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Lack of Atherosclerotic Lesion Progression on Severe Hyperlipidemic Rabbits

DIMAS ANDRIANTO[1]*, SULISTIYANI[1,2], LATIFAH KOSIM DARUSMAN[2], HASIM DANURI[1]

[1]Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Dramaga Campus, Bogor 16680, Indonesia

[2]Biopharmaca Research Center, Bogor Agricultural University, Jalan Taman Kencana No. 3, Bogor 16151, Indonesia

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In human, coronary heart disease causes by severe pathological atherosclerosis. In this study, we established animal model to study atherosclerosis caused by hyperlipidemia. This study therefore was undertaken to define the effect of increasing atherosclerosis risk factor, include body weight as well as age, cholesterol concentration and dietary fat in rabbit chow, and time of treatment. Male *New Zealand White* rabbits were divided into 4 groups; Group I and III were consisted of 2 months rabbit were fed with standard rabbit chow. To introduce atherosclerosis, the chow for Group II was contained 0.25% cholesterol and 5% palm oil; whereas the chow for group IV was contained 0.5% cholesterol and 5% coconut oil to induce higher atherosclerotic lesion. Results showed that group II and IV developed hyperlipidemia. However, aortic cholesterol concentration in those groups did not different significantly (P > 0.05). We suggest that low carbohydrate composition in diet, 50% lower compared to the previous researches, was able to increase high-density lipoprotein (HDL) concentration. This study demonstrated the complex interactions between low carbohydrate diet and cholesterol metabolism and the dramatic effects of reducing atherosclerosis risk factor; however, even though hyperlipidemic condition was achieved, total plasma cholesterol HDL ratio was maintained low.

Key words: atherosclerosis, hyperlipidemia, rabbit, low carbohydrate diet

INTRODUCTION

The number of patient suffering from coronary heart disease is increasing rapidly. World Health Organization reports in 2007 showed that the second mortality was due to heart disease, which affects 8 millions people, prior to cancer and followed by stroke and AIDS (WHO 2007). Hyperlipidemia prevalence in Jakarta, monitored from metabolic syndrome, is up to 26.6% and mostly females. Hyperlipidemia caused by unhealthy lifestyle and food and the risk was higher in people above 40 years due to blood hypertension, diabetes mellitus, and smoking habit.

Researches related to coronary heart disease, hyperlipidemia, and atherosclerosis has been done widely in order to find the medication or to study the effect of certain condition and substance in the diseases. Several experimental animal models have been used to carry on researches in atherogenesis mechanism or to challenge hypolipidemic agents; those are spider monkey (*Macaca fascicularis*), rat (*Rattus novergicus*), and rabbit (*Oryctolagus cunniculus*). Spider monkey is the best animal model in lipid research due to its similar metabolism with human. Nevertheless, the use of this animal is strictly regulated because it is known to be endangering species (Grundy 1991).

Rat is also a broadly used animal model in lipid research. The fact that the animal lack of gallbladder is then make a problem in the difficulties to increase plasma cholesterol concentration in order to make it achieve hyperlipidemic state. These are due to the efficient cholesterol conversion to colic acid in rat (Goodwin *et al*. 1982).

Rabbit seems to be the most suitable animal for some kind of research in hyperlipidemia and atherosclerosis, since its availability and minimum cost in its nursery (Smith & Mangkoewidjojo 1988). In addition, rabbit has normal low-density lipoprotein (LDL) level, i.e. only 17 mg/dl, lower compared to the other species (Grundy 1991).

Hyperlipidemia and atherosclerosis associated researches have been done intensively and still will become interesting field to carry out. These are due to the cholesterol and atherogenesis mechanism are remains obscure, although many theories are widely accepted. Furthermore, there are many hypolipidemic agents suspect that has not been verified, especially the natural one.

Previous researches in the field come up with high deviation since there is no standard in establishing hyperlipidemia and atherosclerosis in rabbit. This study therefore was undertaken to define the effect of increasing atherosclerosis risk factor, include body weight as well as age, cholesterol concentration and dietary fat in rabbit chow, and time of treatment. They revealed that, contrary to expectations, atherosclerosis was not developed although animals have been achieving hyperlipidemic state.

