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Structured Phospholipids from Commercial Soybean Lecithin Containing Omega-3 Fatty Acids Reduces Atherosclerosis Risk in Male *Sprague dawley* Rats which Fed with an Atherogenic Diet

Jaya Mahar Maligan, Teti Estiasih, Joni Kusnadi

***Abstract*—**Structured phospholipids from commercial soybean lecithin with oil enriched omega-3 fatty acid form by product of tuna canning is alternative procedure to provides the stability of omega-3 fatty acid structure and increase these bioactive function in metabolism. Best treatment condition was obtain in 18 hours acidolysis reaction with 30% enzyme concentration, which EPA-DHA incorporation level was 127,47 mg/g and incorporation percentage of EPA-DHA was 51,04% at phospholipids structure. This structured phospolipids could reduce atherosclerosis risk in male Sprague dawley rat. Provision of structured phospholipids has significant effect ($\alpha = 0.05$) on changes in lipid profile, intima-media thickness of aorta rats (male Sprague dawley) fed atherogenic diet. Structured phospholipids intake can lower total cholesterol 78.36 mg/dL, total triglycerides 94,57 mg/dL, LDL levels 87.08 mg/dL and increased HDL level as much as 12,64 mg/dL in 10 weeks cares. Structured phospholipids intake also can prevent the thickening of the intima-media layer of the aorta.

***Keywords*—**Structured phospholipids, commercial soybean lecithin, omega-3 fatty acid, atherosclerosis risk.

I. Introduction

ONE of the most important fatty acids for health is omega-3 fatty acid. Oil product of tuna fish (*Thunnus sp*) canning contains omega-3 fatty acid which is equivalent to omega 3 fatty acid in cod liver oil [1].

Omega-3 fatty acid product of tuna canning has limited stability of the low oxidation, therefore it need to look for the alternative of carrier agent of omega 3. The most stable omega-3 fatty acid is in form of phospholipids. Thus, the incorporation of omega-3 fatty acid to the structure of phospholipids is one of alternative to increase the stability. So, for health, there are double usages of structured phospholipids [2]. From the previous study, showed that the structured phospholipids which obtained from 18 hours acidolysis reaction with 30% enzyme concentration has the highest EPA-DHA fatty acid incorporation level and incorporation percentage [3]. The used of commercial soybean lecithin is more profitable as the carriers of omega-3 fatty acid, the price is cheaper and the quantity is higher also the process is not too difficult. The methods to make oil enrich omega-3 fatty acids product of tuna canning based on the crystallization of solvent in low temperature [4] which is more safety than urea crystallization which produced ethyl carbamate compound / urethane, which are dangerous and carcinogenic [5].

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The method to corporate phospholipids of commercial soybean lecithin and omega-3 fatty acid was enzymatic acidolysis reaction, used lipase *R. miehei* [6].

Omega 3 fatty acids have hypocholesterolemia effect, such as omega 3 fatty acids of fish oil produce protective effect on aorta histopathology through hypolipidemia effect and decreased the thrombosis event [7]-[9]. Soybean lecithin will induct the reducing of plasma cholesterol in hypercholesterolemia rats and atherosclerosis lesion [10] [11].

Based on several studies mentioned above, it is important to study the potential of structured phospholipids in reducing the risk of atherosclerosis by in vivo testing in experimental rats. Tests were carried out by administering a structured phospholipid intake in the male white rat (*Rattus norvegicus*) *Sprague dawley* strain which fed with atherogenic diet for 10 weeks periods. The parameters observed include the examination of lipid profile (total cholesterol, total triglycerides, LDL and HDL level), histopathological examination and calculation of intima-media thickness (IMT) of the aorta.

