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The Effect of Tuna Fish Oil (*Thunnus albacares*) on the Total Cholesterol, LDL Cholesterol, HDL Cholesterol and the Triacylglycerol Level on Hypercholesterolemia Rats (*Rattus norvegicus)*

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ABSTRACT
Setting a daily diet is the first step in tackling the risk of CHD, which is due to the increasing levels of cholesterol and fat. This can be done by consuming fish that is a natural source of ω-3 fatty acids, EPA and DHA. One of the products processed from the extraction of fish, especially fatty fish is tuna fish oil that almost 40% of the total fat content is ω-3 PUFAs are EPA and DHA. This study aimed to determine the effect of Tuna fish oil (Thunnus albacares) against the total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol levels in the blood of hypercholesterolemia rats (Rattus norvegicus). The fish oil was extracted from fish waste Tuna with a reflux method using chloroform, and the levels of EPA and DHA were determined using a gas liquid chromatography. The samples were 40 male rats. Five rats were used as a normal control group, and 35 rats were subsequently given a high-cholesterol diet for 6 weeks to achieve a state of hypercholesterolemia. Five rats from the experiment group were taken as data for hypercholesterolemia. Of 35 rats, 30 were divided into 3 groups of 10 rats: 1) a control posttest group (P0), receiving the treatment of a high-cholesterol diet, 2) the first treatment group (P1), which was given a high-cholesterol diet + 1 dose (0.5 ml) of Tuna fish oil and 3) the second treatment group (P2), which was given a high-cholesterol diet + 2 doses (1 ml) of Tuna fish oil. The treatment was given for four weeks and the data were then analyzed using descriptive statistics and ANOVA test, followed by LSD test. The results showed that the Tuna fish oil supplementation can reduce the total cholesterol, the LDL cholesterol and the triacylglycerol levels significantly with $p < 0.05$. It can also increase the HDL cholesterol levels significantly with $p < 0.05$.

Key words: *Hypercholesterolemia, Tuna Fish Oil, total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerol.*

INTRODUCTION

Blood circulatory system diseases (SSD), according to ICD-10, has been ranked first in 2000 as the leading cause of death and is a major health problem in Indonesia. The results of Household Health Investigation, Health Department of Republic of Indonesia, and National Economic Survey and Health Research show that the prevalence of heart diseases in Indonesia from year to year has increased, followed by the increasing number of deaths[1]. Plasma cholesterol level is believed to be the main factor that leads to atherosclerosis which is a major cause of coronary heart disease. Although the levels of total blood cholesterol has a close relationship with atherosclerosis and coronary heart disease, the total cholesterol level is not the most appropriate predictor to determine the possibility of atherosclerosis.

Atherosclerosis is due to the cholesterol in the blood, brought into a few fractions of lipoproteins and each lipoprotein has different roles in the process of atherosclerosis. It is characterized by the accumulation of cholesterol and cholestery[1] esters from plasma lipoproteins to the arterial wall. Furthermore, diabetes mellitus, nephrosis lipids, hypothyroidism and hyperlipidemia increase the levels of VLDL, IDL, the rest of chylomicrons, and LDL in the blood. This is often accompanied by premature and worse atherosclerosis. There is also an inverse relationship between HDL levels and the coronary heart disease. This makes the ratio of LDL cholesterol against HDL is an important predictive parameter. This is consistent with HDL in reverse cholesterol transport that acts as a cholesterol-esterification of free cholesterol, and ester cholesterol which produces carrier to the liver. Consequently, HDL has a very important role in protection against triacylglycerol atherosclerosis and is also recognized as an independent risk factor. Susceptibility to atherosclerosis can be triggered by a diet high in cholesterol. The tendency of the society to changing diet and lifestyle such as smoking, obesity, lack of exercise is also another factor, which plays a role in the coronary heart disease[2]. For people who have high cholesterol levels (hypercholesterolemia), setting the daily diet is the first choice in tackling this problem. Improved diet can be done by consuming some foods that can lower cholesterol levels in the blood such as crude fiber (crude fiber), PUFA, and fish oil[3]. Indonesia is one of the largest producer of fish because the majority of its territory consists of the sea. One type of fish that recently became the backbone of

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Table 1: Rats Test Animal Weight Loss Before Treatment

Group Weight Loss Basics	*N*	X (gram)	SD	P Value
Early	5	170	14,5574	0,00
Hypercholeterolemia	5	242	13,0384	0**