MATERIALS AND METHODS

Animals and Diets. Thirty two male *New Zealand White* rabbits (20 rabbit were 2 months old and 12 rabbits were 4 months old) were purchased from Indonesian Research

*Corresponding author. Phone/Fax: +62-251-8423267,
E-mail: dimas_andrianto@yahoo.com

Institute for Animal Production at Bogor and kept in individual cages in an animal room at room temperature (25 ± 1 °C) under a 12-h dark-light cycle. They were allowed an adaptation period of 2 weeks and then were randomly allocated to four groups (Table 1). Treatments were done until plasma LDL concentration of group II and IV drop off up to approximately 200 mg/dl. During the study period, daily diet of each animal was restricted to 45 g rabbit chow/kg body weight to align constant body weight. After over night fasting, blood samples were taken from the inferior vena cava every 2 weeks. Research was ended when LDL blood plasma concentration in hyperlipidemic rabbit at group II or IV was constant above 200 mg/dl. At the end of research, all rabbits were killed under anesthesia. The aorta was dissected directly under the perfusion of physiological saline and freezing subsequently.

Plasma Lipids Concentration. Total plasma cholesterol (TPC), triglyceride, LDL, and high-density lipoprotein (HDL) were measured using enzymatic cholesterol kit from Randox® spectrophotometrically.

Atherosclerosis Study. The degree of atherosclerosis in aorta was evaluated by the concentration of cholesterol in thoracic and abdominal aorta. Aorta extracted using methods describe by Folch *et al.* (1957). Cholesterol extracted from the previous methods then to be subject for o-phtalaldehide reaction prior to Rudel *et al.* (1973). Reaction mixtures were the analyzed using spectrophotometer at 550 nm.

Statistical Analysis. All values were expressed as means ± standard error, and were analyzed by one way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). $P < 0.05$ was considered statistically significant.

RESULTS

Animals and Diets. There were no differences in all rabbit body weight, TPC, triglyceride, LDL, and HDL at the beginning of the treatment. At the end of the treatment there were no differences in body weight between younger rabbit group (group I and II for 2.1 ± 0.3 and 2.2 ± 0.2 kg) and between the elder one (group III and IV for 3.3 ± 0.4 and 3.3 ± 0.5 kg) as well (Figure 1).

Plasma Lipids Concentration. Initial experiments were undertaken to measure the cholesterol concentration in blood plasma and blood aorta in the two extreme conditions. There were no differences in plasma lipid profiles; TPC, triglyceride, LDL, and HDL at the beginning among rabbits with younger age (group I and II) and among the elder one (group III and IV). Most importantly, as shown in Table 2, the entire lipid profiles at baseline was in the normal range.

As summarized in Figure 2, 3, 4, and 5, no significant change in triglyceride, LDL, TPC, and HDL rabbits of group I and III ($P > 0.05$) that were administered by standard rabbit chow during the research period. Hence, these groups were

Table 1. Research experimental design

Group	Rabbit age (months)	N	Chow composition	Treatment period (weeks)
I	2	10	Standard chow	7
II	2	10	Standard chow with 0.25% cholesterol and 5% palm oil	7
III	4	6	Standard chow	10
IV	4	6	Standard chow with 0.5% cholesterol and 5% coconut oil	10

Standard rabbit chow contain 22.14% carbohydrates, 8.71% fats, and 17.01% proteins.

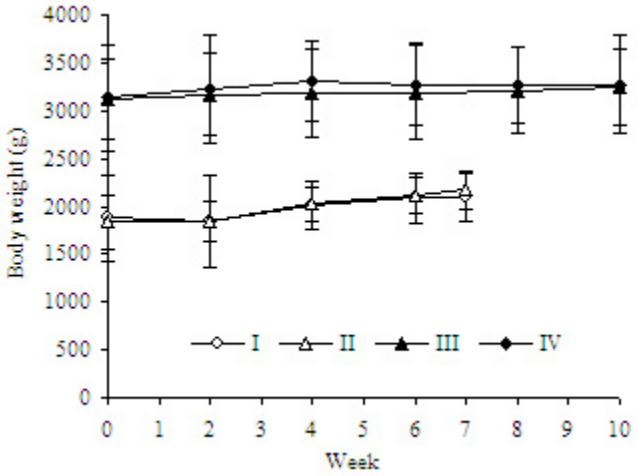

Figure 1. Rabbit body weight along the treatment period.

