THE DECREASE OF FOAM CELL BY DARAPLADIB ADMINISTRATION IN ATHEROSCLEROSIS TYPE 2 DIABETES MELLITUS MODEL SPRAGUE-DAWLEY RATS

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ABSTRACT

Cardiovascular disease is one of the largest global mortality causes. The disease is having a strong correlation with atherosclerosis which is greatly affected by several factors such as metabolic syndrome and diabetes mellitus. Atherosclerosis marked by elevation of pro-inflammatory cells and activity in the arterial wall which then followed with enhancement of several inflammatory mediators concentration. Lp-PLA_2, as one of contributing inflammation mediator hydrolyzes oxidized LDL that has been accumulated in intimal lining which then expands necrotic plaque area. Until now, there has been found a novel treatment of atherosclerosis based on Lp-PLA_2 inhibition mechanism named Darapladib. This study aimed to investigate Darapladib effect on foam cell number reduction.

This true experimental study with post-test only control group design is performed on 30 *Sprague-Dawley* rats that divided into 3 large groups: (1) normal group (n=10), (2) diabetes mellitus type 2 group (n=10), (3) diabetes mellitus type 2 group which given Darapladib (n=10). Each groups then divided again into two time series, 8 and 16 weeks.

The result shows a significant reduction of foam cell number (ANOVA, $p<0,05$) by Darapladib administration in 8 and 16 weeks groups compared to type 2 DM group, but there are no significant difference related to the duration of Darapladib administration 8 and 16 weeks.

From the data above it is concluded that Darapladib is able to reduce aortic foam cell in type 2 diabetes mellitus rat.

Keywords: Darapladib, Lp-PLA_2, Foam Cell, Atherosclerosis, Type 2 Diabetes Mellitus

ABSTRAK

Penyakit kardiovaskular merupakan salah satu penyebab kematian terbanyak di dunia yang sangat erat kaitannya dengan aterosklerosis. Beberapa faktor seperti sindrom metabolik dan diabetes mellitus merupakan predisposisi kuat terjadinya aterosklerosis. Aterosklerosis ditandai dengan disfungsi endotel dan peningkatan jumlah serta aktivitas sel-sel radang dalam dinding pembuluh darah yang kemudian menghasilkan mediator-mediator pro-inflamasi, salah satunya berupa enzim Lp-PLA_2. Enzim tersebut bekerja dengan cara menghidrolisis LDL teroksidasi yang terakumulasi dalam intima sehingga terjadi penarikan makrofag pada lesi dan memicu pembentukan sel busa yang berperan dalam perluasan plak nekrotik. Saat ini telah dikembangkan inovasi terapi aterosklerosis melalui penghambatan enzim Lp-PLA_2 yang bernama Darapladib. Penelitian ini bertujuan membuktikan penurunan jumlah sel busa dengan pemberian Darapladib.

Studi ini merupakan studi eksperimental menggunakan *post-test only controlled group design* yang dilakukan terhadap 30 ekor hewan coba tikus *Sprague-Dawley* dalam tiga kelompok : (1) kelompok normal (n=10), (2) kelompok DM tipe 2 (n=10), dan (3) kelompok DM tipe 2 yang diberi darapladib (n=10). Masing-masing dibagi kembali menjadi dua kelompok berdasarkan serial waktu 8 dan 16 minggu.

Hasil penelitian menunjukkan bahwa terdapat penurunan jumlah sel busa secara bernakna (ANOVA, $p<0,05$) dengan pemberian Darapladib selama 8 dan 16 minggu terhadap kelompok DM tipe 2, namun tidak terdapat perbedaan signifikan terkait dengan durasi pemberian Darapladib 8 dan 16 minggu ($p>0,05$).

Dari hasil tersebut disimpulkan pemberian Darapladib dapat menurunkan jumlah sel busa aorta tikus model diabetes mellitus tipe 2.

