Research Article

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

Putri Diyah Anggraini, Miftahul Mufinadiroh, Hendra Susanto, Betty Lukiati, Sri Rahayu Lestari*

Biology Department, Faculty Mathematics and Natural Sciences, Universitas Negeri Malang, Malang 65145, Indonesia

Article history:

Submission August 2021

Revised August 2021

Accepted October 2021

*Corresponding author:

E-mail: sriahayulestari@um.ac.id

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 of high-fat diet. This research aimed to investigate the effect of single bulb garlic extract (SBGE) on the pro-inflammatory cytokines TNF- α (CD11b⁺TNF- α ⁺) and IL-1b (CD11b⁺IL-1b⁺) 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-1b⁺ 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-1b⁺ 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-1b⁺ in the spleen, and TNF- α in the serum was 100 mg/kg BW.

Keywords: CD11b, HFD, IL-1b, 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-1 β (IL-1 β), 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-1 β (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 NF κ B 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,

How to cite:

Anggraini PD, Mufinadiroh M, Susanto H, Lukiati B, Lestari SR (2022) Potential of single garlic to prevent pro inflammatory macrophage and inflammation in HFD mice. Journal of Tropical Life Science 12 (1): 101 – 106. doi: 10.11594/jtls.12.01.10.

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, Singosari, 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 μ L of FITC anti-mouse/human CD11b Antibody (Biolegend, No. Clone M1 / 70) and incubated for 20 min in a dark place. After incubation, 50 μ L 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 μ L of permeabilization wash buffer and centrifuged at 2500 rpm for 5 min at 10°C. The pellets were added with 50 μ L 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 μ L 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 μ L antigen serum by mixing serum with buffer coating in ratio 1:40 and incubated at 4°C overnight.

The plates were washed with 100 μ L PBS-T two times, then blocked with 100 μ L BSA 1% in PBS for 1.5 h at room temperature. The plates were washed again and then coated with 100 μ L 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 μ L 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 μ L PBS-T. Streptavidin Horseradish (SA-(SA-HRP) enzyme in PBS (1 : 2000) was added and incubated for 1 h at room temperature.

The plates were rewashed and added 100 μ L sure blue Toluene Methyl Benzidine (TMB) for 30

Figure 1. The relative number of CD11b⁺TNF-α⁺ and CD11b⁺IL-1b⁺ in HFD mice after SBGE treatment. (A) Dot plot analysis of CD11b⁺TNF-α⁺; (B) Bar of mean + standard error (SE) of CD11b⁺TNF-α⁺; (C) Dot plot analysis of CD11b⁺IL-1b⁺ (D) Bar of mean + standard error (SE) of CD11b⁺IL-1b⁺.

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

Figure 2. Spleen weight after SBGE treatment for 4 weeks in HFD Mice

Figure 3. TNF- α levels in serum of HFD mice after receiving SBGE for 4 weeks

were markedly reduced after receiving all doses of SBGE ($P < 0.05$). These results indicated that SBGE treatment could prevent increased TNF- α 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- κ B (NF- κ B). Then, NF- κ B triggers the production of pro-inflammatory cytokines such as TNF- α , IL-1b, IL-6 and IL-12 [20]. The current research found that the TNF- α 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- κ B 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 become Ox-LDL [26-30]. The prevention of lipid peroxidation could decrease Ox-LDL levels, macrophage activation and pro-inflammatory cytokine.

SBGE prevents increased production of TNF- α in serum by preventing the activation of nuclear factor- κ B (NF- κ B). Ajoene in single garlic can suppress NF- κ B activation by induction p65 protein. p65 protein will prevent I- κ Ba degradation and NF- κ B will be inactive [29]. NF- κ B is a factor transcription that triggers macrophage, dendritic and T cells to produce cytokine pro-inflammation [31,32]. The suppression of NF- κ B 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, Cxcl10, Cxcl11, 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.

