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The Effect of Darapladip on Lipid Profile, Insulin, Ox-LDL Serum Level and PVAT Thickness At Atherogenesis Development in DM Tipe 2 Rats Model

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Abstract: Atherosclerosis is a chronic inflammation response of cholesterol deposition in artery vascular wall. The most common risk factor for atherosclerosis is diabetes mellitus (DM). DM affects systemically with hyperglycemia condition, increasing free fatty acid (FFA), and insulin resistance. These conditions will trigger oxidative stress oxidizes LDL-c into oxidized-LDL (ox-LDL). Macrophage phagocyte the ox-LDL then it will form the foam cell. In addition, inflammatory process will cause the vascular dysfunction which leads to molecular change in Perivascular Adipose Tissue (PVAT). One of several methods to treat type 2 DM is through inhibition of Lipoprotein-associated Phospolipase A2 (Lp-PLA2) with Darapladib. Lp-PLA2 is very specific to the inflammation in vascular, has a low biologically variability, and has a role in expanding atherosclerotic plaque. This study used post-test only controlled group design. Thirty Sprague Dawley rats were divided into 3 groups which was normal group, type 2 DM model group, and type 2 DM model with Darapladib administration group. Each groups were divided into 2 serials time, 8 weeks and 16 weeks. The Parameters in this study were glucose, lipid profile, insulin, ox-LDL serum, and PVAT thickness. There were shown a significant role of Darapladib on lipid profile, insulin and ox-LDL serum.
Keywords : Darapladib, Atherosclerosis, Type 2 Diabetes Mellitus.

Introduction

Atherosclerosis is one of cardiovascular disease (CVD) which is the most leading problem in developed as well as in developing countries in the world[1]. The risk factors of atherosclerosis most are family history, hyperlipidemia, diabetes mellitus, smoking, hypertension, and lack of antioxidant in daily diet. Diabetes mellitus is characterized by disturbances in insulin secretion, act of its enzyme, or both in which lead to hyperglycemia condition. Clinically, the glucose in bloodstream cannot move efficiently from vascular into cell, so that the glucose level in bloodstream remains high. This will harm the entire organ in the body and certain tissue because of the hyperglycemia[2].

Oxidized LDL (oxLDL) can be formed by several causes, one of them is through non enzymatic glycation. Glycation process can occur in Apo B as well as LDL-c phospholipid. Advanced glycosylation end

products (AGEs) which formed by oxidative stress and hyperglycemia can oxidize LDL-c. Glycated LDL-c will bind with scavenger receptor in macrophage which it can be a foam cell later. The foam cell is a fundamental for forming atherosclerotic lesion[3]. Ox-LDL is involved in early atherosclerotic lesion development through foam cells formation. Foam cell contains several lipid droplet forms foam-like in its cytoplasm. Macrophage expresses a receptor for ox-LDL and bind with it[4].

Lipid accumulation generates reactive oxgen species (ROS) that cause endothelial dysfunction and increase permeability, further can encourage migration of lipoprotein into sub-endothelial space[5]. LDL is very susceptible to modified, especially oxidized to ox-LDL. Monocytes infiltrate toward the plaque then differentiated to macrophages. Macrophages uptake ox-LDL via endocytosis then become foam cell that increase inflammattory response. This condition can lead to the forming of fatty streak, in which is a first step to plaque formation. Death of foam cell lead to forming of necrosis lipid core inside intima[6].

Meanwhile, in the outer wall of adventitia layer has a role in atherogenesis which is perivascular adipose tissue or often abbreviated as PVAT. PVAT is a local adipose tissue deposit surrounding vasculature. PVAT is present throughout the body and has been found to have a local effect in blood vessels[7]. Vascular dysfunction can affect PVAT leading itself increase the production of a several substances such as leptin, adiponectin, resistin, visfatin, HGF, adipokine, TNFα, IL-1, 6, 8), and others. Those substances will increase PVAT thickness and inflame the vascular itself[8]. Vascular dysfunction can be leaded by insulin resistance, hyperlipidemia, hypertension, and metabolic syndrome[8,9]. PVAT dysfunction is a characteristic for chronic obesity because of increasing ROS in endothelium which lead anti-contractile effect loss makes damage in endothelium[8].