Kata kunci: Darapladib, Lp-PLA_2, Sel Busa, Aterosklerosis, Diabetes Mellitus Tipe 2

INTRODUCTION

Cardiovascular disease is the major non-communicable disease mortality cause in Indonesia and the world. WHO stated that 17.5 million people die because of cardiovascular disease in 2012, or 31% of all mortality cause. In 2030, it is predicted that non-communicable disease will be a leading cause of global mortality, and dominated with cardiovascular disease. According to Riskesdas 2013, stroke, as one of major cardiovascular disease, has a relatively high prevalence. It accounts for 7% from total Indonesia population, this number followed with coronary artery disease with 0,5% prevalence [1].

Cardiovascular disease is strongly correlates with atherosclerosis. Atherosclerosis development is marked with increasing intimal fibrosis, fatty plaque formation, smooth-muscle cells proliferation, calcification, and followed with thrombosis as the consequence of fibrofatty plaque rupture. Thrombosis is the main mechanism which lead to the total occlusion of arterial lumen and causing several life-threatening clinical manifestation such as stroke or coronary artery disease (CAD)[2].

Up to now, the main etiology of atherosclerosis remains unknown, but there are several predisposing factors that accelerate the rate of atherosclerosis progression. Atherosclerosis risk factor is divided into two main categories, modifiable and non-modifiable. Increasing age, male gender, and family with cardiovascular history are examples of non-modifiable factor. While modifiable factor consists of sedentary lifestyle, smoking, hypertension, dyslipidemia, obesity, impaired glucose tolerance, and diabetes mellitus [2].

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by marked elevation of blood glucose level as the result of disruption of glucose cellular uptake process (insulin resistance) that followed by pancreatic β cells dysfunction which give rise to insulin hormone insufficiency. Insulin is a hormone secreted by β pancreatic cells and plays a major role in blood glucose level regulation[3]. This metabolic impairment elevates the likelihood of atherosclerosis development by 2-3 times greater[4].

Chronic exposure of high blood glucose to the vascular endothelial cell wall surface will impair it's normal function by inducing superoxide anion and reactive oxygen species (ROS) production within vascular wall. Elevation of oxidative stress then reduces nitric oxide (NO) production as anti-atherosclerosis agent[5]. Chronic diminishment of NO then enhance platelet aggregation, adhesion molecule, leucocyte activation, and pro-inflammatory cytokines which leads to endothelial dysfunction[6,7,8].

Endothelial dysfunction is marked by enhancement of vascular permeability. By this mechanism, low density lipoprotein (LDL) is prone to be accumulated within subendothelial space[6]. LDL deposition then modified into oxidized low density lipoprotein (OxLDL) by pro-oxidant agents and results in further elevations of cytokines and chemotactic agents which promotes recruitement of pro-inflammatory cells such as macrophage, neutrophil, monocyte into site of lesion at subendothelial space and tunica intima[9].

Monocyte then enters subendothelial and tunica intima that immediately differentiate into macrophage. OxLDL then phagocytized by macrophage through scavenger receptor that induce foam cell as precursor of fatty streak which marked initial atherosclerosis plaque formation process[10,11].

Within tunica intima, macrophage and another inflammatory cells such as mast cell and lymphocyte produce *Lipoprotein-phospholipase* A_2 (Lp-PLA_2) within atherosclerotic necrotic core. Lp-PLA_2 is an enzyme that plays an important part in atherosclerosis. A study revealed that Lp-PLA_2 has a pro-inflammatory characteristic and increase the tendency of disruption of the plaque which induce thrombus formation. This condition then leads to many lethal consequences as the distal organ perfusion is being compromised[12].

Up to now, atherosclerosis management in DM type 2 patient is based on tight control of

glucose level using biguanide group of oral antidiabetes (OAD) medicine. The most common used type from this group is metformin, but routine use of this drug may cause any dangerous effect such as hypoglycemia that leads to deterioration of consciousness level until coma[13]. In addition to its dangerous effect, a clinical trial study focusing on this matter revealed that there weren't found any significant correlation between tight glucose control and mortality rate reduction correlates with diabetes macrovascular complication[12]. Besides metformin, another pharmacology agent commonly used is statin, this treatment is especially given for diabetes patient with characteristic of more than 40 years of age and cholesterol level ≥135 mg/dL. However, statin consumption also enhance the adverse effect risk. In 2012, food drug administration (FDA) release a statement that statin is proved to increase HbA1c and fasting blood glucose. Hence, statin administration for DM patient remains controversial[13].

