THE MECHANISM OF ALPHA LIPOIC ACID ON REDUCING THE MDA LEVEL AND MCP-1 EXPRESSION IN ENDOTHELIAL DYSFUNCTION OF HYPERCHOLESTEROLEMIA RAT (*Rattus norvegicus*) MODEL

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ABSTRAK

Disfungsi endotel merupakan kondisi awal dari aterosklerosis dan penyakit pembuluh darah lainnya di mana salah satu faktor risiko hiperkolesterolemia. Kadar kolesterol darah dikaitkan dengan peningkatan produksi spesies oksigen reaktif (ROS). Peningkatan ROS produksi dapat menyebabkan peningkatan stres oksidatif yang pada gilirannya mengakibatkan disfungsi endotel. Asam alpha lipoic (ALA) adalah salah satu senyawa antioksidan yang telah dikembangkan dan dipelajari. Dalam penelitian ini kami menemukan bahwa penggunaan ALA di Rattus norvegicus tikus signifficantly menurunkan kadar kolesterol total pada dosis 60 mg/kgBB (p = 0.020). ALA juga menghambat ekspresi monosit chemoattractant protein-1 (MCP-1) pada dosis 60 mg/kgBB (p = 0,044) dan mengurangi pembentukan Malondialdehyde (MDA) pada dosis 120 mg/kgBB (p = 0,009), yang merupakan tahap awal pengembangan aterogenik dan prognosis kejadian, dengan demikian, ALA dapat mengurangi risiko kerusakan lebih lanjut pada endotel. (FMI 2016;52:154-159)

Kata kunci: *hiperkolesterolemia, disfungsi endotel, MDA, MCP-1, alpha lipoic acid*

ABSTRACT

Endothelial dysfunction is an initial condition of atherosclerosis and other vascular diseases where one of the risk factors is hypercholesterolemia. Blood cholesterol levels is associated with an increase in the production of reactive oxygen species (ROS). The increasing of ROS production can cause increased oxidative stress which in turn resulting in endothelial dysfunction. Alpha lipoic acid (ALA) is one of the antioxidant compound that has been developed and studied. In this study we found that the use of ALA in Rattus norvegicus rats signifficantly lower the total cholesterol levels at dose 60 mg/kgBW (p=0.020). ALA also inhibit the expression of Monocyte Chemoattractant Protein-1 (MCP-1) at dose 60 mg/kgBW (p=0.044) and reduces the formation of Malondialdehyde (MDA) at dose 120 mg/kgBW (p=0.009), which is the initial stage of the atherogenic development and prognosis of events, thus, ALA can reduce the risk of further damage to the endothelium. (FMI 2016;52:154-159)

Keywords: *hypercholesterolemia, endothelial dysfunction, MDA, MCP-1, alpha lipoic acid*

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INTRODUCTION

Cardiovascular disease is leading cause of death in worldwide. In 2012 as many as 17.5 million people died because of it, 7.4 million deaths are caused due to coronary heart disease and 6.7 million deaths due to stroke. The death rate is expected to continue to increase until 2030 and could reach 23.3 million people (WHO 2015). One risk factors for the development of cardio-vascular disease is hypercholesterolemia. In this condition, were also found an increase in levels of total cholesterol, low-density lipoprotein (LDL) and low levels of high-density lipoprotein (HDL). High levels of cholesterol in the blood associated with the increased production of reactive oxygen species (ROS) that play a role in many redox reactions due to the high reactivity.

ROS one of which consists of free radicals which are a group of atoms containing unpaired electrons section and the outer shell electron configuration is very unstable, hence the free radicals will rapidly react with other molecules in order to achieve a stable configur-ation. Levels of LDL and free radicals in hyphercholes-terolemic conditions can trigger oxidation reaction known as lipid peroxidation. Polyunsaturated fatty acids (PUFAs), particularly arachidonic acid and linoleic acid is the main target of LDL oxidation from free radicals. Presence of free radicals is too short to be measured directly, so the products are used in the measurement stability. Lipid peroxidation produces several products including lipid hydroperoxide (LOOH) as the main product and various aldehydes as a by product (second-ary product) such as malondialdehyde (MDA), which

also have important roles in the development and prognosis of atherogenic events (Yang et al 2008, Levitan et al 2010, Niki 2014).

