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The Effects of Elicited Soybean (*Glycine max*) Extract on Hematopoietic Cells of High Fat-Fructose Diet Balb/C Mice Model

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Abstract

High Fat-Fructose Diet (HFFD) triggers various metabolic problems including obesity. Obesity leads to a chronic low-grade inflammation and is associated with the unbalanced production of hematopoietic cells. In the condition of obesity, hematopoietic stem cells (HSC) are more likely to differentiate into granulocytes rather than erythrocytes as a response to inflammation. Modified soybeans are well-known to have higher compounds than the raw soybeans. One of the soybean modification processes is known as elicitation. In this study, soybeans are exposed to stressors such as *Saccharomyces cerevisiae* and light to increase the beneficial compounds inside. The elicited soybean extract (ESE) contains several anti-inflammatory and anti-oxidant compounds which are useful for the hematopoietic system. This study is aimed at determining the effects of ESE on hematopoietic cells; erythrocytes lineage (TER-119$^+$CD34$^+$, TER-119$^+$VLA-4$^+$, TER-119$^+$), and granulocytes lineage (Gr-1$^+$). In this study, twenty-eight three-week old female Balb/C mice were used. They were fed HFFD for twenty weeks, and were given ESE oral treatment for four weeks. The level of hematopoietic cells was analyzed using flow cytometry. The present study found that HFFD decreased the level of TER-119$^+$CD34$^+$, TER-119$^+$, and TER-119$^+$VLA-4$^+$ level, and increased the Gr-1$^+$ level. ESE significantly increased the TER-119$^+$CD34$^+$, TER-119$^+$ and TER-119$^+$VLA-4$^+$ level (p<0.05) and decreased the Gr-1$^+$ level (p<0.05) in the HFFD-treatment group. These results show that the potential use of ESE as an anti-inflammatory agent can improve the hematopoietic system in the HFFD-diet mice model.

Keywords: Elicited soybean extract; High fat-fructose diet; Anti-inflammatory; Hematopoiesis; Erythropoiesis, Granulopoiesis

1. Introduction

High fat consumption is known to cause over-nutrients that lead to obesity. Obesity is related to a low-grade chronic inflammation which may interfere with multiple systems in the body, above all the hematopoietic system (Benites *et al*., 2014). The hematopoietic system starts from the differentiation of hematopoietic stem cells (HSC) in the bone marrow into mature blood cells, both red and white blood cells (Hoffbrand and Moss, 2011).

Molecules CD34, TER-119 and VLA-4 are involved in erythropoiesis, while Gr-1 is involved in granulopoiesis. CD34 is a transmembrane protein which is expressed in hematopoietic progenitor cells. CD34 is also known as a specific marker for stem cells both in humans and in mice, but is not expressed in peripheral blood cells (Garland *et al*., 1997; Knowles, 2001). The TER-119 molecule is a specific marker for erythroblast and mature erythrocyte. TER-119$^+$ reacts with erythroid at different stages of development from proerythroblast to mature erythrocytes (Kina *et al*., 2000; Elliot *et al*., 2009). VLA-4 is a specific marker expressed in reticulocytes but not in mature red blood cells (Hines *et al*., 2014). Gr-1 is a specific marker for the development of granulocytes in the white blood-cell production (granulopoiesis). Gr-1 is expressed in myeloid cells through the development of granulocytes in the bone marrow (Ribechini *et al*., 2009). The expressions of Gr-1$^+$ in bone marrow determine the number of leukocytes circulating in the peripheral blood (Lee *et al*., 2011).

Obesity leads to a chronic low-grade inflammation that increases the production of pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF) and Interleukin (IL), which play a role in the HSC damage and hematopoiesis disruption (Chen *et al*., 2010; Cortez *et al*., 2013). TNF-α causes cell membrane disintegration that leads to cell death of the hematopoietic cells (Jurisic *et al*., 2011). In addition, pro-inflammatory cytokines would increase the HSC proliferation to differentiate into granulocytes in response to the inflammation and can interfere with erythropoiesis (Grigorakaki *et al*., 2011; Kraakman *et al*., 2014).