Therefore, innovation and development of atherosclerosis specific therapy is needed. In the last decade, there is developed a novel pharmacology atherosclerosis therapy by active, selective, and reversible inhibition of Lp-PLA_2. By Lp-PLA_2 activity retardation, then atherosclerosis progression induced by ROS is inhibited, also fibrous capsule thinning and further necrotic plaque expansion also retarded. Accordingly, atherosclerotic plaque tend to be more stabile from plaque rupture and thrombosis.

All this time, experimental studies in order to evaluate darapladib effects on diabetes mellitus type 2 is limited. Concerning to this matter, any further studies are needed. The aim of this study is to evaluate darapladib effects on reduction of foam cell in atherosclerosis type 2 diabetes mellitus *Sprague-Dawley* rats.

MATERIALS AND METHODS

Experimental Design

This study is a laboratory experimental using Randomized Post-Test Only Controlled Group design *in vivo*. Six to seven weeks old male *Sprague-Dawley* rats were purchased from *Institut Pertanian Bogor* (IPB), the rats were weighed 150-160 gram, has an active movement, healthy, clean fur, and clear eyes are used for the study. To obtain variability from the rats as experimental samples then *Sprague-Dawley* rats divided into six groups by simple randomization.

The study started at October, 6th 2014, when rats are firstly given high fat diet (HFD) and Darapladib. The study completed at February, 11th 2015, marked by sacrifice of last rat groups. Then, aorta were extracted to get analysed for foam cell number histopathologically.

Materials

Instruments needed for this study are standard cage, electronic scale, needle syringe, glucometer, gavage, surgery equipments (surgical board, pins, scalpel, organ container), rinsing equipment, microscope, and computer equipped with software Dot Slide Olyvia™.

Materials needed for this study are male, 6-8 weeks old *Sprague-Dawley* rats weighed 150-200 grams, standard diet, high fat diet, water, streptozotocin (STZ) 30 mg/kg, Darapladib 20 mg/kg, ketamine 15-20 mg/kg, formaline 10%, Hematoxyline and Eosin staining, and ELISA kit.

Experimental Procedure

The study is intiated with ethical clearance obtainment. Then, it followed with preparation of materials needed for the further phase. Rats are selected according to inclusion and exclusion criterias which has been set before. *Sprague-Dawley* rats then acclimatized for 7 days while given normal diet AIN-95M. After that, rats were randomized to be grouped into three major experimental groups, consists of normal group (negative control/ N), diabetes mellitus group (positive control/ DM), diabetes mellitus group with 20 mg/kg Darapladib administration (DM+DP). The groups are divided into two series of time, eight and 16 weeks. Hence, there are six groups in total with five rats in each group.

Diabetes mellitus type 2 induction then performed by high fat diet (HFD) administration followed with streptozotocin (STZ) injection intraperitoneally. HFD were given for a couple of weeks for each DM rats which are either going to be given Darapladib or not. By the end of second week, STZ injected to set insulin dysfunction condition. After one week, fasting blood glucose, plasma insulin, and insulin resistance were examined to ensure that the induction process has been succeeded.

Darapladib is then administered to rats that have been determined as diabetes mellitus beforehand. Diabetes mellitus in rats defined by HOMA-IR score above 1.716. 20 mg/kg of Darapladib were given throughout 8 and 16 weeks [14,15].

By the end of 8 and 16 weeks, rats euthanized by injecting ketamine 15-20 mg/kg intraperitoneally. After that, aorta are extracted and rinsed by phosphate-buffered saline (PBS) and paraformaldehyde (PFA) solution, aorta then preserved using 10% formalin. Subsequently, fixed aorta tissue processed using automatic tissue processor and parafinized into paraffin block. The paraffin block then inserted into microtome to be cut. The slide then floated in waterbath and placed on object glass. Hematoxylin and eosin staining then performed and slide preparation ended with mounting.

