**Research Article**

**Potential of Single Garlic to Prevent Pro Inflammatory Macrophage and Inflammation in HFD Mice**

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**ABSTRACT**

A high-fat-enriched diet causes an increase in the level of oxidized LDL (Ox-LDL) in the blood. The presence of Ox-LDL will activate macrophages to secrete pro-inflammatory cytokines and lead to severe inflammation. Single bulb garlic has a potential anti-inflammatory effect due to high-fat diet. This research aimed to investigate the effect of single bulb garlic extract (SBGE) on pro-inflammatory cytokines TNF-α (CD11b+TNF-α) and IL-1β (CD11b+IL-1β) in the spleen, spleen weight, and TNF-α secretion in HFD mice. Twenty-four mice were divided into six groups: normal (healthy mice); HFD (HFD mice without any treatment); HFD + Simvastatin (HFD mice receiving simvastatin); HFD + SBGE 100; HFD + SBGE 200; and HFD + SBGE 400 (HFD mice receiving 100, 200, and 400 mg/kg BW of SBGE for 4 weeks). Blood serum was collected at the end of treatment, and macrophage was isolated from the spleen. The relative number of CD11b+TNF-α and CD11b+IL-1β were examined using flow cytometry. SBGE treatment significantly (p<0.05) reduced the spleen weight and the relative number of CD11b+TNF-α and CD11b+IL-1β in the spleen of HFD mice. SBGE treatment also prevents the elevation of TNF-α levels in the blood serum. The optimal dose of SBGE to diminish the relative number of CD11b+TNF-α, CD11b+IL-1β in the spleen, and TNF-α in the serum was 100 mg/kg BW.

**Keywords:** CD11b, HFD, IL-1β, Macrophage, Single Bulb Garlic, spleen, TNF-α

**Introduction**

The consumption of a high-fat diet (HFD) is considered as the major risk of several cardiovascular diseases such as atherosclerosis, stroke, coronary heart disease, and cardiac failure [1,2]. Excess cholesterol levels in blood vessels easily cause the formation of Ox-LDL [3,4,5]. Ox-LDL in blood vessels triggered endothelial and immune cells and led to an increase in the production of pro-inflammatory markers such as tumor necrosis-α (TNF-α) and interleukin-1β (IL-1β), chemokines, and adhesion molecules. Secretions of pro-inflammatory cytokines, chemokines, and adhesion molecules attract circulating monocytes to the intima and differentiate into macrophages. In the intima, Ox-LDL induces the secretion of pro-inflammatory cytokines, including TNF-α and IL-1β (pro-inflammatory macrophage) and become foam cells [6, 7, 8]. Pro-inflammatory cytokine secretion caused inflammation, triggering more macrophages to the intima and inducing the spleen to produce more pro-inflammatory macrophages. The increased number of pro-inflammatory macrophages in the intima and spleen can generate foam cells and promote lipid plaque formation in blood vessels, triggering cardiovascular disease [9].

Single bulb garlic (SBG) is a variety of garlic with a higher alliaceous odor than regular garlic. Single garlic has anti-inflammatory potential by reducing Nfkb and T regulator expression levels and boosting anti-inflammatory cytokines (IL-6, IL-10, and TGF-β) [10, 11]. SBG has several organosulfur compounds, including allicin, allicin, and ajoene, which will act as primary antioxidants [12, 13, 14] by reducing ROS, SOD, cholesterol total, and LDL in HFD mice [15,16]. Therefore,
the present study aimed to investigate the effect of single bulb garlic extract (SBGE) on the number CD11b+"TNF-α" and CD11b+"IL-1b" in the spleen and spleen weight and TNF-α level in the HFD mice model.

**Material and Methods**

**Experimental animals**

Twenty-four male Balb/c mice (Mus musculus) of about eight weeks weighing 34 ± 2 g were purchased from CV Jasa Kurnia Pratama, Sisingamangaraja, Indonesia. Before initiation of the experiment, mice were acclimatized for one week. Mice were housed in a standardized mice cage maintained under a controlled temperature room (26°C). During this period, mice have received a normal diet and water ad libitum. The Research Ethics Committee approved all procedures of Brawijaya University, Indonesia (approval number: 880-KEP-UB).

**Extraction of Single Bulb Garlic**

SBG was extracted using the maceration method with 70% ethanol. Single bulb garlic was obtained from Ngandas Village, Malang Regency, and Sarangan Village, Magetan Regency. SBG was then cleaned and air-dried. SBG was milled to a fine powder and then extracted with 70% ethanol for 3 × 24 h. The ratio of garlic and solvent was 1:2. The filtrate was evaporated using a rotary vacuum evaporator at 60°C to obtain a semi-solid sample.