Note: ** Significant to the confidence level p <0.01

Indonesian export commodities is Tuna. This is due to the strong demand from overseas markets and is supported by large potential Tuna fish in the Indonesian waters, including North Sulawesi. One of the products processed from the extraction of fish, especially in fatty fish and widely used in food and non-food industries is fish oil. Fish oil produced in Indonesia consists of fish liver oil and fish body oil. The raw materials of fish body oil originate from a byproduct of fishmeal processing and canning. These were mostly used for non-food industry as mixed fodder, tanners, paints and inks. Fish oil is the main source of ω-3 EPA and DHA which can reduce blood cholesterol, prevent clotting of blood platelets in order to avoid blockage of blood vessels, prevent the growth of cancer cells, and reduce mortality due to the coronary heart disease. Fish have different fat content from that of other animals; it is high in polyunsaturated fatty acids (Poly Unsaturated Fatty Acid)[4]. Tuna fish oil contains approximately 34.2% ω-3 PUFA, of which 27.3% is docosahexaenoic acid (DHA), 37% are eicosapentaenoic acid (EPA) and 3.2% are dokosapentaenoat acid. Dietary administration of ω-3 PUFA in fish oil is better because EPA and DHA are absorbed more than the fish diet[5]. A decrease in plasma cholesterol levels can be improved by an increase in the acceleration of the turned over cholesterol or by its excretion. Some efforts to decrease the blood cholesterol levels are the use of polyunsaturated fatty acids (PUFAs) in the diet, which has been investigated very intensively[6]. The amount or levels of PUFA can lower cholesterol levels in a hypercholesterolemic state. The decrease in cholesterol levels caused PUFA to stimulate the excretion of cholesterol and bile acid formation. This is because the metabolism of ester cholesterol formed from polyunsaturated fatty acids by the liver and other tissues is more quickly. Therefore, the replacement (turn over) and cholesterol excretion is greater[2]. There have been many studies on the relationship between consumption of fish oil to reduce the number of deaths and the increasing heart disease. Several epidemiological studies, such as those performed among Eskimos, Japanese fishermen, the Dutch and Norwegians revealed that populations that consume relatively large amount of fish have low coronary heart disease incidence. Consumption of fish usually causes a decrease in VLDL, LDL and plasma triacylglycerol which is mainly due to a decrease in VLDL synthesis of liver and the VLDL degradation by the lipoprotein lipase enzyme[7-9]. Hence this study aimed to determine the effect of Tuna fish oil "Thunnus albacares" on the total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol in the blood levels of hypercholesterolemia rats "Rattus norvegicus".

MATERIALS AND METHODS

The laboratory experimental research used the separate sample pretest - posttest control group design. The tools were blender, electric heater, magnetic stirrer, analytical balance, reflux, evaporator, column chromatography, gas helium, glass laboratory equipment, a mouse cage equipped with a bottle for the provision of drinking water, a feeding tube number 8,5 cc of syringe needle, 26 gauge, centrifuges, incubators, and spectrophotometers. The materials were chloroform, magnesium sulfate anhydrous pa, Reagents MPR 1 no. 1442341, Reagents MPR 2 no. 1442350, Reagents MPR 3 no. 236 691, distilled water, Reagents precipitant MPR 2 no. 543 004. The supplementary Reagents for cholesterol screening with CHOD-PAP method were MPR 1, MPR 2, MPR, MPR Reagent 1 no. 726 290 containing PVS and accelerator, and Reagents I no. 701 912.

Tuna Fish Oil Extraction

The Tuna fish oil was obtained by refluxing the chest and abdominal cavity contents of tuna using chloroform and by evaporating the solvent. After that the levels of fatty acids EPA and DHA were further characterized and determined[12].

Effectiveness Test

The animals used as samples in this investigation were 40 male rats "Rattus norvegicus", aged 3 months, with the weights of 200 grams. The rats were acclimatized for 7 days under laboratory conditions and were given the standard feed for 8 weeks. Of the 40 rats, 5 rats were treated as a normal control group for the data base. 35 rats were subsequently given a high-cholesterol diet for 6 weeks to achieve a state of hypercholesterolemia. After that, 5 rats were used for hypercholesterolemia data. 30 rats that were made hypercholesterolemia were then divided into 3 groups, consisting of 10 rats. The first group was a posttest control group (P0) in which the rats were given a high-cholesterol diet until the completion of the treatment. The second group was the treatment group 1

Table 2: Weight Test Animal Rat After Treatment

Group Weight Loss Treatment		N	X (gram)	SD	P Value
P_0	Before treatment	10	248,00	14,18	0.000**
	After reament	10	284,80	12,43	
P_1	Before treatment	10	257,50	18,45	0,000**
	After treatment	10	277,10	13,57	
P_2	Before treatment	10	255,50	16,41	0.000**
	After treatment	10	283,00	14,55	