References

1. Jang, G. J. *et al.* (2018) 'Metabolomics analysis of the lipid-regulating effect of allium hookeri in a hamster model of high-fat diet-induced hyperlipidemia by UPLC/ESI-Q-TOF mass spectrometry', *Evidence-based Complementary and Alternative Medicine*, 2018. doi: 10.1155/2018/5659174.
2. Silva Afonso, M. *et al.* (2018) 'Molecular pathways underlying cholesterol homeostasis', *Nutrients*, 10 (6), pp. 1–18. doi: 10.3390/nu10060760.
3. Yurina, V. *et al.* (2019) 'Prolonged-heated High-Fat Diet Increase the Serum LDL Cholesterol Level and Induce the Early Atherosclerotic Plaque Development in Wistar Rats', *Journal of Tropical Life Science*, 9(1), pp. 9–14. doi: 10.11594/jtls.09.01.02.
4. Ayala, A., Muñoz, M. F. and Argüelles, S. (2014) 'Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal', *Oxidative Medicine and Cellular Longevity*, 2014. doi: 10.1155/2014/360438.
5. Trpkovic, A. *et al.* (2015) 'Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases', *Critical Reviews in Clinical Laboratory Sciences*. Informa Healthcare USA, Inc, 52(2), pp. 70–85. doi: 10.3109/10408363.2014.992063.
6. Cochain, C. and Zernecke, A. (2017) 'Macrophages in vascular inflammation and atherosclerosis', *Pflügers Archiv European Journal of Physiology*. Pflügers Archiv - European Journal of Physiology, 469(3–4), pp. 485–499. doi: 10.1007/s00424-017-1941-y.
7. Moriya, J. (2019) 'Critical roles of inflammation in atherosclerosis', *Journal of Cardiology*. Japanese College of Cardiology, 73(1), pp. 22–27. doi: 10.1016/j.jjcc.2018.05.010.
8. Borges Da Silva, H. *et al.* (2015) 'Splenic macrophage subsets and their function during blood-borne infections', *Frontiers in Immunology*, 6(SEP). doi: 10.3389/fimmu.2015.00480.
9. Raggi, P. *et al.* (2018) 'Role of inflammation in the pathogenesis of atherosclerosis and therapeutic interventions', *Atherosclerosis*. Elsevier Ltd, 276, pp. 98–

