Histologic response of aortic *Rattus norvegicus* male strain wistar hyperlipidemia after giving kersen fruits juice and extract lakum leaves

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Abstract. People lifestyle who tend to consume high fat diet is one of the risk factors causing cardiovascular diseases such as atherosclerosis and coronary heart disease. The aims of this study were to analyze the effect of kersen fruits juice and extract lakum leaves on lumen width and thickness of aortic wall and to find out the histological features of male *Rattus norvegicus* white rats due to hyperlipemia. The study used a Completely Randomized Design (CRD) consisting of 5 treatments with 4 replications, i.e. the control group (P0), the standard feeding group (P1), the hyperlipid feed group+0.2 mL/200gBB kersen juice (P2), the hyperlipid feed group+40mg / 200gBB extract lakum leaves (P3) and hyperlipid feed group+0.18mg / 200gBB simvastatin (P4). Hyperlipid feeds were given for 28 days. Data were analyzed by ANOVA followed by Duncan Test at a confidence level of 95, while histological features were observed directly using photomicrograph with 400x magnification and were seen aortic lumen narrowing, foam cells and intracellular lipid accumulation in smooth muscle. The results showed that the addition of kersen fruits juice and extract lakum leaves gave a significant effect on lumen width and thickness of the aortic wall (p <0.05). Histological features show the presence of foam cells and accumulation of smooth muscle lipids in the control and group treatments with hyperlipemia feeding. The conclusion of this study is that the addition of kersen juice fruits and extract lakum leaves can reduce atherosclerotic lesions by eliminating foam cells and proliferating smooth muscle cells so the size of the aortic lumen width returns to normal.

1. Introduction

Alteration in eating patterns and lack of physical activity in modern lifestyles can lead to energy imbalances and can cause hyperlipidemia. Hyperlipidemia is a disease caused by impaired fat metabolism, characterized by increased levels of lipids in the blood, and one of major risk factor for cardiovascular disease. Based on WHO data in 2008, 17.3 million people worldwide died caused by cardiovascular disease and are estimated to increase to 23.6 million in 2030 [1]. Based on these data, efforts are needed to reduce cardiovascular disease caused by hyperlipidemia.

Cardiovascular disease begins with endothelial damage. Damage to the endothelial layer through the regulation of the formation of hyperlipidemia is initiated by the presentation of free radicals against LDL. Oxidative stress causes a decrease in endothelial dysfunction and excessive production of Reactive Oxygen Species (ROS) will oxidize extracellular LDL. This LDL oxidation will produce proatherosclerosisproinflammation particles which is the development of atherosclerosis. LDL oxidation will be taken up by macrophages which are passed down through scavenger receptors. LDL oxidation...
will die because of phagocytosis by macrophages, accumulates to form foam cells. This foam cells which will form aggregate and over time will form a layer of fat, then the fat layer thickens the aortic wall and will be clearly seen in the aortic histological appearance by displaying abnormal widening of the aortic wall [2]. Research by Busia et al. [3], states that Wistar rats fed a high-fat diet for 4 weeks visible foam cells in the intima tunic layer and tunica media. Furthermore, atherosclerosis can also cause hypertension which causes an increase in muscle work that results in cardiomegaly.

Lakum leaves (Cayratia trifolia L.) and kersen fruits (Muntingia calabura L.) contain flavonoid compounds, saponins, tannins, squalene and alkaloids [4]. These substances are rich antioxidants. Free radical transport is the main mechanism of antioxidants in lipid peroxidation chain reactions. If the active ingredients that act as antioxidants can reduce ROS effectively, it can prevent the occurrence of LDL oxidation in the endothelium, so that this mechanism is effective in reducing atherosclerotic lesions. This study aims to look at the effect of administration of kersenfruits juice and lakumleaves extractwidth of aortic lumen and aortic wall thickness and to know the histological picture of male Rattus norvegicus white rat due to hyperlipidemia.

2. Material and methods
This research conducted at the Laboratory of Structure and Function of Animals, Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang. The stages of making aortic histology preparations carried out at the Pathology Laboratory of the Faculty of Veterinary Medicine, Gajah Mada University, Yogyakarta.