The slides are examined under microscope with 400x magnification. Foam cells are identified as pale cells with blue nuclei within tunica intima. Foam cells counting was done by more than one examiner using double blind technique in order to diminish bias and obtain a more objective result. To diminish any error probability, DotSlide Olyvia™ is used to prevent any overlap in foam cell counting.

Data Analysis

Data attainment and analysis is done by the end of the study. Data processing and analysis then performed using software statistical product and service solution (SPSS) 23rd version with 0.05 probability value (p=0.05) and 95% reliability level (α=0.05).

Data analysis initiated with normality test, Saphiro-Wilk (total sample number <50) and homogeneity Levene's test with p value above 0.05 is declared as normally distributed and having normal homogeneity. Therefore, the data fulfills the requirement to be analyzed further to parametric repeated ANOVA test. ANOVA test then continued to Post-Hoc least significant difference (LSD) test in order to discover significancy level between the study groups.

RESULTS

Hereby presented supporting data included fasting blood glucose, insulin resistance, oxidated LDL (OxLDL), and lipid profile of *Sprague-Dawley* rats.

Fasting Blood Glucose, Plasma Insulin, and Tissue OxLDL Level in Each *Sprague-Dawley* Rats

Study Groups	Normal 8 Weeks	Normal 16 Weeks	DM 8 Weeks	DM 16 Weeks	DM + DP 8 Weeks	DM + DP 16 Weeks
Fasting Glucose Level (mg/dL) ($\bar{x}$± SD)	91,6± 7,16^{ab}	79,6±14,63^{a}	128±15,01^{bc}	147,8±58,22^{c}	103,2±13,72^{abc}	101,8± 19,07^{abc}
Plasma Insulin (ng/mL) ($\bar{x}$± SD)	4,664±0,425	5,124± 0,297	12,017±1,781	40,220±4,1661	5,704±0,573	8,327±0,859
Tissue OxLDL (ng/mL) ($\bar{x}$± SD)	1,3476± 0,1556^{a}	2,314± 0,270^{ab}	21,0984± 2,8885^{g}	34,049±1,927^{h}	4,440±0,6214^{bc}	8,415±1,645^{d}

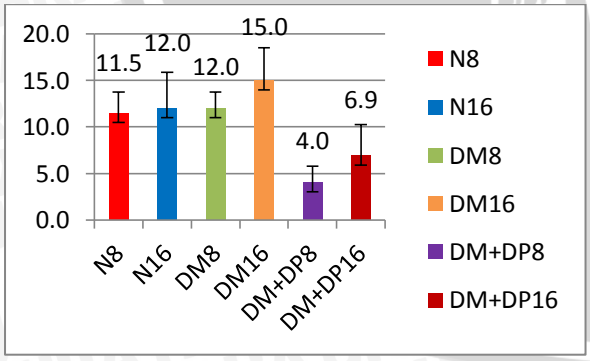
Insulin Resistance by HOMA-IR Formula in Each *Sprague-Dawley* Rats

Study Groups		Normal 8 Weeks	Normal 16 Weeks	DM 8 Weeks	DM 16 Weeks	DM + DP 8 Weeks	DM + DP 16 Weeks
Before DP administration	HOMA-IR ($x\pm$ SD)	0,641 ± -	0,638 ± 0,041	2,001 ± 0,073	6,458 ± 0,603	1,747 ± 0,674	3,061 ± 1,051
	Interpretation	Normal	Normal	Insulin Resistance	Insulin Resistance	Insulin Resistance	Insulin Resistance
After DP administration	HOMA-IR ($x\pm$ SD)	0,486 ± 0,067	0,462 ± 0,079	1,551 ± 0,496	2,967 ± 1,701	0,647 ± 0,141	0,954 ± 0,142
	Interpretation	Normal	Normal	Normal	Insulin Resistance	Normal	Normal