Soybeans are rich in a variety of nutrients that have several health benefits. The modification of soybeans can

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increase the nutrient contents, and/or can generate new nutrient compounds. Elicitation is one modification process through which soybeans are exposed to biotic and/or abiotic stress in order to generate secondary metabolites called glyceollin, and to increase the isoflavone content (Feng *et al*., 2007; Park *et al*., 2010; Aisyah *et al*., 2013). Glyceollin is also known as an herbal medicine for several diseases, such as cancer, hyperglycemia, and hypercholesterolemia (Park *et al*., 2012; Schexnayder and Stratford, 2015). In addition, glyceollin and isoflavone have anti-inflammatory effects which are associated with the regulation of Nitric Oxide (NO) and inducible nitric oxide synthase (iNOS) (Hämäläinen *et al*., 2007; Kim *et al*., 2011). This study investigates the effects of elicited soybean extract (ESE) on hematopoietic cells TER-119$^+$CD34$^+$, TER-119$^+$VLA-4$^+$, TER-119$^+$ and Gr-1$^+$ expression in high fat-fructose-diet (HFFD) mice.

2. Materials and Methods

2.1. Elicited Soybean Extract

In this study, the soybeans were obtained from the Indonesian Legumes and Tuber Crops Research Institute (ILETRI). The soybeans were elicited using *Saccharomyces cerevisiae* according to Kim *et al*. (2011) with several modifications (Kim *et al*., 2011). A total of one-hundred grams of soybeans was sterilized using 70 % ethanol for ten minutes, and were then rinsed with sterile distilled water four times. The sterilized soybeans were then soaked in sterile distilled water for twenty-four hours at the room temperature. After twenty-four hours of soaking, the soybeans were grown on cotton media moistened with distilled water. Every one-hundred grams of soybens were inoculated with 7.5 mL *Saccharomyces cerevisiae* with a concentration of 10^7. After that, the soybeans were covered with a plastic wrap and were put under a light bulb for sixteen hours per day for three days (according to Aisyah *et al*. (2013) (with slight modification). The extraction was performed using 300 mL of 80% ethanol for every one-hundred grams of soybeans. In the next step, the ethanol was evaporated from the extract. The extract was then frozed dried to obtain the paste extract of ESE.

2.2. Animals

This research used twenty-eight three-week old female mice of broodstock obtained from The Integrated Research and Testing Laboratory, Gajah Mada University. The animals were grouped into seven groups containing four mice in each. The normal-diet (ND) group (K1), a normal diet group which were treated with ESE dose of 104 mg/kg BW (P1), and the high fat-fructose diet (HFFD) group (K2) served as a control in this study. We also used an anti-cholesterol drug Simvastatin dose of 2.8 mg/kg BW (P2) as a comparison (Mahmoud *et al.*, 2013). The ESE treatment doses in this study were 78 mg/kg BW (P3), 104 mg/kg BW (P4), and 130 mg/kg BW (P5). All protocols in this study were approved by University of Brawijaya Ethics Committee (Reg. No. 647-KEP-UB).

2.3. High Fat-Fructose Diet (HFFD) and Elicited Soybean Extract (ESE) Treatment

The composition of the high fat-fructose food consisted of 30% fructose, 10% carbohydrate, 35% Hi Gro551 (starter pellets for pigs contain of 73.9% carbohidrate, 20.5% protein, 5% fat, 1.6% fiber, vitamins and minerals by Charoen Pokphand Company, Indonesia), 17% cow fat, and 8% yolk cholesterol. The three-week old female mice were given HFFD diet for twenty weeks, and were then given ESE treatment of the following doses; 78 mg/kg BW, 104 mg/kg BW, and 130 mg/kg BW; and Simvastatin dose 2,8 mg/kg BW for four weeks by oral administration.

