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Potential Antihyperlipidemia Effect of Lactoferrin in Hyperlipidemia-Induced Male Sprague–Dawley Rats

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Abstract

Background: Hyperlipidemia is a condition that is characterized as an increase in total cholesterol and triglyceride levels in the blood. Lactoferrin is a protein that can serve as an antioxidant. This study aims to determine whether lactoferrin can reduce total cholesterol and triglyceride levels.

Methods: This study used 24 Sprague–Dawley rat strains, which were divided into six groups: normal group; negative control; positive control; and dose groups 1, 2, and 3. The normal group was given standard feed, whereas the other group was given high cholesterol and fat. The positive control group and dose groups 1, 2, and 3 were given 1.5 mg/kg BW of simvastatin and 100, 200, and 400 mg/kg of BW lactoferrin, respectively. After 6 weeks, total cholesterol and triglyceride levels were measured.

Results: This study showed that lactoferrin doses of 100, 200, and 400 mg/kg BW could significantly reduce total cholesterol and triglyceride levels ($p < 0.05$). Lactoferrin could also significantly reduce activated Kupffer cell and steatosis area in the liver ($p < 0.05$).

Conclusions: Lactoferrin can reduce total cholesterol and triglyceride levels. Thus, further research is needed to address the existing bias and confirm that lactoferrin can reduce cholesterol and triglyceride levels.

Keywords: cholesterol, hyperlipidemia, lactoferrin, sprague–Dawley, triglyceride

INTRODUCTION

Hyperlipidemia is a common cause of atherosclerotic plaque formation in coronary arteries.[1] An increase in total cholesterol and triglyceride levels shows hyperlipidemia.[2,3] Hyperlipidemia, also known as dyslipidemia, is a common condition, which is associated with cardiovascular diseases, with elevated plasma low-density lipoprotein (LDL) cholesterol being the 8th leading risk factor of death in 2019.[4] Approximately 4.4 million deaths and 98.62 million disabilities were related to high-plasma LDL-cholesterol. Based on data from Indonesia Basic Health Research 2018, 35.9% of people in Indonesia aged 15 years and above have abnormal lipid profiles, of which 5.9% have a high level of LDL, 22.9% have a low level of high-density lipoprotein, and 11.9% have a high level of triglyceride.[5]

Hyperlipidemia could be divided into primary hyperlipidemia and secondary hyperlipidemia. Primary hyperlipidemia is due to heredity, whereas secondary hyperlipidemia is due to low physical activity, smoking, diabetes mellitus, nephritic syndrome, hypothyroidism, and drug use. Complications from hyperlipidemia condition include atherosclerosis, coronary artery disease, myocardial infarct, and ischemic stroke.[6] At present, statin is a common medication for treating hyperlipidemia; however, this medication still has side effects such as myalgia.[7,8] Many types of research are conducted on the basis of this condition, such as lactoferrin research in reducing lipid profile levels.[9]

Lactoferrin is an iron-binding glycoprotein in the body. Mammals such as cows, horses, dogs, and humans can produce lactoferrin.[10] Lactoferrin plays an important role in the physiological function of the human body, such as antimicrobial, antiviral, immune system enhancement, and antioxidants.[10] Antioxidants from lactoferrin can reduce total cholesterol and triglyceride levels in the blood as well as the steatosis area in the liver. The high affinity for the binding of lactoferrin to iron in the body can prevent the production of free radicals during the Fenton reaction, thereby inhibiting the oxidation of lipoproteins in the body.[11] A study conducted by Nozari *et al.* showed that bovine lactoferrin reduces total cholesterol and triglyceride levels in male rats fed with high-cholesterol diet.[12] However, no *in vivo* studies in Indonesia had been conducted to assess the potential

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application of lactoferrin in reducing total cholesterol and triglyceride levels in hyperlipidemia-induced male rats. Thus, this paper aims to examine the potential application of lactoferrin as antihyperlipidemia in hyperlipidemia-induced male Sprague–Dawley male rats.[12]

METHODS

All experiments performed in this study were approved by the ethical review committee of School Medicine and Health Sciences—the Atma Jaya Catholic University of Indonesia on January 20, 2021 (protocol number 01/01/KEP-FKIKUAJ/2021), and all procedures were in accordance with international standards for animal experimentation. This experimental study was conducted from April 2021 until June 2021 at Pharmacology Laboratory and Animal House School of Medicine Atma Jaya Catholic University of Indonesia, Jakarta. Bovine lactoferrin was purchased from Xi'an Ruisaen Biotechnology Co., Ltd., China, as water-soluble pinkish-white crystals with purity of 99% (RSN201119).

