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The effect of avocado seed extract (Persea americana Mill.) on MDA levels and the number of foam cells in atherosclerotic rats

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ABSTRACT

Hypercholesterolemia increases the risk of atherosclerosis (ATH). Avocado seeds (*Persea americana Mill.*) are proven to have antihypercholesterol and antioxidant activities. The effect of these two activities on malondialdehyde (MDA) and foam cell formation in the ATH stage is unknown. This study aims to determine the effect of avocado seed extract (ASE) on malondialdehyde and coronary artery foam cells in male Wistar rats ATH models. This experimental laboratory research applied pretest posttest with control groups design that distributed 24 male Wistar rats into 4 groups. The KN group is a healthy rats; the KP group was given an atherogenic diet for 14 days; group P1 was given an atherogenic diet for 14 days, continued giving ASE 250 mg/kg weight/day for 14 days; and P2 groups were given an atherogenic diet together with ASE 250 mg/kg weight/day for 14 days. MDA levels were measured by the TBARS method and foam cells were observed under a microscope (400x). The data were analysed by using SPSS. Wilcoxon test for MDA levels ($p>0.05$), One Way-ANOVA and Bonferroni post-hoc test for foam cell ($p=0.001$) showed KN (mean$\pm$SD;15.67$\pm$12.97) significantly different against KP (102.50$\pm$32.66); P1 (67.33$\pm$17.50); and P2 (54.17$\pm$8.47; $p=0.001$; $p=0.001$; and $p=0.020$), KP was significantly different from P1 and P2 ($p=0.039$ and $p=0.003$), while P1 was not significantly different from P2. Giving ASE has no effect on MDA levels, but ASE has a protective and curative effect on the formation of foam cells.

Keywords: Persea americana Mill., Atherosclerosis, Malondialdehyde, Foam cells.

INTRODUCTION

The epidemiological transition of disease in Indonesia has led to an increase in the prevalence of non-communicable diseases [1]. Heart and blood vessel disease are ranked first in contributing to the mortality rate in Indonesia [2]. ATH causes heart disease and stroke. The ATH process in the early stages is characterized by early abnormalities in endothelial cells, formation of foam cells and fatty streaks, formation of fibrous cap, to the process of plaque rupture [3]. ATH control is done by efforts to reduce levels of good cholesterol with pharmacological therapy which generally uses statin drugs [4] and non-pharmacologically with herbal therapy. One of the herbal ingredients that began to

be developed is avocado seeds (*Persea americana Mill*.). Control of ATH is expected to increase community productivity, reduce the burden of health costs, and even reduce the risk of heart disease and stroke.

Based on *in vivo* research, ASE has been shown to significantly reduce total cholesterol in hypercholesterolemia mice [5]. Besides the role of ASE as an antihypercholesterol agent, several other studies have shown that ASE has the benefits of ASE as an anti-inflammatory, analgesic [6] and antidiabetic [7]. This is due to ASE having high antioxidant activity, which reaches 93% [8].

Oxidative reactions in the body are able to produce Reactive Oxygen Species (ROS), which if the body's defense mechanism is not able to reduce the free radical, oxidative stress will occur. Oxidative stress can cause lysis of cell membranes or body tissues that contain lipids so that cell damage occurs [9]. Modification of LDL by free radicals forming oxidized LDL (Ox-LDL) produces foam cells that can cause thickening of the arteries and lipid peroxidation produces MDA compounds [10]. Toxic effects due to ROS can be suppressed by enzymatic antioxidant activity, such as Superokside Dismutase (SOD), Glutathione peroxidase (GSH-Px) and Catalase (CAT) [11]. Non-enzymatic antioxidants, such as vitamins, minerals, and phenol compounds are also needed as free radical electron donors and increase enzymatic antioxidant activity [12].

Based on phytochemical tests avocado seeds are known to contain compounds, such as saponins, flavonoids, and tannins. Besides avocado seeds are proven to contain fiber and various minerals that are beneficial to health [13]. The total phenol content in avocado seeds is higher in round green varieties than other varieties, reaching 73.11% [14]. This study aims to look at the effect of giving ASE to changes on MDA levels and the number of foam cells in male wistar rats induced atherogenic diets. ASE is given in 2 ways. The P1 group was given after induction of an atherogenic diet to see the curative effect while in the P2 group it was given together with an atherogenic diet to see the protective effect.

METHODS

This research is a true experimental study with pretest posttest with control group design. Samples were selected using simple random sampling. Twenty-four male Wistar rats were selected based on inclusion criteria, i.e. 8-10 weeks old, body weight 150-200 gr with normal anatomy and activity. Adaptation was carried out for 7 days then the rats were randomized into 4 groups, namely 2 control groups and 2 treatment groups. The process of raising, intervening, and terminating the samples were carried out at the Laboratory of Animal Biology, Semarang State University.