II. Methods

A. Material and Tools

The materials used in this research were commercial soybean lecithin (PT. Panadia), fish oil (by product of tuna canning – PT. Aneka Tuna Indonesia), and Lipase *Rhizomucor meihei* (Sigma Co.). The chemicals used were mixed fatty acids standard, phospholipids standard BF3-methanol 14%, methylcloride, NaOH, benzene, KOH, HCl, acetone, SDS, phosphate buffer pH 7, hexane, sulfuric acid, TLC (silica gel G60 F254 as adsorbent), chloroform, methanol (p.a. Merck), ethanol, hexane, acetone, aquadest, dry ice, nitrogen, sucrose, CMC, Soybean oil, cholic acid, palm oil, yellow eggs, beef lard, formalin, NaCl, ethanol, xylol, hematoxylen-eosin (HE staining), paraffin, normal saline, propylene glycol, gelatin, cholesterol reagent kit (cholesterol, triglyceride, HDL & LDL precipitant, Diasys.co).

The tools used were water bath shaker, gas chromatography (Shimadzu), TLC Scanner (Shimadzu), TLC development tank, densitometer, oven, UV lamp, glassware, magnetic stirrer, rotavapour, pHmeter, microsyringe, refrigerator, filter flask, table balance, digital balance, freeze centrifuge, spectrophotometer, vortex, nitrogen gas sprayer, dissecting kit, feeding needle, spuit injection, microtube, incubator, microtome, microscope, haemocytometer, and microhematocrite.

B. Research Design

This research was conduct by provision of structured phospholipids to male rat (*Rattus norvegicus*) *Sprague dawley* strain. There were 5 treatments to the rats during the 10 weeks experiment periods. In every treatment includes 4 rats. The treatments were:

P0 : standard AIN-93M diet
P1 : atherogenic diet
P2 : atherogenic diet + commercial soybean lecithin
P3 : atherogenic diet + oil enriched omega 3 fatty acid
P4 : atherogenic diet + structured phospholipids

C. Analysis Methods

The analysis methods which are used are analysis of profile by thin layer chromatography phospholipids [12], profile and level of omega 3 fatty acids by gas chromatography (*in situ* transmethylation methods [13]). Analysis of lipid profile used Diasys reagent kit (total cholesterol, total triglyceride, HDL and LDL level), histopathology analysis of aorta and determination of intima-media thickness of aorta [14].

III. Results and Discussion

A. Lipid Profile Analysis

The lipid profile analysis was done to rat blood serum in three stage periods. It was done before adaptation period, after adaptation period (week 0) and every two weeks during 10 weeks periods of treatment. The average of lipid profile (cholesterol, triglyceride, HDL and LDL level) of rat during the maintenance can be seen on Figure 1,2,3 and 4. Then, the changing of lipid profile lipid average during the treatment can be seen on Table I.

1. Total Cholesterol

In this research, rat was fed with atherogenic diet (standard AIN-93M [15] modified by adding cholesterol, beef lard and cholic acid) which can increase the total cholesterol after period of adaptation during one week (week 0).

It emphasized the total cholesterol in composition of atherogenic feed about 2000 mg/kg able to increase the total cholesterol. Adding 200 mg of cholesterol in 100 g feed can increase the cholesterol serum before the treatment up to hypercholesterolemia [16].

On Fig. 1, it could be seen on diet treatment of P1 group (positive control- atherogenic diet), the average of total cholesterol increase continuity from 106.15 mg/dL to 253.64 mg/dL in the end of treatment. In the diet treatment of P2 group, the average total cholesterol was decreased, the average total cholesterol about 233.57 mg/dL in the end. In P3 and P4 group treatment, the total cholesterol began decreased on the second week of treatment; it was about 134.21 mg/dL and 192.26 mg/dL in the end of the treatment period.