2.1. Tools and materials
The tools used in this study were: plastic mouse cages with 30 pieces of wire caps, drinking water bottles and 30 pieces of plastic feed containers, gastric sonde, ovens, filter paper, mortar and pestle, digital scales, thermometers diagnostic system kits (diasys), 3 ml syringes, centrifuges, micropipets, diagnostic system kits, cotton, surgical instruments, paraffin tanks, tapes, freezers, microtomes, waterbaths, measuring cups, Olympus microscopes, dropper drops, spectrophotometers, cholesterol measuring devices Cobalt series, masks and gloves.

The materials used in this study were 30 male white rats (Rattus norvegicus) Wistar strain, kersenfruits (Muntingia calabura L.), lakum leaves (Cayratia trifolia L.), simvastatin, ethanol solvent, aquades, chloroform, physiological saline (NaCl), 10% BNF solution, hematoxylin and eosin dyes, paraffin, 96% alcohol, xylol, absolute alcohol, rat blood serum, rat heart organ, blood lipid profile examination material enzymatic methods, commercial type pellet feed complete grain 594, high-fructose hyperlipidemia feed, and dry rice husk.

2.2. Procedures
Rat cages were prepared as many as 30 pieces with materials made of plastic with a length of 35.5 cm, width of 30 cm, and height of 12.5 cm accompanied by a wire cover. Each cage is given a plastic feed container and drinking water that is hung upside down. The experimental animals used in this study were 30 male Wistar strain white rats, aged 3 months and weighing ± 200 g. The mice were placed in 30 cages to be acclimated for one week. Rats were kept at cage temperatures 28-34°C.

Lakum leaves were taken from the semi-old leaves (the third to fifth leaves of the shoot) and then cleaned. The clean lakum leaves are dried in a 40°C oven to obtain a 10% moisture content. The dried sample then soaked in 96% ethanol solvent for 2 x 24 hours. The maceration results are then filtered, the filtering results in the form of filtrate are then evaporated using a rotary evaporator at a temperature of 80 C. The extract obtained is in the form of a paste. The making of a stock solution of lakum leaves extract was carried out by dissolving 10g of the lakum leaves extract with 50ml of distilled water. For each treatment of a lakum leaves extract, 0.2 ml is taken from the stock solution.

Kersenfruits used in this study is ripe fruits, with a red characteristic, and there are no defects or scratches on the fruits. Kersenfruits that has been cleaned crushed using mortar and pestle. Grinding results containing kersen juice are then filtered using filter paper. Making the stock solution of kersen
juice is done by grinding the cherries and taking the juice. Kersen juice that has been filtered then stored in the freezer and adjusted the temperature when it will be used.

Making simvastatin stock solution is done by mashing simvastatin, then weighing as much as 45mg and dissolving in 50ml of distilled water. For each simvastatin treatment, 0.2 ml of the stock solution is taken. Stock solution is made once during the treatment and stored in the refrigerator.

Before starting the treatment stage, checking the status of hyperlipidemia must be done by checking the blood lipid profile. Checking the lipid profile uses the CHOD-PAP method with the Diagnostic system kit (Daisy’s). Blood is drawn from the tip of the tail, the first drops are removed and the next drops are dropped into a diasys device, then the blood lipid profile will appear on the screen.

Nonhyperlipidemia feeding was carried out in the negative control group (P0), as much as 15g of feed was provided. Feeding hyperlipidemia as much as 15g per day was carried out in the treatment group (P1-P4) of 24 animals for 28 days. Every 2 times counted on the 4th day and 7th the remaining remaining feed was counted for the calculation of rat feed consumption. The first treatment was given standard feed without given kersen juice and leaves extract (P0). The next treatment, namely the provision of HFD (High Feed Diet) which has been induced by triglycerides to the training groups (P1, P2, P3, P4) for 28 days. In P1, rats were only given HFD without any treatment, P2 were given HFD which provided kersenfruits extracts of 0.2 ml / 200gBB, P3 was mice that were given HFD protection of 0.2 ml / 200gBB lakum extractand P4 rats thosewere given HFD and simvastatin 0.18ml / 200gBB.