Note: ** Significant to the confidence level p <0.01

In this study, all the rats were turned into a state of hypercholesterolemia to provide some lard into the feed. This was done in order to investigate the effect of Tuna fish oil in decreasing the levels of the total cholesterol, the LDL cholesterol, and the blood triacylglycerol levels as well as increasing the HDL cholesterol levels. From the weight and measurement analysis, the weight gain of rats in the early treatment group was revealed. At the end of the treatment, the weight gain of the rats in each group was measured before and after administering the Tuna fish oil. Measurement results also showed that there was no significant increase in the weight gain of the tested animals after administering the Tuna fish oil in the experimental group as compared to the P1, P2 and P0 groups (high-fat diet without giving Tuna Fish Oil). Measurement of body weight of rats could detect differences in calorie intake to determine whether there were differences in weight gain between the groups of rats. Based on the weight measurement results, it can be concluded that the rats' body weight would increase at each step of the research. However, the results of weight measurement at the end of the study revealed no significant difference in weight gain between the groups. Besides, there was no difference in the calorie intake in each group and that Tuna fish oil supplementation did not really affect the appetite of the rats. To determine the achievement of hypercholesterolemia condition, the levels of total cholesterol, the LDL cholesterol, the HDL cholesterol and triacylglycerol were measured after the administration of a high-fat diet by adding some lard in a high-fat diet, in accordance with, ITB formula for 6 weeks[13]. The statistical calculations results showed a significant difference in terms of the total cholesterol, the LDL cholesterol, and the levels of triacylglycerol between the groups of rats before and after the administration of high-fat diet. Nevertheless, there was no significant difference between HDL cholesterol levels before and after the administration of a high-fat diet. These results are consistent with studies that have been done before that; that is, the food formula given might raise the levels of the total cholesterol, the LDL cholesterol and triacylglycerol levels and might lower (P1) whereby the rats were given a high-cholesterol diet + 1 dose (0.5 ml) of Tuna fish oil. The last group was the treatment group 2 (P2); the rats in this groups were given high-cholesterol diet with 2 doses (1 ml) of Tuna fish oil. The treatment was given for 4 weeks using a feeding tube. The animals tested were given high-cholesterol diet and drinking water. The total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol levels were determined by CHOD-PAP, PVS, CHOD-PAP and GPO-PAP methods and were measured using a spectrophotometer at 500 nm wavelength. The weighing and the measurement of the total cholesterol of the tested animals, their LDL cholesterol, HDL cholesterol and triacylglycerol levels were done three times; that is, before the rats were fed a high-fat diet, after they achieved hypercholesterolemia condition or before the treatment, and after the administration of Tuna fish oil diet or after the treatment.

Table 3: Comparison of Weight Rats Test Animal Treatment Group With Control Group

Variable	Group	P Value
Initial Body Weight	P_0 vs P_1	0,207
	P_0 vs P_2	0,317
	P_1 vs P_2	0,788
Final Body Weight	P_0 vs P_1	0,215
	P_0 vs P_2	0,769
	P_1 vs P_2	0,339

RESULTS

The results of the weight gain analysis after the tested rats reached hypercholesterolemia condition (before treatment) can be seen in Table 1.

Furthermore, the results of the data analysis of the rat's body weight after the administration of Tuna fish oil (after treatment) can be seen in Table 2 and Table 3. Based on the analysis, there was no significant difference in the weight loss between the rats in P1 group and in the control group (P0), and between the second treatment group (P2) and the control group ($p > 0.05$). There is also insignificant difference in weight gain between groups P1 and P2 at the beginning and after the group treatments.

Laboratory examination

The obtained total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol levels after hypercholesterolemia condition (before treatment) can be seen in Table 4. The results showed that there are significant differences in terms of the total cholesterol, LDL cholesterol, and triacylglycerol levels between the groups of rats before and after the administration of a high-fat diet ($p < 0.05$). However, there were no significant differences between HDL cholesterol levels before and after the administration of a high-fat diet ($p > 0.05$). The results of the total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol levels after the administration of Tuna fish oil diet shown in Table 5 and Table 6.

DISCUSSION

HDL cholesterol levels in the blood of the rats. The effect of Tuna fish oil on the total blood cholesterol levels of rats could be revealed by comparing P0, P1 and P2 groups. The data obtained showed that the overall Tuna fish oil supplementation could reduce the total cholesterol levels in each treatment group. The results showed that in these groups, there was a very significant difference between group P0 (X = 86.30; SD = 7.56 mg / dl), group P1 (X = 67.50; SD = 3.95 mg / dl) and group P2 (X = 60.20 SD = 3.08 mg / dl). Diets rich in polyunsaturated fatty acids (PUFAs) would lower cholesterol levels, especially when saturated fatty acids (SAFA) were substituted with polyunsaturated fatty acids (PUFA).