Male Sprague–Dawley rat strains (N = 30) aged 5 weeks, with 150–200 g body weight, were obtained from the Indonesian Food Drug Administration Laboratory Service (certificate no. 02/LHP/SKKH/IV/2021). Subjects were calculated on the basis of the degree of freedom or resource equation method formula. The minimum sample size of at least four Sprague–Dawley rats was obtained for each group based on the formula. This study used a sample of four rats per study group. The total number of samples in each study group was added by 10% to five rats to overcome the dropout. Subjects were randomly divided into six experimental groups. The first group was the normal group fed a normal diet. The second group was composed of hypercholesterolemic rats fed a high-cholesterol diet (HCD) containing 15% sucrose, 5% cow's fat, 80% quail egg yolk, and 0.01% propylthiouracil (PTU). The third group was given orally HCD and 1.5 gr/150 g BW simvastatin therapy. The fourth, fifth, and sixth groups were given HCD and lactoferrin doses of 100, 200, and 400 mg/kg BW, respectively. All groups were fed HCD for 3 weeks except for the normal group, and then the third group was given simvastatin. The fourth, fifth, and sixth groups were given lactoferrin for 3 weeks. After induction of hyperlipidemia, all groups except for the normal group were still given HCD for another 3 weeks.

After 6 weeks of research, all rats were necropsied. Two milliliters of rat blood were drawn through the rat's heart and placed into the Eppendorf tube. We kept the sample for around 45 min until the blood clotted, and then the sample was centrifuged for around 30 min with 1500 g of power on 400C. Next, we collected 10 µL of serum and placed into the plastic cuvette. Afterward, 1000 µL of reagent kit was mixed with 10 µL of the sample in the plastic cuvette, and then the mixture was incubated for 10 min at 20 °C–25 °C or for 5 min at 37 °C. Absorbance was read within 60 min against reagent blank at a wavelength range of 500–546 nm. Total cholesterol levels were measured by cholesterol FS (10') reagent (manufactured by *DiaSys Diagnostic Systems GmbH*, Germany). However, triglyceride levels were measured by triglyceride FS (10') reagent (manufactured by *DiaSys Diagnostic Systems GmbH*, Germany).

The total cholesterol level was measured as follows:
Cholesterol (mg/dL) = $\frac{Abs\ Sample}{Abs\ Standard}$ × Cholesterol Standard (mg/dL)

The triglycerides level was measured as follows:
Triglyceride (mg/dL) = $\frac{Abs\ Sample}{Abs\ Standard}$ × Triglyceride Standard (mg/dL)

All livers were fixed using formaldehyde and collected to the anatomic pathology laboratory to slice the tissue samples and stain with hematoxylin and eosin. Steatosis in the liver was scored on the basis of the method of Kleiner *et al.* This method determined steatosis based on the following percentage: grade 0, <5% steatosis; grade 1, 5–33% steatosis; grade 2, 33–66% steatosis; grade 3, >66% steatosis.[13] The activated Kupffer cell was scored in accordance with the method of the Arsad *et al*. This method was used to measure activated Kupffer cells in sinusoid, which were graded as follows: grade 0, 0% activated Kupffer cell; grade 1, <30% activated Kupffer cell; grade 2, 30–50% activated Kupffer cell; grade 3, >50% activated Kupffer cell.[14] Three microscopic fields of view were selected for each slide, and histopathological changes were analyzed under a light microscope with 400× magnification.

SPSS for MacOs (SPSS, Chicago, IL, USA) version 28 was used for statistical analyses. Data were shown as means ± SD. One-way ANOVA was used to test established variance analysis. Tukey post hoc test was used to assess the significance of differences among groups (if the homogeneity of variables and normality distribution was assumed). $P < 0.05$ was considered statistically significant. Data for activated Kupffer cell and steatosis were analyzed by Kruskal–Wallis test followed by Mann–Whitney test to compare group differences.

