

Atherogenesis inhibition by darapladib administration in dyslipidemia model Sprague-Dawley rats

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ABSTRACT

Background: Atherosclerosis is a chronic inflammation disease that is caused by the interaction between monocyte and endothelial injury in tunica intima. One of the major factor of atherosclerosis is dyslipidemia. Chronic dyslipidemia, especially hypercholesterolemia, can directly alter endothelial cell through reactive oxygen species (ROS) production that oxidizes low-density lipoprotein (LDL) to become oxidized LDL (Ox-LDL). Proinflammatory cytokines, the products of perivascular adipocyte tissue (PVAT), may draw macrophage. Macrophage then engulfs Ox-LDL and becomes foam cell within tunica intima. Lipoprotein-associated phospholipase A₂ (Lp-pLA₂) is an enzyme that cleaves Ox-LDL to become proatherosclerotic products. Darapladib, an Lp-pLA₂ inhibitor, is expected to inhibit atherosclerotic lesion progressivity. **Aims and Objective:** To know the effects of darapladib on Ox-LDL level, PVAT thickness, and foam cell number. **Materials and Methods:** This study used *in vivo* posttest controlled group design with two time series. Thirty male Sprague–Dawley rats divided into two group based on time series (8 weeks and 16 weeks). Each time serial was divided into three groups which were: standard diet group ;high-fat diet group; and dyslipidemia model with darapladib administration group with dose of 200 mg/200 g body weight (BW). The parameters that was measured in this study were lipid profile [total cholesterol, LDL/very-low-density lipoprotein (VLDL), and high-density lipoprotein (HDL)], Ox-LDL level, number of foam cells, and PVAT thickness. **Result:** Ox-LDL level and foam cell number decreased significantly ($p = 0.000$ and $p = 0.005$, respectively), while PVAT thickness did not show significant difference ($p = 0.912$). **Conclusion:** In this, study, it has been proven that darapladib decreases Ox-LDL levels and foam cell numbers but not in PVAT thickness, even though a decreasing pattern was observed histologically. Further study needed to know the optimum dosage of darapladib administration.

KEY WORDS: Darapladib; Lp-PLA₂; Ox-LDL; Foam Cell; PVAT; Dyslipidemia

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INTRODUCTION

Cardiovascular disease (CVD) is mostly developed from atherosclerosis. Recently, CVD is the leading cause of mortality worldwide, which contribute to 31% of death worldwide.^[1] There are several factors that increase the chance of a person to develop atherosclerosis. These factors are smoking, hypertension, obesity, inactivity, kidney disease, diabetes, and dyslipidemia.^[2]

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Dyslipidemia is a major established risk factor of atherosclerosis.^[3] It has become a worldwide major health problem. Approximately 98.9 millions of Americans that older than 20 years reveal dyslipidemia. Mortality and morbidity from dyslipidemia result from clinical complications. These complications are mostly developed from atherosclerosis, such as ischemic heart disease, stroke, and peripheral artery disease.^[4,5]

One of the emerging theories for the atherosclerosis formation is involved with the activity of lipoprotein-associated phospholipase A₂ (Lp-pLA₂). Lp-pLA₂ is an enzyme that produces potent proinflammatory substances. Lp-PLA₂ is produced by monocyte or macrophage in inflammation process, including atherosclerosis.^[6] The excessive intake of high calories diet that comes along with dyslipidemia condition may increase the accumulation of low-density lipoprotein (LDL). In endothelial lesion, LDL undergoes oxidative modification to become oxidized LDL (Ox-LDL), as a result of the activities of macrophage and endothelial cells. Lp-PLA₂ that has been activated by macrophage and Ox-LDL will hydrolyze the Ox-LDL into two potent proinflammatory products [lysophosphatidylcholine (Lyso-PC) and oxidized nonfree fatty acid (Ox-NEFA)].^[7-9]

