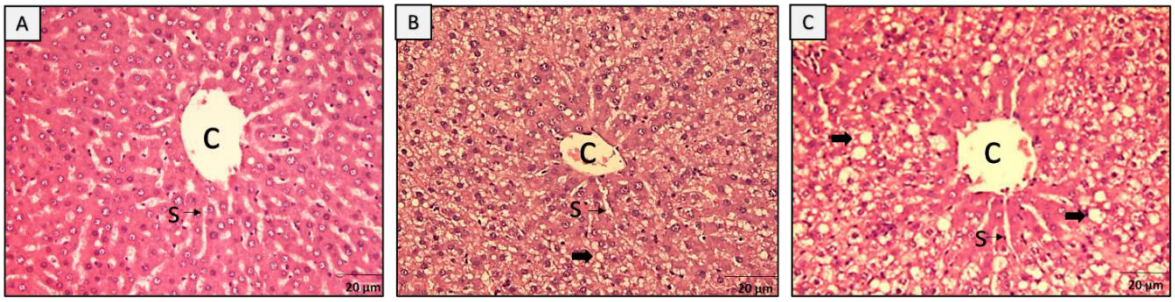

FIGURE 3. H&E-stained sections of the liver in (A) normal, (B) HCD 1, and (C) HCD 2 groups at 40x magnification. (A) The normal group demonstrated no significant change in the appearance of the liver tissue, normal arrangements of hepatocytes, portal triad, and well-spaced sinusoids. In HCD1, severe degenerative changes were observed, including macrovascular and microvascular steatosis and fused hepatocytes in the centrilobular, midzonal, and periportal regions. In HCD2, total degenerative changes and the presence of inflammatory cell infiltration were detected, with high steatosis compared to (B), macrovascular and microvascular steatosis, fused hepatocytes at the centrilobular, midzonal, and periportal regions. The thick arrow indicates the fat cells in the liver tissues. C – central vein, S – sinusoid, bold arrow – lipid vacuoles.

body weight compared to the normal group. All relative organ weights showed no significant change except for the liver weight of the HCD 2 group compared to the normal group.

Biochemical analysis

Lipid profile analysis for each group is presented in Figure 1, showing total cholesterol, LDL cholesterol, non-HDL cholesterol, total/HDL cholesterol, triglycerides, and HCD cholesterol as parameters.

The lipid profile measurements show significant differences between the HCD 1 group for total cholesterol, LDL cholesterol, non-HDL cholesterol, and total/HDL ratio compared to the normal group (5.3±0.17) mmol/L, (4.2±0.2) mmol/L, (4.8±0.17) mmol/L, (10.2±0.35) and (1.43±0.29) mmol/L, (0.9±0.2) mmol/L, (0.93±0.29) mmol/L, (2.77±0.58), respectively. The lipid profile measurements showed significant differences between the HCD 2 group for total cholesterol, LDL cholesterol, non-HDL cholesterol, and total/HDL ratio compared to the HCD 1 group (1.87±0.07 mmol/L, 0.8±0.01 mmol/L, 0.93±0.29 mmol/L, and 3.6±0.1 mmol/L, respectively). No significant changes were observed between the treatment group for triglycerides and HDL cholesterol compared to the normal group.

Atherosclerosis and coronary risk index

Figure 2 shows the atherogenic and coronary risk index. The atherogenic and coronary risk index calculations show significant differences between HCD 1 (8.08±0.33) and (10.19±0.38) compared to the normal group (1.22±0.56) and (2.76±0.56), respectively. There were significant changes observed between the HCD 2 (1.54±0.13) and (3.59±0.01) groups and the HCD 1 group for both atherogenic and coronary risk indexes, respectively. HCD 1 shows a significant increase for

