Effect of Single Garlic Oil for Homeostasis of CD4+CD25+ Immuno-regulatory T Cells Controlling Hypercholesterolemia

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Abstract. This study aims to explore the alternation expression of NFkB, activation degree of naïve T cells, and role of regulatory T cell. Hypercholesterolemia mouse model was created by giving a high-fat diet (HFD) for 45 days, and then they were treated by 12.5 mg/kg BW, 25 mg/kg BW and 50 mg/kg BW SGO for 30 days. The end of the treatment, the mouse was sacrificed, and the spleen was isolated to analyze cell surface molecules and regulatory T cells using flow cytometry. The data were analyzed by Cell Quest software and tested by statistical analysis One Way ANOVA Analysis. The results showed that a high-fat diet decreased the number of regulatory T cell and also decreased the expression of NFkB on CD4+ T cell in hypercholesterolemic mice. Moreover, the SGO did not show a significant effect on increasing the number of naïve T cells expressing CD62L molecule. Thus, the administration of SGO might inhibit activation of the NFkB pathway and decrease the stimulation of effector cell development that plays a role in the inflammatory pathway.

Keywords: Hypercholesterolemia, Immunoregulatory, Single garlic oil, Homeostasis, CD4+CD25+

1. Introduction

Atherosclerosis is an arterial chronic inflammatory disease by producing vessel wall plaques is driven by systemic hypercholesterolemia and local accelerated immune responses [1-5]. The atherosclerotic plaque is characterized by an accumulation of vascular smooth muscle cells (VSMCs), foam cell formation, inflammatory cells recruitment, and production of reactive oxygen species (ROS) [6,7,2]. The early pathogenesis of atherosclerosis was indicated by expression of vascular cell-adhesion molecule 1 (VCAM1) on the endothelium as a response to bioactive lipids ox-LDL that have been trapped within the arterial wall [3,7]. Inflammation in atherosclerosis is caused by primarily oxidized low-density lipoprotein (LDL) directly interacting with pro-inflammatory signal pathways in vascular cells and macrophages [8]. Oxidized-LDL (ox-LDL) can be engulfed by aortic macrophages, which become sub-endothelial accumulations of cholesterol-engorged macrophages forming lipid-laden foam cells [9,10,7].

The initiation and progression of atherosclerosis are associated with components of both innate and acquired immune systems [11]. The immune system is activated in the process of atherosclerosis progression, and immune cells, such as T cells, B cells, and dendritic cells (DCs) are present within
atherosclerotic lesions and contribute to disease development [10]. Macrophages constitute the dominant cell population on atherosclerotic plaques; they can generate oxygen radicals and proteases and can intake non-specific immune responses mediated by scavenger receptors [10,12]. To initiate specific immune responses, macrophages can present exogenous antigens to T lymphocytes (CD4+ T cells and CD8+ T cells) [12]. Development, activation, and differentiation of T lymphocytes are regulated by the nuclear factor-kappa B (NF-kB) [13]. The activation of the NFkB pathway can mediate the induction of a multitude of responsive genes that contribute toward the inflammatory phenotype role in atherogenesis [1,14].

Garlic has pharmacological effects attributed to the presence of pharmacologically active sulfur compounds including thiosulfinate (allicin), diallyl trisulfide, allyl methyl trisulfide, diallyl disulfide, ajoene, and others, which show anticancer, antioxidation, anti-inflammation, immunomodulatory, antimicrobial, hypoglycemic, cardiovascular protection, and preventing other chronic diseases associated with aging [14-16]. Garlic compound, especially allicin has a protective effect of reducing lipid content in the arterial wall on atherosclerosis by suppressed ox-LDL [17]. Garlic is capable of preventing oxidative modification of DNA, protein, and lipids, such as ox-LDL by scavenging reactive oxygen species (ROS) and increasing the cellular antioxidant enzymes [16,18]. The present study was designed to investigate the effect of garlic compound on immunological function in hypercholesterolemia mice models with the aim to explore the alteration expression of NFkB, activation degree of naïve T cells, and role of regulatory T cell.

2. Experimental Methods

2.1. Animal
Twenty four mice (Mus musculus) of male Balb C strain with aged three months old and 38-gram bodyweight were placed in the standard cage and maintained in the free pathogen room. The administration of daily intake was done by ad libitum and regular diet treatment.

2.2. Induction of Hypercholesterolemia with High Fat Diet (HFD)
After a one week adaptation period, four mice were given a normal diet, while the other twenty were given a high-fat diet (HFD) for 45 days. HFD feed consists of feed of Hi-Grow Medicated 551 30%+ duck egg yolk 10% + coconut oil 30% + wheat flour 5% + corn 24.9% + cholic acid 0.1%.

2.3. Oral Treatment with Single Garlic Oil
Hypercholesterolemic mouse model was divided into six experimental groups, i.e., standard chow, mice treated by HFD (negative control), positive control with HFD + simvastatin, HFD + SGO dose 12.5 mg/kg BW, HFD + SGO dose 25 mg/kg BW, and HFD + SGO dose 50 mg/kg BW. The administration of this HFD combined with SGO was performed for 30 days. In the next step, the mice were dissected, and the spleen organ was stored in the fridge for further laboratory test. All procedures were approved by the ethics committee of Universitas Brawijaya Indonesia with certificate number 880-KEP-UB.