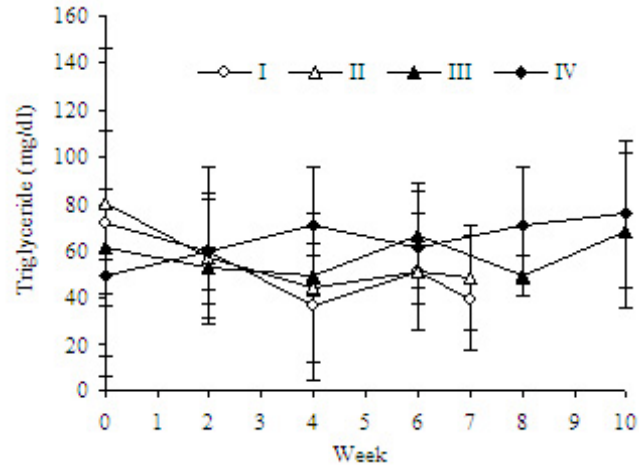

Figure 2. Rabbit triglyceride concentrations along the treatment period.

Table 2. Rabbit lipid profiles at the baseline of the research

Group	Lipid analysis			
	Cholesterol	Triglyceride	LDL	HDL
I	41.10 ± 31.61ab	72.00 ± 39.27a	0.00 ± 0.00a	20.41 ± 6.78a
II	44.29 ± 14.00abc	80.26 ± 65.74a	1.76 ± 2.76a	20.66 ± 6.02a
III	62.02 ± 24.30bc	61.56 ± 25.08a	12.08 ± 15.36b	39.81 ± 7.07b
IV	67.11 ± 22.506c	49.38 ± 7.52a	12.30 ± 16.86b	46.21 ± 12.70b

Value followed by similar alphabet were not significantly different based on DMRT at the á of 5%.

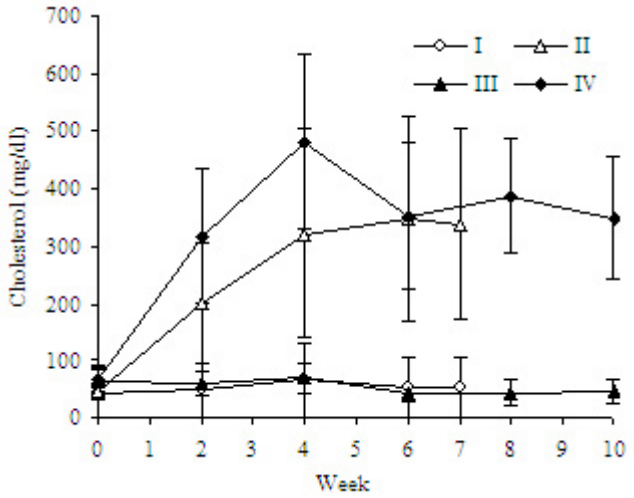

Figure 3. Rabbit total plasma cholesterol concentrations along the treatment period.

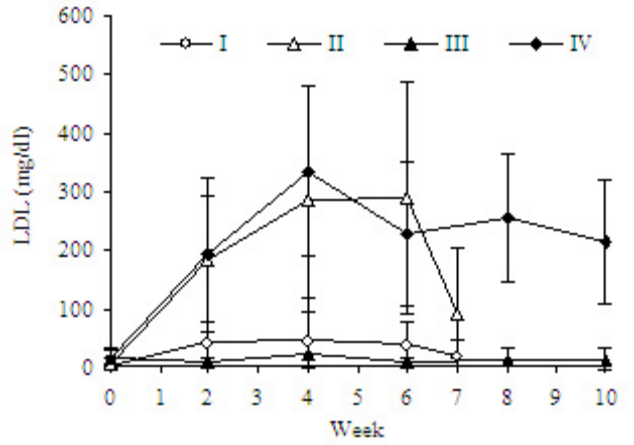

Figure 4. Rabbit cholesterol LDL concentrations along the treatment period.

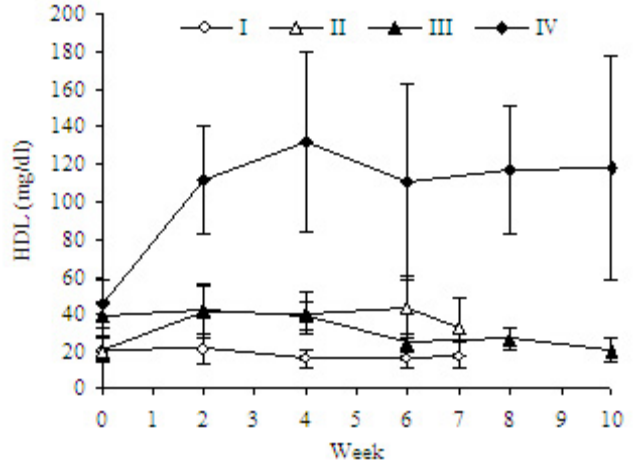

Figure 5. Rabbit cholesterol HDL concentrations along the treatment period.

also maintaining its lipid profile within the normal range during the research.