High levels of ROS activates mitogen-activated pathway protein kinases (MAPK) and nuclear factor-kappa B (NF-B). NF-B is a transcription factor that plays an important role in the expression of molecules involved in the activation of endothelial cells. MAPK would degrade the I-B from p50-65 heterodimer, then translocated into the nucleus to NF-B is activated and generate proinflammatory factors such as IL-6, IL-8, VCAM-1, ICAM-1, ET-1, E-selectin and chemoattractant such as MCP-1 (Caterina et al 2007, Lee et al 2010). Monocyte chemoattractant protein-1 (MCP-1) is a group of chemokines that regulate the migration and infiltration of monocytes into the vessel wall and then differentiate into macrophages. This macrophage activation will take oxLDL through scavenger receptors and turn into foam cells. Furthermore, chemokines and other inflammatory cytokines are released to trigger the further recruitment of monocytes and causing endothelial damage (White et al 2010).

Increasing evidences proved that the use of antioxidants such as vitamins E and C showed a decreased level of ROS, NO enhancement, and improvement in endothelial function observed from vasorelaxation function. This indicates the administration of antioxidants can provide improvements in endothelial function and prevent the development of cardiovascular disease (Pratic 2005, Munzel et al 2010). One antioxidant that has been developed and studied for their effects on cardiovascular disease is alpha lipoic acid (ALA). This compound can provide antioxidant activity in the oxidized form of lipoic acid (LA), 1,2-dithiolane-3-pentanoic acid and the reduced form dihydrolipoic acid (DHLA) with EC50 values of 0.39 mol/l. Until now, the role of ALA as an antioxidant in overcoming the lipid peroxidation and its influence on chemokines, especially MCP-1 remains unclear. Through this research we may explain the role of ALA as an antioxidant in inhibiting endothelial dysfunction in animal models of hypercholesterolemia by evaluating the expression of monocyte chemoattractant protein-1 (MCP-1) and malondialdehyde (MDA).

MATERIALS AND METHODS

Materials used includes: alpha lipoic acid (PT.Simex Pharmaceutical Indonesia), cholesterol crystal (Sigma-Aldrich), polyclonal antibody rat MCP-1 bs-1101R (Bioss USA), histofine kit for immunohistochemistry (Biocare USA), propilenglikol, distilled water, formaldehyde 10%, trichloroacetic acid (TCA), sodium

hydroxide (NaOH), tiobarbiturat acid (TBA), HCl. Diets high in cholesterol with cholesterol composition of 200 mg/KgBW and I mL quail egg and 10% of coconut oil.

In the present study animals used were male Sprague-Dawley rats in the age of 8-12 weeks (with body mass about 150-250 grams) obtained from Laboratory of Animals, Faculty of Pharmacy, Airlangga University. The animals were housed in a controlled a room equipped with controlled lighting (12 hours of lighting and 12 hours of darkening) at 30 ± 1°C. All procedures were reviewed and approved by Animal Care and Use Committee (ACUC) at Airlangga University.

Rats were divided into five groups namely naive group, the hypercholesterolemia (HC) group and 3 groups of hypercholesterolemia and ALA (HC+ALA). Naive group was fed with standard diet while others were given high-cholesterol diet for 14 days. After 14 days of induction, the HC+ALA group received 3 doses of ALA at 30; 60 or 120mg/KgBW for 14 days while the HC group was given the vehicle (propilenglikol 50%). Blood cholesterol level were measured on day 0, 14 and 28. After 28 days of the study, blood from all of the groups were taken intracardiacaly to determine the level of MDA. Rats aortic tissue were also collected to determine the expression of MCP-1.

Determination of blood cholesterol levels

Blood samples were collected from the tail vein of rat for determination of blood cholesterol levels using Easy Touch® GCU (Blood Glucose/Cholesterol/Uric Acid Multi-Function Monitoring System).