2.4. Isolation of Lymphoid Cells and Flow Cytometry Analysis
Spleen was washed with sterile Phosphate Buffer Saline (PBS) and put in a petri dish containing sterile PBS. The spleen was pressed by using a syringe holder. Single cell solution was filtered with a sterile wire and put into a 15 mL conical tube. Suspension in the conical tube was added with PBS up to 10 mL and then put in a centrifuge, centrifugation in 2500 rpm, 4°C, for 5 minutes. The supernatant was removed, and the obtained pellet was resuspended in one mL of sterile PBS. Single cell suspension stained with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD25, and PE-conjugated anti-mouse CD62L. For intracellular staining, the cells were stained with PE/Cy5-conjugated anti-mouse NFk-B, and the staining was performed with a Cytotox/Cytoperm Kit. The cells were ready for analysis according to the parameters that had been set in the flow cytometry FACS Calibur machine.
2.5. Statistical Analysis
The obtained data were analyzed by Cell Quest software and tested by statistical analysis One Way ANOVA Analysis with P < 0.05%. All results were presented as a mean of ± SD values in each group.

3. Results and Discussion
High-fat diet (HFD) can trigger the formation of atheroma plaque in mice, with higher plasma cholesterol. HFD can increase body weight and circulating triglyceride concentrations with the extreme degrees of hypercholesterolemia. Hypercholesterolemia animals are useful models for studies on Atherosclerosis and treatment as well [19-21].

![Figure 1. The expression of NFkB in CD4+. A) Normal mice diet. B) Mice with HFD as a negative control. C) Mice with diet HFD treated by simvastatin as a positive control. D) Mice with HFD + 0.5% garlic extract). E) Mice with HFD + 1% garlic extract. F) Mice with HFD + 2% garlic extract.](image)

The administration of HFD showed an increase in NFkB expression at each treatment level compared with normal mice. Figure 1 shows that there was no statistically significant difference in NFkB expression in all of the treatments with HFD. The transcription factor NF-kB is one of the critical regulators of inflammation, immune responses, and cell survival by increasing the expression of specific cellular genes. NFkB can mediate the induction of various inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8, and TNF-α, many of which have played a role in atherogenesis. Activation of NF-κB can induce activation of iNOS (inducible nitric oxide synthase) catalyzes the formation of a significant amount of NO (nitric oxide), which plays a vital role in the pathogenesis of a variety of inflammatory diseases, such as atherosclerotic [1,14].

The administration of SGO showed the decrease in NFkB expression at all treatment levels compared with negative control. The lowest decrease in NFkB expression occurred in mice with diet HFD treated by 1% garlic extract from 0.47% to 0.29% (Figure 1). The active sulfur compound in garlic has the potential to be anti-inflammatory, which can inhibit the expression of NFkB by downregulation of NF-kB signaling [14, 15].
CD4+CD25+ T regs play crucial roles in the suppression of atherosclerosis by down-regulating inflammatory reactions [22]. In this study, CD4+CD25+ T regs decreased in all groups of mice with HFD administration. Figure 2 shows a decrease in CD4+CD25+ T regs in normal mice compared with mice model hypercholesterolemia from 1.78% to 1.05%. In general, natural T regs contains in much lower amounts in atherosclerotic plaques (1%–5% of all T cells) than in normal arterial tissue or inflammatory skin lesions (~25% of all T cells), suggesting that atherosclerotic plaques disturbed the local tolerance protection [8,23].

CD8+ T cells are the dominant leukocyte infiltrate in atherosclerotic lesions [4], but the role of CD8+ T cells in atherosclerotic is less clear [3,24]. In this study, the administrator of garlic extract in atherosclerotic mice model showed the increase in the activation of CD8+CD62L+ T cells, but it was not significant. The percentage of naïve T cells decreased in mice with the HFD diet compared with the normal mice from 4.48% to 4.18%. The amount continued to decline after the treatment with garlic extract at the level of 0.5% and 2%, with the values of 3.57% and 4.16% (Figure 3).
Figure 3. Profile of Naïve T Cells decrease in the mice with diet HFD which was not significant. A) Normal mice diet. B) Mice with HFD as a negative control. C) Mice with HFD + simvastatin as a positive control. D) Mice with HFD + 0.5% garlic extract. E) Mice with HFD + 1% garlic extract. F) Mice with HFD + 2% garlic extract.

4. Conclusion
The oral administration of SGO to hypercholesterolemia mouse model decreased the number of regulatory T cell and might inhibit the activation of the NFkB signaling pathway on CD4+ T cell. Also, SGO treatment decreased the stimulation of effector cell development that plays a pivotal role in the inflammatory pathway related atherogenesis.

Acknowledgments
We would thank Bambang Pristiwanto for assisting flowcytometry analysis. This research is funded by The Ministry of Research, Technology, and Higher Education of Republic Indonesia no. Contract 3.4.8/UN 32.14/LT/2017.

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