2.4. Bone Marrow Isolation

Mice were dissected after four weeks of treatment, then both femurs of each mouse were isolated. The bone-marrow isolation procedures were based on Khasanah *et al*. (2015) protocol. Bone marrow cells were isolated by cutting both ends of the femur and were then flushed out using 1 mL of phosphate buffered saline (PBS) in 1 mL syringe. The flushed-cells were then collected into centrifuge tubes, adding 10 mL of PBS, and were then centrifuged at 4500 rpm, 10°C, for five minutes. Pellet was resuspended in 1 mL of PBS for antibody staining procedures.

2.5. Immunostaining

Antibody staining in this study included fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD34, FITC-conjugated rat anti-mouse Gr-1, Phycoerythrin (PE)-conjugated rat anti-mouse VLA-4, and PE-conjugated rat anti-mouse TER-119. A mixture of 250 µL of cell suspension and 350 µL of PBS were centrifuged at 4500 rpm, 10°C for five minutes. Pellet was resuspended in 50 µL PBS and specific antibodies, and was then incubated in the ice box for twenty minutes. Flow cytometry analysis was performed to determine the expression of TER-119$^+$CD34$^+$, TER-119$^+$VLA-4$^+$, TER-119$^+$ and Gr-1$^+$ cells.

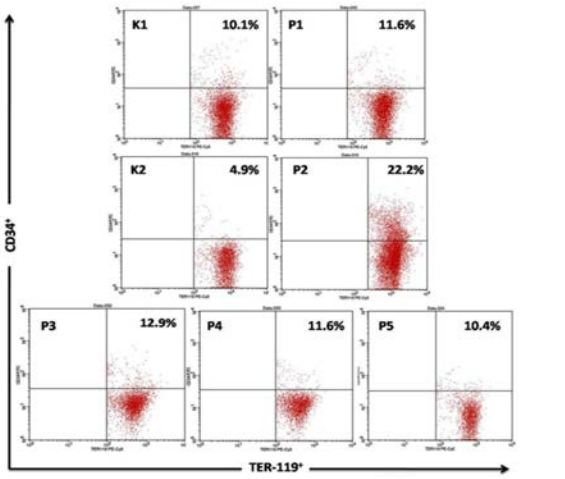
2.6. Data Analysis

Data were analyzed by the BD Cell Quest PRO™ software and were then analyzed statistically using SPSS (version 16.0) for Windows using one-way ANOVA. Significant data were further tested using Duncan test.

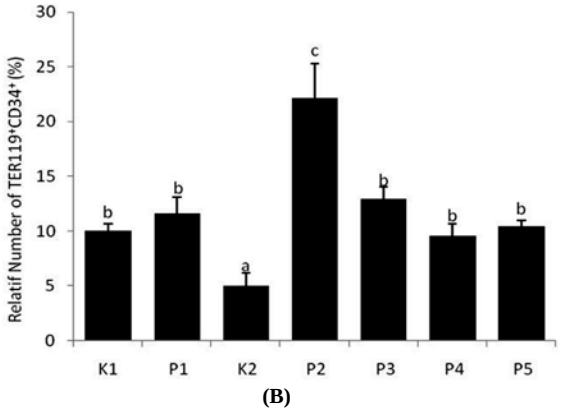
3. Results

3.1. Elicited Soybean Extract (ESE) on Red Blood Cells Progenitor TER-119+CD34+

The level of progenitor cells TER-119$^+$CD34$^+$ significantly decreased after the HFFD administration for twenty-four weeks (4.9%) ($p < 0.05$) compared to normal (10.1%) (Figure 1). All ESE doses increased the level of TER-119$^+$CD34$^+$ (12.9%, 11.6%, 10.4%, respectively) and the highest number of TER-119$^+$CD34$^+$ cells was found in the HFFD-mice treated with Simvastatin (Figure 1). Moreover, the ESE of all doses and the Simvastatin administration normalize the relative number of TER-119$^+$CD34$^+$ cells.