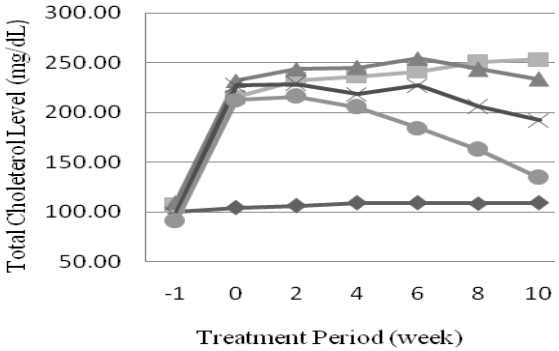

Fig. 1 Total cholesterol level of rat's treated by standard diet AIN-93M / P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (**X**), and atherogenic diet + structured phospholipids/P4 (●)

Omega 3 fatty acid and soybean lecithin in structured phospholipids give synergic hypercholesterolemia effects. Phospholipids can decreased the plasma cholesterol by influenced absorption of cholesterol in intestine. Giving soybean phospholipids can improve the activity of LCAT enzyme [17]. Soybean phospholipids increasing the activity of lecithin-cholesterol acyltransferase (LCAT) in rat, it disturbed the absorption of cholesterol on ileum and increases the excretion of steroid through feces [18].

The mechanism of hypercholesterolemia omega 3 fatty acid was it could reduced the activity of HMGCoA-reductase [6], [19] and Acyl-coenzymA : cholesterol acyltransferase enzyme (ACAT) [20], also arranged the regulation of SREBP (Sterol Receptor Element Binding Protein) in reducing the regulation of gen PPARα (peroxisome proliferator activated receptor - alpha) [21] .

Omega 3 fatty acid contains of phospatidylcoline which can supressed the activity of fatty acid synthase significantly and increased the activity of carnitine palmytoil transferase and peroxisomal β-oxidation [22].

TABLE I
CHANGES IN LIPID PROFILE OF RATS DURING THE PERIOD OF TREATMENT

Parameter	Time Period	P0	P1	P2	P3	P4
Cholesterol Total	Week 0 (mg/dL)	104,42	215,64	231,92	226,99	212,56
	Week 10 (mg/dL)	109,42	253,64	233,57	192,26	134,21
	Average (mg/dL)	4,85^{c}	38,00^{c}	1,64^{bc}	-34,72^{b}	-78,36^{a}
Triglyceride Total	Week 0 (mg/dL)	64,36	114,65	109,57	117,97	104,9
	Week 10 (mg/dL)	68,55	162,28	111,90	94,44	84,92
	Average (mg/dL)	4,20^{b}	50,63^{c}	2,33^{b}	-23,52^{a}	-94,57^{a}

HDL Level	Week 0 (mg/dL)	57,12	52,56	56,67	55,26	55,96
	Week 10 (mg/dL)	56,31	43,82	54,46	57,64	68,60
	Average (mg/dL)	-0,87[b]	-8,74[c]	-2,21[b]	2,39[a]	12,64[a]
LDL Level	Week 0 (mg/dL)	34,44	140,15	153,34	148,14	135,70
	Week 10 (mg/dL)	39,26	176,77	156,73	115,73	48,62
	Average (mg/dL)	4,82[d]	36,32[cd]	3,39[c]	-32,41[b]	-87,08[a]

Note: Data are the average value of four replications
Values accompanied by different notations indicate significant differences in Tukey test (α = 0.05)

2. Total Triglycerides

The provision of structured phospholipids contain of omega 3 fatty acid in which rat which fed with atherogenic diet could reduced the average of total triglyceride up to 95, 74 mg/ dL, besides giving only omega3 fatty acid reduced about 23,52 mg/dl (Fig. 2). It was different with giving phospholipids which not able to reduce the average of total triglyceride. It could increased the total triglyceride about 2,33% during the maintenance along 10 weeks.