There are several methods that can be done as a step of palliative therapy for type 2 diabetes mellitus. One of them is through inhibition of Lipoprotein-associated Phospholipase A2 (Lp-PLA2). Lp-PLA2 is very specifically to the inflammation in vascular, has a low biologically variability, and has a role in atherosclerotic plaque inflammation. Lp-PLA2 is one of intracellular enzyme that secrets phospholipase which can hydrolyzed ester sn-2 phospholipid in cellular membrane and lipoprotein. A factor that differ unstable or stable atherosclerotic plaque is the presence of active inflammation cells and increase of Lp-PLA2 level in unstable plaque. New finding suggests that Lp-PLA2 can be a substantial risk factor on atherosclerotic plaque formation and on its rupture[10]. One of drugs which act through selective inhibition of Lp-PLA2 is Darapladib[11].

Darapladib known has it effects to prevent the formation of atherosclerotic plaque on vascular endothelium. This study had been conducted to determine the effects of Darapladib orally to glucose, insulin plasma, ox-LDL level, and PVAT thickness in 2 time serials, which were 8 weeks and 16 weeks.

Experimental

Animal models

This study used male Sprague Dawley Rats as animal model with 4 weeks old and weight around 150-200 grams. The samples were obtained from Bogor Agricultural University, Bogor, Indonesia. These rats were divided into three groups; normal group (N); Type 2 DM Model groups (DM) which fed with High Fat Diet (HFD) and Streptozotocin STZ injection intraperitoneal low dose 35 mg/KgBW, and type 2 DM model with Darapladib administration group (DMDP). Each group was divided into two serial time which was 8 weeks (early phase) and 16 weeks (late phase). The used of animal modelsin this research had been approved by Ethical Clearance Committee Brawijaya University (No. 229-KEP-UB).

Darapladib was obtained from Glaxo Smith Kline. Samples were given Darapladib orally 20mg/Kg body weight once daily in duration according the time serial groups given.

Lipid Profile Measurements

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

Fasting Glucose Measurement

The rats were fasted for 24 hours before checking the rats blood glucose level. Blood glucose measurement was performed with AccuCheckTM kit. The blood serum was obtained from the distal part of the rat tail and checked with AccuCheckTM. The result was defined with mg/dl.

Insulin Plasma Measurement

The concentrations of Insulin in Rats' blood serum were measured with Sandwich ELISA using Rat INS (Insulin) ELISA kit (Cat. No. E-EL-R2466). The first step of Sandwich ELISA was started with coating the antigen. The standard and samples were filled into the antigen-coated wells for each 100 µL per well. Then, the wells were incubated in temperature of 37^{o}C for 90 min. After draining the remains of the unbounded with antigen, 100 µL of Biotynilated Detection Antibody working solution was added into each well. Then, wells were incubated for an hour in 37^{o}C. After that, it was aspirated and washed with wash buffer for five times. And then, TMB substrates were added into each well as much as 90 µL and incubated for 15 min in 37^{o}C (avoided it from sun light). For stopping the reaction, 50 µL stop solution were added into each well. After 5 min, the result was red with ELISA reader with the wavelength for 450 nm.

Ox-LDL Measurement

Ox-LDL was measured using rats blood serum as samples. Rats had been fasted a day before the serum blood obtained. Ox-LDL Level 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 was put into well that are coated with antibody before then incubated in 37^{o}C for 90 min. The residual antigen that was not bind with antibody were disposed. After that, 100 µL Biotin-antibody was added into each well. Incubated each well in 370C for 1 h. The fluid in well was aspirated and washed by wash buffer for 3 times. Next, 100 µL HRP-avidin was added into each well and incubated in 37^{o}C for 30 min. Repeated the aspiraiton and washing processfor 5 times. 90 µL TMB substrate was added to each well then was incubated in 37^{o}C for 15 min in light-free area.50 µL stop reaction was added for stopped the reaction. Each well was read 5 min later with ELISA reader with 450 nm wavelength.

