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Original Article

Lowering Inflammation Level by Lp-PLA2 Inhibitor (Darapladib) in Early Atherosclerosis Development: *in vivo* Rat Type 2 Diabetes Mellitus Model

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ABSTRACT

Background: Type 2 Diabetes Melitus (T2DM) is a condition of insulin resistance that causes extensive tissue damage due to vascular inflammation and oxidative stress. Lipoprotein-associated phospholipase A2 (Lp-PLA2) has anti-inflammatory role as it hydrolyzes atherogenesis mediators such as oxidized LDL (Ox LDL) and platelet activating factor (PAF) but in contrast, it has pro inflammatory effect as it produced lysophosphatidylcholine (lysoPC) and oxidized fatty acid (oxFA). **Methods and Results:** This study aimed to measure inflammation marker of T2DM *in vivo* model with Lp-PLA2 selective inhibitor (darapladib) treatment. It used true experimental laboratory and post test only with control group design using 30 spraque dowley rats that is divided into 3 main groups: normal, T2DM, and T2DM with darapladib (DMDP) administration group. Each group are divided into 2 serial treatment times: 8-weeks and 16-weeks intervention. So, there are 6 groups total with 5 rat in each group. Parameter measured was ox-LDL, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin 6 (IL-6), PAF, perivascular adipose tissue (PAT) thickness and also blood glucose, lipid profile, and insulin plasma level. ANOVA test result showed that darapladib were significantly (p: 0.000) lowering tissue and blood ox-LDL level, iNOS, PAF, IL-6 and PAT thickness on T2DM *in vivo* model. **Conclusions:** Darapladib proved to have anti inflammation role on T2DM model.

Key words: Type 2 diabetes mellitus, Inflammation, Oxidative stress, Lipoprotein-associated phospholipase A2, Darapladib.

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INTRODUCTION

Cardiovascular disease (CVD) has become the leading cause of death in both developed and developing countries. The global mortality rate of cardiovascular disease has reached 13.4 millions since 2007 and is expected to increase to 23.6 millions in 2030.[1] Atherosclerosis is the most common cause of CVD.[2]

Type 2 Diabetes mellitus (T2DM) is one of major classical risk factors for atherosclerosis.[3] Insulin resistance in T2DM causes extensive tissue damage due to vascular inflammation and oxidative stress.[4] Hyperglycemic environment caused by T2DM increased Reactive Oxygen Species (ROS) production.[5]

Enzymatic activity and expression of lipoprotein associated phospholipase A2 (Lp-PLA2) was studied as a biomarker of CVD.[6] Lp-PLA2's anti-inflammatory role demonstrated by the ability of this enzyme to hydrolyze atherogenesis mediators such as oxidized LDL (oxLDL) and platelet activating factor (PAF). But, the products of these molecules such as lysophosphatidylcholine (lysoPC) and oxidized fatty acid (oxFA) have pro-inflammatory, proliferative and pro-atherogenic effect.

A new pharmacological therapy or atherosclerosis that selectively inhibit Lp-PLA2 was being developed substrate. Darapladib reduce caspase-3 and caspase-8 activity and inhibits macrophage apoptosis induced by ox-LDL.

Several studies stated that darapladib showed significant results in inhibiting atherosclerosis process.[7,8,9,10] However, there are several opinions about darapladib usefulness.[11] So, this study aims to determine epression of inflammation marker of T2DM *in vivo* model with darapladib treatment.

MATERIAL AND METHODS

Study group

This study used 4 weeks old male Sprague Dawley rats and weighted about 150-200 grams. They were obtained from Bogor Agricultural University, Indonesia. 30 rats were divided into three groups; normal (N); T2DM fed with High Fat Diet (HFD) and low dose Streptozotocin (STZ) intraperitoneal injection of 35 mg/kg BW, and T2DM with darapladib administration group (DMDP). Each group was divided into two serial times: 8 weeks (early phase) and 16 weeks (late phase). So there are 6 groups total with 5 rats in each group. Darapladib was obtained from Glaxo Smith Kline. Samples were given Darapladib orally 20mg/Kg body weight once daily according the time serial groups given.

The normal rats food contained total energy Calories (kcal/g) of 3.43 (67% carbohydrate, 21% protein and 12% fat), while the HFD contained total calorie energy of 5.29 (58% fat, 17% carbohydrate and 25 % protein). Measurements of parameters were done at the Central Laboratory of Biological Sciences, Brawijaya University, Malang, Indonesia. Slicing and staining samples were done at Pathological Anatomy Laboratory, Faculty of Medicine University of Brawijaya, Malang, Indonesia

Blood glucose measurements.