RESULTS

All data were analyzed by one-way ANOVA and then Tukey post hoc test. The result of one-way ANOVA showed a significant difference among all groups ($p < 0.05$). The effect of bovine lactoferrin treatment on total cholesterol and triglyceride alteration in experimental animals is shown in Table 1. The total cholesterol and triglyceride levels were significantly higher in the HCD group than in the normal group ($p < 0.05$). Total cholesterol and triglyceride levels were significantly lower in bovine lactoferrin treatment in all dose groups than in the HCD group ($p < 0.05$). In addition, no significant changes were

observed between the normal group and all bovine lactoferrin dose groups. No significant difference was also found between all bovine lactoferrin dose groups and the positive control group. Similarly, no significant difference was observed among dose groups 1, 2, and 3. Dose group 2 had the lowest total cholesterol level and triglyceride level compared with the other dose groups; however, dose group 3 had the highest total cholesterol level and triglyceride level compared with the other dose groups.

All lobes (right lobe, left lobe, quadrate lobe, and caudate lobe) from the liver were examined in this study. Histopathological analysis showed that rats from the HCD group had fatty livers. However, lipid accumulation reduces in groups administered with lactoferrin. This condition was supported by a higher steatosis score in the HCD group compared with the normal group (Table 2; $p < 0.05$). The lactoferrin treatment group had significantly lower histopathological steatosis scores than the control group (Table 2; $p < 0.05$) Lipid accumulation can increase oxidative stress, characterized by the activation of Kupffer cells in the liver. The activated Kupffer cell score was significantly higher in the HCD group than in the normal and lactoferrin treatment groups (Table 2; $p < 0.001$). No significant difference in activated Kupffer cell score was found among the normal group, positive control group, and groups administered with lactoferrin (Figure 1). Many activated Kupffer cells were found in negative control rats (Figure 1A) compared with the normal group (Figure 1B). Activated Kupffer cells were also shown to be minimal in the positive control group (Figure 1C), dose 1 group (Figure 1D), dose 2 group (Figure 1E), and dose 3 group (Figure 1F). As shown in the picture, lactoferrin dose 2 group has least activated Kupffer cells compared with the other lactoferrin dose groups.

TABLE 1. Effects of bovine lactoferrin on total cholesterol and triglyceride in male rats

Parameter	Normal	HCD	HCD + 1,5 mg/150 g BW Simvastatin	HCD + 100 mg/kg BW bLf	HCD + 200 mg/kg BW bLf	HCD + 400 mg/kg BW bLf
Total Cholesterol (mg/dL)	84,440 ± 36,641[a]	157,514 ± 40,010[b]	76,435 ± 2,470[a]	73,043 ± 28,765[a]	71,604 ± 23,572[a]	78,781 ± 23,093[a]
Triglyceride (mg/dL)	39,741 ± 16,380[a]	70,818 ± 14,385[b]	36,044 ± 8,819[a]	35,669 ± 10,983[a]	31,747 ± 7,140[a]	39,927 ± 17,381[a]

HCD: high-cholesterol diet; bLF: bovine lactoferrin.
Different letters in the same row indicate a significant difference ($p < 0.05$).

TABLE 2. Effects of bovine lactoferrin on activated Kupffer cells and steatosis score in the liver

Parameter	Normal	HCD	HCD + 1,5 mg/150 g BW Simvastatin	HCD + 100 mg/kg BW bLf	HCD + 200 mg/kg BW bLf	HCD + 400 mg/kg BW bLf
Activated Kupffer Cell Score	0.83 ± 0.389[a]	2.42 ± 0.515[b]	1.08 ± 0.515[a]	0.75 ± 0.452[a]	0.42 ± 0.515[a]	1.17 ± 0.577[a]
Steatosis Score	0.00 ± 0.000[a]	2.58 ± 0.515[b]	1.58 ± 0.669[c]	1.33 ± 0.888[c]	1.00 ± 0.739[c]	2.00 ± 0.739[c]

HCD: high-cholesterol diet; bLF: bovine lactoferrin.
Different letters in the same row indicate a significant difference ($p < 0.05$).