**Induction of high-fat diet**

The normal group was received standard pellets (Comfeed), while the HFD group were received a high-fat diet containing 30% Hi-Grow Medicated, 10% duck egg yolk, 30% corvette, 5% wheat flour, 24.99% corn, and 0.01% colic acid for four weeks. All ingredients were mixed into a circle with a size 7 g approximately.

**Experimental design**

HFD group were divided into 4 subgroups: 1) HFD: mice without any treatment, 2) HFD + simvastatin: HFD mice received simvastatin at dose 2.6 mg/kg BW, and 3) SBGE + 100, SBGE + 200 and SBGE + 400: HFD mice received single bulb garlic extract (SBGE) at a dose of 100, 200 and 400 mg/kg BW, respectively for 4 weeks. At the end of treatment, mice were sacrificed and removed lymphatic organs. The spleens were crushed and added with PBS to isolate the lymphocytes.

**Isolation of macrophage and flow cytometry analysis**

The isolation of macrophages from the spleen was conducted to determine the relative number of CD11b+"TNF-α" and CD11b+"IL-1b". The spleen organ was crushed with the syringe base and ± 5 ml PBS, then transferred into 15 ml propylene. Homogenates were centrifuged at 2500 rpm for 5 min at 10°C and then the supernatant was removed. Pellets were added 50 µL of FITC antimouse/human CD11b Antibody (Biolegend, No. Clone M1 / 70) and incubated for 20 min in a dark place. After incubation, 50 µL of the fixative solution was added to the sample and incubated for 20 min at 4°C, in a dark room. The suspension was then added to 500 µL of permeabilization wash buffer and centrifuged at 2500 rpm for 5 min at 10°C. The pellets were added with 50 µL of APC anti-mouse/rat TNF-α Antibody (Biolegend, No. Clone TN3-19.12) and Anti-mouse/rat IL-b antibody (clone 11n92, PerCP, No. catalog LS-C184794), and then incubated with for 20 min at 4°C in a dark room. The homogenate was then added with 400 µL PBS and transferred to the flow cytometry cuvette.

**ELISA assay**

TNF-α levels were measured in blood serum using enzyme-linked immunosorbent assay (ELISA) assay. 24-well plates were coated with 20 µL antigen serum by mixing serum with buffer coating in ratio 1:40 and incubated at 4°C overnight.

The plates were washed with 100 µL PBS-T two times, then blocked with 100 µL BSA 1% in PBS for 1.5 h at room temperature. The plates were washed again and then coated with 100 µL antibody primer (Cat. No. SC-52749, Santa Cruz Biotechnology, USA) in BSA 1% (1:1000) for 2 h at 37°C.

The plates were rewashed and then coated with 100 µL secondary antibody (Anti-Rat TNF-α) in BSA 1% (1:1000). The plates were incubated for 60 min at room temperature and washed with 100 µL PBS-T. Streptavidin Horseradish (SA-SHRP) enzyme in PBS (1 : 2000) was added and incubated for 1 h at room temperature.

The plates were rewashed and added 100 µL sure blue Toluene Methyl Benzidine (TMB) for 30
minutes at room temperature in a dark room. The plates were washed, added 100 μL HCl 1N for stop reaction and incubated for 15 minutes in a dark room. The absorbance levels were measured by ELISA microplate reader at 450 nm and substituted in the standard equation to determine TNF-α levels (Bio-Rad Benchmark, Japan).

Statistical analysis
The relative number of CD11b+TNF-α+, CD11b+IL-1b+, TNF-α in serum and spleen weight were analyzed using one-way variance analysis (ANOVA). The post-doc test used Duncan Multi-level Range Test (DMRT) to calculate the significance of the difference between the groups. P-value < 0.05 was considered as significant different.

Results and Discussion
The relative number of CD11b+ TNF-α⁺ and CD11b+ IL-1b⁺

The effect of SBGE treatment in the relative number of CD11b+TNF-α⁺ and CD11b+IL-1b⁺ HFD mice was shown in Figure 1. Induction of HFD for four weeks could increase the relative number of pro-inflammatory macrophages. The relative number of CD11b+TNF-α⁺ of HFD mice were markedly decreased (P < 0.05) after receiving 100 and 200 mg/kg BW of SBGE compared to another dose of SBGE (Figure 1 B). Figure 1 showed that SBGE dose 100 and 200 mg/kg BW could prevent an increase of TNF-α in the spleen caused by HFD. Interestingly, the relative number of CD11b+IL-1b⁺ also reduced in all SBGE groups (P < 0.05). These results suggested that all SBGE doses prevent increased macrophages that express IL-1b in HFD mice.