Figure 2 showed that rabbits in group II and IV which was consumed atherogenic diet with low carbohydrate increased in LDL plasma level within similar patterns from the baseline to the forth week. We end the research for the younger rabbits in group I and II in week 7 due to the LDL plasma concentration in group II was drop down dramatically from the forth week to sixth and seventh weeks for 68 and 75% compared to the forth week. The treatment of group III and IV was continued up to ten weeks.

Total plasma cholesterol (TPC) concentration of group II and IV resulting similar patterns during the research, which was increased significantly ($P < 0.05$) from the first week to the forth week and then was constant until the research was ended (Figure 3). At the end of the research, TPC group I, II, III, and IV were 51.43 ± 36.71, 338.43 ± 165.29, 44.38 ± 20.84, and 349.14 ± 106.52 mg/dl, respectively.

The same patterns were also occurred in HDL concentration from group II and IV, which was increased significantly ($P < 0.05$) from the first week to the forth week and then stay constant until the research ended (Figure 4). At the end of research, HDL concentration in each group were 17.36 ± 6.80, 33.05 ± 15.74, 20.68 ± 6.35, and 118.42 ± 59.51 mg/dl, respectively.

Figure 5 showed that triglyceride plasma concentration during the research tend to be fluctuated, but the fluctuation was in the normal range for the entire groups involved. Triglyceride concentration for group I, II, III and IV at the end of research each were 39.73 ± 27.07, 48.59 ± 22.26, 68.69 ± 32.92, and 75.88 ± 31.08 mg/dl, respectively.

Atherosclerosis Study. All experimental animals was completed the study and tolerated the treatment in well condition. During the research, there were no significant changes in the body weight and triglyceride concentration ($P > 0.05$). In response to atherogenic rabbit chow treatment, TPC, LDL, and HDL increase in group II and IV respectively, but there was no relation to high TPC and LDL, atherosclerosis plaques did not occurred (Table 3).

There was no significant difference between aortic cholesterol concentrations in all groups ($P > 0.05$); in conclusion, to those the atherosclerosis plaque was not formed in group II and IV. Cholesterol concentration in thoracic aorta for each group were 8.16 ± 4.14, 13.46 ± 3.61, 7.34 ± 2.44, and 11.00 ± 2.71 mg/g, all values were measured in mg cholesterol per dry weight of thoracic aorta.

DISCUSSION

This study illustrate that hyperlipidemia does not, as expected, always lead to atherosclerosis, rather, the treatment applied initiates a series of more complex events that affect cholesterol balance in the whole animal. Cholesterol plaque formation in aortic vessel wall is undetectable, and the high cholesterol pool in LDL is replaced by a compensatory increase in HDL plasma concentration of the atherogenic fed animals. The results were consistent for the group II and IV. These findings illustrate that atherosclerotic lesion can be prevent using the reverse cholesterol pathway, and provide detailed information on how of these cholesterol pathways is balanced to maintain cholesterol homeostasis in the whole organism.

Cholesterol and oil plays the central role of making hyperlipidemic state and induce atherosclerotic lesion (Hur *et al*. 2005). Clearly, these two processes are interrelated. In any animal on a relatively high cholesterol diet, most of the input of sterol to the liver comes from the diet while a lesser amount is derived from de novo synthesis in whole cell (Ross *et al*. 1977). In the recent state, the rate of net input must be balanced by an equal rate of net sterol output from the liver as cholesterol itself or as bile acid. While small amounts of cholesterol can be excreted through the skin and as steroid hormones, the majority is disposed of through the gastrointestinal tract as either neutral or acidic fecal sterol (Ozben 1989).

To a limited degree, the liver can compensate for a change in these rates of net sterol flux. For example, hepatic cholesterol synthesis may be reduced under circumstances where there is an increase in sterol absorption from the diet or diminished bile acid synthesis. However, once hepatic synthesis approach zero, further increases in net cholesterol delivery to the liver are associated with an expansion of the cholesteryl ester pool, suppression of LDL receptor activity, an increase of LDL cholesterol production rate, and a rise in the circulating LDL cholesterol concentration (Boger *et al*. 1997). Furthermore, LDL will become oxidize and draw macrophages, which then turn to foam, cell in aortic vessel wall. As a result, atherosclerotic plaque has been starting to developed (Libby *et al*. 2002).