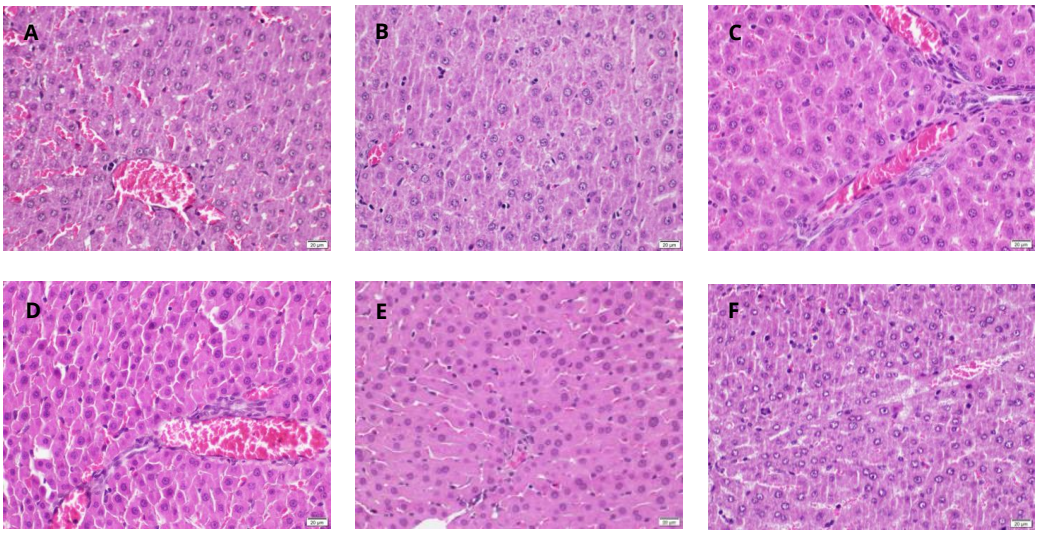

FIGURE 1. Histopathologic examination of rat liver. (A) negative control shows several activated Kupffer cells, (B) normal control (C), positive control, (D) dose 1 (E) dose 2, and (F) dose 3 shows minimal activated Kupffer cells.

DISCUSSION

This study analyzes antihyperlipidemia from lactoferrin by measuring blood total cholesterol and triglyceride levels and performing microscopic liver analyses. Histopathological analyses measured the amount of activated Kupffer cells and steatosis score in rat liver. The results show that bovine lactoferrin treatment could decrease total cholesterol and triglyceride levels in male Sprague–Dawley rats, commonly at risk of cardiovascular diseases.

High-sucrose consumption can increase lipid profile levels such as total cholesterol, triglycerides, VLDL, NASH, NAFLD, and insulin resistance. Insulin resistance is a condition where insulin's ability to suppress glucose and VLDL production is impaired; thus, TG will be released to the bloodstream through VLDL lipoproteins, leading to dyslipidemia.[15] Another food that can induce hyperlipidemia is cow's fat because 100 g of cow's fat contains 126 mg of cholesterol.[16] PTU can also induce hyperlipidemia by inhibiting the thyroid hormone. Thyroid hormones will activate the LDL receptor-related protein 1 (LRP1). LRP1 is a receptor that binds lipid-conjugated apolipoprotein E (ApoE) and internalizes triglyceride-rich lipoproteins containing ApoE, such as chylomicron remnants and LDL remnant, thereby contributing to the clearance of chylomicron. The consumption of PTU will reduce the expression of LRP1 receptors, thereby increasing the LDL levels in the bloodstream.[17]

Lactoferrin promotes lipid absorption through the stimulation of bile acid synthesis. The lactoferrin pathway inhibits the production of TNF-α.[18] TNF-α is a proinflammatory cytokine that inhibits the expression of HNF4A.[19] HNF4A is a gene that activates the CYP7A1 enzyme.[20] The HNF4A gene makes instructions for making a protein, namely, hepatocyte nuclear factor-4 alpha.[21] This protein contributes to the development of the liver, kidney, and intestine; however, CYP7A1 is a catalyzed enzyme for converting cholesterol to bile acids.[22] By phosphorylating AMP-activated protein kinase (AMPK), lactoferrin serves as an agonist of AMPK, which is a protein that regulates energy homeostasis and coordinates metabolic pathways, thereby balancing nutrient supply with energy demand. Two enzymes (FAS and ACC) and genes (SERBP1) are controlled by AMPK.[23] SERBP1 is a mediator of hepatic lipogenesis, which is over-induced in obese animals; however, FAS and ACC are the key enzymes for lipid synthesis, and ATGL is the main enzyme for triglyceride degradation.[24–26] The supplementation of LF can decrease the expression of SERBP1, inhibit the protein expression of FAS and ACC, and elevate the protein expression of ATGL. Therefore, lactoferrin decreases lipid synthesis and increases the degradation of lipid in the liver.[23,27] These research results are consistent with a study conducted by Faridvand *et al.*, who stated that bovine lactoferrin can reduce lipid profiles such as total cholesterol and triglyceride levels in rats induced with a HCD. This lipid profile–lowering effect follows the role of lactoferrin as an antioxidant in inhibiting lipid synthesis in the body.[28] Nakamura *et al.* reported that lactoferrin could significantly reduce hepatic cholesterol levels in rats by increasing cholesterol excretion through interaction with bile acids.[9]