This study consisted of 4 groups. The KN group is a healthy mouse; the KP group was given an atherogenic diet for 14 days; group P1 was given an atherogenic diet for 14 days, continued giving ASE 250 mg/kg weight/day for 14 days; and P2 groups were given an atherogenic diet together with ASE 250 mg/kg weight/day for 14 days. All groups were given standard BR-2 Confeed feed and drinking *ad libitum*. Induction of an atherogenic diet in the form of initial injection of adrenaline of 0.006 mg intravenously to a lateral vein in the tail on the first day followed by administration of 5 mg duck egg yolk for 14 days. Avocado seeds used are local products from Bandungan Subdistrict, Semarang Regency with round green varieties. Making ASE using maceration method with 80% ethanol solution then extract is given 1(one) time a day using gastric sonde. Before taking blood, rats fasted for 8-10 hours. MDA levels were examined before and after the intervention using 2 cc of blood taken from the eye's retro-orbital plexus. MDA levels were measured using the TBARS method with a spectrophotometer at a wavelength of 545 nm. At the end of the study, rats were given inhaled clhorofoam and then terminated to take their coronary arteries. Staining preparations with Hematoxylin Eerlich-Eosin (HE) and observing foam cells using a microscope (400x). The independent variable in this study was treatment of ASE. The dependent variable is the measurement of MDA levels and observation of the number of foam cells. The data obtained were analyzed using SPSS 20.

RESULTS

Analysis of malondialdehyde level

The results of the analysis of MDA levels in the studied group can be seen in Table 1.

Table 1. Effect of avocado seed extract on malondialdehyde level

	Malondialdehyde level (Mean±SD) nmol/ml				P value
	KN	KP	P1	P2	
Pretest	0.025±0.035	0.012±0.008	0.024±0.025	0.012±0.017	0.994**
Posttest	0.010±0.009	0.025±0.010	0.017±0.022	0.013±0.012	0.100**
Δ	-0.015±0.036	0.013±0.015	-0.007±0.022	0.001±0.022	0.272**
P value	0.600*	0.116*	0.686*	0.715*	

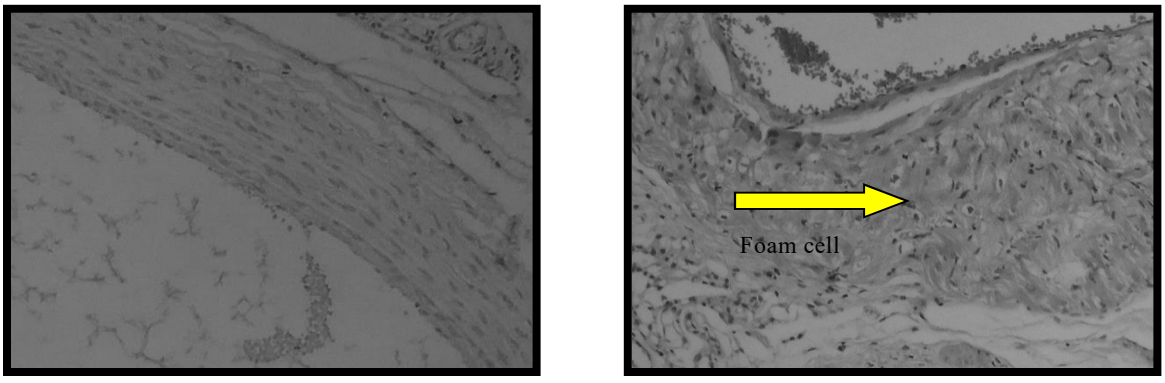
*Wilcoxon test, significant difference (p<0.05)