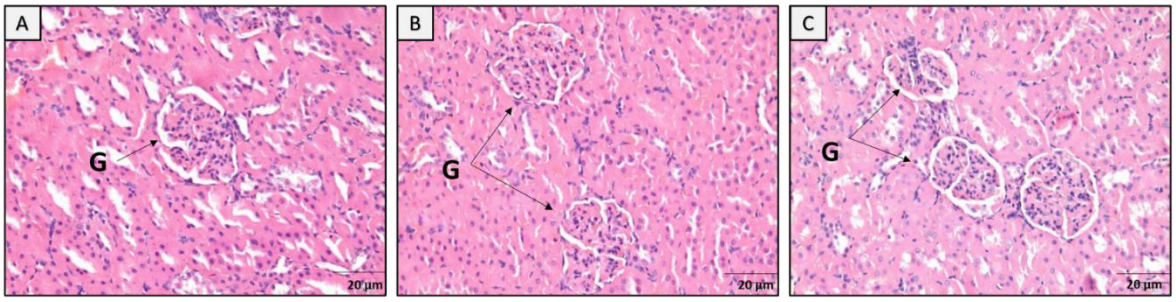

FIGURE 4. H&E-stained sections of the kidney in (A) normal, (B) HCD 1, and (C) HCD 2 groups at 40x magnification. The normal group (A) and HCD 1 group (B) demonstrated no significant change in the morphology of the tissue, normal kidney morphology, intact glomerular and Bowman's capsule, normal epithelial layer of Bowman's capsule and kidney tubules, and normal arrangements of kidney podocytes. In (C), moderate degenerative changes were observed, including degeneration of the glomerulus, intact kidney podocytes, Bowman's space increase, and intact structural arrangements. G – Glomerulus.

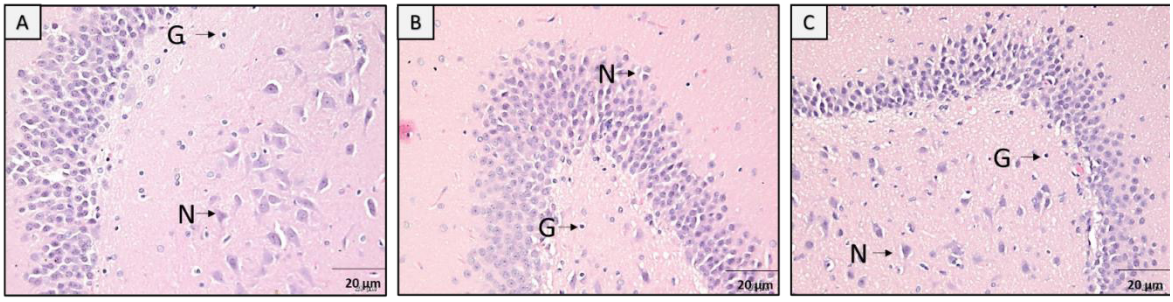

FIGURE 5. H&E-stained sections of the brain in (A) normal, (B) HCD 1, and (C) HCD 2 at 40x magnification. All groups demonstrated no significant change in the morphology of brain tissue, normal brain morphology, or normal glial cells. No degenerative changes were observed. N – neuron, G – glial cell.

both indexes. No significant changes were detected between the normal and HCD 2 groups.

Histologic characterization

Figure 3 shows the histological section of the liver for each group under H&E staining at 40x magnification. The normal group showed a normal histological liver structure in terms of hepatocyte cell arrangement and normal sinusoids. The HCD 1 and HCD 2 groups showed severe degenerative changes and the appearance of macro- and macrovascular steatosis in hepatocytes. Fused hepatocytes were observed throughout the liver lobules at the centrilobular, midzonal, and periportal regions.

Figure 4 shows histological sections of the kidney for each group under H&E staining at 40x magnification. The normal and HCD 1 groups showed normal histological structure, including a normal glomerulus and Bowman's space. HCD 2 shows moderate degenerative changes in the glomerulus and structural arrangements.

Figure 5 shows histological sections of the brain for each group under H&E staining at 40x magnification. All groups showed normal histological structure, including normal neurons and glial cells.

Discussion

The incidence of hypercholesterolemia, which is currently on the rise, leads to many CVD-related diseases (Rodrigues et al., 2021). Therefore, an animal model of hypercholesterolemia is needed to understand the mechanism involved and thus develop a better treatment for hypercholesterolemia. Due to the many diet recipes created by many researchers, it is difficult to determine the best diet for the induction of hypercholesterol. However, many diet recipes include fat-absorptive agents in their diet (Hassan et al., 2023). Therefore, this study aims to investigate the effects of cholic acid in the diet recipe in inducing hypercholesterolemia in Sprague Dawley rats.

The HCD 2 group showed a significant increase in the percentage of body weight change compared to the

normal group. HCD 1 showed no significant change in body weight compared to the normal group. All groups were given the same amount of food to ensure that only the compound of food was responsible for induction.

