The present study aimed to evaluate the effect of single-bulb garlic oil (SGO) on toll-like receptors 3 and 4 (TLR3 and TLR 4) and nuclear erythroid factor-like 2 (Nrf2) signaling pathway resulted from a high-fat diet and its underlying mechanism. Twenty-four Balb/c mice allocated into six groups: 1) N: mice fed with standard chow; 2) HFD: mice fed a high-fat diet for 45 days without any treatment; 3) HFD + Simv: mice fed a high-fat diet for 45 days and treated with simvastatin; 4–6) HFD + SGO 100, 200, 400 (mice fed a high-fat diet for 45 days and treated with single-bulb garlic oil at dose: 100, 200, and 400 mg/kg body weight for 30 days), respectively. At the end of treatment, spleen and hepar were isolated. The flow cytometry analysis was performed to analyze the relative number of nrf2, superoxide dismutase (SOD), malondialdehyde (MDA), TLR3, TLR4 and interleukin (IL-17). The results showed that HFD induction significantly reduced Nrf-2 and antioxidant enzyme levels. Furthermore, HFD induction increased TLR3 and TLR4 signaling and IL-17 production. Interestingly, 200 mg/kg BW of SGO increased the relative number Nrf-2 followed by SOD and HO-1 elevation at a dose of 100 mg/kg BW. SGO100 notably decrease the relative number of TLR3 (CD11b+TLR3+) and TLR4 (CD11b+TLR4+). The production of IL-17 by CD4 and CD8 were also reduced after receiving SGO at 200 mg/kg BW. This study suggests that the protective effect of SGO treatment on HFD mice was achieved by modulating TLR-Nrf2 cross-talks and decreasing IL-17 production. Our findings support a potential beneficial role of SGO for treating metabolic disease caused by a high-fat diet.

1. Introduction

Metabolic syndrome is dramatically rising worldwide (Faulds and Dahlman-Wright, 2012), which need optimal medication to treat this disease (Chooi et al., 2019). The metabolic syndrome includes diabetes mellitus, hyperlipidemia, cardiovascular disease, non-alcoholic fatty acid liver, and hypertension, commonly caused by an excessive fat diet and aging. The long-term consumption of a high-fat diet induces abnormal lipid distribution and blood lipid disorder and leads to obesity, a significant constituent of metabolic syndrome. The metabolic syndrome is recently associated with low-grade systemic inflammation, and the molecular mechanism is still unknown. However, several studies revealed that overconsumption of fat would increase plasma lipopolysaccharide (LPS) (Faulds and Dahlman-Wright, 2012).

Obesity has been projected as stimuli that activate the toll-like receptors (TLRs) pathway. TLRs is closely related to macrophage and dendritic cells and activate various inflammatory pathway (Shaikh et al., 2015). A high-fat diet (HFD) could induce endotoxemia, followed by increased expression of Toll-like receptor 3 (TLR3) and NF-κB in the circulating mononuclear cells (Ghanim et al., 2009). The involvement of TLR 3 in the chronic inflammatory cause by fat-enriched diet also well studied. Toll-like receptor 3 (TLR3) is a pathogen pattern recognition receptor involved in chronic inflammation in infection response. TLR3 has been reported to affect glucose and lipid metabolism as inflammatory mediators (Wu et al., 2015). Toll-like receptor 4 (TLR4) also contribute to inflammatory response due to HFD feeding. The expression of TLR4 has raised in arterial walls after receiving HFD. Cao et al. (2018) revealed that TLR4 mediates HFD induced increase...
in body weight, inflammation, and lipid accumulation in the aorta via the PPARγ/ABCG1 pathway. Therefore, targeting TLR3 and TLR4 signaling is a promising option in treating disease with metabolitc syndrome.

Obesity is also characterized by the high storage of fatty acids in adipose tissue (Upadhyay et al., 2018). The adipose tissue of the animal model of obesity expresses a high level of pro-inflammatory cytokines, including tumor necrosis factor (TNF) α, interleukin (IL)-1, and IL-6, which followed by infiltration of macrophage into adipose tissue (Maury and Brichard, 2010). Interleukin-17 (IL-17) is a crucial marker of inflammatory progression of some liver diseases caused by a high-fat diet (Xu et al., 2013). Shen et al. (2017) reported that a fat-enriched diet could induce the elevation of IL-17A cytokines and accelerate the hepatic steatosis through the JNK-PPARδ pathway. The inhibition of IL-17 production HFD-mice could improve liver function, decrease hepatic lipid accumulation, and decrease pro-inflammatory cytokines levels (Xu et al., 2013).