Plasma MDA level assay

MDA standards was made by hydrolyzing 1,1,3,3-tetraethoxypropane (TEP) with acid (1 mol TEP equivalent to 1 mol MDA). TEP stock solution was made with 25.0 mL TEP diluted with distilled water to 100.0 mL (1 mM). MDA primary standard solution was prepared by pipette 1.0 mL of stock solution TEP then added 1N HCl to a volume of 50.0 mL (20 m). Working standard solution was prepared by diluting the standard solution into 5 concentration of MDA: 25; 10; 5; 1; and 0.5 µM. 0.5 mL of blood plasma was put into a test tube, 0.5 mL of trichloroacetic acid (TCA) 20% was added and homogenized. Then added 0.5 mL of 1% Na-TBA and 1N HCl solution to a volume of 10.0 mL were homogenized. The solution was incubated in a water bath at a temperature of 90-950C for 30 minutes. Rapid cooling in the centrifuge were perform later at a speed of 3000 rpm for 15 minutes. Colorless solution obtained was measured by spectrophotometer at a wavelength of 528 nm.

Immunohistochemistry and scoring MCP-1 expression

MCP-1 protein expression were evaluated by immuno-histochemistry analysis on rat's aortic tissue, using anti-human MCP-1 antibody. Examination were carried out at Pathology Anatomy Laboratories, Soetomo Teching Hospital's, and the Institute of Tropical Disease, Airlangga University.

Statistical data analysis

Significant difference of MDA levels mean value was determined by One-way ANOVA, while significant difference of blood cholesterol level mean value was determined by two-way ANOVA and significant difference of Allred Score System data expression of MCP-1 was determined by non-parametric Kruskal-Wallis and post hoc Mann-Whitney. Statistical significance was accepted at $p < 0.05$.

RESULT

ALA decreases blood cholesterol level in hypercholesterolemia rat model

Administration of ALA at 30, 60, or 120 mg/kgBW for 14 days reduced total blood cholesterol level in rats. By two way ANOVA test, we found that there were significant differences between the groups receiving high-cholesterol diet compared to the normal group ($p < 0.001$). Further test with post hoc test Tukey HSD showed that total cholesterol levels of HC group did not differ significantly compared to dose group at 30 mg/kg BW ($p = 0.248$), but significantly different compared to HC+ALA 60 group ($p = 0.020$) and HC+ ALA 120 group ($p = 0.045$). Furthermore, there was significant difference on blood cholesterol level of HC+ALA 30 group compared to HC+ALA 60 and HC+ALA 120 groups ($p < 0.001$) (Fig. 1).

Effect of ALA on plasma MDA level

Administration of ALA for 14 days reduced the plasma MDA level in induced HCD group ($p < 0.001$). There was no significant differences observed ($p = 0,266$) between the HC+ALA 30 and HC+ALA 60 groups, but HC+ALA 120 group were found to be significantly different ($p < 0.001$). While the HC+ALA 60 groups did not differ significantly compared with HC + ALA 120 ($p = 0.044$).
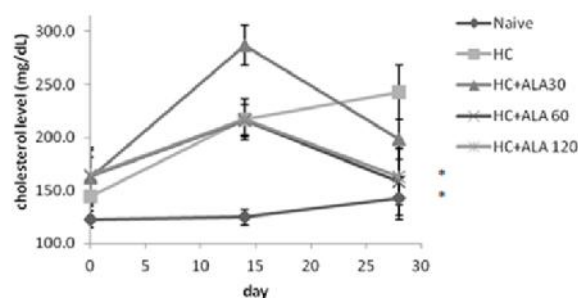

Fig 1. Effect of ALA on blood cholesterol level in rats. HCD induced every day for 28 days. ALA (30; 60; 120 mg/kg BW) administered by oral gavage for 14 days, 14 days after HCD induction. *$p < 0.001$ vs HC+ALA 30 group
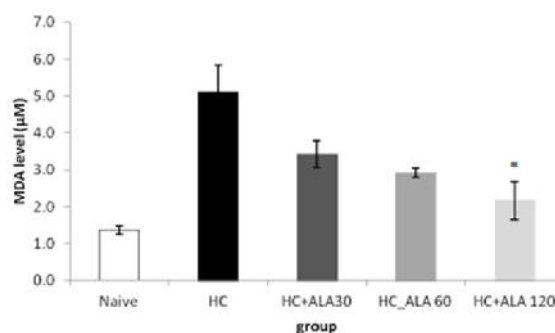

