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Elaborate evaluation of serum and tissue oxidized LDL level with darapladib therapy: A feasible diagnostic marker for early atherogenesis

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ABSTRACT

Objective: To compare oxidized low density lipoprotein (oxLDL) levels in serum and vascular wall of Sprague-Dawley rats, identify their patterns in 8 weeks and 16 weeks of dyslipidemia induced by high fat diet, compare foam cells in aorta of each group and investigate lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) role in atherosclerosis by darapladib administration.

Methods: This study generated in twenty-four Sprague-Dawley rats. Rats were divided into 6 groups, which were received normal diet (normal group), high fat diet and high fat diet plus darapladib therapy for both 8 weeks and 16 weeks. Surgeries were performed at Week 8 and Week 16 to take the blood serum and aortic tissue. Level of oxLDL in serum, oxLDL aortic tissue, foam cell amount in aortic tissue, and Lp-PLA$_2$ expression in aortic tissue were measured.

Results: There were significant differences in oxLDL level in serum, aortic tissue and foam cell amount ($P < 0.05$). There was no significant difference in Lp-PLA$_2$ expression in aortic tissue. OxLDL in serum and aortic tissue had a very strong correlation ($r^2 > 0.9$, $P < 0.05$). This study also composed an equation for oxLDL level in aortic tissue prediction. Factorial ANOVA found that there was a significant difference of oxLDL level in the interactions between duration and location, location and treatment, and also duration, location and treatment ($P < 0.01$). Administration of darapladib was able to reduce levels of oxLDL in serum, aortic tissue and foam cell significantly ($P < 0.05$, $P < 0.05$ and $P < 0.01$, subsequently).

Conclusions: OxLDL level is location-dependent and duration-dependent. As a feasible early diagnosis, we can predict oxLDL level in aortic tissue by its level in serum. Though Lp-PLA$_2$ expression was unsignificant, Lp-PLA$_2$ inhibition by darapladib can reduce oxidative stress and inflammation in atherogenesis.

1. Introduction

Dyslipidemia is one of the independent risk factors of atherosclerosis [1]. Imbalanced lipid in blood is more likely to undergo oxidation process. Oxidized low density lipoprotein (oxLDL) is one of the product [2]. High level of LDL has been proven as an atherosclerosis risk factor by playing a role in vascular lesion formation by its accumulation in subintimal layer of aorta or large artery [3]. Oxidation of LDL is mediated by cell, subsequently composing a strong prooxidant oxLDL [4]. OxLDL initiates chemoattractant release, which in this way attracts macrophage migration into the lesion, and triggers formation of reactive oxygen species (ROS), leading to oxidative stress, and the death of vascular smooth muscle cells [5]. In other words, oxLDL triggers chronic inflammatory process, therefore activating the vicious inflammation cycle in the body [6].

OxLDL accumulation in vascular wall evoke atherosclerosis plaque formation [7]. But it is not ethically legal to examine

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oxLDL level in vascular wall of human. So, many researchers chose to examine its level in serum or plasma to describe progression of atherosclerosis [8–10]. Comparison study for oxLDL level in serum and aorta is rarely done before, even though measurement of its level in serum as a marker in cardiovascular disease is widely used nowadays.

Darapladib is newly emerging potential drug for atherosclerosis treatment. This drug works by selectively inhibiting lipoprotein-associated phospholipase A_2 (Lp-PLA_2) [11]. Though Lp-PLA_2 had been established as a marker of atherosclerosis, up to now its role in atherogenesis is still ambigous [12]. Many researchers believe that Lp-PLA_2 had a proinflammatory effect by breaking down oxLDL into lysophosphatidylcholine and oxidized nonesterified fatty acid; both have proinflammatory property [13]. In this way, darapladib can reduce proinflammatory effect of Lp-PLA_2 [14]. But when tested in clinical trial, darapladib was failed to reduce inflammation process [15]. Lp-PLA_2 is also believed to act as an anti-inflamatory agent by hydrolyzing platelet activating factor, thus reducing activated platelet and subsequently preventing vascular abnormalities [16].

In this study, we want to compare oxLDL levels in serum and vascular wall of Sprague-Dawley rats, identify their patterns in 8 weeks and 16 weeks of dyslipidemia induced by high fat diet and compare foam cells in aorta of each group. This study also investigates Lp-PLA_2 role in atherosclerosis by darapladib administration.

2. Materials and methods

This research was conducted at the Central Laboratory of Biological Sciences, Bioscience and Pathology Anatomy Laboratory of Brawijaya University from August 2014 to March 2015. All experimental procedures involving animals were conducted in accordance to guidelines of the animal care of the Bioscience Department, Math and Science Faculty of Brawijaya University and approved by Institutional Ethics Committee of Brawijaya University (Number 229-KEP-UB). This study is the part of Lp-PLA_2 study.