Single -bulb garlic (Allium sativum L.) is a promising herbal medicine to treat several inflammatory diseases, including hypertension, hypercholesterolemia, diabetes, arteriosclerosis, and cancer. Garlic has many bioactive compounds, including alliiin, allicin, allicyl-sulfides, ajoene, and 1,2-vinyldithiin, responsible for several pharmacological activities (Martins et al., 2016). The other compound in garlic, such as steroids, terpenoids, flavonoids and other phenols, also have a therapeutic effect in inflammatory process through regulatory T cells activation and anti-inflammatory cytokine IL-10 and TGF-β (Lestari and Rifa’i, 2018). Single bulb garlic oil with hexane solvent also modulate the expression of TNF-α and IFN-γ through NF-kB expression. The naïve T cells level also improved in HFD-mice after receiving a hexane extract of single-bulb garlic for 45 days (Lestari et al., 2020). The bioactive compound of garlic also increases antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase (Taha et al., 2013). Several studies have explored different forms of single bulb garlic extract such as aqueous, hexane, and dried powder. However, the reviews of the ethanolic extract of SGO in preventing metabolic disorder is still limited.

Several studies have been trying to find the antagonist of TLR, which may benefit from treating obesity or other metabolic diseases. TLR2 and TLR4 signaling have a lot of evidence of their involvement in obesity compared to TLR3. The mechanisms linked between TLR and Nrf2 in obesity are not fully understood. Hence, in the present study, the effect of single-bulb garlic oil (SGO) on antioxidant status (Nrf2 and related target gene, including SOD-1, and HO1) and their cross-talk with TLR3 and TLR4 signaling and pro-inflammatory cytokines IL-17 were elucidated to investigate more detailed mechanisms in the improvement of enriched-fat diet.

2. Materials and methods

2.1. Ethical approval

The animal procedures were permitted by the Research Ethics Committee, Brawijaya University, Indonesia (Approval no. 880-KEP-UB).

2.2. Preparation of SGO

Single-bulb garlic was provided by the farmer in Ngadas District, Poncokusumo, Malang Indonesia. Single-bulb garlic was extracted by the maceration method using 70% ethanol. In brief, 1 kg of fresh single-bulb garlic was cleaned, dried and milled to a powder. The powdered samples were soaked in 70% ethanol (sample/solvent ratio: 1:2) for 3 days at room temperature. Samples were filtered and concentrated by a rotary evaporator. SGO was then stored at 4 °C until further use.

2.3. Animals and diet treatment

Twenty-four male Balb/c mice (Mus musculus), weighing 28–31 g, were kept under a 12/12 h light/dark cycle at 22 °C. Mice had free access to food and water ad libitum. Mice were obtained from CV. Kurnia Jasa Pratama Pusat, Singosari, Malang, Indonesia. The composition of HFD fed was referred to previous research by Lestari and Rifa’i (2018), which consisted of Hi-Grow Medicated 551 feed (30 %), duck yolk (10 %), coconut oil (30 %), wheat flour (5 %), corn (24.9 %), and cholic acid (0.1 %). HFD fed was given for 4 weeks.

After 1-week acclimatization, mice were divided randomly into 6 treatments groups (n = 4): control group: mice fed with standard chow; HFD: mice fed a high-fat diet for 45 days without any treatment; HFD + Simv: mice fed a high-fat diet for 45 days and treated with simvastatin at a dose of 2.6 mg/kg body weight; HFD + SGO100: mice fed a high-fat diet for 45 days and treated with single garlic oil at dose 100 mg/kg body weight; HFD + SGO200: mice fed a high-fat diet and treated with single garlic oil at dose 200 mg/kg body weight; HFD + SGO400: mice fed a high-fat diet and treated with single garlic oil at dose 400 mg/kg body weight. SGO treatment was orally administered every day for 30 days.