(A)



(B)

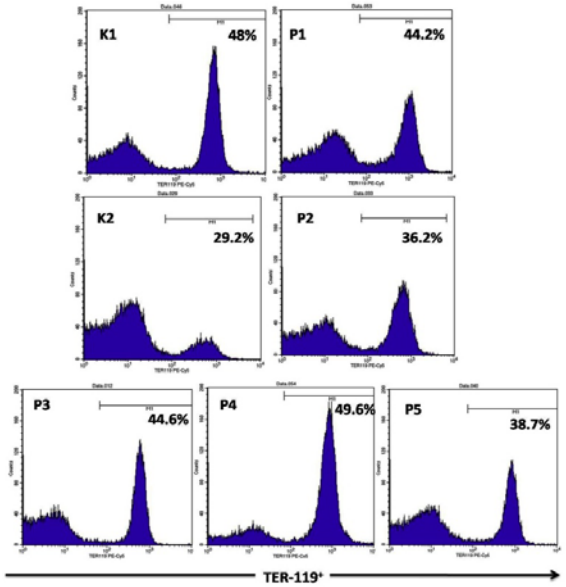
Figure 1. Elicited soybean extract (ESE) improved the level of red blood-cell progenitor TER-119$^+$CD34$^+$ in the bone marrow. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) Flowcytometry analysis of TER-119$^+$CD34$^+$ expression in bone marrow cells. All ESE doses and Simvastatin increased the level of TER-119$^+$CD34$^+$ (B) TER-119$^+$CD34$^+$ expression in each group analyzed by One-way ANOVA. ESE improved the hematopoietic system by increasing the level of TER-119$^+$CD34$^+$ in all doses compared to the HFFD control. Letters above each bar indicate a significant difference.

3.2. Elicited Soybean Extract on Erythropoiesis

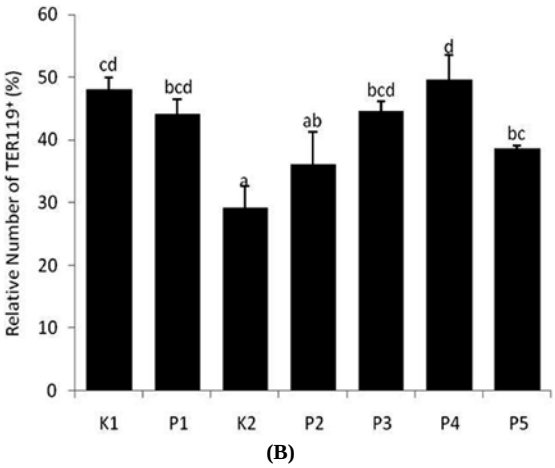
This study also observed the expression of erythroid TER-119$^+$ and reticulocytes TER-119$^+$VLA-4$^+$ in bone marrow cells. The HFFD administration for twenty-four weeks decreased the level of erythroid TER-119$^+$ (29.2%) compared to the normal group (48%) ($p < 0.05$). The administration of ESE from low to high doses was able to improve erythropoiesis by increasing the level of erythroid TER-119$^+$ significantly in the HFFD group ($p<0.05$) (44.6%, 49.6 %, 38.7 %, respectively) (Figure 2).

Moreover, the TER-119$^+$VLA-4$^+$ level is also decreased in the HFFD group (40.6 %) compared to the normal group (63.9 %) ($p < 0.05$) (Figure 3). The ESE dose of 104 mg/kg BW which was given to the normal group increased the level of TER-119$^+$VLA-4$^+$ (54.1 %) but with no significant difference from normal and HFFD groups. The HFFD mice were given ESE doses of 104 mg/kg BW and 130 mg/kg BW which significantly increased the expression of TER-119$^+$VLA-4$^+$ (79.8 %, 70.2 %, respectively) ($p<0.05$), while the 78 mg/kg BW increased the level of TER-119$^+$VLA-4$^+$ approaching the normal group (50%). The HFFD mice that were given 2,8 mg/kg BW of Simvastatin also had the level of TER-119$^+$VLA-4$^+$ increased in the HFFD group (79.4%) ($p<0.05$) (Figure 3).