**Kruskal Wallis test, significant difference (p<0.05)

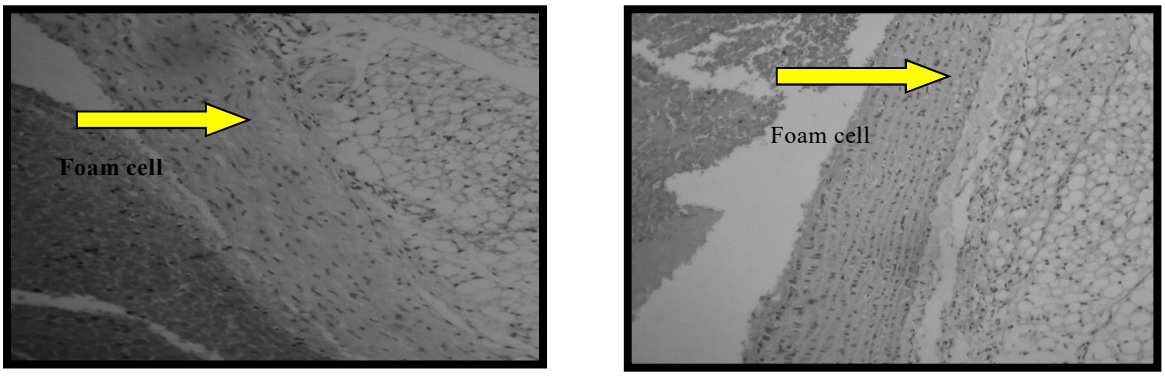
The results of the descriptive analysis of MDA levels obtained the lowest mean of MDA levels before the intervention is on the KP and P2 groups, while the highest is KN group. Meanwhile after the intervention, the lowest MDA level was found in the KN group and the highest is KP group. The KN group had the largest decrease in MDA levels, while the KP group had the largest increase in MDA levels. The normality test using Shapiro Wilk on MDA levels showed MDA levels were not normally distributed with p value < 0.05 in the KP group (p = 0.029), P1 (p = 0.041), and P2 (p = 0.014) so that the data analysis used nonparametric test. Abnormal data distribution on MDA levels was reported as outliers in the sample. The mean of MDA level before the intervention showed no significant difference between the treatment and the control groups (p = 0.994). After the administration of an atherogenic diet, there was an significant increase in MDA levels in the KP group (p = 0.116). P1 group given atherogenic diet for 14 days and then given ASE for 14 days showed a decrease in MDA levels. While P2 group who were given an atherogenic diet together with ASE for 14 days showed an increase in MDA levels, but the increase was less when compared to the KP group. Nevertheless, the results of the Kruskall Wallis test showed that there were no differences in the mean of MDA levels in the treatment and control groups (p = 0.272) (Table 1). The results of this study indicate that there is no effect of ASE on MDA levels.

Analysis of Foam Cell

The results of microscopic observations of foam cells can be seen in Fig.1.



(A) **(B)**



(C) **(D)**

Fig.1. Results of observation of artery coronary : (A) KN group (B) KP Group, endothelial injury and formation of foam cells are visible (C) P1 Group and (D) Group P2 there are foam cells but there are fewer (HE-microscope 400x)

The results of the analysis of the number of foam cells can be seen in Table.2.

Table 2. Effect of avocado seed extract on foam cell

Group	Average of Foam cell (Mean±SD)	P value		
		KP	P1	P2
KN	15.67±12.97	0.001*	0.001*	0.020*
KP	102.50±32.66	(-)	0.039*	0.003*
P1	67.33±17.50		(-)	(-)
P2	54.17±8.47			

*significant difference ($p<0.05$)

The results of the descriptive analysis of the number of foam cells, obtained the highest number of foam cells is in the KP group while the least is in the KN group. The normality test using Shapiro Wilk on the number of foam cells shows that the data is normally distributed in each group so that it is analyzed using parametric tests. The Levene test shows the data is homogeneous ($p = 0.065$). Different test on the number of foam cells between research groups using One-Way-ANOVA showed significant differences (p value = 0.001). In the post hoc Bonferroni test the results showed that there were differences in the number of foam cells in the control group and the treatment group, while the P1 group was not significantly different from the P2 group (Table.2). The results of this study indicate that there is an effect of ASE on the number of foam cells.

DISCUSSION

Oxidative reactions in the body can produce ROS that are reactive and easily react with biomolecules such as proteins, lipids, carbohydrates, and DNA [15]. So if endogenous antioxidants are not able to reduce excess ROS, it causes oxidative stress as the main cause of endothelial dysfunction which is the beginning of atherosclerosis. A high cholesterol diet also contributes to MDA levels because of the precursor compounds forming MDA, namely arachidonic acid, which is found in the phospholipid layer on the outer membrane of LDL [16]. Oxidative stress triggers lipid peroxidation to produce MDA compounds that are mutagenic

The results of this study indicate that giving atherogenic diets for 14 days causes an increase in MDA levels in the KP group. P1 group given ASE after atherogenic diet for 14 days showed a curative effect in the form of decreased MDA levels. While the P2 group that was given ASE together with the atherogenic diet showed a protective effect in the form of an increase in MDA levels which was less when compared to the KP group. Nevertheless, the results of this study showed no significant difference in MDA levels between the treatment and control

groups (Table 1). These results are in line with research conducted on patients who experience oxidative stress due to obesity [17].