Ox-LDL is capable of inducing monocyte adhesion to endothelial cells. During the accumulation of lipid, macrophage infiltrates to lipid deposition and leads to the thickening of intima. Macrophage uptakes Ox-LDLs via endocytosis and then transport them to lysosomes to be degraded. But, Ox-LDLs are less susceptible to degradation. Thus, the cholesterol will be degraded slowly. This condition will induce the macrophage to transform into a foam cell.^[10] Inside the sclerotic plaque, Lp-pLA₂ stimulates expression of several adhesion molecules, monocyte recruitment, macrophage activation, macrophage transformation into foam cell, and several cytokines. By these actions, sclerotic plaque area expands, and inflammatory activation leads the necrosis inside lipid core, which become an unstable plaque.^[11]

Meanwhile, perivascular adipocyte tissue (PVAT), which is an adipose tissue surrounding the blood vessels, is also involved in the pathogenesis of atherosclerosis, atherothrombosis, and plaque rupture.^[12] The mechanisms of PVAT in atherosclerosis are to increase the production of adipokines and proinflammatory cytokines, which increase the production of monocyte chemoattractant protein (MCP)-1 and interleukin-6.^[13] The proinflammatory cytokines from PVAT can diffuse into surrounding structures and cause the effects at two places, which are the endothelial and other nearby tissues. Effects on endothel cause an endothelial dysfunction (owing to declining production of NO), hypercoagulable (owing to increased regulation of tissue factor and plasminogen activator inhibitor-1), an increase in chemotaxis, and monocyte adhesion to endothelium (owing to upregulation of MCP-1 and expression adhesion molecules). While on the adjacent tissue, cytokines and adipokines causes influx of macrophages in the arterial wall tissue and proliferation of smooth muscle cells.^[12]

The pharmacological management of atherosclerosis that is caused by dyslipidemia is currently focusing on maintaining the lipid profile, such as total cholesterol (TC), high-density lipoprotein (HDL), and LDL at desirable levels. Recently, Lp-PLA₂ has become

a promising target of intervention in the management of atherosclerosis.^[14] One of the drugs that have an inhibition mechanism of Lp-PLA₂ is Darapladib.^[15]

Darapladib is a Lp-PLA₂ inhibitor developed by GlaxoSmithKline (GSK) to become a drug for atherosclerosis. Although its pharmacokinetic effect and minimal interaction with other drugs has been proven, darapladib turned out to be failure in last phase III clinical trials.^[16,17] This made darapladib an interesting drug for research to explore its true pharmacological feature and effect in the future. In this article, we report the effects of darapladib on lipid profile, Ox-LDL level, number of foam cells, and PVAT thickness in Sprague-Dawley rats that were given high-fat diet.

MATERIALS AND METHODS

Darapladib

Darapladib was supplied by GlaxoSmithKline in the form of powder. Darapladib was administrated to the animal models daily via oral gavage in 8 and 16 weeks. High-fat diet (HFD) was given to the rats during the experiment.

Study Group

Thirty Sprague-Dawley male rats (*Rattus norvegicus*), 6–8 weeks of age and weighing 100–200 g, were obtained from Bogor Agricultural University, Bogor, Indonesia. These rats were divided into three groups: a standard diet group; HFD group; and a group fed with HFD and given a darapladib orally with 20 mg/200 g body weight (BW) (GlaxoSmithKline). Each group was divided into two time series: 8 and 16 weeks. This study got ethical clearance from the Animal Care and Use Committee (Number 229-KEP-UB).

Lipid Profile Measurements

The lipid profiles were measured in the blood serum of rats by counting the levels of lipids using a EnzyChrom™ Kit from BioAssay Systems.