Tissue Sampling

Tissues from the rats were observed in the end of the study after surgically removed aortic tissues and blood samples via cardiac puncture.

PVAT Thickness Measurement

Measurement of PVAT thickness started after the aortic tissue slide preparation. Aortic tissues were performed paraffin and then sliced thinly. After that, sliced tissues were placed in slide to be performed deparaffinised. Next, slides were stained with Hematoxillin-Eosin. Histopathological slides was observed using a microscope with 400x magnification. Then, it was measured of PVAT thickness using dotslide software Olyvia™. 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

This study used Analysis of Variance (ANOVA) to determine the effects of Darapladib on glucose, lipid profile, insulin, ox-LDL serum, and PVAT thickness in two time serial 8 weeks and 16 weeks. And then, continued with Post hoc test using the Duncan method to determine the differences of each groups. Statistical Software Product and Service Solutions (SPSS) software version 22 (IBM Corporation, 590 Madison Avenue, New York, USA) were used to gain data.

Results and Discussions

The blood glucose level and lipid profile (shown in Table 1) led to sustained elevation of glucose and cholesterol levels because the induction of Type 2 DM with STZ and HFD. This condition will cause some systemic effects such as Oxidative stress, activation of protein kinase C, and activation of RAGE. These

changes impair the endothelial function, increasing vasoconstriction, promote thrombosis, and increase inflammation. The continuity of these events will eventually lead to atherogenesis process[12].

The ox-LDL level in the various treatment groups ranged from 0,154 to 5.920 ng/mL. The Normal group from the first measurement (aged 8 weeks) had the lowest level of ox-LDL (ranged from 0.154 to 6.277 ng/mL), while the highest level of ox-LDL were in the DM group from the second measurement (aged 16 weeks; ranged from (5.585-6.277 ng/mL). ANOVA test with a 95% confidence level showed that administration of Darapladib had a significant effect ($p<0.0005$) on reducing the levels OxLDL. Post hoc test with Duncan method indicated that the levels of ox-LDL in each group differed significantly from each other.

Repeated ANOVA test with 95% confidence level showed that administration of Darapladib had an insignificant effect ($p=0.354$) to 8 weeks and 16 weeks time serial in the PVAT thickness. The average of PVAT thickness in various group was 494.709 µm. The lowest average of PVAT thickness was 330.18 µm, which was in DMDP16 group. The highest average of PVAT thickness was 752.7967 µm which was in DMDP16 too. There were sure a large diversity in the DMDP16. This results in p values for the PVAT thickness to be higher than the probability value of $p<0.050$ ($p=0.354$) and cannot be further processed by post hoc Duncan.

In addition to support the data, this study also measure lipid profile (TC, HDL-c, and LDL-c), fasting blood glucose and insulin plasma. Repeated ANOVA test with 95% confidence level showed that administration of Darapladib had an insignificant effect ($p<0.05$) in profile lipid and insulin plasma. But, the result showed that there was an insignificant probability value ($p>0.05$) in fasting blood glucose.

Table 1. Parameters Measurements (Lipid Profile, Fasting Blood Glucose, Insulin Plasma, PVAT Thickness, Ox-LDL)

Parameter	Treatment Group						*p*(ANOVA *p*<0,05)
	8 weeks			16 weeks			
	Normal	DM	DM+DP	Normal	DM	DM+DP	
Lipid Profile							
1. TC (mg/dL)	72,799 ±4,045	123,002 ±2,863	97,960 ±1,704	56,560 ±5,434	111,720 ±7,299	98,853 ±1,207	*0.004
2. HDL-c (mg/dL)	34,739 ±8,312	4,958 ±0,415	15,936 ±1,207	35,767 ±1,676	13,963 ±0,871	20,793 ±2,764	*<0.0005
3. LDL/VLDL (mg/dL)	48,831 ±5,065	95,531 ±8,664	85,919 ±6,839	19,241 ±3,670	88,246 ±6,227	61,518 ±6,034	*<0.0005
Fasting Blood Glucose (mg/mL)	91,6 ±7,162	128 ±15,016	103,6 ±13,722	79,6 ±14,639	147,8 ±58,225	101,8 ±19,070	0.577
Insulin Plasma (ng/mL)	81.476 ±3.671	5.096 ±0,788	6,126 ±0,967	9,875 ±0,305	4,431 ±0,744	6,789 ±0,622	*0.019
PVAT Thickness (µm)	488.0691 ± 90,1514	565.5426 ± 109,3565	534.5253 ± 163,5874	505.5600 ± 110,6899	552.9846 ± 36,2119	421.3560 ± 79,4164	0,354
Ox-LDL (ng/mL)	0,215 ±0,064	4,622 +0,212	0,991 +0,119	0,489 +0,355	5,920 +0,317	1,948 +0,184	*<0,0005