In this study, polyunsaturated fatty acids (ω-3 PUFA), namely EPA and DHA, contained in the fish oil supplementation were given, and PUFAs were also administered to substitute the SAFA. Mechanisms of cholesterol-lowering diet containing ω-3 PUFAs were established because ω-3 PUFA might increase cholesterol

Table 4: Levels of total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol levels Hypercholesterolemia Rats Test Animal Group (before treatment)

Basic Group		N	X (mg/dl)	SD	P Value
Total Cholesterol	Before Hypercholestrol	5	63,40	2,07	0,000**
	After Hypercholesterol	5	83,00	4,18	
LDL Cholesterol	Before Hypercholestrol	5	21,60	2,70	0,006**
	After Hypercholesterol	5	29,00	3,54	
HDL Cholesterol	Before Hypercholestrol	5	40,00	7,45	0,654
	After Hypercholesterol	5	38,20	4,38	
Triacylglycerol	Before Hypercholestrol	5	64,80	10,11	0.018*
	After Hypercholesterol	5	84,00	10,30	

Note: * Significant to the confidence level p <0.05
** Significant to the confidence level p <0.01

Table 5: Levels of total cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol levels Rats Test Animal groups after treatment

Variable	Group Treatment	N	X (mg/dl)	SD
Total Cholestrol	P_0 (Control)	10	86,30	7,56
	P_1 (TFO 0,5 ml)	10	67,50	3,95
	P_2 (TFO 1 ml)	10	60,20	3,08
LDL Cholesterol	P_0 (Control)	10	29,20	3,49
	P_1 (TFO 0,5 ml)	10	22,30	4,08
	P_2 (TFO 1 ml)	10	14,10	2,69
HDL Cholesterol	P_0 (Control)	10	38,70	2,11
	P_1 (TFO 0,5 ml)	10	42,40	2,95
	P_2 (TFO 1 ml)	10	43,40	3,37
Triacylglycerol	P_0 (Control)	10	83,60	9,00
	P_1 (TFO 0,5 ml)	10	60,40	10,04
	P_2 (TFO 1 ml)	10	44,00	9,72

Note: TFO = Tuna Fish Oil

Table 6: Comparison of Total Cholesterol Levels, koleterol LDL, HDL cholesterol and triacylglycerol levels Treatment Group

Variable	Group	P Value
Total Cholesterol	P_0 vs P_1	0,000**
	P_0 vs P_2	0,000**
	P_1 vs P_2	0,029*
LDL Cholesterol	P_0 vs P_1	0,079*
	P_0 vs P_2	0,000**
	P_1 vs P_2	0,000**
HDL Cholesterol	P_0 vs P_1	0,035*
	P_0 vs P_2	0,003**
	P_1 vs P_2	0,818
Triacylglycerol	P_0 vs P_1	0,009**
	P_0 vs P_2	0,000**
	P_1 vs P_2	0,003**

Note: * Significant to the confidence level p <0.05
** Significant to the confidence level p <0.01

excretion in the stool. It could also change the composition of the fatty acids contained in lipoproteins. As a result, the fluidity of lipoproteins would increase, and would affect the activity of lipolytic enzymes. In addition, it could increase the speed of synthesis and VLDL catabolism[10]. The amount of maintained PUFA levels in the blood would lower cholesterol levels because PUFA stimulated the excretion of cholesterol into the intestine and stimulated the oxidation of cholesterol to bile acids. This was a result of the metabolism of ester cholesterol formed more quickly by PUFA in the liver and other tissues, leading to the replacement (turn over) and greater excretion of cholesterol. Other evidence suggests that the effects of the decline were caused by a shift in the distribution of cholesterol in the plasma to the network due to the increased speed of catabolism of LDL. The increased speed catabolism of LDL was due to the increasing number of LDL receptors by polyunsaturated fatty acids. In addition, endogenous cholesterol was formed from the conversion of acetyl CoA to mevalonate acid, which was then converted into squalene and finally formed cholesterol. Feedback inhibition of the formation of cholesterol actually happened in mevalonic acid formation step. Mevalonic was acid derived from two molecules of acetyl CoA, contained in fat metabolism pathways. The main controls were located on the HMG-CoA synthesis enzyme cholesterol which catalyzed the formation of mevalonate. The formation of mevalonic acid catalyzed by the HMG-CoA enzyme was the enzymatic reaction, influenced by food control. If the cholesterol was derived from food, the synthesis of cholesterol in the liver and intestines rose to meet the needs of the tissues and organs. Feedback barriers affect cholesterol synthesis by inhibiting the activity of HMG-CoA enzyme formed and rapidly increasing the inactivation of the enzyme. Fish oil containing ω-3, EPA and DHA, in addition to its effect on cholesterol synthesis and the increased degradation of