Measurement of Ox-LDL

Ox-LDL level was measured by Sandwich ELISA method using Rat OX-LDL ELISA kit (Cat. No. E-EL-R0710). The first step is antigen coating; 100 µL standard and sample were put into wells that were coated with antibody before incubation at 37°C for 90 min. The residual antigen that did not bind with antibody were disposed. After that, 100 µL biotin-antibody was added into each well and incubated at 37°C for 1 h. The fluid in well was aspirated and washed by wash buffer for three times. Next, 100 µL HRP-avidin was added into each well and incubated at 37°C for 30 min. The aspiration step was repeated, and washing process done for five times. TMB substrate (90 µL) was added to each well and then incubated at 37°C for 15 min in light-free area. To stop the reaction, 50 µL stop solution was added. Each well was read 5 min later with ELISA reader at 450 nm wavelength.

Tissue Sampling

Tissues from the rats were observed in the end of the study after surgically removing aortic tissues and blood samples via cardiac puncture. The blood samples were preserved in the Laboratorium Sentral Ilmu Hayati, Brawijaya University. The aortic tissues were fixated in 10% formalin % in the Laboratory of Pathology and Anatomy, Brawijaya University, Malang, Indonesia.

Foam Cell Measurements

Observation of foam cell started with slide preparation. The aortic tissue was stained by Oil Red O staining. Then, tissues were blocked and cut and were deparaffined. After that, hematoxylin-eosin (HE) staining was performed. The study begun with scanning of histopathological slides using a microscope at 400 × magnification, viewed, and then counted using dotslide software.

PVAT Measurements

Observation of PVAT started with slide preparation. The aortic tissues were blocked and cut and were deparaffined. After that, HE staining was performed. The study begun with scanning of histopathological slides using a microscope with 400 × magnification, viewed, and then measured using dotslide software. The PVAT thickness values were gathered from the sum of the smallest, medium, and largest thickness in PVAT and then divided by three from each sample.

Statistical Analysis

Repeated ANOVA test was used to determine the effects of darapladib on Ox-LDL, number of foam cells, and PVAT thickness. This analysis was continued by *post hoc* test using the Duncan method to detect differences in parameters in each treatment group. SPSS software, version 16 (IBM Corporation, New York, NY), was used for data analysis.

RESULTS

Table 1 gives the results of the analyzed parameters using the repeated ANOVA (p value < 0.05) and *post hoc* Duncan tests. The parameters were measured in all treatment groups to observe the effects of the darapladib administration. The significance of the darapladib administration was determined after p values had been taken from the measurements of the parameters that were compared with the standard p value of the repeated ANOVA test.

The administration of darapladib showed significant effect on decreasing of some parameters that were connected with vascular injury. Darapladib was beneficial in lowering the levels of Ox-LDL, lipid profiles (LDL), and numbers of foam cells. Darapladib was also beneficial in increasing the level of HDL (repeated ANOVA, p < 0.05). The highest parameters value (except HDL) of treatment groups were found in the group that received HFD for 16 weeks and the lowest values of the

parameters (except HDL) were found in the group that received standard diet for 8 weeks (Ox-LDL and foam cells value) and in the group that received standard diet for 16 weeks (LDL value). The highest level of HDL was found in the group that received HFD for 16 weeks and lowest in group that received standard diet for 8 weeks.

However, the decreasing of PVAT showed a different insignificant pattern after being analyzed with the repeated ANOVA test (p < 0.005). The highest thickness of PVAT was found in the group that received HFD for 16 weeks and the lowest thickness of PVAT in the group that received darapladib for 16 weeks. These disorder made the p value for the PVAT higher than the probability value of p < 0.005 ($p = 0.916$) and could not be further processed using the *post hoc* Duncan test to determine the difference between the treatment groups. Although the difference was not statistically significant, the result showed that darapladib can greatly reduce the thickness of PVAT, because, in the group that received darapladib for 8 and 16 weeks, a lowest thickness of PVAT was observed than that in the groups that received the HFD for 8 and 16 weeks.