Darapladib is a reversible inhibitor for Lp-PLA2 enzyme, so there is no hydrolysis of PAF, 1-myristoyl-2-(4-nitrophenylsuccynyl) PC, and others. Darapladib is formed from two hydrogen bonding interactions with Tyr160 and Gln352 side chain and a couple of pi-pi interaction with aromatic and aliphatic hydrophobic remainder of Lp-PLA2[13].

Darapladib has proven good results on the expression of Lp-PLA2 atherosclerosis in vivo. The effects Darapladib was decreasing of inflammatory cells and the plaque area, but increasing the anti-inflammatory effects. Then there are also changes in markers of inflammation of Darapladib administration towards rat model of HFD, which is a decrease in; the serum Lp-PLA2 activity, in pro-inflammatory cells (such as Hs-CRP, IL-6, MCP-1, VCAM-1 and TNFα); and in macrophages atherosclerotic lesions[14].

Darapladib has been shown to be beneficial in treating type 2 DM with atherogenic lesions in artery[15,16]. Lp-PLA2 inhibition with Darapladib halted the unabated necrotic core expansion which leads to cardiovascular events in high-risk patients such as type 2 DM patient[16]. A study carried by Wilensky et al. indicated that Darapladib reduced coronary inflammatory gene expression, which was associated with decreased incidence of necrotic cores and significant preservation of coronary media. The study used male Yorkshire pigs induced by STZ to induce type 2 DM and fed with HFD for 1 month to induce a hypercholesterolemic state[17].

In this study, blood glucose and insulin plasma were measured in rats that had been fed a high cholesterol diet and were determined. The blood glucose is high in DM groups because of STZ injection and HFD-induced insulin resistance. As in the lipid profile (Table 1), it was shown that TC and LDL-c was high but low of HDL-c levels because of HFD. The HFD were given in the first 8 weeks before induced with STZ, after that, it continued until 8 weeks to 16 weeks (depended on time serial group). These data supported that the rats in DM groups as well in DMDP groups was a valid type 2 DM model.

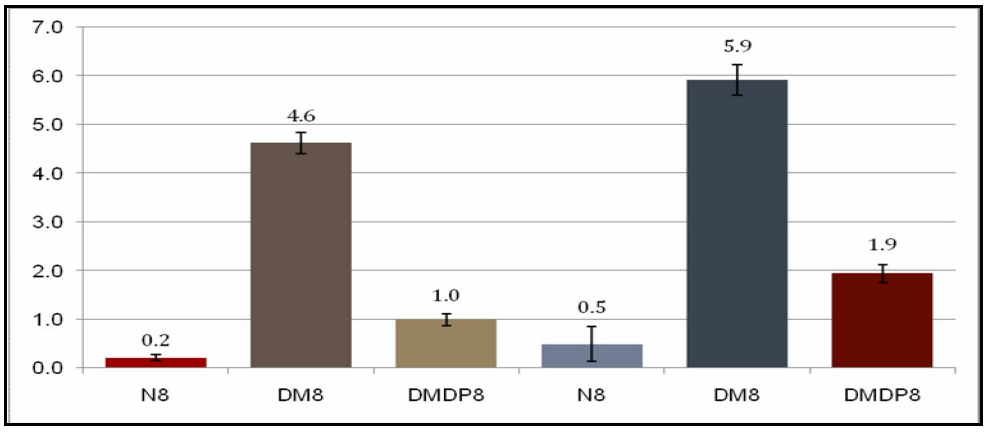

Figure 1. Effect of Darapladib on ox-LDL level in different trial groups of Sprague Dawley Rats (shown in $\bar{x}$ + S.D. ; ng/ml).