2.1. Darapladib

Darapladib was purchased from GlaxoSmithKline. It was administered to the animal models daily as much as 20 mg/kg of rat body weight. The control groups were administered placebo *per os*. High fat diet was given as much as 26 g/day with 62% Comfeed PARS composition, 20% flour, 0.2% cholic acid, 1% cholesterol, and 16.8% margarine.

2.2. Study design

Sprague-Dawley rats (4 weeks old and 150–200 g body weight) were obtained from Bogor Agricultural University, Indonesia. After acclimatization period, rats were divided into 6 groups with randomization method, which were negative control, positive control and darapladib groups for 8 weeks (N8, DL8, and DLDP8, subsequently), and the negative control, positive control and darapladib groups for 16 weeks (N16, DL16, and DLDP16, subsequently). The negative control group was given a normal diet while positive control group and darapladib group were given high fat diet. Surgeries were performed at Week 8 and Week 16 to take the blood serum and aortic tissue.

2.3. Lipid profile measurements

Serum lipid profiles were measured by colorimetric method using EnzyChrom™ AF HDL and LDL/VLDL Assay Kit (E2HL-100) from BioAssay Systems.

2.4. Measurement of Lp-PLA_2 expression in aortic tissue

Aortic tissue samples were cut and stained with platelet-activating factor acetylhydrolase polyclonal antibody (bs-1451R Bioss Inc.). The luminescences then were measured by Olympus FluoView software (version 1.7A) (Olympus Corporation, Tokyo, Japan).

2.5. Measurement of oxLDL level

Levels of oxLDL in aortic tissue and serum were measured by sandwich ELISA method using Rat oxLDL ELISA kit (Cat. No. E-EL-R0710) with proper recommended protocol, and subsequently measured at 450 nm wavelength.

2.6. Foam cell measurements

The aortic tissue was stained by Oil Red O staining. Tissue was subsequently blocked, sliced, deparaffinized and stained with hematoxylin-eosin staining. Foam cell was then counted manually under a microscope with 400× magnification.

2.7. Statistical analysis

We used SPSS software, version 16 (IBM Corporation, New York) for data analysis. Dependent *t*-test was used to compare oxLDL level in serum and aorta. Independent *t*-test was used to compare 8 weeks and 16 weeks treatment, also to compare group which received darapladib treatment and those who did not receive darapladib treatment. Factorial ANOVA was then performed to compare oxLDL based on location, duration, and darapladib treatment. One-way ANOVA was used to determine the effects of darapladib on number of foam cells and Lp-PLA_2 expression. This analysis was continued by *post hoc* test using the Duncan method to detect differences in parameters in each treatment group. After that, we analyzed the pattern of oxLDL in serum and aorta, and formulated an equation to predict oxLDL level in aorta with its level in serum.

3. Results

3.1. OxLDL in serum and aorta

Serum levels of oxLDL in the group which received normal diet were lower compared to high fat diet group (Table 1). Table 1 demonstrates that administration of darapladib was able to reduce levels of oxLDL in serum significantly compared to the positive control groups. This pattern was clearly illustrated in the 8-week and 16-week treatment.

Levels of oxLDL in aortic tissue in the normal diet group were lower compared to the group given high fat diet (Table 1). Administration of darapladib was able to reduce levels of oxLDL in aortic tissue significantly compared with the DL group given a high fat diet. This pattern was clearly illustrated in both treatment duration (8 weeks and 16 weeks) (Table 1).

Table 1
Tissue Lp-PLA_2, serum and tissue oxLDL, and foam cell in each group.