DISCUSSION

Atherosclerosis is a chronic inflammatory disease in which the interaction of monocytes with luminal endothelium leads to atherosclerotic alteration of arterial intima.^[18] In addition, atherosclerosis involves the complex process between Ox-LDL and vascular smooth muscle cells.^[19] The inflammatory cells, such as monocytes, macrophages, T cells, and mast cells, secrete enzyme Lp-pLA₂ and circulate primarily bound to LDL. Within the atherosclerotic plaque, Lp-pLA₂ hydrolyzes Ox-LDL particles leading to the formation of Lyso-PC and Ox-NEFA. These products are believed to contribute to atherogenesis and plaque destabilization through propagation of the inflammatory cascade and to endothelial dysfunction, necrolysis, and apoptosis.^[20] The main risk factor of atherosclerosis is dyslipidemia. Dyslipidemia is a major term that refers to a number of lipid disorders. The basic categories of dyslipidemia include low HDL, elevated LDL, elevated very-low-density lipoprotein, elevated triglyceride, elevated cholesterol, excess lipoprotein (a), and mixed lipid disorders.^[21]

Darapladib is a novel, selective, reversible orally active inhibitor of Lp-pLA₂ activity. Metabolism of darapladib is fundamentally mediated by cytochrome p450 (CYP) 3A4, with lesser contribution of other enzymes.^[22] Darapladib inhibits phospholipase activity from Lp-pLA₂ and shows good enzyme selectivity towards Lp-pLA₂. This drug has the IC₅₀ value of 0,25 nM. The IUPAC name of darapladib is *N*-[2-(diethylamino)-ethyl]-2-[2-[(4-fluorophenyl)methylsulfanyl]-4-oxo-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-1-yl]-*N*-[[4-[4-(trifluoromethyl)phenyl]phenyl]methyl]acetamide.^[23] *In vitro* studies suggest that Lp-pLA₂ inhibition can inhibit at least some of the inflammatory and proatherogenic effects of LysoPC and OxNEFA. The selective inhibition of Lp-pLA₂ activity prevents the generation of Lyso-PC and Ox-NEFA, resulting in the

Table 1: Measurement and analysis of parameters by ANOVA

Parameter	Treatment groups						p (ANOVA p < 0.05)
	Normal diet 8 weeks	High Fat Diet 8 weeks	HFD + Darapladib 8 weeks	Normal diet 16 weeks	High Fat Diet 16 weeks	HFD + Darapladib 16 weeks	
Lipid profile Total Cholesterol (mg/dl)	72.799 ± 4.045	115.555 ± 3.110	81.355 ± 3.975	55.550 ± 5.434	117.774 ± 4.498	101.955 ± 7.977	0.049
HDL (mg/dl)	34.739 ± 8.312	8.354 ± 2.057	20.017 ± 0.355	35.757 ± 1.575	18.145 ± 0.889	21.400 ± 5.057	0.000
LDL/VLDL (mg/dl)	48.831 ± 5.055	88.195 ± 3.075	50.337 ± 2.543	19.241 ± 15.545	102.142 ± 15.545	55.509 ± 11.330	0.000
Thickness of PVAT (µm)	455.55 ± 94.3454	555.85 ± 84.5305	494.72 ± 154.2432	478.90 ± 73.0025	580.94 ± 100.4355	388.35 ± 35.1524	0.915
Ox-LDL (ng/ml)	0.215 ± 0.054	1.935 ± 0.112	0.329 ± 0.050	0.489 ± 0.355	2.022 ± 0.124	0.585 ± 0.082	0.000
Number of foam cell (cell/slide)	8.0000 ± 13.7355	11.0000 ± 5.5833	2.7500 ± 4.2720	17.7500 ± 5.3774	25.0000 ± 7.0355	8.8000 ± 5.2530	0.005

Data are presented as mean ± standard deviation (range) values. All the values of the parameters have been corrected into International Standard of Mathematics (decimals).

*p < 0.05 indicates statistically significant difference.