Rats were put into a closed container and anesthetized using cotton that had been moistened with chloroform until fainting. The rats then dissected until reached the heart. The next step was taking rat blood using 3 ml syringe. The position of the needle forms an angle of 45oC to the rat. When the needle is positioned correctly, blood is drawn slowly. The blood sample is then allowed to stand for 30 minutes then centrifuged at 1500 rpm for 5-10 minutes to get blood serum. The serum is separated from blood cells and inserted into a sample cup using a micropipette for subsequent testing of the blood lipid profile through the CHOD-PAP method using a diagnostic system kit (Daisy’s). After that, the heart organ is removed, washed with physiological NaCl solution then weighed. The heart organ along with other organs are soaked in a bottle containing 10% BNF to be made in the preparation of heart preparations in the Pathology Laboratory of the Faculty of Veterinary Medicine UGM.

Measuring lumen width and aortic wall thickness of rats were observed through photomicrograph with 100x magnification,then calculations were performed using the DP2-BSW application on photomicrograph. Observation of atherosclerotic lesions is done by observing the presence or absence of foam cells, accumulation of smooth muscle cells, macrophages or thrombus in the intima tunica layer or tunica media at 400x magnification. The experimental design used in this study was a completely randomized design (CRD) with 5 types of treatment, each treatment consisting of 6 replications. The data obtained were analyzed with normal distribution patterns and homogeneity of the data. Differences between groups are known by conducting ANOVA (Analysis of Variance). If there are significant differences between groups, followed by Duncan's test with a significance level of 5% [5].

3. Result and discussion

The results of this study in the form of aortic lumen width and aortic wall thickness can be seen in Table 1. The analysis results of the effect of graining kersen fruits juice and lakum leaves extract of the aortic lumen width and aortic wall thickness showed significantly different results (p <0.05). This shows that administration of kersen juice (Muntingiacalabaura) and ethanol extracts of lakum leaves (Cayratia trifolia) for 4 weeks can reduce atherosclerotic lesions by removing foam cells and preventing smooth muscle cells from proliferating so the size of the lumen width and aortic wall thickness return to normal.
Table 1. Average of aortic lumen width and aortic wall thickness of rats after administration of lakum leaves extract and kersen fruits juice for 28 days

| Parameter                     | Treatment          |
|------------------------------|--------------------|
|                              | P0                |
|                              | $\overline{X} \pm SD$ |
| Aortic Lumen Width (μm)      | 259.54b ± 33.59    |
| Aortic Wall Thickness (μm)   | 85.12b ± 17.39    |
|                              | P1                |
|                              | 124.41a ± 32.34    |
|                              | 89.79b ± 20.46    |
|                              | P2                |
|                              | 211.84ab ± 83.89   |
|                              | 50.79a ± 14.96    |
|                              | P3                |
|                              | 253.22b ± 75.06    |
|                              | 48.54a ± 12.69    |
|                              | P4                |
|                              | 179.732b ± 53.67   |
|                              | 45.82a ± 24.2    |

Based on the results of measurement and analysis test with ANOVA the effect of granting kersen fruits juice (*Muntingia calabura* L.) and lakum leaves extract (*Cayratia trifolia* L.) there are differences in the size of lumen width and aortic wall thickness between treatments. The administration of 0.2 mL / 200 gBB / day of kersen fruits juice solution, 40 mg / 200 gBB / day of lakum leaves extract and 0.18 mg / 200 gBB / day of simvastatin given for 28 days had a significant effect on decreasing lumen width and aortic wall thickness mice induced hyperlipidemia. Narrow aortic lumen width caused by hypercholesterolemia. In this condition, the amount of excess LDL in the blood vessels and the amount of HDL decreases. Excess LDL in the blood vessels will cause endothelial dysfunction. Increased endothelial permeability causes LDL to easily enter the tunica media and intima tunica layers.

The average value of the aortic lumen width of P0, P2, P3 and P4 groups is wider than group P1. The thickness of the aortic wall is opposite to the width of the aortic lumen where the treatment group P1 shows the aortic wall that is thicker than P0, P2, P3 and P4. According to Handayani and Prijadi[6], oxidized LDL will stimulate the formation of substances that can attach and attract monocytes through the endothelial layer and intima. Oxidation is one of the changes that occur in LDL in the intima. This can occur as an action of reactive oxygen substances and prooxidant enzymes derived from activated endothelium or smooth muscle cells, or it can also be from macrophages that penetrate blood vessel walls. Perfectly oxidized LDL can convert macrophages into foam cells. Foam cells will form IGF-1 which is a type of growth factor that will trigger the proliferation of smooth muscle cells from tunica media to tunica intima. Foam cells and smooth muscle migration will be interconnected and form clots that get bigger which results in narrowing of the lumen of blood vessels. After that, the foam cell will die and remove the lipid component, this lipid component is the beginning of atherosclerosis.