A dose of 20 mg/kg body weight STZ was administered prior to the first measurement of blood glucose to induce T2DM. T2DM was diagnosed after measurement of the blood glucose level with a GlucoDR blood glucose test meter (All Medicus Co. Ltd, Dongan-gu, Anyang-si, Korea). T2DM was diagnosed after obtaining random blood glucose levels >200 mg/dL.

Measurement of lipid profile levels

Lipid profiles were measured using rat blood serum and calculate total cholesterol, HDL (High Density Lipoprotein), and LDL (Low Density Lipoprotein) using EnzyChrom™ kit which is a Bioassay system.

Measurement of insulin resistance

Insulin in rat's blood plasma was measured using sandwich ELISA by using Rat INS (insulin) ELISA kit (Cat. No. E-EL-R 2466). The results were expressed by ng/mL units. Forthe conversion of plasma insulin levels of ng/mL to IU/L, used WHO formula by dividing the result by 0.0347, as 1 IU is equivalent to 0.0347 mg/L.[13] Measurements of insulin resistance can be performed with HOMA-IR (homeostatic model assessment-insulin resistance) formula especially in rat, which require data from fasting glucose and plasma insulin levels by the following formula:[14]

$$HOMA - IR = \frac{\text{FBS x FINS}}{14,1}$$

Explanation:

HOMA-IR : Homeostatic Model Assessment-Insulin Resistance

FBG : Fasting Blood Glucose (mmol/L)

FINS : Fasting Insulin Plasma (μU/mL)

The interpretation from HOMA-IR calculation is if the result>1,716 then it can be categorized as insulin resistance with a sensitivity of 83.87% and a specificity of 80.56% (95%confidence interval).[15]

Biochemical tests

Interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase (iNOS) level were measured by immunofluorescence of aortic tissues that were previously fixated with PHEMO buffer (68 mM PIPES, 25 mM, HEPES, pH 6.9, 15 mMEGTA, 3 mM MgCl2, 10% [v/v] dimethyl sulfoxide containing 3.7% formaldehyde and 0.05% glutaraldehyde) and were processed by imumunofluoresence labeling with anti-rat antibody Lp-PLA2 using rhodamin secondary antibody and anti-rat antibody IL-6 using fluorescein isothiocyanate (FITC) secondary antibody (BIOS Inc., Boston, MA, USA). These parameters were observed with confocal laser scanning microscopy (Olympus Corporation, Tokyo, Japan) and were quantitatively analyzed using Olympus Fluo View software (version 1.7A; Olympus Corporation).

Ox-LDL was measured using rat's aorta tissue as samples whereas PAF measurement used blood plasma sample. Rats had been fasted a day before the sample obtained. Ox-LDL level was measured by Sandwich ELISA method using Rat ox-LDL ELISA kit (Cat. No. E-EL-R0710). PAF ELISA kit use Cat. No.MBS722041.

Ethics

We obtained ethical approval for the animal treatment and experimental processes in this study from the Animal Care and Use Committee Brawijaya University Number 229-KEP-UB

Statistical Analysis

The one-way analysis of variance (ANOVA) test was used to determine the effects of time series factor, treatment of darapladib and the interaction of time and treatment Darapladib on OxLDL, PAF, IL-6, perivascular adiopse tissue (PAT) and TNF-α level in T2DM Spraque Dowley rats. This analysis was followed by the post hoc test using Duncan method to detect differences in parameters of each intervention. SPSS software version 20 (IBM Corporation, New York, NY, USA) was used for data analysis.

RESULTS

Lipid profile and fasting glucose level are shown in table 1. There are significant effects of darapladib administration effect in lowering total cholesterol, HDL cholesterol, non HDL cholesterol and fasting blood glucose level at both 2 serial time treatments.Inflammation marker result are shown in table 2. DMDP group has lower Ox-LDL level (in blood and tissue) than T2DM group at both 8 weeks and 16 weeks serial time treatments. One way ANOVA test shows a significant value ($p<0.05$; $p=0.000$) for darapladib in lowering OxLDL level in T2DM model at 2 serial time treatments.