Based on this study, HCD 1 (2% cholesterol + 0.5% cholic acid) is more effective in inducing hypercholesterolemia than HCD 2 (2% cholesterol + 30% ghee) because of cholic acid function. Ghee and cholesterol powder, although showing increased body weight, cannot effectively induce hypercholesterolemia without cholic acid. The cholic acid in HCD 1 helps with the absorption of cholesterol from the intestines into the blood system, thus increasing the level of total cholesterol and bad cholesterol in the blood (Devi and Singh 2017; Woollett et al., 2004). Sterols are only marginally soluble in aqueous systems and depend on the detergent characteristics of bile acids for dispersion in the intraluminal environment. Bile salts are necessary for the solubility of the lipolytic products of fat digestion, which include cholesterol.

Ghee, which is a type of clarified fat, is mostly used by researchers to induce obesity and diabetes (Nadig et al., 2021; Veteläinen et al., 2006). Ghee can increase the body weight of research subjects significantly for obesity and diabetic studies; however, the use of ghee alone failed to induce the markers of hypercholesterolemia. With the use of ghee, the HCD 2 group showed high-fat accumulation in the abdominal area, which is known as visceral fat, compared to the normal and HCD 1 groups.

The lipid profile test shows increasing total cholesterol, non-HDL cholesterol, LDL cholesterol, and total/HDL ratio in the HCD 1 group. The HCD 2 group showed a near normal ratio of all markers. Hypercholesterol diet groups showed a slight increase in triglycerides, but the results were not significant. All groups showed the same level of HDL cholesterol. This result shows that the diet composition used in HCD 1 only increases bad cholesterol levels but not good cholesterol levels, which creates the best model for experimental disease.

Liver histology shows greater damage in the HCD 2 group, in which the blood cholesterol level does not show any changes compared to normal. Both hypercholesterol groups showed severe degenerative changes, macrovascular and microvascular steatosis, and fused hepatocytes at the centrilobular, midzonal, and periportal regions. The kidney histology of the normal and HCD 1 groups showed no degenerative changes, but the HCD 2 group showed moderate degenerative changes, especially degeneration of the glomerulus with intact kidney podocytes, an increase in Bowman's space, and intact structural arrangements. HCD 2 shows an abnormal lipid profile in severe degenerative changes in the liver and kidney because of fat cell buildup but does not interfere with the lipid and cholesterol profile (Basheer et al., 2023).

Brain histology shows normal layers of the cerebral cortex, the basophilic appearance of neurons with intact supporting cells (glial and oligodendrocytes, astrocytes), prominent nucleoli of neurons, white matter with myelinated axons and supporting cells, and a normal arachnoid and subarachnoid space with a superficial brain ventricle. This result is closely related to the blood-brain barrier function that heavily restricts the movement of molecules, ions, and cells between the blood and the CNS (Daneman and Prat 2015). This shows that the diet recipe used in our study did not disrupt the integrity of the blood-brain barrier and thus did not have any effect on brain morphology.

An important metric that can be used independently to estimate the risk of heart disease is the atherogenic and coronary risk index. Changes in the lipid profile increase the risk of developing atherosclerotic and CVD problems. The atherogenic and coronary risk index of the HCD 1 group shows a significant increase compared to the normal and HCD 2 groups, suggesting that hypercholesterolemia is the major risk factor that can lead to cardiovascular diseases such as atherosclerosis and coronary heart disease (Ardiana et al., 2022; Suman et al., 2016).

We acknowledge that n=3 is a small sample number for blood parameters. However, this research is a pilot study and a sample size of 3 is a standard for pilot study. It is important to reduce the number of animal studies following the 3R Principle in animal research. In addition, the SEM is small meaning that the randomly chosen sample for blood work showed similar blood parameters across the animals.

Conclusion

This research reveals the efficient dietetical recipe for inducing hypercholesterolemia for future research. The HCD 1 group, which consisted of 2% cholesterol and 0.5% cholic acid, showed that cholic acid enhanced the hypercholesterol parameters based on the lipid pro-

file analysis and histology of liver and kidney damage. Cholic acid is the key ingredient in hypercholesterolaemic study compared to HFD alone. The combination of cholic acid in HFD enhances cholesterol absorption by cholesterol solubilization in micelles thus facilitating the absorption in the small intestine. A diet rich in cholic acid increases the risk of CVD, hypertension, and urinary albumin levels. Therefore, this molecule is very important in hypercholesterolemia induction.

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Conflict of interest

All authors declare no conflicts of interest in this paper

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