Results of Duncan test showed no significant difference in both the aortic lumen width and aortic wall thickness in the treatment P2 with P3, P2 with P4, and P3 with P4. This shows that kersen fruits juice, lakum leaves extract and simvastatin have almost the same effectiveness in decreasing atherosclerotic lesions in the aorta which is thought to be due to the performance of antioxidant substances. The group of rats with the administration of kersen fruit juice, lakum leaves extract and simvastatin showed lower wall thickness and heart aortic endothelium compared with wall thickening in the group of rats given hyperlipid feed.

The observation of aortic lumen width preparation of 100x magnification with Hematoxylin-Eosin (HE) staining can be seen through the following pictures and descriptions:
Figure 1. Histological features of aortic lumen width. A: control group (P0), B: hyperlipidemic feeding group without treatment (P1), C: hyperlipidemia feeding group + kersenfruits juice (P2), D: hyperlipidemia feeding group + lakum leaves extract (P3), E: group feeding hyperlipidemia + simvastatin (P4). Narrowing of the aortic lumen in groups P0 and P1. Note: (a) plaque (b) rupture (c) thrombus

The observation results of the aortic wall preparations of tunica media 400x magnification with Hematoxylin-Eosin (HE) staining can be seen through the pictures and descriptions below:

Figure 2. Histological features of the aortic wall thickness. A: control group (P0), B: hyperlipidemic feeding group without treatment (P1), C: hyperlipidemia feeding group + kersenfruits extract (P2), D: hyperlipidemia feeding group + lakum leaves extract (P3), E: hyperlipidemia feeding group + simvastatin (P4). Narrowing of the aortic lumen in groups P0 and P1. Note: (a) foam cell
Based on Figure 1A and 1B it can be seen that the aortic lumen in the P0 and P1 groups have narrow lumen while in P2, P3, and P4 there is no narrowing of the lumen. The results in Figure 1 part B which is a histological feature of P1 aorta even show the rupture of blood vessels and produce thrombus. The aorta in P0 and P1 are thickens the aortic wall due to proliferation of smooth muscle cells and the presence of foam cells that produce atheroma plaque, showing the development of atherosclerosis, whereas in P2, P3 and P4 there is no atheroma plaque and thickening of the aortic wall. This result shows that those aorta don’t have atherosclerosis.

Excessive fat consumption can cause hyperlipidemia with increased levels of LDL cholesterol. Hyperlipidemia is an increase in cholesterol or triglyceride levels, the cause of hyperlipidemia is the result of intake of high fat diet, saturated fat, and excessive calories. Hyperlipidemia can also interfere with endothelial function through increased formation of radical oxygen species that deactivate nitric oxide. Chemical changes in fat triggered by free radicals produced in macrophages or endothelial cells in arterial walls will result in oxidized LDL. LDL oxidized (LDL-ox) then captured by macrophages through the scavenger receptor continuously and macrophages turn into foam cells, also triggering migration of smooth muscle cells from the media into the intima and triggering the proliferation of smooth muscle cells to the tunica intima. These foam cells then unite to form fatty streak (fatty spots) so it is considered a precursor of atheroma. According to Sukandar[7], narrowing of the aortic lumen width is caused by the proliferation of smooth muscle cells, extracellular matrix production, and lipid accumulation in the aortic wall, so the aortic wall will thicken and force the lumen.

In the formation of atherosclerotic lesions further as shown in Figure 1 part B (group P1), fat streak develop into intermediates and advanced lesions that form fibrous layer. This fibrous layer is a mixture of leukocytes, debris, foam cells and free lipids that form a necrotic nucleus, as well as the accumulation of calcium into fibrous plaque that causes hardening. If there is a platelet attachment to the edge of the atheroma, it can cause thrombosis. These atheroma fat patches can cause bleeding, ulceration, calcification, and myocardial infarction.

