ROLE OF LACTOFERRIN ON THE HISTOPATHOLOGICAL CHANGES OF HYPERLIPIDEMIC-INDUCED FATTY LIVER IN RATS

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Abstract

Introduction: Hyperlipidemia is a condition that may lead to many illnesses. The worldwide prevalence of hyperlipidemia is still relatively high. Lactoferrin is a glycoprotein found in mammals and fishes' epithelial mucosa cells, also known for its protective roles.

Objective: This study aims to discover the histopathologic changes in rat's liver after the intervention of 100, 200, and 400 mg/kgBW lactoferrin.

Method: This study involved 30 Sprague-Dawley rats divided into six groups: normal, negative control, statin-treated, dose 1, dose 2, and dose 3. The normal group was given standard feed, while the other groups were hyperlipidemic induced by a high cholesterol diet. The intervention for the statin-treated group was 1.5 mg/kgBW statin, while dose 1, 2, and 3 were respectively given 100 mg/kgBW, 200 mg/kgBW, and 400 mg/kgBW of lactoferrin. After six weeks, all rats were dissected, and the livers' histopathologic slides were scored with Manja Roenigk scoring.

Result: Lactoferrin improved the fatty liver condition. The statistical test results showed that 200 mg/kgBW of lactoferrin caused a significant change in the rats' liver structures ($p < 0.05$).

Conclusion: 200 mg/kgBW of lactoferrin significantly improved the histopathologic structure of fatty liver.

***Keywords**:* Histology, Hyperlipidemia, Lactoferrin, Liver, Sprague-Dawley Rats

Introduction

Hyperlipidemia or increased lipid level in blood is a medical condition that may lead to many other diseases. According to a survey conducted by the World Health Organization (WHO) (1) in 2008, the global prevalence of increased cholesterol level is 39%. A survey done by Indonesian Basic Health Research in 2018 stated that 7.6% of Indonesian citizens have high total cholesterol level, 13.8% have high triglyceride level, and 0.8% have very high triglyceride level (2). Many things can lead to hyperlipidemia. Some of them are genetic disorders, certain drugs consumption, unhealthy lifestyle, and other medical conditions. If left untreated, hyperlipidemia can increase the risk of cardiovascular diseases, such as coronary heart disease, cerebrovascular disease, aneurism, and non-alcoholic fatty liver disease (3, 4).

The pharmacological therapy that is widely utilized in treating hyperlipidemia is statins (4). Statin consumption, however, comes with side effects, such as myalgia, rhabdomyolysis, liver function disorder, and an increased risk of diabetes mellitus (5, 6). These side effects led to the search for other drugs or substances that can be considered an alternative to statins.

Lactoferrin is a glycoprotein produced by the mucous membrane of the epithelial cells in various mammals and fishes (7). Lactoferrin can be extracted from animal milk and breast milk (8). According to Giansanti et al. (7) and Morishita et al. (9), other than its protective functions, lactoferrin also manifests the feature of lowering high cholesterol level in the blood and the risk of atherosclerosis. The process is suspected to be related to increased cholesterol excretion through feces (10).

Lipid infiltration in the liver, which may lead to other complications, is often found in hyperlipidemic patients (11). This phenomenon can be observed through histopathological changes in the liver, such as fatty vacuolization, lipid deposition in the cytoplasm, and hepatocyte degeneration (12). With this knowledge, the authors are interested in learning lactoferrin's histopathologic effect on Sprague-Dawley rats' livers. The aim is to prove that lactoferrin can improve hyperlipidemia microscopically.

Materials and methods

The research began after the ethical clearance (03/05/KEP-FKIKUAJ/2021) was obtained from Atma Jaya Catholic University of Indonesia's Ethical Clearance Committee. This research occurred in Atma Jaya Catholic University of Indonesia School of Medicine's Animal House and Anatomic Pathology Laboratory, Jakarta, from January to November 2021. Lactoferrin, with a purity level of 99%, was purchased from Xi'an Ruisaen Biotechnology Co., Ltd, China. Subjects of 30 male Sprague-Dawley rats of five weeks old and 0.15-0.2 kgBW (kilograms body weight) were purchased from Indonesia's National Agency of Drug and Food Control (NADFC) laboratory.