Fig 2. Effect of ALA on blood cholesterol level in rats. HCD induced for 28 days. ALA (30; 60; 120 mg/kg BW) administered by oral gavage for 14 days, 14 days after HCD induction. Plasma MDA level determined by spectrophotometry method at λ 528 nm. *$p < 0.001$ vs HC+ALA 30 group.

Effect of ALA on the expression of Protein MCP-1

The percentage of cells expressed and the color (brown) intensity caused by the interaction between MCP-1 protein on the surface of endothelial with polyclonal antibody rat MCP-1 (1: 200) were assessed to describe the activity of the protein. Fig 3 Shows the weakening expression of MCP-1 on the surface of endothelial characterized by the decreasing intensity of the brown color along with the increased dose of ALA. There was significant difference in HC group compared to the treatment groups at ALA 30 mg/KgBW ($p = 0.015$), at 60 mg/KgBW ($p = 0,002$), and at ALA 120mg/KgBW ($p = 0.002$).
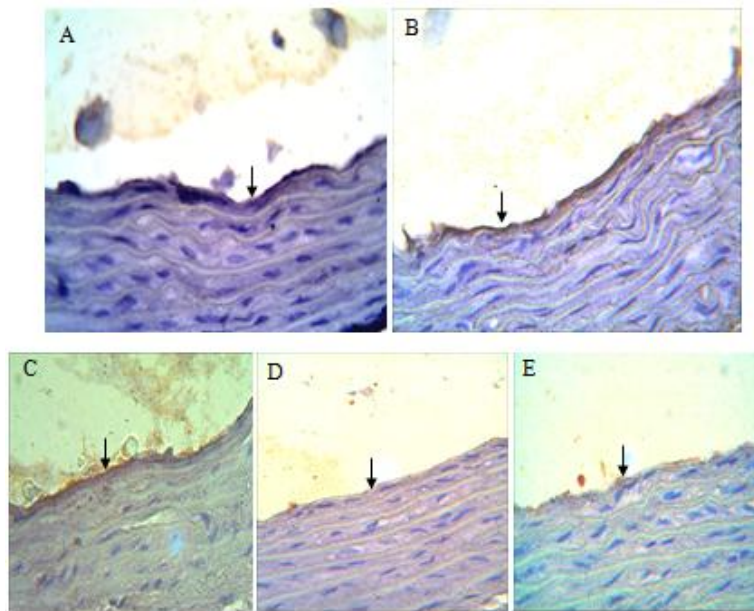
Fig 3. The Expression of MCP-1 in Endothelial Aortic tissue with imunohistochemical Staining, 400x; (A) naive group, (B) HC group, (C) HC+ALA 30 mg/kg BW group, (D) HC+ALA 60 mg/kg BW group, (E) HC+ALA 120 mg/kg BW group.

DISCUSSION

Using a rat model driven by diet induction toward hypercholesterolemia, results of this study expands the current knowledge base regarding the protective properties of ALA with several novel observations. ALA supplementation in high cholesterol fed animals reduced total cholesterol level to <200 mg/dL compared with the HC animals (p<0.001) (Fig 1). The reduction in blood total cholesterol in the ALA-supplemented animals has been reported in multiple previous pre-clinical studies. This hypocholesterolemic response was associated with a reduction in hepatic PCSK9, an essential regulator of hepatic LDL receptor turnover and serum LDL-C concentrations, and serum PCSK9 concentrations (Carrier et al 2014). ALA probably capable to initiate LDL receptor synthesis in the liver which in turn increase the uptake of cholesterol back to the hepatic system and increase synthesis of apoprotein A component for reversed cholesterol transport (Amom et al 2008, Zulkhairi et al 2008).

