Immunological Responses in Female Rats Model with Anemia Treated with Snake Fruit Seed Flour Extract

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Abstract. Natural substances become popular for alternative treatment of human diseases including anemia. A recent study has reported that administration of snake fruit seed flour (SSF) is able to increase hemoglobin level in rat model with anemia but it needs higher amount of SSF. In addition, SSF contains 3.71% protein/100g that potentially results in hypersensitivity. The aim of this study was to evaluate the side effects of SSF extract on leucocytes number and its differential account (granulocytes, lymphocytes, and monocytes) in rat model with anemia. We used 14 female rats model with anemia, aged 8-10 weeks, and had ± 200 g body weight (BW). After rats model with iron deficiency anemia (IDA) has been established, female rats were randomly divided into 2 groups: control (C) group only received a low iron diet, and treatment (T) group received a low iron diet and 1.75g/kg BW/day SSF extract for 14 days. Leucocytes number was measured using a veterinary haematology analyzer. Collected data were analyzed using the independent T-test with p value <0.05. Female rats in the C group had the mean leucocytes number higher than the T group, but not statistically different (p=0.073). From differential count, greater numbers of granulocytes and monocytes were observed in the C group, compared to the T group (p=0.301;p=0.34). In contrast to granulocyte and monocytes number, the mean lymphocytes number in the C group were statistically different than the T group (p=0.046). Overall, administration of SSF extract was able to decrease leucocytes number and differential count. Administration of 0.175g/100g BW/day SSF extract does not increase immunological responses in female rats model with anemia.

Keywords: Anemia, Leucocytes, Immunological responses, Snake fruit seed extract

1. Introduction
Iron deficiency is commonly found in some susceptible age groups such as children, female adolescents, and pregnant women [1,2]. Based on a global survey, the prevalence of anemia is 1.93 million people or 27% in 2013 and 59.5% anemia cases are due to iron deficiency [3,4]. In Indonesia, the prevalence of anemia belongs to moderate category. According to data of Riskedas (2013), the prevalence of anemia in children above 1 years old is 21.7% and 23.9% in women, higher than men.
(18.4%) [5,6]. Meanwhile, the prevalence of anemia in pregnant women has increased from 37.1% in 2013 to 48.9% in 2018 [7].

Some studies have indicated that iron deficiency anemia (IDA) has conflicting results toward the immune system. In general, IDA can be due to increase of iron requirement, to decrease of iron intake or absorption or to increase of iron loss [8]. As a results, hemoglobin levels reduce below the normal range and it changes morphology of red blood cells [9]. A recent study conducted in 200 respondent (182 female and 18 male), age 32-52 years with IDA shows that total leucocytes number and its differential account are in normal range [10]. In contrast to this study, Samanta and Senapati (2018) reported that 30 respondent (19 female and 11 male), age 20-40 years with nutritional anemia have higher total leucocytes number than that of control group, but it is not statistically different [11]. In addition, hypoxia and ischemia are strongly associated with anemia, which lead to increase of white blood cells [12,13,14].

Iron-fortified foods and iron supplementation are the most recommended therapy for IDA [15, 16, 17,18]. Administration of iron tablet frequently has some side effects, such as abdominal pain, vomiting, and diarrhea, so that some anaemic patients do not complete their treatment. Now days, natural substances become popular for an alternative treatment of human diseases including anemia. A recent study has reported that administration of 18.6 g/kg BW/day SSF in rat model with anemia can restore hemoglobin levels after 4 weeks treatment [19]. It is not surprising that SSF administration can be used for anemia treatment because 100 g SSF contains 26.8 mg iron, 4.56 mg zinc, and 0.88 mg vitamin C. If this dose of SSF administration is converted to human with anemia, it will need high amount of SSF. In order to decrease the amount of SSF, we have extracted SSF by using a maceration method with ethanol solvent. Therefore, the aim of this study was to evaluate the side effects of SSF extract on total leucocytes number and its differential account in rat model with anemia.

2. Methods
An experimental laboratory with post test control group design was conducted in this study during 24 days in May 2019. Approval of this study protocol was obtained from The Ethics Committee of Medical Research, Faculty of Medicine, Universitas Sebelas Maret, Surakarta (No. 165/UNS27.6/KEPK/2018). Female Wistar rats, which used in this study were got from Laboratory Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta.

2.1. Preparation of SSF Extract
Snake fruit seeds, which were waste of processed fruit production in three home industries at Turi district, Sleman Regency, Yogyakarta, were used as raw material for making SSF extract. We modified the existing method to extract SSF, using maceration with ethanol solvent [20]. SSF extract was prepared at Food Engineering Laboratory, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta. Iron level in SSF extract was measured using Atomic Absorbance Spectrometry (AAS) in Research Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah, Surakarta.

2.2. Rat Model with Iron Deficiency Anemia Care
Wistar rats were kept in clean plastic cage with a wire and controlled environmental conditions in the Animal Research Centre, Laboratory for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta. The room was maintained at 27-29°C, 12 hours light and 12 hours dark, and 70-90% humidity [21]. Rats were given 10% BW of AIN-93M standard diet, and distilled water ad libitum. All foods and drink were provided by the Nutrition Laboratory. Development of rat model with IDA was adopted from Susanti et al (2017), giving by a low-iron diet (iron depletion from AIN-93M standar feed) for 10 days [22].