ANOVA = Analysis of Variance; HFD = High Fat Diet.

inhibition of monocytes chemotaxis and the protection of macrophages against cell death.^[24] In addition, selective inhibition of Lp-pLA₂ activity can inhibit the subsequent progression to advanced lesions. Treatment with darapladib has been found to reduce the coronary atherosclerosis, reduce lesion with Lyso-PC content, and downregulate the expression of proinflammatory genes in coronary arteries in animal model.^[25,26]

Lipid profile in rats that had been fed with HFD were determined. Group of rats that had been fed with HFD, for 8 or 16 weeks, showed significant lipid levels that indicated dyslipidemia condition based from LDL and HDL levels. TC level was insignificant to indicate dyslipidemia condition, although an obvious pattern of reduction as a result of darapladib treatment was found. This lipid condition signify the existence of dyslipidemia in rats that was fed with HFD.

Repeated ANOVA test shown significant result of Ox-LDL level reduction (p < 0.05) between group that was given HFD and darapladib for 8 weeks and group that was given HFD and darapladib for 16 weeks. This signify the effect of darapladib on the level of Ox-LDL in rats. Darapladib, with its mechanism to inhibit Lp-pLA₂, can reduce the macrophage uptake of Ox-LDL.^[27] This theories correlate with research that state that daily administration of darapladib for 24 weeks can reduce Lyso-PC, that is, the proinflammatory derivation of Ox-LDL, which is created when the oxidative modification of LDL occurs.^[26] It indicates that darapladib can lower the activity and level of Ox-LDL. According to our research, the level of Ox-LDL in group that was given HFD and darapladib showed reduction pattern than group that was given HFD alone. It shows that darapladib is capable of lowering the level of Ox-LDL in HFD condition.

Post hoc Duncan test showed that there were no significant difference in Ox-LDL level between groups that were given HFD for 8 weeks and for 16 weeks. This finding correlates with theories that stated about few indication of Ox-LDL level modulation by age.^[28] On the contrary, there was no significant difference of Ox-LDL levels between group that was given HFD and darapladib and group that was given standart diet, whether it is for 8 or 16 weeks. This indicated that daily administration of darapladib can lower the Ox-LDL level of Sprague-Dawley rats under dyslipidemia condition until the level reached close to Ox-LDL level of group that was given standard diets (Figure 1).

Repeated ANOVA test showed a significant difference in the reduction of number of foam cell (p < 0.05) between HFD and darapladib group for 8 weeks and HFD and darapladib group for 16 weeks. This results show that darapladib exhibit effect on the foam cell in rats. According to another experimental study, it was shown that administration of darapladib daily for 6 weeks reduced lesion plaque and macrophages at arcus aorta area.^[29] This reduction was caused by darapladib mechanism that blocked Lp-PLA₂ activity, which in turn blocked the formation of foam cell. In another study, the expression of RNA Lp-PLA₂ showed that the formation of foam cell begun by production of Lp-PLA₂.^[30] It proved that darapladib had blocked Lp-PLA₂

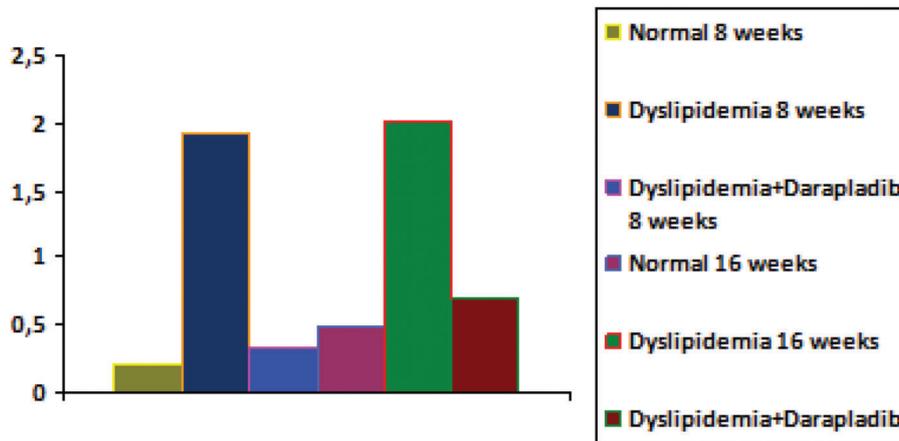


Figure 1: Effect of darapladib on Ox-LDL level at each groups (ng/mL).