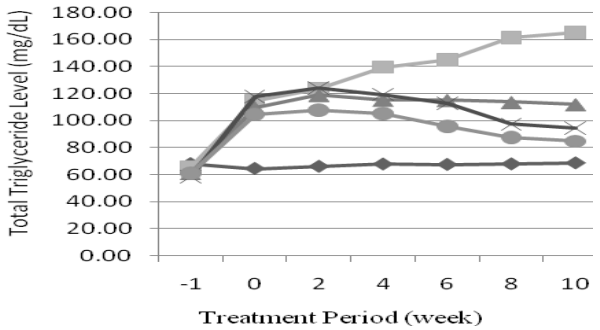

Fig. 2 Total triglyceride level of rat's treated by standard diet AIN-93M/P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (**X**), and atherogenic diet + structured phospholipids/P4 (●)

The effect of reducing of the total lipids was possible because of the increasing of transport of lipid from blood to liver. Diet enriched phophatidylcholine from soybean lecithin will reduce the absorption cholesterol in ileum and decreased the cholesterol [23]. It controlled the biodynamic changing of lipid, by regulation of cholesterol homeostasis and fatty acid through the process of reducing the synthetic of cholesterol and fatty acids beside it increase the cholesterol oxidation became bile salt which increased the secretion of lipid.

Fish oil could reduce the level of triglyceride plasma significantly [24]. Omega 3 fatty acid increased the effect of hipotriglyseridemia by suppressed lipogenesis in liver by reduce the level SREBP-1c (*Sterol Receptor Element Binding Protein*–1c), it increased the regulation of oxidation fatty in liver and muscle through activation PPAR (*Peroxisome Proliferator-Activated Receptor*) and increased the rate of change of glucose become glycogenby pressing the regulation of HNF-4α (*Hepatocyte Nuclear Factor*-4α)[25]. Simultaneously, omega 3 fatty acid pressed the regulation of gen which coded protein which stimulated the fatty synthesis and increase the regulation of gen which protein coded that stimulated the fatty acid oxidation.

3. HDL Level

Phospholipids contains of omega3 fatty acid in rat's diet able to increase the average of HDL level about 12,64 mg/dL during ten weeks, besides omega 3 fatty acid only increased about 2,39 mg/dl. It compared to phospholipids input during the maintenance, reducing of the average HDL level about 2.21 mg/dl. The average of HDL level could be seen on Fig. 3.

The provision of phospholipids came from diet stimulated the production of HDL. CaCO2 cell in ileum will secreting ApoA-I which contains lipoprotein HDL [17]. The phospholipids which came from diet in ileum will incorporated directly in HDL then will increase the production of intestinal HDL.

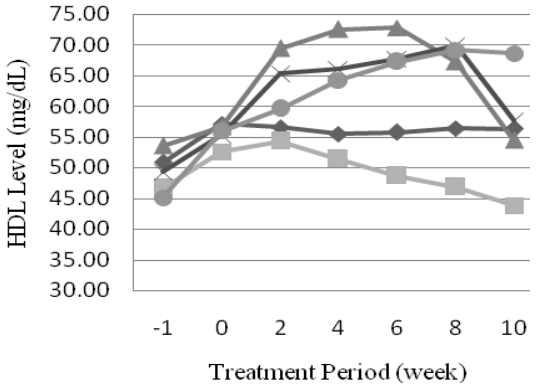

Fig. 3 HDL level of rat's treated by standard diet AIN-93M/P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (**X**), and atherogenic diet + structured phospholipids/P4 (●)

DHA will increase the HDL level and HDL2 in heperlipidemia [26]. Omega-3 fatty acid, especially EPA and DHA was the inhibitor cholesteryl ester transfer protein (CETP). CETP is hydrophobic glycoprotein plasma which was synthesized by liver, its function transfer cholesterol ester [27]. CETP is the mediator to transfer cholesteryl ester from HDL become LDL or VLDL. The obstruction of CETP will increase the HDL level and support the transport back reaction of cholesterol.

4. LDL Level

The average of LDL rat which are given feed standard input tend to stable, between 35,77-39,29 mg/dL during maintenance. Atherogenic diet intake was able to increase the average of LDL level from 46, 88 mg/dl up to 176, 77 mg/dl.