2.4. Lymphocyte and liver isolation

At the end of treatment, mice were dissected by neck dislocation. The liver and spleen were isolated. Both organs were washed using Phosphate Buffer Saline (PBS) and weighted. Then, each organ was crushed clockwise on a Petri dish containing 1 mL PBS. Spleen suspension was then centrifuged at 2500 rpm for 5 min at 4 °C. Homogenate added with 1 mL PBS and re-centrifuged at 2500 rpm at a temperature of 4 °C for 5 min. The supernatant was discarded, and the pellet was then stained with a specific antibody.

2.5. Immunostaining and flow cytometry analysis

For extracellular staining, the obtained pellet from the spleen was stained with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8 and FITC-conjugated anti-mouse CD11b and then incubated at 4 °C for 20 min in a dark place. For intracellular staining, samples were added with Cytotix/Cytoperm (BD-Biosciences Pharmingen) and then incubated at 4 °C for 20 min in a dark place. Then, 500 μL of intracellular staining permeabilization wash buffer was added into samples and then centrifuged at 2500 rpm for 5 min at 4 °C. The obtained pellet from the spleen was stained with PerCP/Cy5.5-conjugated rat anti-mouse IL-17, PE-conjugated rat anti-mouse TLR3 and PerCP/Cy5.5-conjugated rat anti-mouse TLR4. While the obtained pellet from liver was stained with FITC-conjugated anti-mouse Nrf2, FITC-conjugated anti-mouse SOD, and FITC-conjugated anti-mouse HO-1. Each sample was then incubated at 4 °C for 20 min in a dark place. After incubation, samples were then analyzed using flow cytometry (BD Biosciences FACs Calibur™). Data were analyzed using BD CellQuest Pro™ to obtain the relative number of each parameter.
2.6. Statistical analysis

Data were expressed as mean ± SE. The significant among the group were analyzed using one-way ANOVA followed by Tukey post hoc test with p < 0.05. The experiment was performed in four replications.

3. Results

3.1. Effect of SGO on body weight and liver weight of HFD mice

Bodyweight gain, liver and spleen weight of animals fed HFD are shown in Table 1. The consumption of a high-fat diet for 45 days resulted in significant increased of body weight compared to healthy mice (N). The study found that there was no significant difference in final body weight among simvastatin and SGO groups. The administration of HFD led to a significant elevation of the liver and spleen weight of HFD mice compared to healthy mice. Treatment with SGO 200 and 400 mg/kg body weight could attenuate the liver weight in HFD mice and not significantly different with the simvastatin group. The low dose of SGO (100 mg/kg BW) could decrease the spleen weight, but did not reach the normal spleen weight.

3.2. SGO increase the relative number of Nrf-2, SOD-1 and HO-1 in HFD mice

To evaluate the role of SGO on oxidative stress, hepatic Nrf2 status and another related antioxidant enzyme were determined. The results showed that the relative number of Nrf2 in hepar of normal mice was 29.44%. However, HFD induction caused severe oxidative stress in the liver, as indicated by significantly (p < 0.05) reduced in the relative number of Nrf2 (11.90%). Interestingly, SGO at a dose of 200 and 400 mg/kg BW could enhance the relative number of Nrf2 by 18.19% and 17.20%, respectively (Fig. 1A). These treatments had the same effect with simvastatin as drug control in enhancing Nrf2 in hepar of HFD mice. However, the value did not reach the control group (Fig. 1B).

The relative number of SOD-1 decreased by 17.53% in the hepar of HFD mice (p < 0.05) compared to the control group (27.27%) (Fig. 2A). All dose of single-bulb garlic oil treatment has a significant effect on SOD-1 level in the liver of HFD mice (Fig. 2B). SGO at dose 100 mg/kg BW exhibited a high increase in SOD-1 level in the hepar by 34.81% compared to other treatments and the value same as the simvastatin group (30.80%).

The induction of HFD was associated with 20.91% reduction in expression of HO-1 in the hepar of HFD mice compared to the control group (37.94%) (Fig. 3A). The single-bulb garlic oil treatment group (SGO100 and SGO400) demonstrated a high elevated expression of HO-1 in the liver by 34.62 and 34.24 %. However, the value did not reach the control group. All SGO treatment groups did not reach the control group. All SGO treatment groups remained higher as compared to the simvastatin group (Fig. 3B).