activity and, thereby, blocked the formation of foam cell. According to this study, number of foam cell in HFD and darapladib group showed reduction pattern than HFD group and normal diet group. It can be concluded that darapladib is capable to reduce the number of foam cell in HFD condition.

On the basis of *post hoc* Duncan test, it has been found that there is a difference in foam cell number between normal diet group for 8 weeks and normal diet group for 16 weeks. This difference may be caused by the age of rats, which are younger than normal diet group for 16 weeks. One of the risk factor of

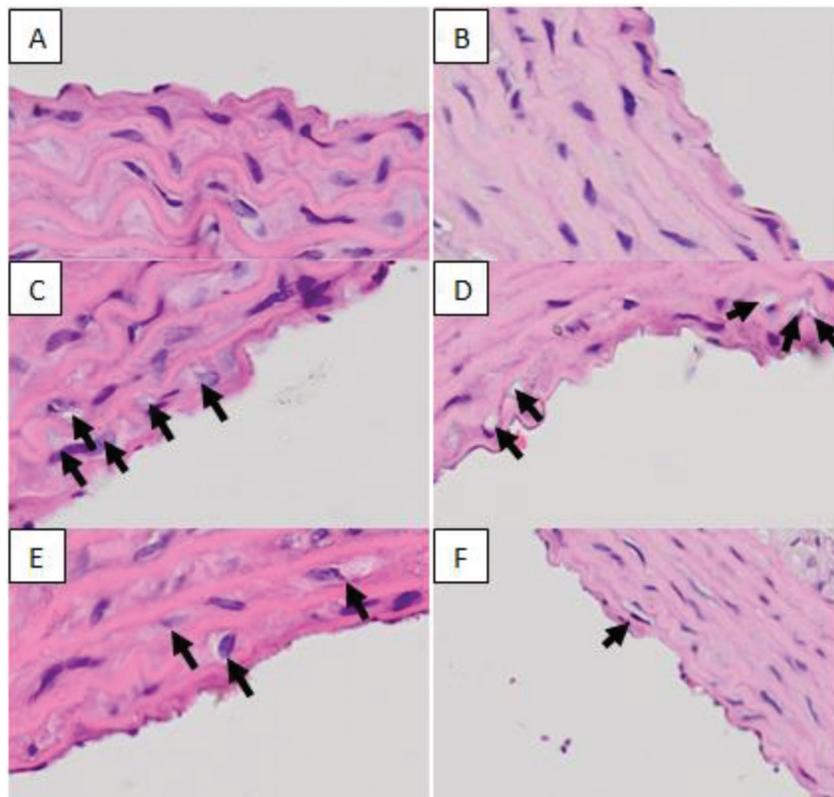


Figure 2: Foam cells in different treatment groups of rats: (A) normal diet group 8 weeks; (B) normal diet group 16 weeks; (C) high-fat diet group 8 weeks; (D) high-fat diet group 16 weeks; (E) high-fat diet and darapladib (20 mg/200 gBW) group 8 weeks; and (F) high-fat diet and darapladib (20 mg/gBW) group 16 weeks.