TNF-α and iNOS expression at 8 weeks show higher level of TNF-α and iNOS in DMDP group than DM group. 8 weeks treatment of darapladib does not significantly ($p>0.05$) decrease TNF-α and iNOS level at T2DM

Table 1: Lipid profile and fasting glucose level

Variable	Group	8 weeks Mean± SD	16 weeks Mean± SD
Total cholesterol (mg/dL)	Normal	72.80 ± 4.05^{b}	56.56 ± 5.43^{a}
	DM	123.00 ± 2.86^{d}	111.72 ± 7.30^{cd}
	DMDP	97.96 ± 1.70^{c}	98.85 ± 3.25^{c}
HDL (mg/dL)	Normal	34.74 ± 8.31^{f}	35.77 ± 1.68^{f}
	DM	4.96 ± 0.41^{a}	13.96 ± 0.87^{bc}
	DMDP	15.94 ± 1.21^{bcd}	20.79 ± 2.76^{e}
Non HDL (mg/dL)	Normal	49.83 ± 5.06^{b}	19.24 ± 3.67^{a}
	DM	95.53 ± 8.66^{de}	88.25 ± 6.23^{de}
	DMDP	85.92 ± 6.84^{d}	61.52 ± 6.03^{c}
Fasting blood glucose (mg/dL)	Normal	91.60 ± 7.16^{ab}	79.60 ± 14.64^{a}
	DM	128.00 ± 15.02^{cd}	147.80 ± 58.23^{d}
	DMDP	103.60 ± 13.72^{abc}	101.80 ± 19.07^{abc}

Note: Trials with duncan multiple range test (DMRT). Multiple similar letter notation show the difference between two mean value and not significant at α = 0.05

Table 2: Inflammation marker

Variable	Group	8 weeks Mean ± SB	16 weeks Mean ± SB	P Value
Tissue Ox LDL (pg/mL)	Normal	1.35 ± 0.16[a]	2.31 ± 0.27[ab]	0.000
	DM	21.10 ± 2.89[h]	34.05 ± 1.93[i]	
	DMDP	4.44 ± 0.62[c]	10.02 ± 0.78[e]	
Blood OxLDL (pg/mL)	Normal	0.22 ± 0.06[a]	0.49 ± 0.36[bc]	0.000
	DM	4.62 ± 0.21[f]	5.92 ± 0.32[g]	
	DMDP	0.99 ± 0.12[d]	1.95 ± 0.18[e]	
TNF- (pg/mL)	Normal	1283.12 ± 55.08[c]	792.55 ± 91.29[ab]	0.094
	DM	826.36 ± 263.81[ab]	927.84 ± 228.53[b]	
	DMDP	853.13 ± 115.48[ab]	845.63 ± 100.01[ab]	
iNOS (pg/mL)	Normal	555.65 ± 139.28[a]	687.56 ± 217.82[ab]	0.064
	DM	664.52 ± 345.43[ab]	775.48 ± 96.66[ab]	
	DMDP	672.54 ± 174.51[ab]	640.88 ± 153.16[a]	
IL-6 (pg/mL)	Normal	716.09 ± 42.19[ab]	670.56 ± 158.24[ab]	0.022
	DM	742.94 ± 174[ab]	792.13 ± 323.33[ab]	
	DMDP	634.61 ± 199.93[a]	731.63 ± 76.43[ab]	
PAF (pg/mL)	Normal	0.79 ± 0.11[a]	0.96 ± 0.12[ab]	0.000
	DM	1.26 ± 0.15[cd]	1.96 ± 0.08[f]	
	DMDP	1.21 ± 0.25[c]	1.48 ± 0.33[de]	
PAT (μm)	Normal	501.42 ± 67.90[abc]	478.90 ± 63.22[abc]	0.000
	DM	470.78 ± 128.9[abc]	608.59 ± 74.09[c]	
	DMDP	541.83 ± 150.46[bc]	420.71 ± 51.08[ab]	

Note: Trials with duncan multiple range test (DMRT). Multiple similar letter notation show the difference between two mean value and not significant at α = 0.05

model. But the different results are shown at 16 weeks of treatment. 16 weeks treatment of darapladib decreases TNF-α and iNOS level in T2DM group.

IL-6 expression shows that DMDP group has lower level of IL-6 than DM group at both 8 weeks and 16 weeks serial time treatments. However one way ANOVA test shows an unsignificant value (p>0.05; p=0.022) for darapladib in lowering IL-6 level at T2DM model.

PAF expression shows that DMDP group has lower level of PAF than T2DM group at both 8 weeks and 16 weeks serial time treatments. One way ANOVA test show a significant value (p<0.05; p=0.000) for darapladib in lowering PAF level in T2DM model at both 2 serial time treatments.