In the erythroid lineage, all doses of ESE were capable to normalize the level of hematopoietic cells of the HFFD mice from the early stage up to the late stage. In the early stage of erythroid development, ESE was capable to normalize the level of progenitor red blood cells. ESE also normalized the level of red blood-cell precursors in the late development of erythroid lineage.



(A)



(B)

Figure 2. The level of erythroid cells TER-119$^+$ increased after ESE treatment. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) TER-119$^+$ expression in bone marrow cells. All doses of ESE and Simvastatin increased the level of TER-119$^+$ in HFFD mice (B) TER-119$^+$ expression comparison between each group analyzed by One-way ANOVA. All doses of ESE improved the level of erythroid TER-119$^+$ in the HFFD-fed mice. Letters above each bar indicate a significant difference.

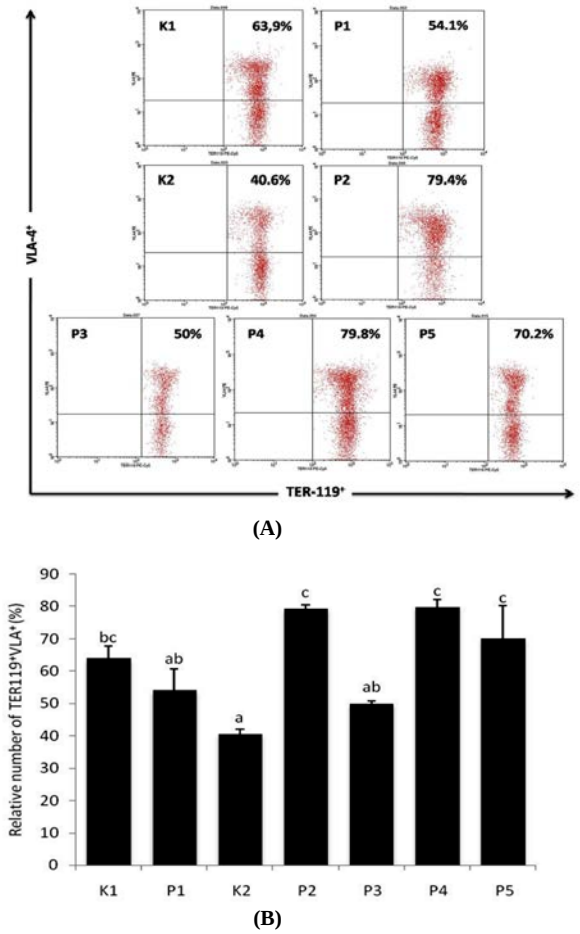


Figure 3. The level of reticulocyte cells TER-119$^+$VLA-4$^+$ increased after ESE treatment. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) Flowcytometry analysis of TER-119$^+$VLA-4$^+$ expression in bone marrow cells. All doses of ESE and Simvastatin increased the level of TER-119$^+$ VLA-4$^+$ in the HFFD-fed mice (B) The bars show the expression of TER-119$^+$VLA-4$^+$ in each group. Simvastatin and all doses of ESE improved the level of erythroid TER-119$^+$VLA-4$^+$ in the HFFD-fed mice. Letters above each bar indicate a significant difference.