2.3. Study Design
A total of 14 female Wistar rats aged 8-10 weeks, had ± 200 g body weight (BW), and were healty condition were used in this study. Once rats model with IDA has been established, they were randomly
divided into 2 groups: control (C) and (T) treatment groups. Anemic rats in the C group only received a low iron diet, whereas anemic rats in the T group received a low iron diet and 1.75g/kg BW/day SSF extract for 14 days. The administration of SSF extract proportionally correlated with daily iron requirement and was given to rats using a 3 ml gastric canule every morning [19].

2.4. Data Analysis
Blood samples were taken from orbital vein for hematological studies and collected using vials with EDTA anticoagulant. Total leucocytes account was measured using a veterinary haematology analyzer at the Animal Hospital Prof. Soeparwi, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta. The independent t-test was used to analyze the differences of total leucocytes, granulocytes, lymphocytes, and monocytes between C and T groups with p value <0.05.

3. Results and Discussion
We developed IDA in 14 female rats by depletion of iron in the rats standard diet for 10 days. Table 1 showed basic characteristics of female rats with IDA. The average of BW in the C group (184.14±3.97 g) was significantly lower than the average of BW in the T group (193.29±3.35 g) with p= 0.001. Additionally, female rats in the C group had 9.65±0.57 g/dl haemoglobin level and lower than the average of haemoglobin level in the T group 9.88±0.49 g/dl, but not significantly different (p=0.444).

IDA can affect the body weight of rats, that caused by reduced enzyme activity and oxygen supply to cells, so that decrease in cell energy and disturb body's metabolism [23].

Table 1. Characteristics of Female Rats Model with IDA.

| Characteristics | C Group | T Group | p-value |
|-----------------|---------|---------|---------|
| BW (g)          | 184.14±3.97 | 193.29±3.35 | 0.001   |
| Hemoglobin (g/dl) | 9.65±0.57  | 9.88±0.49  | 0.444   |

In general, we found that female rats in the T group treated with SSF extract for 14 days had lower leucocytes, compared to female rats in the C group (Figure 1a-d). Higher mean total leucocytes account (13.78±4.5 x10^3/μl) was observed in female rats of C group, compared to female rats of T group (10.05±2.19 x10^3/μl) with p=0.073. From differential count, the average of granulocytes, lymphocytes, and monocytes in the C group was greater than the average of granulocytes, lymphocytes, and monocytes in the T group. However, a significant difference was only found in the average of lymphocytes (p=0.046).

Study by Singh (2010) reported in 20 patient with nutritional anemia, total number of leucocytes in the anemia group was higher than the control group, but not statistically different, and on differential count, there was an increase in the number of neutrophils, without changes in the number of lymphocytes [12]. This study indicate the same result with study by Samanta and Senapati (2018), reported that 30 respondent (19 female and 11 male), age group of 20–40 years with nutritional anemia, showed that total leucocytes number in anemia group is higher than control group, but statistically not significant [11]. However, these results are conflicting with the study by Burak et al (2016), a study conducted on 200 respondent (182 female and 18 male), age 32-52 years with IDA showed total leucocytes number and its differential number are in normal range [10]. Our study indicated the same result with Samanta and Senapati’s, total leucocytes number in C group was higher than T group, but it was not statistically different.
Anemia is associated with hypoxia and ischemia, an increase in total leukocytes (although still within the normal range) is associated with an increased risk of heart disease, and can be a sign of haematological stress syndrome [14,10]. Differential leukocytes that play a role as a marker of ischemic heart diseases is neutrophils to lymphocyte ratio (N/L ratio) [11].

Increased leucocytes number can occur because erythrocyte production is impaired due to a decrease in iron levels. The erythropoiesis process is inhibited because hypoferremia maintains the amount of iron for other metabolic needs during infection, thereby increasing glucocorticoid activity to increase the release of many nucleated leukocytes from the bone marrow. In addition, inflammatory cytokines directly suppress the erythropoiesis process, increase leucocyte production, and activate macrophages to improve erythrophagocytosis [12,11,24].

Granulocyte is a leukocyte group consisting of neutrophils, esinophils and basophils. Neutrophils will increase in response to inflammation. Our study showed granulocytes number of C group was higher than the T group, but statistically not significant. Iron deficiency will affect the regulatory
function of T-Lymphocytes and immune cells. The N/L ratio is an inflammatory marker of IHD, myocardial infarction, solid tumour, and colon cancer [13,14,25,26]. The study by Gosai et al (2013), showed no significant difference of total leucocytes number in anemia and control group, but in the anemia group the N/L ratio was significantly higher than control group, this was due to the neutrophils numbers in anemia group significantly increase than control group [14]. Another study by Samanta and Senapati (2018), also reported an increase in the N/L ratio significantly in the anemia group compared to the control group [26]. However, in this study it has not been known whether the increase in leucocytes and lymphocytes in the control group will increase the risk of incidence IHD, MI and etc, because no neutrophils number is performed.

The role of monocytes in anemia cannot be determined with certainty. Inflammatory cytokines including TNF-α, IL-1 and Interferon-γ play a role in inhibiting the erythropoesis [27]. The study by Librets et al (2011), mice with overproduction of interferon-γ caused mild to moderate normocytic anemia. Interferon-γ activates macrophages in the splenic red pulp causing a 50% reduction in erythrocytes. Increased interferon-γ is done by induction of viruses or intracellular pathogen infection, which causes increased production of monocytes and macrophages and suppresses the erythropoesis process [28].

4. Conclusion
Administration of 1.75g/kg BW/day SSF extract for 14 days not increases leucocytes number in female rats model with anemia. Lymphocyte number in female rats significantly higher in C group, while, total leucocyte, granulocytes, and monocytes amount in C group insignificantly different with T group. We require further investigation whether or not administration of SSF extract can result in allergic responses.

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