The subjects were then divided into six groups of five rats. The number of subjects per group was calculated with the Degree of Freedom formula or The Resource Equation Method. To overcome possible dropouts, we added 10% or equal to one rat per group. The research was conducted for six weeks with the group labelled as normal, negative control, statin-treated, dose 1, dose 2, and dose 3. The normal group was not hyperlipidemic-induced nor given intervention; hence it was only given standard feed. The other five groups were hyperlipidemic induced for 21 days by feeding the rats with 1.5 g/150gBW (gram body weight) of high cholesterol and fat diet, which consisted of 80% quail egg yolk, 15% sucrose and 5% beef fat along with 0.01% 1 ml/150gBW of propylthiouracil solution each day (13).

At the end of the induction stage, the total blood cholesterol level was tested using blood strip test to ensure that the rats had become hyperlipidemic. Dose 1 group then received 100 mg/kgBW lactoferrin as intervention, dose 2 group received 200 mg/kgBW, and dose 3 group received 400 mg/kgBW per day. The negative control group did not receive any intervention, and the statin-treated group received statin as much as 1.5 mg/kgBW per day. The intervention occurred for 21 days.

After six weeks, all rats were dissected. Their livers were then collected and sent to the Anatomic Pathology laboratory to be made into histopathological slides with Hematoxylin-Eosin stain. The microscopical photos were taken using OLYMPUS cellSens software. In each slide, five fields were chosen, and twenty cells of each field were then observed under light microscope with 400x magnification. We consulted the results to a pathologist.

The degree of liver destruction was determined semi-quantitatively by using Manja Roenigk scoring. The minimal score is 100 and the maximum score is 400. The number of hepatocyte damage was calculated on five fields of view in each preparate. The parameter for determining hepatic cell damage was conducted by comparing the rats' livers' structural changes and finding signs of histopathological changes, including degeneration and necrosis. A normal cell will be scored 1, parenchymal degeneration 2, hydropic degeneration 3, and necrosis 4. Parenchymal degeneration is when the hepatocyte appears cloudy with cholesterol. Hydropic degeneration is when the hepatocyte swells up and hence appears bigger, yet there is no color change. Lastly, necrosis is manifested by no nucleus or undifferentiated cell membrane (14). The obtained data are shown in mean standard deviation (SD) and analyzed with SPSS 23. The statistical test used in this study was one-way ANOVA with Tukey post-hoc to see the significance between groups. A value of $p < 0.05$ is considered significant.

Results

Figure 1 shows the histopathological structure of all groups. Figure 1A is the normal group, where most of the hepatocytes appear normal. Figure 1B is the negative control group, where most hepatocytes underwent parenchymal degeneration, and some had gone through necrosis. Figure 1C, 1D, and 1E show less parenchymal degeneration than figure 1B. Figure 1F is the third dose group which manifests parenchymal degeneration, hydropic degeneration, and necrosis.

These histopathological changes align with the mean of all groups' Manja Roenigk scoring, which is shown in Figure 2 and Table 1. From the one-way ANOVA test, it is known that there is a significant mean difference between all groups ($p < 0.05$). The analysis then proceeded with Tukey post-hoc test, which showed a substantial difference between the negative control and normal group's mean ($p < 0.05$). Meanwhile, the mean between the negative control and statin-treated group's scoring does not show any significant difference ($p > 0.05$). Among the groups which received lactoferrin, only the dose 2 group (200 mg/kgBW) shows a significant difference towards the negative control group ($p < 0.05$). All rats in groups that received lactoferrin showed significantly lower Manja Roenigk scores when compared to the rats receiving a normal diet ($p < 0.05$). However, these groups do not show any significant changes compared to the statin-treated group ($p < 0.05$).

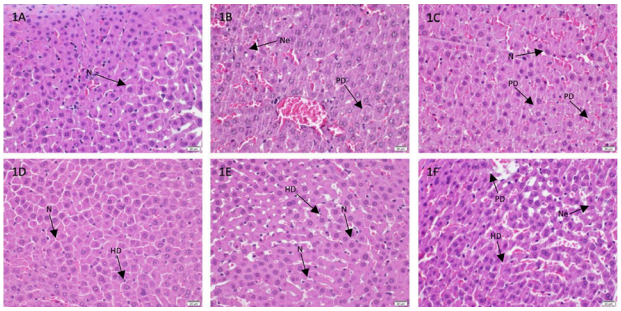

Figure 1: Lactoferrin effect on livers' histopathologic structure in rats. Sprague-Dawley male rats were given high cholesterol diet, received statin therapy of 1.5 mg/kgBW, and were treated with lactoferrin for 21 days. Representative histological findings of hematoxylin and eosin (H&E) stains in liver sections taken from normal, negative-control, statin-treated, 100 mg/kgBW, 200 mg/kgBW, and lastly, 400 mg/kgBW of lactoferrin-treated groups. N = normal, PD = parenchymatous degeneration, HD = hydropic degeneration, Ne = Necrotic