In the present study, we examined the plasma cholesterol profiles and cholesterol concentration in thoracic and abdominal aorta. As expected, TPC and LDL plasma cholesterol concentration increase rapidly in those who fed the atherogenic diet (Figure 3 & 4). Although of the LDL in blood plasma has been increasing far above its normal level, atherosclerotic plaque has not yet been developed (Table 3). The increasing of HDL concentration correspondingly (Figure 5) caused these, thus it reduce atherosclerosis risk factor due to HDL role in reverse cholesterol transport (Ansell *et al*. 2005).

Grundy (1991), Momuat *et al.* (2001), Andriani (2004), and Kurosawa *et al*. (2005) showed that LDL has negative correlation with HDL. Due to its opposite role, this was broadly accepted. Hur *et al.* (2005), Hong *et al.* (2007) and this research come up with different result in which LDL and TPC increase will elevate HDL level. Several possibilities may become background of these findings. Hur *et al.* (2005) explained that HDL responses might vary from one individual to other. The elevation of HDL during research by Hong *et al.* (2007) was something unexpected either, which may be resulted from the decrease of HDL receptor at the hepatocyte surface.

Table 3. Rabbit aortic cholesterol concentration at the end of research

Group	Aortic cholesterol concentration (mg/g dry weight)	
	Thoracic aorta	Abdominal aorta
I	8.16 ± 4.14a	7.54 ± 3.34a
II	13.46 ± 3.61a	8.59 ± 4.11a
III	7.34 ± 2.44a	7.34 ± 2.44a
IV	11.00 ± 2.71a	11.00 ± 2.71a

Value followed by similar alphabet were not significantly different based on DMRT at the á of 5%.

The current studies, therefore, took advantage of the used en extreme design in inducing atherosclerosis. Because of its consistency in the undeveloped atherosclerotic plaque of group II and IV (Table 3), hence we conclude that there is a factor that can be controlled in order to have better result in the forward research on atherosclerosis, such as controlling the nutrition in diet. Contrary to the hypotheses by Hur *et al.* (2005) and Hong *et al*. (2007), undeveloped atherosclerotic plaque in the research may come from the rabbit chow used. In addition, TPC and LDL concentration group II and IV rose above normal, so there is nothing wrong with the cholesterol and oil in the atherogenic rabbit chow.

To evaluate what caused the increase of HDL concentration in the present research, we evaluated the standard chow and compared it with standard chow used by Andriani (2004) and Azima (2004). We found that the carbohydrate concentration in our standard chow was only about 50% lower compared to those in Andriani (2004) and Azima (2004) (Table 4).

Several research in human showed that low carbohydrate in diet have a correlation in the elevated HDL concentration of hyperlipidemic patient. Brehm *et al.* (2003) proved that 15% carbohydrate, 28% protein, and 60% fat (20% saturated fat) intake for 6 months will elevate HDL concentration for 13%, it was also noted that β-hyroxybutirate, one of the ketone bodies, plasma concentration does not increase during this low carbohydrate diet. While Volek *et al*. (2003) confirmed that 10% carbohydrate, 29% protein, and 60% fat (41% saturated fat) for 4 weeks will increase HDL concentration for 33% and decrease TPC HDL ratio for 13%.

Detailed mechanism behind the process is still unclear, however it was suggested that β-oxidation and Crebs cycle run out efficiently so the ketone bodies concentration in plasma does not increase. Moreover, hepatocyte will elevate the production rate of pre-pro HDL and lipoprotein lipase activity (LPL) in extra hepatic tissue will increase (Volek *et al*. 2003).

Elevated activity of LPL will increase the uptake of triglyceride from triglyceride rich lipoprotein by extra hepatic tissue. Consequently, triglyceride rich lipoprotein will also lost its surface component such as free cholesterols, apolipoproteins, and phospholipids as well. HDL will absorb these particles through the activity of various proteins in HDL surface.

In conclusion, we reported that low carbohydrate concentration in atherogenic diet may prevent atherosclerosis by increasing HDL plasma concentration, even though

Table 4. Composition of standard chow compared to Andriani (2004) and Azima (2004)

Composition	Standard chow	Andriani 2004	Azima 2004
Carbohydrates	28.14	51.05	58.35
Fats	8.71	3.00	7.77
Proteins	17.01	18.00	17.81
Fibers	12.00	4.00	10.42
Ash	22.94	na	6.62
Waters	11.20	na	9.45
Calorie (kcal)	259.00	303.20	374.57

na: not available.

hyperlipidemic condition has been achieved. These outcomes emphasize the importance of obtaining quantitative data about nutrition composition in order to find the best standard rabbit chow to held hyperlipidemia and atherosclerosis researches.

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