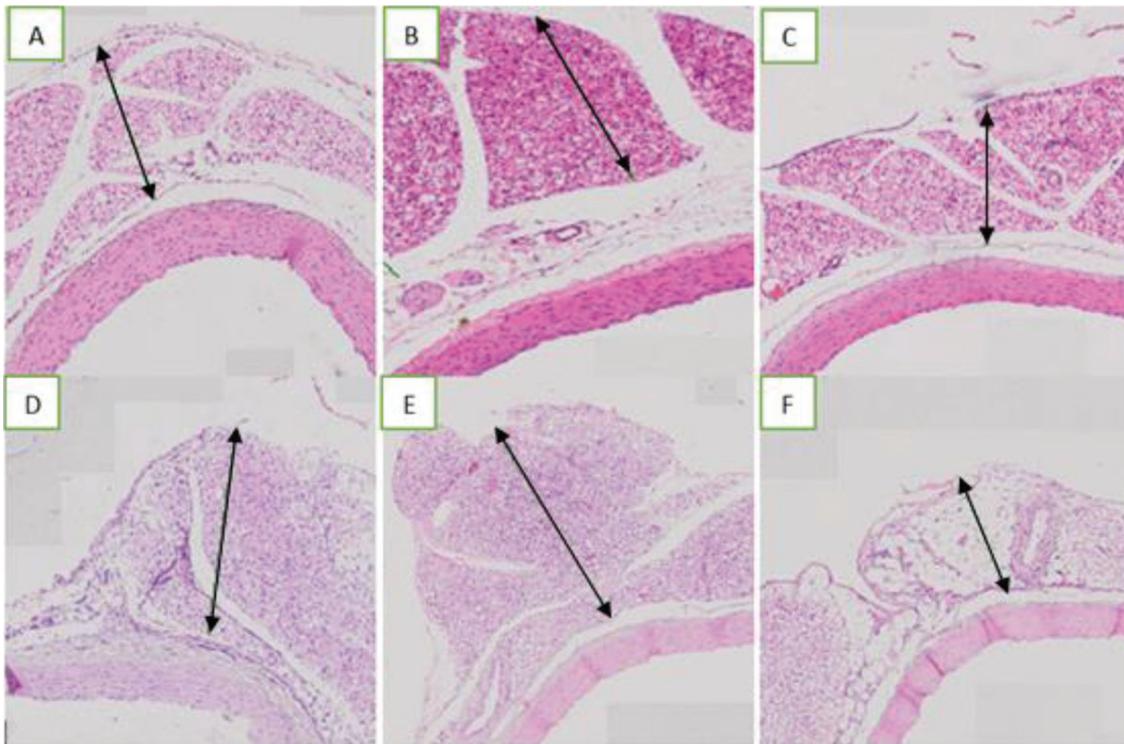


Figure 3: PVAT in different treatment groups of rats fed: (A) normal diet group 8 weeks; (B) normal diet group 16 weeks; (C) high-fat diet group 8 weeks; (D) high-fat diet group 16 weeks; (E) high-fat diet and darapladib (20 mg/200 gBW) group 8 weeks; and (F) high-fat diet and darapladib (20 mg/gBW) group 16 weeks.

atherosclerosis is aging.^[31] Meanwhile, numbers of foam cell in rats in normal diet group for 8 weeks, HFD and darapladib group for 8 weeks, and HFD and darapladib group for 16 weeks were insignificant. It means that treatment with darapladib daily was able to reduce the number of foam cell in HFD and darapladib group until it reached closer to the number of foam cell in normal diet group (Figure 2).

Repeated ANOVA test shows that administration of darapladib exhibits an insignificant role to decrease the thickness of PVAT in Sprague–Dawley rats with hypercholesterol diet. This may occur as a result of less precise dosing of darapladib to reduce the thickness PVAT in this experiment. However, the average of PVAT thickness in darapladib-receiving group showed a lowest thickness of PVAT than group that did not receive darapladib. It can be concluded that darapladib also possess an effect on reducing the thickness of PVAT (Figure 3).

CONCLUSION

Darapladib has the ability to inhibit the enzyme Lp-pLA₂, which can reduce Ox-LDL levels and number of foam cells. It can also exhibit decreasing pattern of PVAT thickness in Sprague–Dawley rats that received HFD. Future studies are recommended to explore effect of darapladib in relation to other parameters of atherosclerosis.

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