Oxidative stress will occur in hyperglycemic rat. This will lead to an increase of reactive oxygen species (ROS), while bonded with native LDL carrier of Lp-PLA2 will lead to the formation of an oxidized LDL. Then Lp-PLA2 will hydrolyze the ox-LDL into two pro-inflammatory products, which are lyso-phosphatidylcholine (lyso-PC) and oxidized free fatty acid (ox-FA). Lyso-PC and ox-FA will cause a variety of things, such as increased adhesion molecules, apoptosis, and proliferation of vascular smooth muscle cell (VSMC) [18].

ANOVA results showed that administration of Darapladib had a significant role in decreasing the blood ox-LDL level in Sprague Dawley rat with type 2 DM. Post hoc test with Duncan method showed that the levels of OxLDL in each group are differed significantly from each other. From the previous studies, it has been known that Darapladib has the ability as a selective inhibitor for Lp-PLA2 receptor. By inhibiting the receptor of Lp-PLA2, it also inhibits the formation of lyso-PC and ox-FA which is derived from ox-LDL[19].

Atherosclerosis progresses through intracellular lipid accumulation within macrophage, leading to foam cell formation and necrotic core growth[20,21,22]. Once modified and taken up by macrophage, LDL activates the foam cells[20]. Foam cell occur in the early stage of atherosclerosis. Triggered by endhotelialdysfnction, lipid

accumulation happens inside the tunica intima. The monocyte-derived macrophages will eventually phagocyte the lipid and form the foam cell. A fibrous cap which consists vascular smooth muscle cell and endhotelialepithel will engulf the foam cell and form the atheroma[23]. High level of blood glucose increases the chance of atheroma plaque ruptures. Diabetic endhotelial cells promote cytokines that will eventually lead to a decreasing synthesis of collagen by vascular smooth muscle cells. Lack of collagen in plaque's fibrous cap may ruptures the plaque more readily[12]. Based on repeated ANOVA test, it showed an insignificant result in foam cell between 8 weeks and 16 weeks time serial group. There was not an effect of darapladib between these group, so it could not differ in post hoc test either.