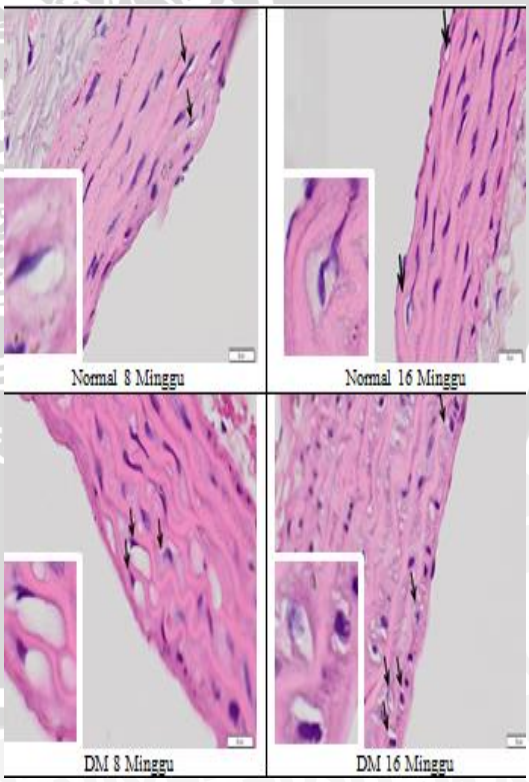
Blood Lipid Profile Level in Each *Sprague-Dawley* Rats

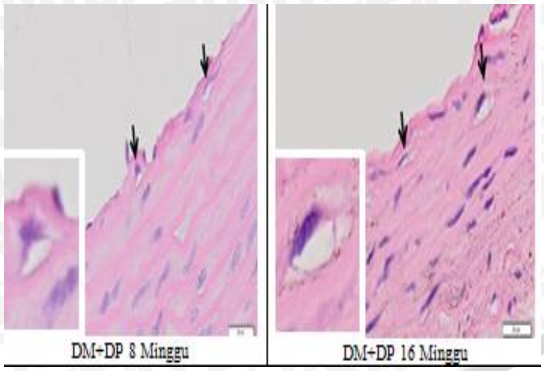
Study Groups	Normal 8 Weeks	Normal 16 Weeks	DM 8 Weeks	DM 16 Weeks	DM + DP 8 Weeks	DM + DP 16 Weeks
Total Cholesterol (mg/dL) ($x\pm$ SD)	72,79±4,045^{a}	56,56 ±5,43^{a}	123,00 ±2,8^{d}	111,72±7,29^{cd}	97,96±1,70^{bc}	98,85±3,249^{bc}
HDL (mg/dL) ($x\pm$ SD)	34,73±8,31^{d}	35,76±1,67^{d}	4,95 ±0,41^{a}	13,96±0,87^{b}	15,93±1,21^{bc}	20,79±2,76^{c}
LDL/VLDL (mg/dL) ($x\pm$ SD)	49,83 ±5,06^{b}	19,24±3,67^{a}	95,53±8,66^{c}	88,24 ±6,22^{c}	85,91 ±6,83^{c}	61,51 ±6,03^{b}

Foam cells identification and counting were done after slide preparation using paraffin block and Hematoxylin and Eosin Staining. Foam cells identified histopathologically under microscope and Dot Slide Olyvia™ within whole aorta high power field. Foam cells are the pale cells with blue nuclei in tunica intima. The result is recorded as cells/aorta. Hereby presented average foam cells value in each groups:



Average Foam Cells Value in Each *Sprague-Dawley* Rats (cells/aorta)





Histopathology image of Aorta using Hematoxylin and Eosin Staining in Each *Sprague-Dawley* Rats

Acquired data then analysed using SPSS 23rd version. Normality Saphiro-Wilk test showed that the data is normally distributed with significancy level for each 8 weeks series of time p=0.224 and p=0.483 for 16 weeks series of time (p>0.05). Therefore, data analysis continued to *Levene's* homogeneity test, from the result it is known that the data is having the same variance with p=0.0336 in 8 weeks group and p=0.308 in 16 weeks group (p>0.05). Hence, requirements of repeated ANOVA test have been fulfilled. From repeated ANOVA test, it is acknowledged that there are significance different within different time series with p=0.000 (p<0.05). Analysis then followed by Post-Hoc LSD test to identy difference between study groups

From Post-Hoc LSD analysis, foam cells average number within 8 and 16 weeks time series shown a significant difference between DM 8 weeks group with DM plus Darapladib administration within the same time serial. Significant difference also found in DM 16 weeks group and DM plus Darapladib administration within the same time serial, but there are no significant difference between Darapladib administration for 8 and 16 weeks.