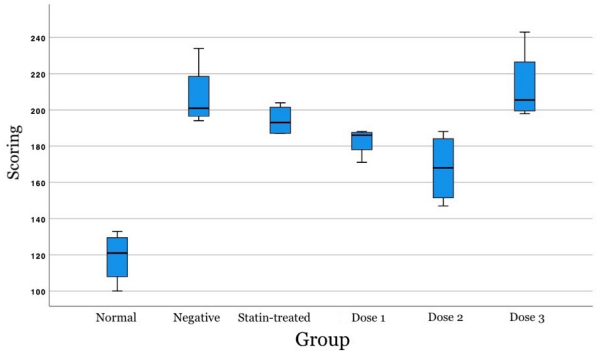

Figure 2: Histopathological analysis of fatty liver rats was evaluated by Manja Roenigk Scoring, with 100 being the lowest score for a normal structure, 200 for parenchymatous degeneration, 300 for hydropic degeneration, and the maximum score is 400 for necrosis. Data are shown as the mean, n = 5 rats per group

Table 1: Post Hoc Analysis Between All Groups' P-value

	Normal	Negative	Statin-treated	Dose 1	Dose 2
Normal					
Negative	< 0.001				
Statin-treated	< 0.001	0.833			
Dose 1	< 0.001	0.270	0.898		
Dose 2	0.004	0.022	0.210	0.752	
Dose 3	< 0.001	0.996	0.552	0.117	0.008

The hyperlipidemia induction in this study was successfully achieved, as seen in Figure 1. The statistical test shows significance between the negative control and dose 2 group, indicating that the rats' livers which were given 200 mg/kgBW of lactoferrin, improved histopathologically. The dose 2 group also has the lowest mean compared to the other lactoferrin-treated groups, which means dose 2 is the most optimal dose to improve the fatty liver condition in this study. Meanwhile, dose 1's effectivity is not much different from dose 3's, and both groups did not work as optimally as the dose 2 group.

Discussion

The results of this study are supported by the study that was conducted by Aoyama et al. (15), which stated that lactoferrin possesses the potential to improve hyperlipidemic condition in animal testing. The mechanism of lactoferrin itself is believed to be related to the increased cholesterol excretion through bile acid. This is proven by the study conducted by Ling et al. (16), which also stated that lactoferrin plays a role in the deposition of cholesterol in a rat's liver.

According to Hessin et al. (17), lactoferrin administration in the range of 0 to 200 mg/kgBW dose has no side effects. A review by Superti (18) stated that excessive dosing of lactoferrin has side effects, such as proinflammation. This phenomenon is also discussed by Nguyen et al. (19), stating that lactoferrin in high doses could cause a lesion on the colon, shorten the small intestines' villi, and increase the ratio of Bax/Bcl-2 and HIF-1α levels. A high dose is not parallel to increased receptors amount on the cells' surface; hence it does not lead to increased absorption of lactoferrin. The unbound lactoferrin has a higher chance of binding with TLR4 or TNF receptor, which will later lead to inflammation, hence a worse structure than the untreated rats' livers.

Despite the histopathological improvement found in the dose 2 group, the structure did not manage to return to normal. This is supported by the study conducted by Aoyama et al. (15) where the cholesterol level of the lactoferrin-treated group did not reach the normal groups. The scoring means of dose 1 and dose 2 group are also lower than those of the statin-treated group. The histopathological improvements found in both groups also show that intervention with lactoferrin did not manifest a significant change compared to statin given group. Nonetheless, statin remains as the first-line choice pharmacotherapy in hyperlipidemia cases (20).

This study had some limitations; hence, further study is necessary. We experienced difficulty obtaining the rats' blood samples for the cholesterol level screening before the intervention. This was caused by the high viscosity of the rats' blood due to the high fat level. The lactoferrin was also administered with the high-cholesterol diet feed due to the difficulty of administering it alone. Any possible reaction between lactoferrin and high cholesterol diet itself hasn't been discussed in any study. The duration of the study was also relatively short to observe the long-

term effect that could arise from statin and lactoferrin intervention.

Conclusion

This study shows significantly less parenchymal degeneration in the histopathological structure of the rats' livers after 200 mg/kgBW lactoferrin treatment.

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Competing interest

The authors report no conflict of interest.

Ethical clearance

We obtained approval from the Ethics Committee of Atma Jaya Catholic University of Indonesia, registered under 03/05/KEP-FKIKUAJ/2021.

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