The provision of phospholipids was not able to reduce the average of LDL, it was increased from 153,34 - 256,73 mg/dL during ten weeks treatment periods. In other hand, the provision of omega 3 fatty acid and structured phospholipids which contains omega 3 fatty acids could reduce the average of LDL level during ten weeks periods. The reducing of LDL average was 148,14 - 115,73 mg/dl (P3) and 135,70 - 48,62 mg/dL (P4 group). The average of LDL content during 10 weeks treatment periods could be seen on Fig. 4. Giving lecithin to the hypercholesterol rat could decrease the level of VLDL, IDL and LDL plasma also increase the level of HDL plasma. The changing of lipoprotein plasma maybe because of the role of lecithin which influenced the activity of lecithin:cholesteryl acyl transferase (LCAT), which increased the conformation of HDL plasma [28]. Omega 3 fatty acid in diet P3 and P4 reduced the average of LDL of mice. It caused by the role of omega 3 fatty acid in managed the lipogenesis which caused synthesis of VLDL decrease so the LDL level also decreases. Fatty acid disturbed the process of SREBP which stimulated the fatty acid oxidation.

Peroxidation omega 3 fatty acid improving the degradation of apolipoproteinB meanwhile reduces the secretion of VLDL and LDL [26].

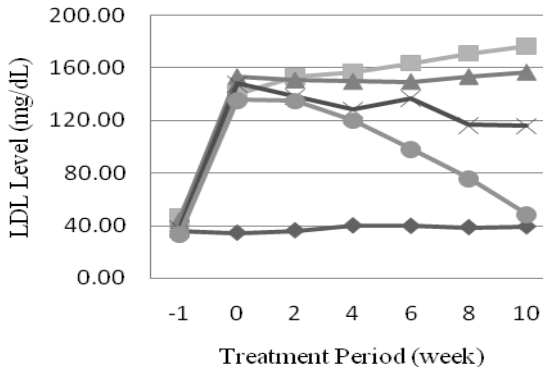

Fig. 4 LDL level of rat's treated by standard diet AIN-93M/P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (**X**), and atherogenic diet + structured phospholipids/P4 (●)

B. Analysis of Rat's Aorta Histopathology and Intima-Media Thickness

1. Analysis of Rat's Aorta Histopatology

Results of hematoxylen-eosin staining in the aorta cross section and examination at 400x magnification light microscope could be seen on Fig. 5. It can conclude that the microscopic thickness of the aortic intima-media layer of P0 treatment group (standard diet AIN-93M) is the smallest compared with other treatment groups.

The composition of the innermost layer of the aorta close to the lumen or direct contact with blood is the tunica intima is relatively flat not bumpy. It can be assumed that the arrangement and number of endothelial cells in aortic intima areas are still well preserved.

Boundary layer between the tunica intima with the media is not very clearly visible, and the tunica adventitia is also not clearly visible as well. The formation of foam cells in the tunica intima and smooth muscle cell migration into the tunica media tunica intima of the aorta have not seen.

Endothelial cells are very sensitive to the effects of oxidative stress and dyslipidemia conditions will lead to oxidative stress. This situation occurs due to disturbances of lipoprotein metabolism, are often referred to as the lipid triad, which includes increased levels of VLDL or triglycerides, decreased HDL levels and formation of a more atherogenic LDL [29]-[31]. During the body's metabolism of protein molecules will be modified lipoproteins due process of oxidation, glycosylation and glycosydation with the end result will be an increase of oxidative stress and formation of radical oxygen species (ROS). Beside, sthe modified lipoprotein retention within the tunica intima would trigger atherogenesis.

The exposure of injury to the endothelium would trigger a variety of molecular and cellular mechanisms that induce atherosclerotic lesions. A high level of LDL is the main factor for endothelium and myocytes damage. The ability of oxidized LDL in causing foam cells will initiate atherosclerosis [32].