This study uses ASE which is rich in flavonoids which can be a free radical electron donor and simultaneously increase HDL cholesterol which is able to fight LDL to prevent heart and blood vessel disease [5]. The content of vitamin E in the form of α tocopherol can react with tocopheroxyl radicals so that it can protect cell membranes from lipid peroxidation [18]. In addition, mineral content especially Zn and Cu in avocado seeds can increase catalase and SOD activity because it acts as an enzyme cofactor. [15, 19].

Toxic effects due to free radicals can be suppressed by the presence of endogenous (enzymatic) antioxidants, such as Superoxide Dismutase (SOD), Catalase, and Glutathione Peroxidase (GSH-Px). Endogenous antioxidants act as hydrogen atom donors which can quickly bind to radical compounds so that more stable compounds will be formed [20]. MDA levels are influenced by endogenous and exogenous antioxidant activity that comes from food. *In vivo* studies show there is a strong correlation between MDA levels and SOD [21]. But in this study the SOD activity was not examined.

Although in this study there was no significant reduction in MDA levels, but several other studies have proven that exogenous antioxidants such as flavonoids, saponins, C, and E can improve oxidative stress based on significant decreases in MDA levels [22, 23]. Another mechanism that is able to explain this is that the antioxidant activity of flavonoids depends on the amount of antioxidant concentration that is given. At high concentrations, the antioxidant activity of the phenolic group may disappear, even in fact it is prooxidant [15].

The development of atherosclerosis is inseparable from the concentration of total plasma cholesterol [9]. Induction of an atherogenic diet can trigger the accumulation of excess fat in tissues so that adipocytes produce proinflammatory cytokines such as TNF-α, IL-1, and IL-6 which are potent stimulators to produce oxygen and nitrogen which are reactive [24]. The formation of foam cells as an early stage of atherosclerosis was successfully induced: this explains why the mean number of KP cells in the KP group was significantly higher than in the normal control group (Table.2). An atherogenic diet has been shown to trigger atherosclerosis in mice. It is said that cholesterol levels, especially high LDL can increase the possibility of LDL oxidation by free radicals into Ox LDL which can trigger phagocytosis by macrophages to form foam cells [25].

The results of this study indicate that the number of foam cells in the treatment group is greater when compared to the normal control group, but the administration of ASE in both treatment groups can significantly reduce the number of foam cells. In the post hoc test, there were significant differences between the control group and the treatment group. Meanwhile, among groups P1 is not different from group P2. This shows that ASE can be supported by previous research which states that ASE can reduce total cholesterol and triglyceride levels, and significantly increase HDL [26].

ASE affects atherosclerosis through 3 main mechanisms, namely as free radical electron donors, inhibits cholesterol absorption and synthesis, and reduces the inflammatory response. The effect of decreasing the number of foam cells by ASE is related to the content of saponin and tannin compounds. Saponins can bind with bile acids and cholesterol to form micelles thus preventing the absorption of cholesterol in the intestine. This process is assisted by tannin compounds that can bind to proteins lining the intestinal wall [5]. Cholesterol reduction can not be separated from the role of flavonoid compounds, where flavonoids have the ability to inhibit the activity of the enzyme acyl-CoA cholesterol acyltransferase (ACAT) which plays a role in decreasing the esterification of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). Inhibition of the enzyme HMG-CoA reductase causes a decrease in total cholesterol levels and increases the formation of LDL receptors on the surface of hepatocyte cells resulting in an increase in LDL transport from blood vessels to the liver [27]. This causes the total cholesterol level in the blood to be reduced thereby reducing the chance of LDL cholesterol being oxidized to Ox-LDL. Not only phytochemical content, avocado seeds also contain high vitamin C as a free radical scavenger by reacting directly with superoxide and hydroxyl anions, and various lipid hydroperoxides. Vitamin B3 can reduce the production of VLDL in the liver causing a decrease in total cholesterol, LDL,

and triglycerides [28]. While vitamin E also in avocado seeds can reduce free radicals by inhibiting the formation of squalent 2.3 oxides by reacting with oxygen to form alpha tocopherquinone which is stable [29].

Giving ASE simultaneously and after giving an atherogenic diet is proven to reduce the number of foam cells. Future research is required to examine the effect of avocado seed extract on other biomarkers such as SOD, catalase, and Ox-LDL with larger sample sizes. Clinical trials of anti-atherogenic activity of avocado seed extract also need to be carried out.

CONCLUSION

Avocado seed extract 250 mg/kg weight/day for 14 days has no effect on MDA levels, but can provide a protective and curative effect on formation of foam cells.

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