PAT expression at 8 weeks shows a higher PAT thickness at DMDP group than DM group. However, greater results are shown at 16 weeks of treatment. One way ANOVA test shows a significant value (p<0.05; p=0.000) for darapladib in lowering PAT thickness in T2DM model at 16 weeks treatments.

Darapladib administration lowered OxLDL, PAF and IL-6 level in T2DM group at 8 and 16 weeks serial treatment. Whereas 16 weeks administration of darapladib lowered TNF-α, iNOS level and PAT thickness.

Immunofluorescence staining using secondary FITC antibody show qualitative expressions of TNF-α, iNOS and IL-6 at aortic tissue that can be seen from the fluorescence intensity.

Figure 1 shows that DMDP rat at figure (a), (b) and (c) has dim intensity whereas DM rat has moderate-maximum intensity. It shows that darapladib can reduce the expression of TNF-α, iNOS and IL-6 at aortic tissue of DM rat.

Figure 2 shows the comparison of perivascular adipose tissue (PAT) thickness from each group. It shows that DM group at 8 and 16 weeks serial time has a wider range of perivascular adipose tissue than normal group. Furthermore darapladib treatment can reduce PAT thickness at both of 8 and 16 weeks serial time as seen in figure (3C) and (3F).

DISCUSSION

T2DM condition tends to be more dominant in illustrating systemic oxidative stress accompanied by an increase in AGEs that has a major role in the pathogenesis of atherosclerosis. Atherosclerosis has been declared as one of the inflammatory diseases since inflammatory processes play a dominant role in the various stages of the development of atherosclerosis. Those processes include endothelial dysfunction that lead to the increasment of adhesion molecules expression. Smooth muscle and endothelial cells inside atherosclerotic plaque produce pro-inflammatory mediators that stimulate monocyte differentiation process into macrophage. Then macrophage developed into foam cells as the OxLDL uptake process and local inflammatory response released. Intracellular inflammation process (such as IKK and JNK signal) plays an important role in the development of atherosclerosis, which involves genetic transcription factors such as NF-KB, activator protein-1 and early growth response 1 (EGR1).

Insulin resistance at T2DM causes extensive tissue damage due to vascular inflammation and oxidative stress.[4] Hyperglycemic environment causes by T2 DM increased Reactive Oxygen Species (ROS) production.[5] Enzymatic activity and expression of lipoprotein associated phospholipase A2 (Lp-PLA2) was discovered as a biomarker of CVD.[6] Lp-PLA2's anti-inflammatory role demonstrated by the ability of this enzyme to