3.3. SGO decreased TLR 3 in macrophage (CD11b"TLR3")

To determine whether TLR3 are crucial in mediating inflammatory process caused by HFD induction, the activation of TLR3 in CD11b cells was determined. FACS analysis indicated a significant increase in the percentage of CD11b"TLR3" cells circulating in the spleen of mice after HFD induction by 37.86 % (Fig. 4A). These results were correlated with previous findings when inflammatory responsive gene such as TLR3 and IL17 was elevated, so the antioxidant status was decreased. Interestingly, SGO treatment at dose 100 mg/kg BW exhibited a high reduction in the percentage of CD11b"TLR3" cells. The Simvastatin group also showed the same effect as the SGO100 group.

3.4. SGO ameliorate TLR4 expression in macrophages (CD11b"TLR4")

In this study, we also determined the role of TLR4 in mediating inflammatory response due to HFD induction. The expression of TLR4 also remains a crucial marker in the inflammation process. The results manifested that the induction of a high fat diet exhibited significantly (p < 0.05) higher TLR4 expression in macrophages (CD11b) compared to healthy mice (32.04 vs 16.52%). The administration of simvastatin slightly reduced the expression level of TLR4 (CD11b"TLR4") by 27.58%, if compared to the single bulb garlic group. Furthermore, the administration of single bulb garlic showed a significant reduction in TLR4 expression as it recorded at 19.71% at 100 mg/kg BW and 20.05% at 200 mg/kg BW, and 25.89% at 400 mg/kg BW. Interestingly, the effect of single bulb garlic was more pronounced in modulating TLR4 expression compared to the simvastatin group (Fig. 5).

3.5. SGO improves the IL-17 production

It was considering that Nrf2 in the liver is related to the regulation of TLR3 and TLR4 signaling by targeting its inflammatory responsive genes such as pro-inflammatory cytokine IL17. This study showed that the production of IL-17 cytokines was markedly elevated in mice after fed a high-fat diet. The percentage of CD4+/IL17+ in HFD mice tend to increase compared to normal mice (HFD: 16.39% vs N: 7.54%) (Fig. 5A). Treatment with 200 mg/kg BW of SGO exhibited a high decrease in IL-17 production by 1.81% compared to other doses and the simvastatin group. The percentage of CD4+IL17+ at SGO100 and SGO400 group was not significantly different from the simvastatin group (Fig. 6B).

The study observed that there was a notable increase (p < 0.05) in the production of IL-17 cytokines in high fat diet-induced mice compared to healthy mice (6.21 % vs 0.78 %) (Fig. 7). The production of IL-17 cytokines in HFD mice was decreased after receiving simvastatin drug (2.71%). The level of IL-17 was favorably brought to normal in a significant with the administration of SGO at 200 mg/kg BW (1.08%). Similarly, other doses possessed a high reduction in IL-17 production (1.64 % at 100 mg/kg BW and 1.22 at 400 mg/kg BW). It was noticed that the effects of SGO at three comparative doses surpassed the simvastatin effects.

4. Discussion

Nuclear factor erythroid 2 related factor 2 (Nrf2) is a cap-n-collar primary leucine zipper transcription factor that plays a vital role in regulating the cellular antioxidant and decreasing inflammatory stress resulted in tissue and organ homeostasis. Obesity is characterized by accumulating pro-inflammatory cytokines,
which then triggers the activation of pathways correlated with inflammation-induced resistance (Li et al., 2019). Research by Jia et al. (2020) demonstrated that the supplementation of a high-fat diet in tilapia feed causes a weak antioxidant system by reducing the Nrf-2 signaling pathway. Ninety-day feeding with a high-fat diet in tilapia fish also exhibits a high expression level of JNK-NF-κB and TLRs-MyD88- NF-κB pathway, which is known to mediate this pathway the inflammatory response. Similarly, pro-inflammatory cytokine production is also increased, such as IL-1, IL6, IL-8 and IL-10 (Jia et al., 2020). Several studies performed that treatment with a potent Nrf2 activator could decrease body weight, adipose mass and hepatic lipid accumulation in HFD mice compared to Nrf-disrupted mice (Shin et al., 2009). Thus, targeting the Nrf-2 pathway is a promising strategy to decrease inflammation and oxidative stress in obesity (Li et al., 2019). Our results demonstrated that HFD mice noticeably more incredible increased body and liver weight compared to healthy mice. High-fat diets commonly cause weight gain, fat accumulation, and increased fat levels in the liver and serum, resulting in the high risk of non-alcoholic steatohepatitis (NASH) (Hirako et al., 2011).