2. Analysis of Intima-Media Thickness (IMT) Rat's Aorta

The average of intima-media thickness of the aorta in rat which fed the standard AIN-93M diet during the treatment period was 103.81 μm, ranging from 102.96 to 104.86 μm. The thickness average of the aortic intima-media on atherogenic diet treatment groups (P1) was not much different from the group treated atherogenic diet + phospholipid (P2), it was 214.02 μm and 206.52 μm.

While the average thickness of the intima layer of the aorta in the treatment group atherogenic diet + omega-3 (P3) was equal to 140.21 μm are not much different from the treatment of atherogenic diet + phospholipid-structured (P4), which is 131.82 μm. The intima-media thickness (IMT) average of aorta can be seen in Fig. 6.

Soy lecithin intake could increase the activity of paraoxonase (PON) and apolipoproteinA-1 (apoA-1 / apoA-I) in mice that the ApoE gene (ApoE null mice) has been disabled. Increased activity of both led to decreased lesion of atherosclerosis in ApoE-null mice. PON1 acts as an antioxidant that prevents oxidation of LDL during circulation, whereas apoA-1 is a major component of plasma HDL [10].

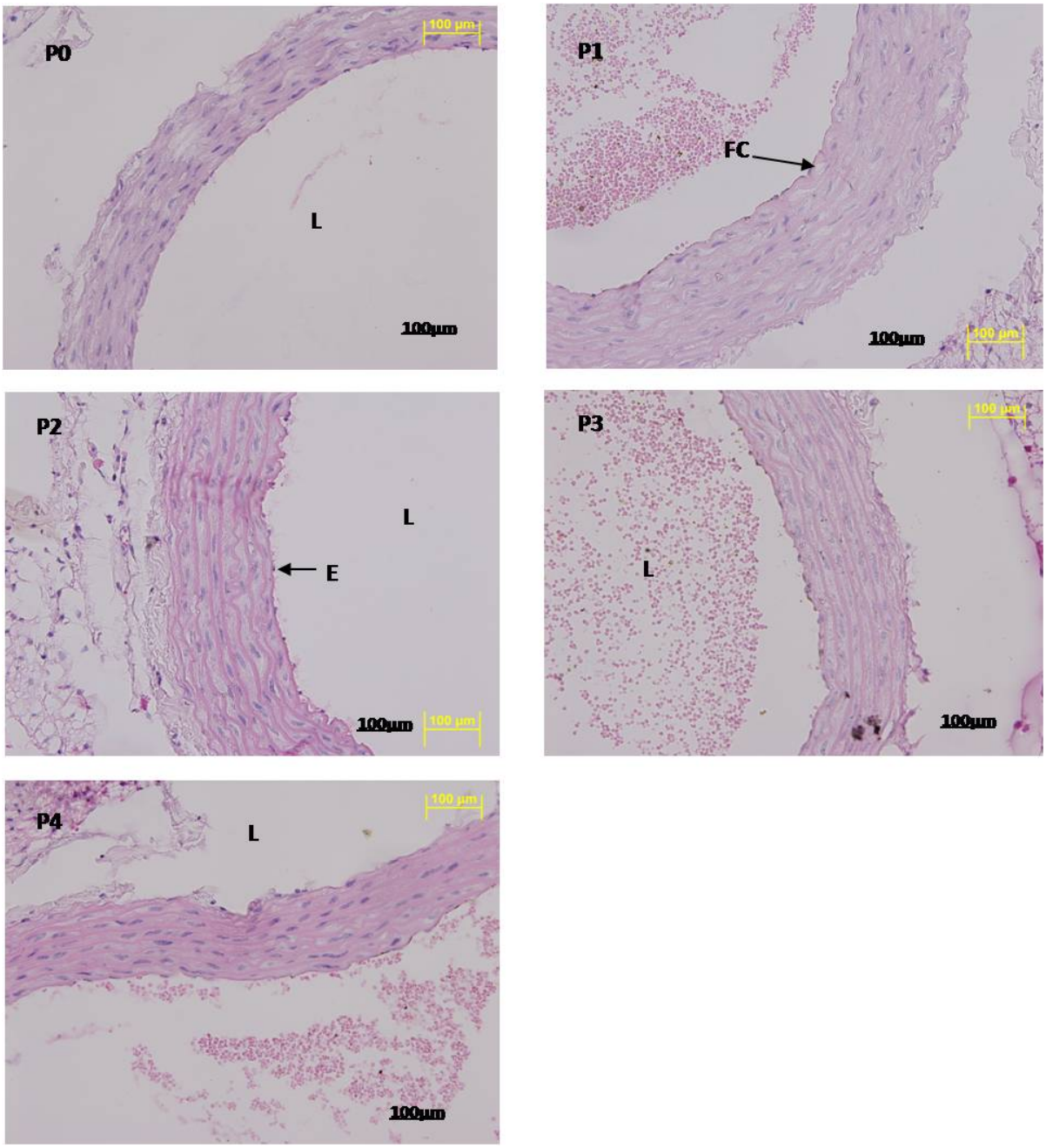

Fig. 5 Rat's aorta cross section (400x magnificent) treated by standard diet AIN-93M (P0), atherogenic diet(P1), atherogenic diet + commercial soybean lecithin (P2), atherogenic diet + oil enriched with omega 3 fatty acid (P3), and atherogenic diet + structured phospholipids (P4). Foam cell (FC), endotel cell (E) and lumen (L)

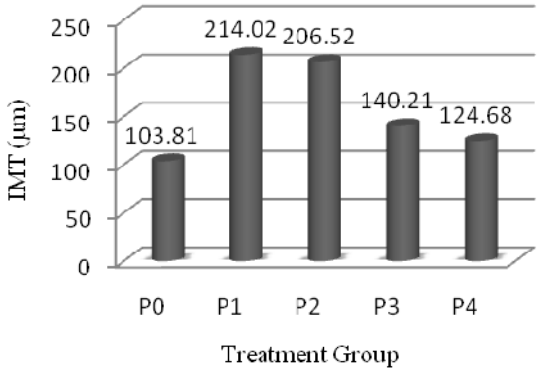