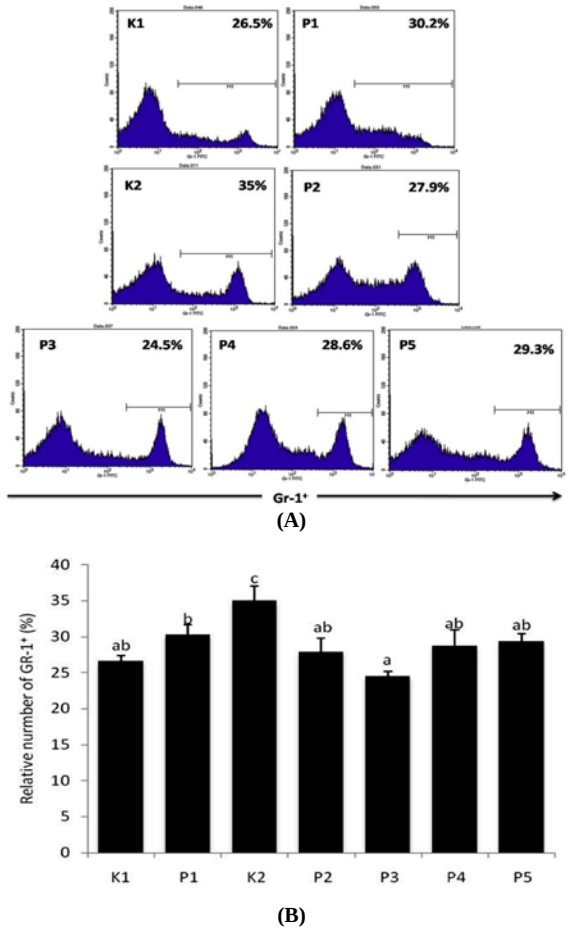


Figure 4. Elicited soybean extract (ESE) treatment decreased the level of granulocyte precursors Gr-1$^+$ in bone marrow cells. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) Gr-1$^+$ expression in bone marrow cells. All doses of ESE and Simvastatin decreased the level of Gr-1$^+$ in the HFFD-fed mice (B) The bars show the expression of Gr-1$^+$ between each group analyzed by One-way ANOVA. Simvastatin and all doses of ESE decreased the level of granulocytes precursors Gr-1$^+$ in the HFFD-fed mice. Letters above each bar indicate a significant difference.

3.3. Elicited Soybean Extract on Granulopoiesis

HFFD feeding increased the level of Gr-1$^+$ (35%) compared to the normal group (26.5%) (p<0.05). ESE in all doses suppressed the expression of Gr-1$^+$ in bone marrow (p<0.05), (24.5%, 28.6%, and 29.3%, respectively). Similar to the ESE results, the administration of Simvastatin dose of 2,8 mg/kg BW also decreased the level of Gr-1$^+$ until close to the normal group level (27.9%) (p<0.05) (Figure 4).

4. Discussion

Nowadays, obesity is a common health problem. The prevelence of obesity involves adults and children and its rates are increasing in most countries arround the world. The lack of physical activity, as well as the high fat-sugar food consumption may increase the occurrence of obesity (Antipatis and Gill, 2001). On the other hand, the consumption of foods with rich nutrient content can reduce the risk of obesity (Troesch *et al*., 2015).

Soybeans contain rich nutrients both in raw, as well as in modified forms. The elicitation of soybeans can increase the isoflavone contents, such as genistein and daidzein and can also produce glyceollin, a new secondary metabolite compound (Feng *et al*., 2007; Park *et al*., 2010; Aisyah *et al*., 2013).

This study shows that the HFFD administration leads to obesity (data not shown) and may be associated with an inflammation that affects the hematopoietic system. The

HFFD-fed mice have impaired red blood-cell development by decreasing the level of red blood-cells progenitors (TER-119$^+$CD34$^+$) and (TER-119$^+$ and TER-119$^+$VLA-4$^+$). The impairment in the hematopoietic system may be related to an inflammation that leads to erythropoiesis impairment and interferes with iron absorption which disrupts the erythroid maturation in red blood-cell production (Ganz, 2005; Martins, 2014). A previous study has shown that the inflammation decreased the regulator complement of red blood-cells, resulting in the lysis of red blood-cells progenitor (Safitri *et al.,* 2017). The inflammation was also marked by cytokines pro-inflammatory production such as TNF-α, IL-1β, and IL-6 (Kraakman *et al*., 2014). One study related to hematopoietic cells showed that after TNF-α treatment, cell membrane transduced many signals for apoptosis and necrosis (Jurisic *et al*., 2011). Another study indicated that TNF-α inhibits CD34$^+$ hematopoietic progenitor cells proliferation (Rusten *et al*., 1994).