DISCUSSION

Average foam cells enumeration in rat's aorta was done histopathologically by Hematoxylin-Eosin staining method under microscope with 400x magnification. Normal groups given standard diet for 8 weeks has 11.5±2.27 cells/aorta of foam cells. While normal 16 weeks group has 12±3.86 cells/aorta of foam cells. The foam cells enhancement expected correlated with arterial walls stiffness and endothelial dysfunction as the result of increasing age[16]. But the increasing foam cells doesn't significant statistically (p=0.112).

DM 16 weeks group has 15.0±3.50 foam cells. This value is higher than DM 8 weeks group which has 12.0±1.76 cells/aorta. Between DM 16 weeks group, there is significant difference between it and both normal 8 weeks group (p=0.000) and 16 weeks group (p=0.014). Foam cells elevation is a consequence of high fat diet administration, increasing age and body weight of rats [17,18]. Presences of those factors are a predisposing factor for metabolic syndrome which can be indicated by increasing blood glucose level, insulin resistance, and lipid profile of the rats. From examination of those factors, DM groups shows an indication of metabolic syndrome by elevation of fasting blood glucose level, insulin resistance, total cholesterol level, and LDL/VLDL level. While HDL level is declined. The value of those parameters is found to be higher comparing to another study groups, especially in DM 16 weeks group.

DM group that given Darapladib 20 mg/kg has a lower foam cells number compared to positive control group from each time series, p=0.000 for eight weeks, and p=0.000 for 16 weeks. Foam cell is elevated from 4±1.76 cells/aorta dan 6.9±3.35 cells/aorta in 8 and 16 weeks group consequently. This result shows that Darapladib administration reduce foam cells number significantly comparing to study group that only given HFD with the same time series. This is due to Lp-PLA_2 inhibitor administration which inhibits expansion of necrotic plaque formation and plaque destabilization. Hence, atherosclerosis progressivity is able to be blocked[19]. The result of this sudy is concur with a trial study which stated that Darapladib administration inhibited necrotic plaque formation rate significantly, compared to groups which given plasebo[20]. In adiition, in positive control group, high fat diet and low-dose streptozotocin administration cause impairs of insulin production by β pancreatic cells[21]. Therefore, hyperglycemic state worsens atherosclerosis condition by protein

and lipid glycosylation, oxidative stress, and protein kinase C (PKC) activation mechanisms[22]. Hence, type 2 DM induction procedure and darapladib administration contributes to difference in DM and DM+DP group foam cells number

Post-Hoc LSD test from 8 weeks negative control and 16 weeks DM group which given Darapladib shows no significat difference (p=0.188). This result indicates, 16 weeks administration of Darapladib diminishes foam cells number until the same as normal-diet-only group for 8 weeks time series.

In DM group with Darapladib administration for 8 and 16 weeks time series, there are found to be no significant difference (p=0.101). This result shows there is no such significant difference between short and long period of Darapladib administration. It is expected that data variation and Darapladib *dose-dependent* characteristic plays a role in this result[23].

CONCLUSION

Darapladib administration orally 20 mg/kg for 8 or 16 weeks is able to significantly reduce the foam cell number of *Sprague-Dawley* rats atherosclerosis type 2 diabetes mellitus.

RECOMMENDATION

1. Further study is needed to evaluate Darapladib effects on atherogenesis in type 2 diabetes mellitus model rat before study in order to enhace reliability level of the result.

2. This study uses 20 mg/kg Darapladib. Therefore, further study with dose variation is needed to reveal an optimum dose to reduce foam cells in type 2 diabetes mellitus model rat.

3. Further study is needed in order to investigate Darapladib administration effect on foam cell number using specific staining method for foam cell or macrophage of immunohistochemistry staining

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