Our data showed that the decrease in total cholesterol and triglyceride levels was not consistent with the increase in lactoferrin dose. 200 mg/kg BW of lactoferrin had the lowest total cholesterol and triglyceride levels; thus, this dose was considered as optimal for decreasing total cholesterol and triglyceride levels. Nozari *et al.* also showed that 200 mg/kg BW of lactoferrin could reduce total cholesterol and triglyceride levels.[12] Hence, further research is recommended to ensure that 200 mg/kg BW is the optimal dose for lactoferrin in lowering total cholesterol and triglyceride levels. In addition, 400 mg/kg BW of lactoferrin had higher total cholesterol and triglyceride levels than the other doses. A high dose of lactoferrin can reduce cell proliferation by inhibiting the activation of extracellular signal-regulated kinase, releasing IL-8 cytokines, increasing NF-Kβ, and activating hypoxia-inducible factor-1α (HIF-1α).[29] Therefore, excessively high lactoferrin dose can increase the inflammatory effect, thereby promoting cholesterol synthesis.

A similar effect was found in microscopic analysis of the liver, where the steatosis area score was significantly lower in the group administered with lactoferrin compared with the HCD group. That is, lactoferrin could be a potential drug to prevent fatty liver. Lactoferrin also has an antioxidant effect, thereby preventing lipid accumulation in the liver. This condition indicates that activated Kupffer cells were significantly higher in the HCD group than in the lactoferrin treatment group. Activated Kupffer cell is a physiological response of the liver to injury or infection. High-fat diets can increase gut permeability and trigger the accumulation of lipopolysaccharides. The binding of LPS to their receptors on Kupffer cell surface promotes the production and secretion of cytokines that recruit T and B lymphocytes and other leukocytes; therefore, activated Kupffer cells can indicate dyslipidemia.[30] Recent studies have also shown that lactoferrin could treat fatty liver and serve as an antioxidant.[31]

Statin is a common class of drugs that reduce cholesterol levels in the blood. Their mechanism of action is primarily via the inhibition of HMG-CoA (hydroxymethylglutaryl-coenzyme-A) reductase, which is the enzyme responsible for the cholesterol biosynthetic pathway. Statin could lower LDL-c and reduce cardiovascular morbidity and mortality, in primary and secondary prevention; however, statin has some serious side effects. Statin toxicity or intolerance is commonly presented as statin-associated

muscle symptoms). Another side effects of statin therapy, which can be more serious, include new-onset type 2 diabetes mellitus, neurological and neurocognitive effects, hepatotoxicity, renal toxicity, and other conditions. Mechanistically, statin toxicity can arise because of HMG-CoA reductase inhibition effects, direct cellular and subcellular effects, or a combination of both. Other possible causes include genetic factors, drug–drug interactions, vitamin D status, and other metabolic or immune effects.[32]

This research still had some limitations from various aspects during the research process. Differences in the amount of normal feeding to rats were found among the groups, and not all food given using a probe could be swallowed completely by rats. The researchers could not assess the average total cholesterol and triglyceride levels before and after the intervention with lactoferrin. Therefore, further research is necessary to measure total cholesterol and triglyceride levels before and after intervention with lactoferrin.

CONCLUSIONS

Bovine lactoferrin could be used in treating patients with hyperlipidemia. In addition, 100, 200, and 400 mg/kg BW of lactoferrin could reduce total cholesterol and triglyceride levels to normal levels.

CONFLICT OF INTEREST

The author stated there is no conflict of interest.

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