Spleen Weight
The induction of HFD for four weeks was significantly increased the spleen weight compared to a normal diet (P < 0.05) (Figure 2). SBGE treatment reduced spleen weight at a dose of 100 and 200 mg/kg BW. SBGE at a 400 mg/kg BW dose significantly increased the spleen weight of HFD mice. These results showed that SBGE treatment for four weeks could prevent increased spleen weight in HFD mice.

TNF-α level in blood serum
The level of TNF-α in blood serum after receiving HFD for four weeks showed significantly increased compared to a normal diet. The results showed that the levels of TNF-α of HFD mice...
were markedly reduced after receiving all doses of SBGE (\( P < 0.05 \)). These results indicated that SBGE treatment could prevent increased TNF-\( \alpha \) levels in serum of HFD mice (Figure 3).

Pro-inflammatory macrophage is mainly known as the main factor that causes foam cells and lipid plaque in the blood vessel. Ox-LDL activates the pro-inflammatory macrophage. Metabolism HFD increases the number of LDL, ROS, and inflammation [14,15]. ROS can trigger lipid peroxidation and convert LDL to Ox-LDL [6,8]. Ox-LDL in the blood vessel will bind CD36 receptor, toll-like receptors (TLRs) 4 and TLR-6 in monocyte [17, 18, 19]. The lysosome will digest Ox-LDL to become cholesterol and fatty acid. Cholesterol and fatty acid triggered transduction signals My88 and TRIF to induce nuclear factor-\( \kappa B \) (NF-\( \kappa B \)). Then, NF-\( \kappa B \) triggers the production of pro-inflammatory cytokines such as TNF-\( \alpha \), IL-1b, IL-6 and IL-12 [20]. The current research found that the TNF-\( \alpha \) level in serum was increased in HFD mice. The high pro-inflammatory cytokine production caused inflammation, triggering monocyte migration in the intima and becoming a macrophage. The macrophage in the intima also produces pro-inflammatory cytokine, generated foam cells and lipid plaque in the blood vessel [21, 22, 23].

The secretion of pro-inflammatory cytokines in the blood also triggers lymphocyte production.
in the spleen. HFD has increased Granulocyte-Macrophage Progenitors (CFU-GM) in the spleen [23]. Our study showed that the production of TNF-α and IL-1b after HFD induction for 4 weeks were raised significantly. Furthermore, HFD induction also increased the number of T cells and B cell memory in the white pulp of the spleen [24]. The high level of lymphocytes such as macrophage, T cells, B cell memory, and CFU-GM could increase the spleen weight.

In our study, the high production of pro-inflammatory cytokines by macrophage in the spleen and TNF-α level in serum can be prevented by SBGE at a dose of 100 and 200 mg/kg BB. Single garlic has decreased LDL, ROS, pro-inflammatory cytokine IL-6, and TNF-α. T reg and NF-κB caused by HFD [10, 11, 15, 16]. SBGE contains allicin that could reduce ROS and prevent peroxidation lipid. Allicin transferred one H+ in ROS to become a stable molecule and prevent LDL becoming lipid. Allicin activated NF-κB (NFκB) by SBGE at a dose of 100 and 200 mg/kg BB. Similarly to what was previously reported [32], the suppression of NFκB activation will be reducing pro-inflammatory cytokine production such as TNF-α in the blood serum.

Treatment with 400 mg/kg BW SBGE could prevent the secretion of TNF-α* by monocyte, T cells, dendritic cells, and neutrophil in the blood vessel, but cannot prevent TNF-α production by macrophage in the spleen. Macrophages secreted the pro-inflammatory cytokine such as IL-1b and TNF-α because chemokine triggered from Ccl3, Cxc10, Cxc11, Ccl25, Cx3cr1, and Ccr7. This chemokine will activate the production of IL-1b and TNF-α [32]. IL-1b has been known to increase the synthesis of TNF-α protein by increasing TNF-α receptors and TNFR1 expression. Meanwhile, the secretion of TNF-α was not triggering the synthesis of IL-1b protein [34]. SBGE at a dose of 400 mg/kg BW could decrease IL-1b in the spleen. Further research is needed to investigate more effects of a high dose SBGE (400 mg/kg BW) in the number of CD11b+ ‘TNF-α’.

Conclusion

SBGE treatment could prevent elevation of TNF-α* in blood serum, CD11b+ TNF-α* and CD11b+ IL-1b+ in the spleen of HFD mice. The optimal dose of SBGE to reduce TNF-α and IL-1b production in HFD mice was 100 mg/kg BW.

Acknowledgment

This research was supported by the Ministry of Research, Technology and Higher Education Republic of Indonesia (grant no. 18.3.22/UN 32.14.1/LT/2021). We thank the Animal Physiology Laboratory of Brawijaya University for assisting the analysis process and the research team members who helped during the research.

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