108. doi: 10.1016/j.atherosclerosis.2018.07.014.
10. Lestari, S. R. and Rifa'i, M. (2018) 'Regulatory T cells and anti-inflammatory cytokine profile of mice fed a high-fat diet after single-bulb garlic (*Allium sativum* L.) oil treatment', *Tropical Journal of Pharmaceutical Research*, 17(11), pp. 2157–2162. doi: 10.4314/tjpr.v17i11.7.
11. Lestari, S. R. and Rifa'i, M. (2018) 'Effect of Single Garlic Oil for Homeostasis of CD4+CD25+ Immuno-regulatory T Cells Controlling Hypercholesterolemia', *Journal of Physics: Conference Series*, 1093(1). doi: 10.1088/1742-6596/1093/1/012018.
12. Chung, L. Y. (2006b) 'The antioxidant properties of garlic compounds: Allyl cysteine, allyl, allicin, and allyl disulfide', *Journal of Medicinal Food*, 9(2), pp. 205–213. doi: 10.1089/jmf.2006.9.205.
13. Rahman, M. S. (2007) 'Allicin and other functional active components in garlic: Health benefits and bioavailability', *International Journal of Food Properties*, 10(2), pp. 245–268. doi: 10.1080/10942910601113327.
14. C, P. B., R, D. A. and Cr, H. (2013) 'Detail Comparative Pharmacognostical Study of Single Bulb and Multi Bulb Lasuna (Garlic).', 02(02), pp. 181–186.
15. Ilmawati, R. R., Gofur, A. and Lestari, S. R. (2019) 'Single bulb garlic oil improves interleukin-6 via decreased reactive oxygen species (ROS) in high-fat diet male mice', *Universa Medicina*, 38(2), p. 100. doi: 10.18051/univmed.2019.v38.100-107.
16. Arifah, S. N. et al. (2020) 'Herbal medicine from single clove garlic oil extract ameliorates hepatic steatosis and oxidative status in high fat diet mice', *Malaysian Journal of Medical Sciences*, 27(1), pp. 46–56. doi: 10.21315/mjms2020.27.1.5.
17. Febbraio, M., Hajjar, D. P. and Silverstein, R. L. (2001) 'CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism', *Journal of Clinical Investigation*, 108(6), pp. 785–791. doi: 10.1172/jci200114006.
18. Itabe, H., Obama, T. and Kato, R. (2011) 'The Dynamics of Oxidized LDL during Atherogenesis', *Journal of Lipids*, 2011, pp. 1–9. doi: 10.1155/2011/418313.
19. Magwenzi, S. et al. (2015) 'Oxidized LDL activates blood platelets through CD36/NOX2-mediated inhibition of the cGMP/protein kinase G signaling cascade', *blood*, 125(17), pp. 2693–2703. doi: 10.1182/blood-2014-05-574491.
20. Chatauret, N. et al. (2014) 'Diet-induced increase in plasma oxidized LDL promotes early fibrosis in a renal porcine auto-transplantation model', *Journal of Translational Medicine*, 12(1), pp. 1–11. doi: 10.1186/1479-5876-12-76.
21. Bae, Y. S. et al. (2009) 'Macrophages generate reactive oxygen species in response to minimally oxidized LDL: TLR4- and Syk-dependent activation of Nox2', *Circulation research*, 104(2), pp. 210–218. doi: 10.1161/CIRCRESAHA.108.181040.
22. Bekkering, S. et al. (2014) 'Oxidized low-density lipoprotein induces long-term pro-inflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(8), pp. 1731–1738. doi: 10.1161/ATVBAHA.114.303887.
23. Charney, L. H. and Vascular, R. B. (2015) 'Macrophages in atherosclerosis : a dynamic balance', 13(10), pp. 709–721. doi: 10.1038/nri3520.Macrophages.
24. Torello, C. O., Paredes Gamero, E. J. and Martins, F. (2016) 'Extramedullary Hematopoiesis in the Spleen of Obese Mice Modulation by the Alga *Chlorella*', *Medicinal & Aromatic Plants*, 05(06). doi: 10.4172/2167-0412.1000275.
25. Gomaa, A. M. S. and El-Aziz, E. A. A. (2017) 'Vitamin D reduces high-fat diet induced weight gain and C-reactive protein, increases interleukin-10, and reduces CD86 and caspase-3', *Pathophysiology*, 24(1), pp. 31–37. doi: 10.1016/j.pathophys.2017.01.003.
26. Bruck, R. et al. (2005) 'Allicin, the active component of garlic, prevents immune-mediated, concanavalin A-induced hepatic injury in mice', *Liver International*, 25(3), pp. 613–621. doi: 10.1111/j.1478-3231.2005.01050.x.
27. Chung, L. Y. (2006a) 'The Antioxidant Properties of Garlic Compounds ', *Journal of Medicinal Food*, 9(2), pp. 205–213.
28. Birben, E. et al. (2012) 'Oxidative stress and antioxidant defense', *World Allergy Organization Journal*, 5(1), pp. 9–19. doi: 10.1097/WOX.0b013e3182439613.
29. Lee, D. Y. et al. (2012) 'Anti-inflammatory activity of sulfur-containing compounds from garlic', *Journal of Medicinal Food*, 15(11), pp. 992–999. doi: 10.1089/jmf.2012.2275.
30. Nimse, S. B. and Pal, D. (2015) 'Free radicals, natural antioxidants, and their reaction mechanisms', *RSC Advances*. Royal Society of Chemistry, 5(35), pp. 27986–28006. doi: 10.1039/c4ra13315c.
31. Aggarwal, B. B. and Shishodia, S. (2004) 'Suppression of the nuclear factor- κ B activation pathway by spice-derived phytochemicals: Reasoning for seasoning', *Annals of the New York Academy of Sciences*, 1030, pp. 434–441. doi: 10.1196/annals.1329.054.
32. Le Rossignol, S., Ketheesan, N. and Haleagrahara, N. (2018) 'Redox-sensitive transcription factors play a significant role in the development of rheumatoid arthritis', *International Reviews of Immunology*, 37(3), pp. 129–143. doi: 10.1080/08830185.2017.1363198.
33. Orecchioni, M. et al. (2019) 'Macrophage polarization: Different gene signatures in M1(Lps+) vs. Classically and M2(LPS-) vs. Alternatively activated macrophages', *Frontiers in Immunology*, 10(MAY), pp. 1–14. doi: 10.3389/fimmu.2019.01084.
34. Di Paolo, N. C. et al. (2015) 'Interdependence between Interleukin-1 and Tumor Necrosis Factor Regulates TNF-Dependent Control of Mycobacterium tuberculosis Infection', *Immunity*. Elsevier Inc., 43(6), pp. 1125–1136. doi: 10.1016/j.immuni.2015.11.016.