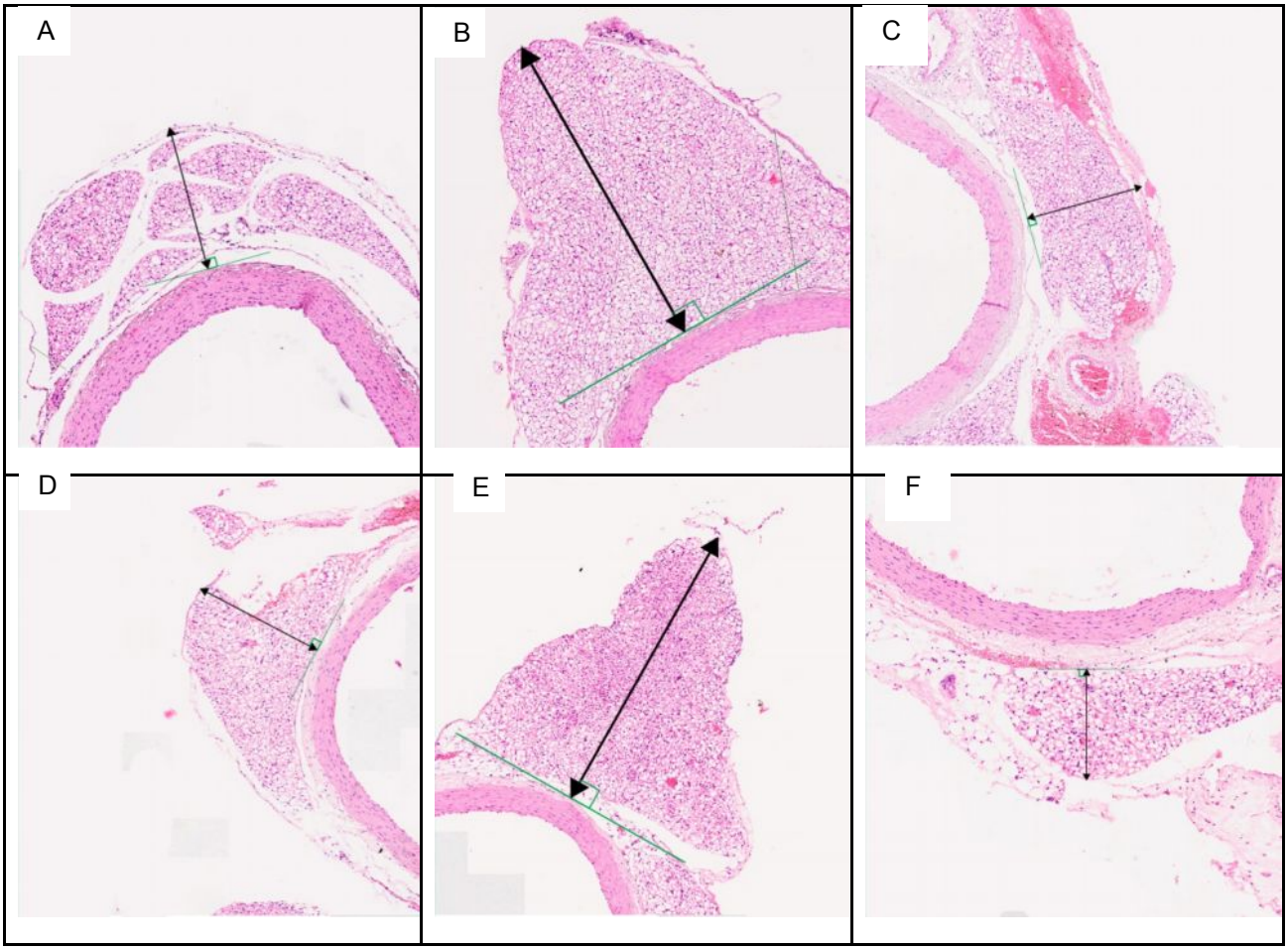

Figure 2. Histological images of PVAT thickness in different trial Groups of Sprague Dawley Rats Using Scan Dot Olyvia™ magnified by 40x.

A. 8 weeks old Normal Control; B. 8 weeks old DM Type 2 Model Group; C. 8 weeks old DM Type 2 Model with Darapladib Treatment Group; D. 16 weeks old Normal Control; E. 16 weeks old DM Type 2 Model Group; F. 16 weeks old DM Type 2 Model with Darapladib Treatment Group. (Black arrow shows PVAT thickness; Green line shows the perpendicular line)

PVAT has a role as endocrine organ by secreting various cytokines such as adipokine, which affects metabolism of energy, insulin sensitivity, inflammation response, and blood flow. In physiologis condition, PVAT produces vasoactive substanses such as adventitium-derived relaxing factor (ADRF), adiponectine, angiotensin (1-7), H_2O_2, leptin, and NO. Those vasoactives have an anticontractility advantage to maintain vascular resistension. The anticontractility effect by PVAT is affected by increasing of it cells (thickness) from its adipose[24].

ANOVA results showed that administration of Darapladib had an insignificant role in decreasing PVAT thickness in atherogenic progression. Post hoc test with Duncan method could not be performed at this state. There was no effect of Darapladib towards 8 weeks and 16 weeks' time serial group with foam cell and

PVAT thickness as a variable. However, there was a decreasing pattern between DMDP and DM groups in each time serial (Figure 2).

Conclusion

There is significant role of Darapladib as a treatment of atherogenic progression in oxLDL. However the result was insignificant in PVAT thickness, but there was a decreasing pattern between treatment groups and model groups.

Conflict of Interest

There is no conflict of interest in this study.

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