Fig. 6 Average of rat aorta's intima-media thickness treated by standard diet AIN-93M (P0), atherogenic diet (P1), atherogenic diet + commercial soybean lecithin (P2), atherogenic diet + oil enriched with omega 3 fatty acid (P3), and atherogenic diet + structured phospholipids (P4)

Fish oil intake in male wistar rats for 4 weeks resulted in a protective effect on histopathological aorta through hipolipidemia effect. Besides the intake of fish oil will decrease the activity of C-reactive protein (CRP) compared with the intake of saturated fatty acids and trans fatty acids. This CRP has effect on tissue inflammation [33].

Thickening of the aorta increases the risk of atherosclerosis, and exacerbated by the onset of inflammation and vascular pressure factor. Omega-3 fatty acids produce various eicosanoids and anti-inflammatory compound that can prevent the thickening of the aortic atherosclerosis and risk further into. Omega-3 fatty acids EPA-DHA in particular is able to prevent the occurrence of atherosclerosis by producing eicosanoids PGE1, PGI2, LXs, anti-inflammatory compounds such as resolvin and IL-4, IL-10 and TGF-β [27].

IV. Conclusion

Provision of structured phospholipids containing omega 3 fatty acids can reduce the risk of atherosclerosis on male *Sprague dawley* rats which fed with atherogenic diet. It may improve lipid profiles by lowering total cholesterol, total triglycerides and LDL levels and increase HDL levels. Besides, structured phospholipids can also reduce the occurrence of thickening of the aorta intima-media layer.

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