In the group of wistar rats with a high-fat diet with administration of kersenfruits juice at a dose of 0.2 ml / 200gBB for 28 days, based on Figure 2 part C, the histological picture of the aortic treatment of hyperlipid feed + kersenfruits juice shows the absence of foam cells in the intima and tunica media layers. This is because of the content of kersen fruits juice containing squalene, vitamin C and various antioxidants such as flavonoids, saponins, tannins and alkaloids so that it can provide a protective effect against hyperlipidemia. The treatment group on the administration of hyperlipid added with a lakumleaves extract (Figure 2 part D) showed no visible results of foam cells, macrophages and accumulation of smooth muscle lipids in the intima tunica and tunica media. According to Perumal et al. [8], lakumleaves extract contains alkaloids, tannins, flavonoids, saponins, phenols, and triterpens. Group of compounds that are thought to have potential as antioxidants in lakum methanol extract include flavonoids, phenolics, and triterpenoids. Phenolic compounds, flavonoids and triterpenoids in their structure contain hydroxyl groups that can donate their hydrogen atoms to free radicals, so phenolic, flavonoid, and triterpenoid compounds have the potential as antioxidants.

Based on Sudargo’s research [9], the ability of kersen juice to reduce total cholesterol is due to high levels of Vitamin C, where vitamin C is an antioxidant that can prevent LDL oxidation. Vitamin C works in reducing cholesterol with the activity of hydroxylace 7α which increases the reaction of cholesterol to bile acids by increasing the speed of cholesterol excretion, after that the excretion of cholesterol will reduce the amount of cholesterol in the blood. Vitamin C can also increase HDL in blood vessels where it can increase the removal of feces and reduce absorption of bile acids that can be converted into cholesterol. In addition, kersen fruits also contains phenols, saponins, tannins and flavonoids.

Flavonoids are reducing compounds that can inhibit many oxidation reactions. According to Puspasari et al. [10], flavonoids work by reducing levels of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which in turn has the effect of reducing cholesterol levels in the body. When cholesterol is transported from the intestine to the liver, HMG-CoA reductase which is responsible for converting acetyl-CoA to mevalonate in cholesterol synthesis will be inhibited so the cholesterol synthesis product by the liver will decrease. Flavonoids also increase the activity of Lecithin Acyl Transferase (LCAT)
which can reduce free cholesterol levels in the blood. Saponins can inhibit the amount of triglycerides in the blood by inhibiting their absorption in the intestine. Saponin inhibits the absorption of cholesterol in the intestine, causing cholesterol to be absorbed which is finally released along with feces. Saponins bind to bile acids and increase the excretion of bile acids in the stool which causes the conversion of cholesterol to bile acids to greatly increase the effort to maintain the bile acid depot. Consequently, LDL receptors from the liver will be raised so that an increase in LDL uptake will be accompanied by a decrease in plasma cholesterol levels. According to Adiputro et al. [11], flavonoids as antioxidants are ROS scavenger which will inhibit the oxidation reaction of LDL. Tannins inhibit the absorption of fat in the intestine by reacting with mucosal proteins and intestinal epithelial cells. In addition, tannins can precipitate mucosal proteins on the surface of the small intestine thereby reducing the effectiveness of cholesterol and fat absorption. Protein and amino acids contained in the feed may be precipitated by tannins so that the absorption of fat from the feed is disrupted.

In the treatment group giving hyperlipidemia added simvastatin dose 0.18 mg / 200 kg body weight, still found a little foam cells in the tunica media, but not as much as in the P1 group. According to Suyatna [12], simvastatin is an effective drug for lowering cholesterol levels which works by inhibiting the enzyme HMG-CoA reductase which functions as a catalyst in the formation of cholesterol. Inhibited HMG-CoA reductase will cause a decrease in cholesterol synthesis and an increase in the number of LDL receptors present in the liver cell membrane so that as a result cholesterol levels in the blood decrease. The presence of carbonyl groups in the structure of simvastatin that can bind to electrons free from reactive radicals is strongly suspected to be related to antioxidant activity.

These active substances were classified as antioxidants. Free radical transport is the main mechanism of antioxidants in lipid peroxidation chain reactions. If the active ingredients that act as antioxidants can reduce ROS effectively, it can prevent the occurrence of LDL oxidation in endothelium, so this mechanism is effective in reducing atherosclerotic lesions [13].

4. Conclusion
Based on the research that has been done, it can be concluded that administration of kersenfruits juice (Muntingia calabura L.) and ethanol extract of lakum leaves (Cayratia trifolia L.) for 4 weeks can reduce atherosclerotic lesions by removing foam cells and preventing proliferation of smooth muscle cells so that the width of the lumen is wide and the aortic wall thickness returns to normal.

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