Our study indicated that HFD induction caused severe oxidative stress in the liver, as characterized by significant (p < 0.05) reduced in the relative number of nrf2, SOD and HO-1 and followed by a significant increase in liver weight. These findings are in line with Badr and Al-Mulhim (2014) which reported that aged garlic extract increases the level of MDA, SOD, catalase (CAT) and glutathione...
peroxidase (GPx) in mice induced with indomethacin. Single bulb garlic plays a role as a chain-breaking antioxidant for scavenging free radicals. The effectivity of ethanol extract of single clove garlic is more optimal than multi clove garlic in preventing hepatic damage in rabbits (Naji et al., 2017). Alliinase acts on alliin (S-allyl cysteine sulfoxide) to produce an antioxidant compound, and allicin effectively scavenges hydroxyl radicals and inhibit LPO (Lawson and Wang, 2005). Our study revealed that SGO200 could alleviate nrf2, while SGO100 and SGO400 also demonstrated a high increase in SOD and HO-1 levels compared to HFD mice. SGO treatment showed a significant decrease in liver weight but did not alter the body weight gain of HFD mice.

Toll-like receptor (TLR) 3 has a significant role in metabolic regulation, including obesity and diabetes type 2. TLR3 and TLR4 contributed to metabolic disorders during the high-fat diet (Strodthoff et al., 2015). The TLR mediated innate immune responses and Nrf2-modulated antioxidant system is contributed to modulate the inflammatory response (Mohan and Gupta, 2018). The TLRs agonists could be performed as Nrf2 inducers and enhance the expression of antioxidant molecules essential to cell homeostasis (Nadeem et al., 2016). Conversely, Nrf2-activator may reduce the TLR expression by activating antioxidant enzymes and suppressing pro-inflammatory mediators (Chen et al., 2017). These two majors signaling pathways may interact differently, and their cross-talk can be manipulated to regulate inflammation (Mohan and Gupta, 2018). Our study revealed that the expression of both TLR3 and TLR4 in HFD mice was reduced after receiving SGO. A low dose of SGO (100 mg/kg BW) possessed a high decrease in TLR3 and TLR4 expression (Fig. 4 and Fig. 5).

Several pharmacological studies performed that modulating the TLR-Nrf2 cross-talks could decrease the inflammatory response. Jurenka (2009) revealed that curcumin could inhibit the TLR

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**Fig. 3.** Hepatic HO-1 expression levels in SGO treatment in mice fed a high fat diet and control group. (a) Histogram FACS analysis showed the relative number of nrf2 in various group; (b) Each bar represents the mean ± standard error (SE). *p < 0.05 indicated a significant different in N and treatment groups compared to HFD group. Note: N: mice fed with normal chow; HFD: mice fed a high fat diet without any treatment; HFD + Simv: mice fed a high-fat diet for 45 days and treated with simvastatin; HFD + SGO 100,200,400 (mice fed a high-fat diet for 45 days and treated with single garlic oil at dose: 100, 200, and 400 mg/kg bodyweight for 30 days), respectly.