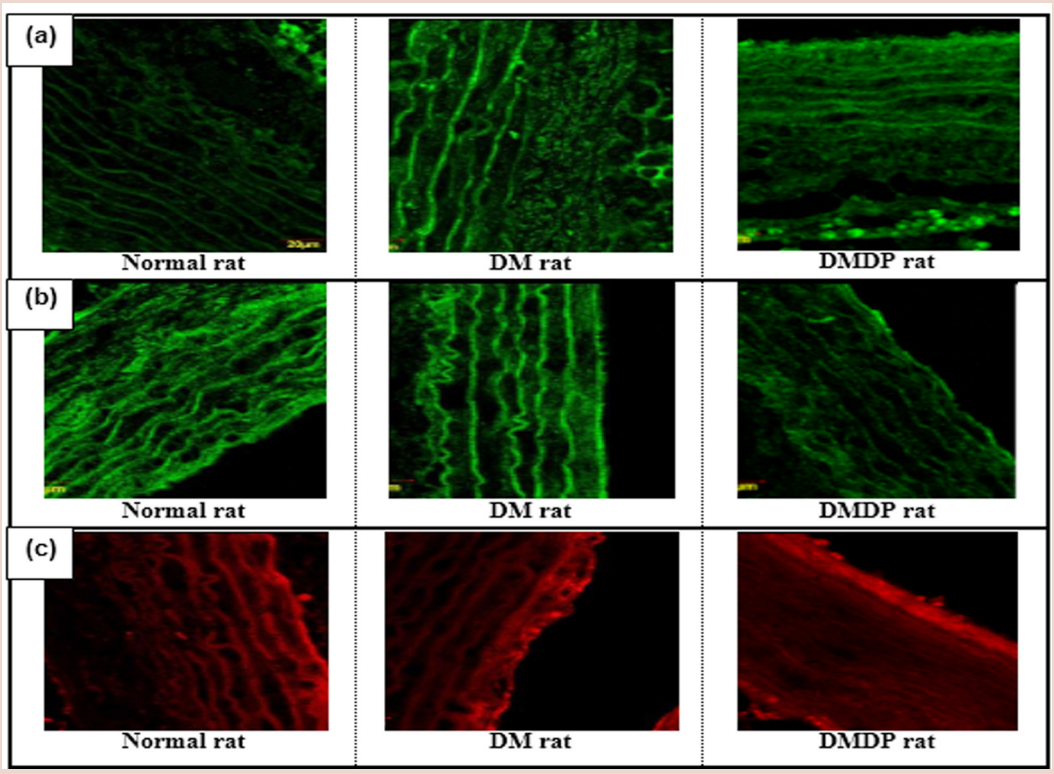

Figure 1: Immunofluorescence results using secondary FITC and rhodamin antibody for (a) TNF-α expression; (b) i NOS expression; (c) IL-6 expression

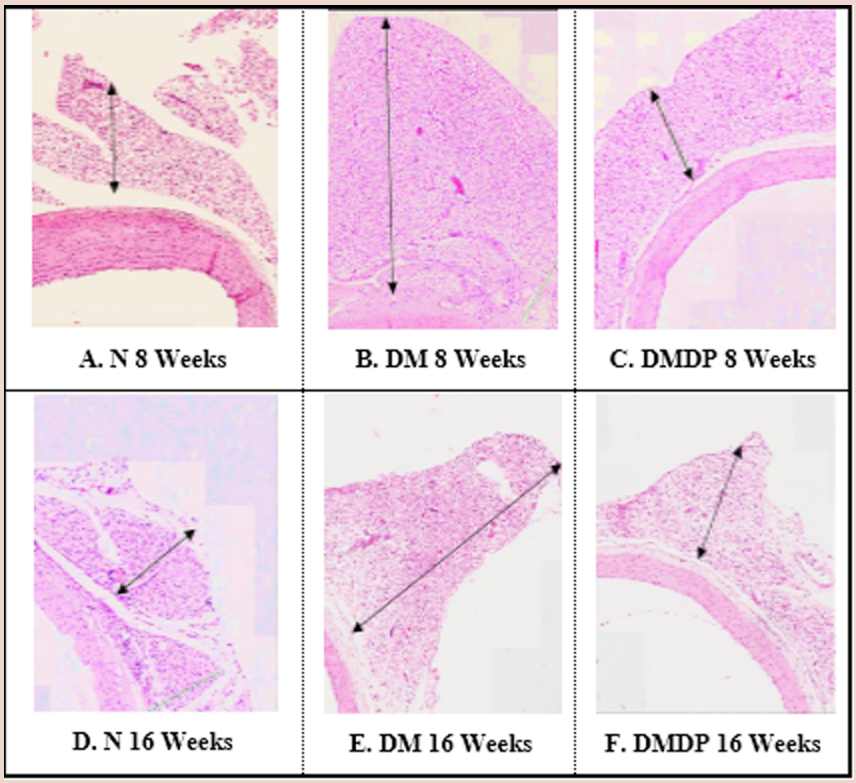

Figure 2: Black arrow shows perivascular adipose tissue (PAT) thickness. (A) Normal 8 Weeks group (N8); (B) Normal 16 weeks group (N16); (C) 8 weeks T2DM group; (D) 16 weeks T2DM group; (E) T2DM group with 30 mg/kg BB administration of DP for 8 weeks (DMDP8); (F) T2DM rat group with 30 mg/kg BB administration of DP for 16 weeks (DMDP16)

hydrolyze atherogenesis mediators such as oxidized LDL (ox LDL) and platelet activating factor (PAF).

Lp-PLA2 through ox-LDL will produce bioactive product such as lyso PC and ox NEFA that able to stimulate pro-inflammatory cytokines formation (especially IL -6, IL-1β and TNF-α).[13-17] Research about atherosclerotic plaques at human found significant association between increased levels of Lp-PLA2 and lyso PC in atherosclerotic plaques with increased expression of IL-6, IL-1β and TNF-α in atherosclerotic plaque.[17] Increased activity of Lp-PLA2 and formation of bioactive products have occurred in an earlier period which responsible for increasing IL-6 expression in the 8 weeks observation group. Other *in vivo* studies reported a significant reduction in IL-6 levels post 50 mg/kg body weight/day DP administration in HFD rat for 6 weeks. Additional studies using experimental animals (pigs) with a T2DM model also found a significant increase in IL-6, IL-1β and TNF-α parallel with the increase in Lp-PLA2 mRNA expression in peripheral blood mononuclear cells (PBMC) and coronary arteries. A selective blocker of Lp-PLA2 administration significantly suppress the expression of IL-6, IL-1β and TNF-α.[18]