Analysis of plasma levels of MDA conducted in rat at the end of the study by spectrophotometric method and MDA measurements performed at a wavelength of 528 nm which is the wavelength of maximum absorbance. The HC group against the naive group differ significantly (p<0.001). This means there has been an increase in the production of MDA after administration of a diet high in cholesterol and indicates that has been an increase in oxidative stress. The higher the MDA levels indicate the level of oxidative stress were higher so the impact on the incidence of endothelial dysfunction is also greater. Increased oxidative stress is characterized by increased levels of MDA and this is in accordance with research conducted by Mohammadi et al (2006) which showed elevated levels of MDA in rabbits that were given high-cholesterol diet for 8 weeks. MDA was also seen increased in liver tissue of rat induced cholesterol diet for 30 days, indicating that hypercholes-terolemia can improve the process of lipid peroxidation (Wu et al 2012).

Alpha lipoic acid (ALA) as an antioxidant shown to prevent oxidative stress by lowering plasma MDA levels in rat that received a high cholesterol diet. Based on Fig. 2 seen any significant difference between the plasma MDA concentration in experimental group (p <0.001). The ability of ALA in lowering plasma MDA concentration is related to its ability as a scavenger of various types of ROS. ALA may scavenge hydroxyl radicals (OH.), hypochlorous acid and oxygen singlets. Thereduction form (DHLA) scavenges superoxide radicals (O2.-), peroksonitrit (ONOO.), hydroxyl radicals (OH.) and peroxyl radicals, thereby preventing the free radical-mediated peroxidation of proteins (Packer et al 1995, Gor caet al 2011).

Alpha lipoic acid (ALA) as an antioxidant shown to prevent oxidative stress by lowering plasma MDA

levels in rat that received a high cholesterol diet. Based on Fig. 2 there was significant difference between the plasma MDA concentration in all experimental group (p <0.001). The ability of ALA in lowering plasma MDA concentration is related to its ability as a scavenger of various types of ROS. ALA may scavenge hydroxyl radicals (OH.), hypochlorous acid and oxygen singlets. The reduction form (DHLA) scavenges superoxide radicals (O2.-), peroksonitrit (ONOO.), hydroxyl radicals (OH.) and peroxyl radicals, thereby preventing the free radical-mediated peroxidation of proteins (Packer et al 1995, Gor caet al 2011). ALA effective-ness in improving endothelial function is also supported by studies using different models. ALA can improve endothelial function by decreased levels of plasma MDA and aortic tissues in diabetes models (Sena et al 2008). On testing in vivo model of LPS-induced, ALA is also known to reduce levels of MDA and H2O2 in tissues of the spleen, brain and heart (Goraca et al 2013).

Fig 3. Shows the weakening expression of MCP-1 on the surface of endothelial characterized by the decreasing intensity of the brown color along with the increased dose of ALA. By two way ANOVA test, there were significant differences in the expression of MCP-1 between HC group and the group treated with various doses (p <0.001). Administration of ALA possibly improve vascular reactivity and inhibit NF- B pathway. Hence the process of gene transcription of pro-inflam-matory or chemoattractant can be altered. ALA admin-istration can also improve the vasorelaxation character-ized by increased phosphorylation of eNOS through the PI3K/Akt in order for the the eNOS enzyme to be activated and NO synthesis could be improved (Shay et al 2009, Gor caet al 2011, Ying et al 2010).

Previous study indicated that supplementation of ALA in high cholesterol fed animals could inhibit the progression of atherosclerosis. The formation of foam cell in the ALA treated group was found to be signifi-cantly lower compare to that of the non-treated group (HC). This data may provide to a new ALA activity in vivo. The inhibition effect by ALA in atherogenesis might be attributable partly to its hypo-cholesterolemic property.

CONCLUSION

Based on the results of research into the effects of alpha lipoic acid (ALA) in animal models of hypercholes-terolemia at a dose of 30mg, 60mg, and 120mg/KgBW, ALA was proven to be effective in preventing the event of endothelial dysfunction in hypercholesterolemic rats

by lowering the levels of plasma MDA level and supressing the expression of MCP-1.

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