VLDL also had an effect on fatty acid synthesis. As a result, it tended to decrease blood cholesterol levels but also to lower blood levels of triacylglycerol[2,8]. Tuna fish oil effect on the levels of LDL cholesterol could be achieved by comparing the P0, P1 and P2 groups. The data obtained show that the Tuna fish oil supplementation could reduce levels of LDL cholesterol which could be revealed by comparing group P0 (X = 29.20 SD = 3.49 mg / dl) and P1 (X = 22.30 SD = 4.08 mg / dl) with P2 (X = 14.10 SD = 2.69 mg / dl). This led to a decrease in the rate of VLDL formation in the liver. Its estimated mechanism, which in addition to the unsaturated fatty acids eicosapentaenoic (20: 5 ω-3) and docosahexaenoic acid (22: 6 ω-3), had the ability to lower the cholesterol levels. Although how it worked was still unknown, some said that it linked to the speed the formation of VLDL and B apolipoprotein in the liver that is necessary for the synthesis of VLDL[11]. The most striking effect after the administration of dietary fish oil was a decrease in VLDL plasma. This was in addition to the increased VLDL degradation by the lipoprotein enzyme. Nevertheless, other opinions argued that the lipoprotein activity may be less important in regulating the levels of VLDL plasma. This decrease was due to a decrease in VLDL synthesis of the liver following then administration of fish oil diet[8,9]. A significant difference was obtained between P0 and P1 groups, which might be due to the ineffectiveness of a 0.5 ml dose of Tuna fish oil to lower LDL holesterol. Tuna Fish Oil effect on the HDL cholesterol levels could be obtained by comparing the P0, P1 and P2 groups. In this study, it showed that there was a significant difference between group P0 (X = 38.70 SD = 2.11 mg / dl) and P1 (X = 42.40 SD = 2.95 mg / dl) and a highly significant difference between P0 group and P2 (X = 43.40 SD = 3.37 mg / dl). HDL was synthesized and secreted by the liver and intestine, and the main function of HDL was to act as a repository for A and E apoprotein, which were needed in the metabolism of chylomicrons and VLDL[2]. Thus because the omega-3 PUFA increased the degradation of VLDL, the A and C apoprotein also increased. Consequently, HDL, which were synthesized and secreted into the plasma, also increased. Furthermore, HDL cholesterol levels between P1 and P2 groups showed a significant difference, probably due to differences in doses given, which had the same ability to increase the levels of HDL cholesterol.Tuna fish oil effect on the blood levels of triacylglycerol in rats could be revealed by comparing the P0, P1 and P2 groups. The data obtained indicated that supplementation with Tuna fish oil in rats could decrease very significantly the blood triacylglycerol levels between P0 group (X = 83.60 SD = 9.00 mg / dl) with P1 (X = 60.40 SD = 10.04 mg / dl) and P2 group (X = 44.00 SD = 9.72 mg / dl), as well as between P1 and P2. From the previous research, it was suggested that the triasilgliserol level among Eskimos who consumed fish was decreased compared to the population of Eskimos who had left the habit of consuming fish[11]. This was because the fish containing ω-3 PUFA might inhibit the synthesis of triacylglycerol in the heart due to the inhibition of the formation of the acetyl CoA carboxylase enzyme, an enzyme controlling circuit lipogenesis. As a result triacylglycerol was not formed by the reaction of free fatty acids with glycerol. Triacylglycerol was synthesized in the liver from fatty acid metabolism of fat and together with VLDL Apo B lipoproteins, it was excreted into the blood circulation. The same results from studies conducted in humans showed that the rising levels of triacylglycerol in serum, after being given foods containing saturated fatty acids, decreased dramatically due to the fish oil containing EPA and DHA. However, this was not the case when the award was dietary PUFA from plants. Another test with the provision of oil that was not derived from fish did not show effects which could lower the levels of triacylglycerol serum[8].

CONCLUSION

Based on the results of this study, it was concluded that the Tuna fish oil supplementation can reduce the total cholesterol, the LDL cholesterol and the triacylglycerol levels significantly with $p<0.05$, and can also significantly increase HDL cholesterol levels ($p <0.05$)

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