Macrophages inside atherosclerotic plaques express iNOS that produce large amount of NO. Some adverse effects are associated with iNOS that it produce excessive peroxinitrite and superoxide. iNOS also has protective role by inhibiting smooth muscle cell proliferation and leukocyte adhesion. Protective effect is derived from the production of NO by basal speed while the cytotoxic effects was derived from NO and peroxynitrite production at higher speed. This imbalance can occur in atherosclerotic plaque due to decreased expression of superoxide dismutase.[16]

Previous research proved that iNOS is expressed by 87% in the atherosclerotic plaque macrophages of patients with acute coronary syndrome while TNF-α is expressed by 50%. Increased iNOS are associated with atherosclerotic plaque instability at advanced stage.[17] The iNOS genes are expressed in macrophages, endothelial cells and vascular smooth muscle cells under inflammatory conditions.[19]

One novelty of these research findings is directly connecting iNOS activity with Lp-PLA2 in T2DM condition. Darapladib administration tends to decrease iNOS expression. This finding shows that darapladib has potency as a broad spectrum anti-inflammatory agent

An increased level of iNOS and TNF-α in this study was more evident in 16 weeks observational group. Macrophages showed increased level as long as the length of treatment time.[20] These findings explain that the longer procedure of pro atherogenic HFD will certainly increase the number of macrophages and activity of Lp-PLA2 that has dominant side of pro inflammatory effect. Increased thickness of PAT is highly associated with classic risk factors for cardiovascular disease (CVD), such as dyslipidemia, hypertension and T2DM.[21] Inflammation of PAT has systemic and local effects which play a major role in proatherogenesis. There are fascia tissues that separate fat tissues in the tunica adventitia of arterial wall that allows direct access from the humoral response and inflammatory mediators to the tunica adventitia. Direct contact with the blood vessel wall is followed by vasa vasorum proliferation and development of early atherosclerotic plaque deposits in subendothel.[23] Metabolic disorder causes PAT becomes hypoxic and dysfunctional as well as it recruits phagocytic cells. Changes in adipocytes size and number of macrophages will decrease the production of protective adipokines and increase the production of pro-inflammatory adipokines such as leptin, resistin, kemerin, vaspin, IL-6, or TNF-α, both on human and mouse adipocytes tissue.[24,25] Similar action of PAT in promoting local inflammatory response closely mimic the activity of Lp-PLA2 in triggering the inflammatory response. This research found DP's ability to suppress the thickness of the PAT in conditions of 16 weeks of T2DM. DP administration led to disconnect Lp-PLA2 activity in oxLDL so that the number of macrophages in oxLDL will be also decreased so it can lower inflammation marker level.[26] The effect of DP in this study closely mimic the effect of anti-oxidants such as super oxide dismutase (SOD, anti IL- 6 and TNF-α which administered to HFD rat and metabolic syndrome model). HFD administration increased PAT, ROS, unpaired eNOS, cytokine production, macrophage infiltration and leptin. Anti-oxidant administration and anti-cytokine significantly reduced the thickness of PAT and inflammation marker. Research in obesehuman also found similar result.[25,26]

CONCLUSION

This study proves the role of darapladib as a powerful anti-inflammatory and potential anti-oxidant to minimize PAT thickness, OxLDL level in aortic tissue and blood, IL-6 and PAF level in T2DM model.

CONFLICT OF INTEREST

None

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ABBREVIATIONS USED

Lp-PLA2: lipoprotein-associated phospholipase A2; T2DM: type 2 diabetes mellitus; Ox-LDL: oxidized low density lipoprotein; PAF: platelet activating factor; LysoPC: lysophosphatidylcholine; OxFA: oxidized fatty acid; DMDP: type 2 diabetes mellitus with darapladib; TNF-α: tumor necrosis factor α; iNOS: inducible nitric oxide synthase; IL-6: interleukin 6; PAT: perivascular adipose tissue; CVD: cardiovascular disease; ROS: reactive oxygen species; HFD: high fat diet; STZ: streotozotocin; HDL: high density lipoprotein; HOMA-IR: homeostatic model assessment-insulin resistance; FBG: fasting blood glucose; FINS: fasting insulin plasma; FITC: fluorescein isothiocyanate.

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