Group		Tissue Lp-PLA_2 (arbitrary unit)	Serum oxLDL (ng/mL)	Tissue oxLDL (ng/mL)	Foam cell (cell/slide)
N8	Mean ± SD	761.551 ± 19.040	0.231 ± 0.062[a]	1.323 ± 0.168[a]	72.00 ± 4.08[a]
	Min–Max	734.970–776.230	0.167–0.292	1.120–1.470	67.00–77.00
DL8	Mean ± SD	1 210.715 ± 231.640	1.973 ± 0.085[c]	12.943 ± 0.565[e]	116.50 ± 4.03[d]
	Min–Max	1 021.130–1 547.880	1.870–2.070	12.150–13.460	106.00–115.00
DLDP8	Mean ± SD	1 069.052 ± 108.070	0.315 ± 0.059[a]	3.519 ± 0.114[c]	86.75 ± 3.09[b]
	Min–Max	937.900–119.950	0.246–0.385	3.410–3.670	84.00–91.00
N16	Mean ± SD	810.872 ± 212.110	0.335 ± 0.090[a]	2.246 ± 0.257[b]	70.50 ± 5.56[a]
	Min–Max	560.770–988.180	0.277–0.467	1.870–2.470	63.00–76.00
DL16	Mean ± SD	874.685 ± 154.320	2.031 ± 0.141[c]	15.631 ± 0.788[f]	98.75 ± 2.75[c]
	Min–Max	687.060–101.340	1.890–2.210	14.840–16.640	96.00–102.00
DLDP16	Mean ± SD	1 142.094 ± 598.510	0.673 ± 0.088[b]	6.750 ± 0.589[d]	78.50 ± 2.88[b]
	Min–Max	622.710–188.510	0.570–0.754	5.980–7.400	78.00–85.00
P value		0.90[*]	0.00	0.00	0.00

[*]: With nonparametric test. Different letters indicate a statistically significant difference (Duncan *post hoc* test, $P < 0.05$). Lipid profile can be seen in our previous publication [17].

The level of oxLDL in serum and aorta had a significant difference ($P < 0.05$). Based on Pearson correlation analysis, correlation between oxLDL in serum and aorta was strong ($R^2 = 0.946$, $P < 0.05$). In Figure 1, we can predict oxLDL level in aorta by its level in plasma in the following equation.

$$y = 6.809x + 0.764$$

where y is oxLDL level in aorta, and x is oxLDL level in serum.

There was no significant difference of 8 weeks duration and 16 weeks duration ($P > 0.05$). Darapladib treatment proved to have an effect on dyslipidemia, with significant difference of DL and DLDP groups in both duration ($P < 0.05$) (Table 1).

Based on factorial ANOVA, there was significant difference of oxLDL when there were interactions between duration and location, location and treatment, and also duration, location and treatment ($P < 0.01$).

3.2. Amount of foam cell

DL8 and DL16 group that received high fat diet had a considerable amount of foam cells than other groups (Table 1, Figure 2). Administration of darapladib for 8 weeks and 16 weeks could significantly reduce the amount of foam cells in the aorta ($P < 0.01$) (Table 1).

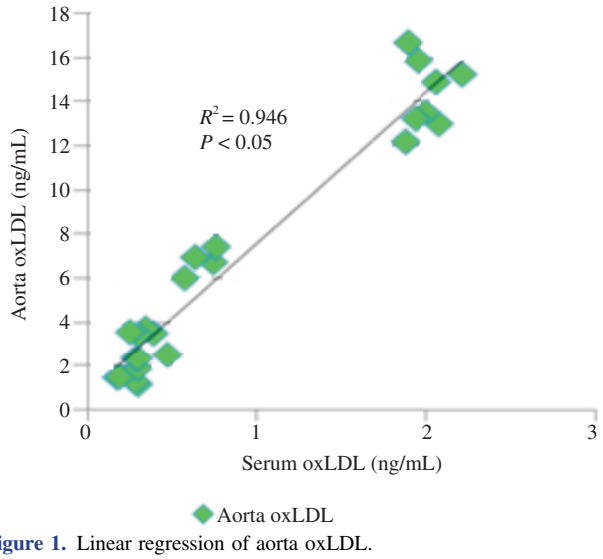

Figure 1. Linear regression of aorta oxLDL.

3.3. Expression of Lp-PLA_2 in aortic tissue

The groups which received normal diet expressed lower level of Lp-PLA_2 than the high fat diet group. In the 8-week treatment group, the difference was significant, but in 16-week group, the difference was not significant. DLDP8 group which was given high fat diet and darapladib for 8 weeks had a lower Lp-PLA_2 expression than the DL8 group which was also given high fat diet for 8 weeks, even though the difference was not significant.