The ESE treatment for four weeks improved the red blood-cells development in the HFFD-fed mice. Every ESE dose normalized the level of TER-119$^+$CD34$^+$ progenitors cells in the HFFD-fed mice. These results are supported by previous studies which stated that genistein has a role in regulating hematopoietic progenitor cells to reduce the risk of DNA damage in the bone marrow cells (Day *et al*., 2013). The expression of red blood-cell precursors, TER-119$^+$ and TER-119$^+$VLA-4$^+$ were normalized as well after four weeks of the ESE treatments. Similar to the ESE treatment, the simvastatin increased the TER-119$^+$CD34$^+$ progenitor cells to a level higher than all doses of ESE treatment, but the expression of TER-119$^+$ and TER-119$^+$VLA-4$^+$ cells have no significant difference compared to the ESE treatment. Simvastatin is well known to have a potential effect on the hematopoiesis improvement through the ability as an anti-inflammatory agent. Previous studies showed that simvastatin has a role as an anti-inflammatory agent, increasing the HSC number through the bone marrow improvement, and increasing erythropoiesis by targeting regulatory pathways of hepcidin and iron (Lefer, 2002; Chang *et al*., 2011; Bajaj *et al* 2015).

Our finding revealed that the effectiveness of the ESE is not only relevent to the red blood- cell early development, but also in the late development. This is indicated by the fact that ESE is able to normalize not only the level of progenitor cells but also the precursors which were decreased after the HFFD treatment. Glyceollin and daidzein in ESE have the potential effects of reducing ferric ion (Fe^{3+}) to be a ferrous ion (Fe^{2+}), thus making it easier to be absorbed (Kim *et al*., 2010). Iron absorption is an important factor in hemoglobin synthesis (Hoffbrand and Moss, 2011). Vitamin C in the soybean improves the iron absorption for hemoglobin synthesis during erythropoiesis and is able to prevent the formation of insoluble and unabsorbable iron (Hallberg *et al*., 1989). However, calcium which is also found in the soybean has a contrastive effect on hematopoiesis. Calcium inhibited the iron absorption for the hemoglobin synthesis by competing with iron to bind with same substrates which have an important role in the iron absorption process (Hallberg *et al*., 1991). This condition may explain why the potential dose of ESE in each parameter is different.

Obesity induces Granulocyte-Colony Stimulating Factor (G-CSF) to stimulate the production of granulocytes and further increase the recruitment of macrophages by adipose cells (Benites *et al*., 2014). Obesity increases Hematopoietic Stem Cell (HSC) differentiation into monocytes (Singer *et al*., 2014). HSC proliferation and differentiation in the bone marrow occur continuously in response to inflammation and decrease the ability to repair themselves (self-renewal) eventually causing imbalanced blood-cell production (Schuettpelz and Link, 2013). This study indicated that the level of Gr-1$^+$ increased significantly in the bone marrow cells of the HFFD-fed mice. Gr-1 is a specific marker for granulocytes in white blood-cell production (granulopoiesis). After the ESE treatment, Gr-1$^+$ level decreased significantly approaching the normal value. This finding can be attributed to the ability of genistein as anti-inflammatory (Kim *et al*., 2011) which reduces the ability of G-CSF to induce the proliferation of HSC occuring continuously due to the inflammation, but does not reduce the ability of G-CSF in accelerating the granulocytes differentiation (Zhou *et al*., 2005; Day *et al*., 2013; Souza *et al*., 2014).

The current study speculated that the ESE contents, such as glyceollin, genistein, daidzein, vitamins among others related to each other in improving the hematopoietic system. Based on the previous explanation, ESE can be used as an alternative medicine because of the effectiveness of its herbal ingredients as anti-inflammatory. Moreover, ESE is more likely to have fewer side effects than synthetic medicines.

5. Conclusion

Elicited soybean extract (ESE) improves the development of hematopoietic cells by increasing the level of TER-119$^+$CD34$^+$, TER-119$^+$, and TER-119$^+$VLA-4$^+$ as well as lowering the level of Gr-1$^+$ in the bone marrow cells of HFFD-fed mice. ESE can be an effecctive anti-inflammatory agent due to its ability to improve the hematopoietic system of obese mice.

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