**Fig. 4.** SGO treatment decreased the expression of CD11b^TLR3^ in mice fed a high-fat diet. (a) Dot plot FACS analysis showed the relative number of nrf2 in the various group; (b) Each bar represents the mean ± standard error (SE). *p < 0.05 indicated a significant different in N and treatment groups compared to HFD group. Note: N: mice fed with standard chow; HFD: mice fed a high-fat diet without any treatment; HFD + Simv: mice fed a high-fat diet for 45 days and treated with simvastatin; HFD + SGO 100,200,400 (mice fed a high-fat diet for 45 days and treated with single garlic oil at dose: 100, 200, and 400 mg/kg bodyweight for 30 days), respectly.
mediated NF-κB signaling, induces Nrf2 expression followed by the activation of HO-1 and GSH and downregulates cyclooxygenase and lipoxygenase pathways and other pro-inflammatory cytokines. Yasui et al. (2015) also revealed that the inhibition of TLR-mediated NF-κB signaling by quercetin could induce nrf2 expression and decrease IL-6, TNF-α, ICAM-1, VCAM-1, COX. Therefore, targeting these two pathways is crucial to modulate inflammation response during HFD. Our study demonstrated that the activation of TLR3 possesses an upregulated expression of the inflammatory marker, including IL-17 produced by CD4 T cells. SGO extracted with ethanol solvent also suppressed the relative number of TLR3 and the production of IL-17 produced by CD4 T cells.

Natural plant product has gained much interest between researchers because it is expected to prevent obesity without possessing side effects. Another study revealed that single bulb garlic oil with hexane solvent effectively prevents hepatic steatosis by triggering the elevation of serum SOD levels and reducing the level of pro-inflammatory cytokines TNF-α in HFD mice. This study also demonstrated a significant decrease in liver weight of HFD mice after receiving single bulb garlic oil for 35 days compared to the statin group as a positive control. The total cholesterol, LDL, and triglyceride were also reduced, followed by increased HDL levels after treatment with single bulb garlic oil (Arifah et al., 2020). Ilmawati et al. (2019) revealed that single bulb garlic oil extracted with hexane exhibited a high decrease in inflammation by reducing ROS, IL-6 level in the aorta, and lymphocyte density spleen and bone marrow of HFD mice. In the molecular studies by Lestari et al. (2019), the organosulfur compounds in single bulb garlic such as s-ajone, z-ajone and allicin play a role in inhibiting the FAS enzyme (a responsible enzyme for lipid biosynthesis). These compounds had the same binding site as Statins in the FAS enzyme (Lestari et al., 2019). The other research also revealed that allicin, alliin and ajoene in single bulb garlic could be considered potent inhibitors for 11β-hydroxysteroid dehydrogenase reductase.
type 1 (11β-HSD1) enzyme. The activity of the 11β-HSD1 enzyme is high in obesity conditions (Nikmaturrohana et al., 2020). In our study, flow cytometry analysis demonstrated that the activation of TLR3, TLR4, and IL-17 were involved in the development of obesity-induced by a high-fat diet. After receiving with single bulb garlic (SGO), activation of Nrf2, SOD-1 and HO-1 was significantly increased, hence, the TLR3, TLR4, and IL-17 signalling pathway was markedly suppressed.

5. Conclusions

In conclusion, our study revealed that the Nrf-2 pathway and antioxidant related genes were adversely impaired in hepatic of HFD-fed mice after 4 weeks of treatment with SGO. The vital mechanism contributing to SGO anti-obesity is the elevation of antioxidant-dependent protein including nrf2, HO-1 and SOD-1 in HFD mice, indicating that this antioxidant mechanism may underlie reduced inflammatory markers TLR3, TLR4, and IL-17. The protective effect of SGO treatment on HFD mice was achieved by modulating TLR-Nrf2 cross-talks and decreasing IL-17 production. Our findings support a potential beneficial role of SGO for treating metabolic disease caused by a high-fat diet.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 7. SGO treatment normalized the production of IL-17 by CD8 T cells in mice fed a high-fat diet. (a) Dot plot FACS analysis showed the relative number of nrf2 in the various group; (b) Each bar represents the mean + standard error (SE). *p < 0.05 indicated a significant different in N and treatment groups compared to HFD group. ns indicated not significant (p > 0.05) compared to N group. Note: N: mice fed with standard chow; HFD: mice fed a high-fat diet without any treatment; HFD + Simv: mice fed a high-fat diet for 45 days and treated with simvastatin; HFD + SGO 100,200,400 (mice fed a high-fat diet for 45 days and treated with single garlic oil at dose: 100, 200, and 400 mg/kg bodyweight for 30 days), respectively.
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