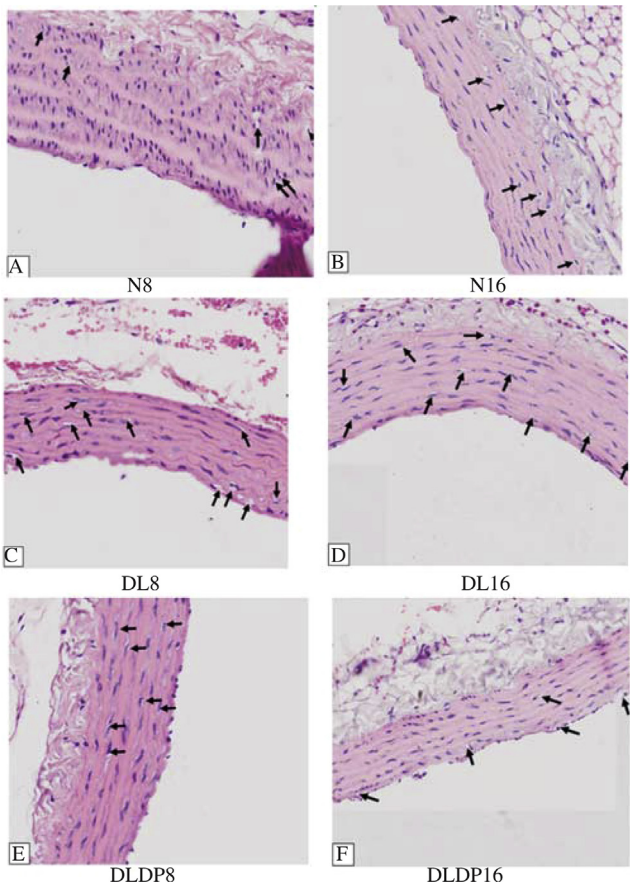

Figure 2. Foam cell in aortic tissue.
A: Normal diet for 8 weeks; B: Normal diet for 16 weeks; C: High fat diet for 8 weeks; D: High fat diet for 16 weeks; E: High fat diet and darapladib therapy for 8 weeks; F: High fat diet and darapladib therapy for 16 weeks. Foam cells were pointed by black arrow.

Another group which was given darapladib for 16 weeks (DLDP16), on the other hand, expressed higher level of Lp-PLA_2 than the other 16-week groups (Table 1).

4. Discussion

High fat diet was proved to increase LDL level in the blood. This theory is in line with lipid profile result in our previous publication [17]. High fat diet was also proved to enhance ROS formation in the body [18]. ROS are produced by immune cells, such as monocyte, as a response to lipopolysaccharide release by intestinal epithelial cells in form of chylomicron [3]. This chronically mild inflammation and oxidative stress mediated by high fat diet are factors that play ultimate role in atherogenesis [19].

Lipid content in diet is normally stored in adipose tissue as an esterified lipid. But, in high fat diet consumption, 40%–50% of lipid content will undergo spillover, contributing to ectopic fat deposition [7]. Hyperlipidemia, ectopic lipid accumulation, and cytosolic lipid droplet can induce ROS production, thus leading to subsequent endothelial dysfunction [7]. ROS had a reciprocal relationship with LDL through oxidation process. It acts by modifying LDL into oxLDL, and oxLDL then will induce more ROS formation [20].

LDL oxidation process is believed to take place in the arterial wall rather than in circulation. It is because the circulation contains high antioxidant content [21]. So, oxLDL level in serum tends to be lower than that in arterial wall. LDL concentration circulation was 30-fold lower than that in atherosclerotic intima [3]. In recent study, we found that oxLDL in serum was 6–7 folds lower than that in aorta of normal and dyslipidemia groups. But darapladib treatment caused a different ratio of oxLDL level in serum and plasma; oxLDL in serum was 10 times lower than that in aorta. On the other side, there was a pattern of oxLDL level in both serum and aorta, which was low in normal diet and darapladib treatment groups and high in high fat diet group. We also found that oxLDL level in both site had a very strong correlation ($R^2 > 0.9$, $P < 0.05$) (Figure 1). From the linear regression analysis, we formulated an equation to predict aorta's oxLDL level based on its level in serum. While measuring oxLDL level in aorta is ethically restricted, this innovation could be used as a feasible diagnosis marker to predict progression of atherosclerosis.

LDL oxidation occurred in 2 phases. In early phase, LDL oxidation marked by few changes in apolipoprotein B100. This process forms minimally oxidized LDL. This process then follows with LDL protein modification phase. As a result, LDL will lose its recognition by LDL receptor. Lipopolisaccharide is fashioned in oxLDL surface then recognized by scavenger receptor such as CD36 in macrophages [4].

Nonesterified fatty acid and oxLDL have a connection with inflammation in atherosclerosis [22]. Both of them could stimulate activation of inflammatory transcriptional factor, such as nuclear factor-κB, thus increasing proinflammatory cytokines expression [7,23]. One of the most important inflammation hallmark in atherosclerosis is monocyte recruitment [4].

Monocytes are recruited to the subintimal layer as a response to oxLDL accumulation [24]. Monocytes then activate into macrophages and express some scavenger receptor such as CD36 and SR-A [25]. Their interaction will stimulate phagocytosis and pinocytosis of oxLDL [26]. In macrophage, oxLDL will hydrolyze into free cholesterol by lysosomal acid lipase enzyme. Free cholesterol had cytotoxic properties, therefore it will undergo reesterification by acetyl-CoA acetyltransferase in endoplasmic reticulum, forming a cholesterol ester. Cholesterol ester was then stored in cytoplasmic lipid droplet [26]. Scavenger receptor for oxLDL does not have negative feedback mechanism. Thus, oxLDL uptake is uninhibited. This will lead to more cytoplasmic lipid droplet accumulation in macrophage cytoplasm, forming the foamy appearance of foam cell [3]. Accumulation of foam cell in aorta/arterial wall is the early sign of atherosclerosis [27]. In this study, the results of foam cell are harmonious with established theory. Rats which consumed high fat diet tend to have more foam cell in aorta, rather than those who consumed low fat/normal diet ($P < 0.01$) (Table 1). Foam cell formation by high fat diet was caused by oppressed expression of enzymes that control cholesterol efflux and reverse cholesterol transport, such as neutral cholesteryl ester hydrolase, ATP-binding cassette transporters A1, ATP binding cassette subfamily G member 1, and scavenger receptor BI [26].

OxLDL and foam cell level tend to be higher in longer duration of high fat diet consumption (Table 1). In oxLDL serum, no significant difference in 8 weeks and 16 weeks treatment may imply the antioxidant role in the circulation. On the other side, aorta tissue does not have antioxidant as much as in the circulation (Table 1). Therefore, oxLDL level in aorta in 8 weeks and 16 weeks high fat diet treatment have a significant difference ($P < 0.05$) (Table 1). In rats which consumed high fat diet for 16 weeks, number of foam cell in aorta is prone to be higher than that in 8 weeks treatment (Table 1). This happened because more severe oxidative stress and inflammation in 16 weeks groups induced more foam cell formation and accumulation in aortic tissue.

Lp-PLA_2 is produced mainly by proinflammatory cells such as monocytes, macrophages, T-lymphocyte and mast cell [28]. Its majority is tied up to LDL in the circulation [29]. Lp-PLA_2 was first discovered by its capacity to hydrolyze platelet activating factor, thus leading to subsequent inactivation of platelet. This fact implies anti-inflammatory effect of Lp-PLA_2 [30]. Lp-PLA_2 also had an ability to hydrolyze oxLDL. This reaction generates lysophosphatidylcholine and oxidized nonesterified fatty acid [31]. Both of them could multiply existing inflammation and oxidative stress. Therefore, Lp-PLA_2 also had a proinflammatory feature [30]. Lp-PLA_2 anti-inflammatory features were proposed to be more in the circulation, rather than in vascular wall tissue. When Lp-PLA_2 diffuse into endothelial wall, its pro-inflammatory properties become more prominent [32].

In the present study, results of Lp-PLA_2 were unsignificant ($P > 0.05$) (Table 1). Nevertheless, in 8 weeks treatment groups, the pattern was still portrayed. Normal diet group had lower Lp-PLA_2 expression, darapladib treatment group had moderate Lp-PLA_2 expression, and the highest Lp-PLA_2 expression was in high fat diet group (Table 1). In 16 weeks treatment groups, darapladib treatment group had the highest Lp-PLA_2 expression (Table 1).

Oral administration of darapladib contributes to its low distribution in tissue. Large proportion of oral darapladib administered is neither absorbed or maintained by first pass metabolism. On the contrary, intravenous administration of darapladib tends to have a slower elimination and higher tissue distribution [33]. This might be the clarification of unsignificant Lp-PLA_2 results in this study. Low tissue distribution of oral darapladib administration merely inactivates few Lp-PLA_2 in aortic tissue.

OxLDL, on the other hand, can activate platelet via its binding with CD36 and lectin-like oxLDL receptor-1 receptors. This interaction is concentration-dependent, as the activation of platelet is rising in parallel with oxLDL level. On the contrary, activated platelet will generate ROS, which will cause another LDL oxidation [34]. This might be the reason why oxLDL and foam cell counted in darapladib treatment groups were low, but still higher than normal.

In conclusion, oxLDL level is location-dependent and duration-dependent. As a feasible early diagnosis, we can predict its level in aortic tissue by its level in serum of rat model. Though Lp-PLA_2 expression was unsignificant, Lp-PLA_2 inhibition by darapladib can reduce oxLDL level in serum and aortic tissue and also foam cell formation. Therefore, oxidative stress and inflammatory process of atherogenesis can be reduced.

Conflict of interest statement

We declare that we have no conflict of interest.

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