The effect of NaFeEDTA-fortified soy milk on the red blood cell counts of male Sprague Dawley rats (*Rattus norvegicus* L.)

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Abstract. This study explored the effect of NaFeEDTA-fortified soy milk on red blood cell counts of male Sprague-Dawley rats (*Rattus norvegicus* L.). Using a completely randomized design (CRD), 25 rats were divided into five groups: the normal control group, which received standard food and water without the addition of soy milk or fortificant; treatment control group, which received extra soy milk without fortificant; and three treatment groups, which received extra soy milk containing NaFeEDTA as a fortificant at 2.7 (treatment group 1), 5.4 (treatment group 2) and 10.8 mg Fe/kg bodyweight (treatment group 3). All the five groups were treated for 21 consecutive days. Rat red blood cell counts were measured using a hematology analyzer. One-way ANOVA and the least significant difference post hoc test showed that after 21 days of consecutive treatment, there was a significant effect on the red blood cell count in all the treatment groups compared with the normal control and treatment control groups. The highest increase in the red blood cell count was detected in treatment group 2 at t21, with a 19.70 % increase compared with the normal control group and 17.27 % compared with the treatment control group.

Keywords: Fortificant, NaFeEDTA, *Rattus norvegicus* L., red blood cell, soya milk

1. Introduction
Iron is the most essential microelement in the body. It is required in blood plasma for erythropoiesis; uptake of iron by erythroid cells (precursors of red blood cells) is mediated by receptors on the cell surface, and the iron is then incorporated into the hemoglobin required by mature red blood cells [1]. Sources of dietary iron generally contain either heme or nonheme iron, each with a different absorption rate. Approximately 25 % of heme iron can be absorbed by the body, unlike only 5 % of nonheme iron [2]. This is because of inhibitor compounds found in foods containing nonheme iron, which binds iron and prevents it from being absorbed by the intestine [3]. Therefore, efforts to increase the bioavailability and absorption of iron are of critical importance.

Indonesians generally obtain their dietary iron from vegetable foodstuffs, such as soybeans. One of the soybean-based foods consumed is soy milk, which is affordable and can be used by individuals with
dairy allergies and lactose intolerance as a protein substitute for cow’s milk [4]. Every 100 g of soy milk contains as high as 0.64 mg of iron [5]. However, soy milk contains nonheme iron, which is less bioavailable than heme iron [2].

One of the efforts taken to improve the bioavailability and absorption of iron is the fortification of various foodstuffs with additional iron. A commonly used iron fortificant is NaFeEDTA. It has several advantages, such as increasing the bioavailability of iron and not changing the color or flavor of the fortified food [6]. In addition, in vivo research conducted by Pranoto [7] demonstrated that the addition of NaFeEDTA to soy-based foods, such as soy milk, can increase the iron levels in the plasma of rats. Munawaroh [8] demonstrated that the administration of roselle (Hibiscus sabdariffa) petal extract containing iron can affect the red blood cell count of rats. Therefore, increased blood iron levels in rats administered NaFeEDTA-fortified soy milk [7] are also expected to affect the red blood cell counts. The objective of the present study was to determine the effect of NaFeEDTA-fortified soy milk on the red blood cell counts of rats.

2. Materials and method

2.1. Materials
Soybeans used in this study were obtained from a supermarket in Jakarta. NaFeEDTA was synthesized by the Inorganic Chemistry Laboratory, Department of Chemistry, University of Indonesia, Depok. The feed given to the test animals during this study was a rat feed pellet product of CV PD Kasman, Jakarta.

2.2. Production of soy milk and fortified soy milk
Soybeans were washed and soaked for 10–12 h, followed by blending and straining. The filtrate was pasteurized using the low temperature long time (LTLT) procedure at 62–71 °C for 30 min and then cooled. The appropriate NaFeEDTA doses were administered in each treatment group: 20.3098, 40.6196, and 81.2391 mg/10 mL of soy milk. Soy milk used during this study was freshly prepared every day.

The dose of the fortificant was determined using the following formula:

$$\text{Added Fortificant (mg)} = \text{Dose Fe in vivo} \times \frac{\text{Mr}^* \text{NaFeEDTA}}{\text{Ar}^{**} \text{Fe}}$$

with *Relative molecular mass, and **Relative atomic mass

2.3. Test animals
Twenty-five male Sprague-Dawley rats (Rattus norvegicus L.) aged approximately 2 months were obtained from the Health Research and Development Agency, Jakarta. They were housed in five plastic cages (27–28 °C with a 12 h light/dark cycle), and each cage contained five rats according to the treatment groups. Rats in the normal control group were only fed standard food and water without the addition of soy milk or fortificant. Rats in the treatment control group were fed extra soy milk without any fortificant. Treatment groups 1, 2, and 3 were fed extra soy milk fortified with NaFeEDTA, with 2.7, 5.4, and 10.8 mg Fe/kg bodyweight, respectively. Food and water were provided ad libitum. Rats were treated for 21 consecutive days, and blood sampling was performed at the start (t0) and end (t21) of the study. The body weight of the rats was measured on each day during the study period, and the volume of soy milk administered was adjusted to their weight, as determined by the following equation:

$$\text{Treatment volume} = \frac{\text{weight of the test animal (g)}}{100 \text{ g}} \times 1 \text{ mL}$$
2.4. Measurement of rat red blood cell count
Blood was drawn from the orbital sinus, placed into an EDTA vacutainer, and homogenized by inverting. The red blood cell count was then measured using a hematology analyzer.

2.5. Statistical analysis
Statistical analysis was conducted using the Statistical Product and Service Solution (SPSS) software for Windows, version 16. One-way ANOVA and least significant difference (LSD) post hoc tests were used to analyze the differences in red blood cell counts between the treatment groups (significance, \( p \leq 0.05 \)).

3. Results and discussion

3.1. Initial red blood cell counts (t_0)
Measurements of the initial red blood cell count (t_0) in all the treatment groups are shown in table 1. The initial counts in the NC, TC, T1, T2, and T3 groups were 7.22 ± 0.11 (× 10^6/μL), 7.16 ± 0.21 (× 10^6/μL), 7.33 ± 0.12 (× 10^6/μL), 7.31 ± 0.10 (× 10^6/μL), and 7.27 ± 0.20 (× 10^6/μL). Statistical analysis indicated that the data were homogeneous, and there were no significant differences between the treatment groups (figure 1).

3.2. Final red blood cell counts (t_21)
Measurements of the final red blood cell count (t_21) in all the treatment groups are shown in table 1. The average red blood cell counts in all the groups were 7.26 ± 0.16 (× 10^6/μL), 7.41 ± 0.12 (× 10^6/μL), 8.10 ± 0.17 (× 10^6/μL), 8.69 ± 0.11 (× 10^6/μL), and 8.29 ± 0.14 (× 10^6/μL). Statistical analysis indicated significant differences between the treatment groups (\( p \leq 0.05 \); figure 1).

Table 1 showed that t_0 in all the groups was in the range of 6.91–7.48 (× 10^6/μL). According to Sharp and Villano [9], the normal range of red blood cell counts in rats is 5.00–10.00 (× 10^6/μL); thus, all the initial red blood cell count data are within the normal range. The mean t_0 and t_21 red blood cell count data in the normal control group ranged from 7.06 to 7.46 (× 10^6/μL). The data show that the red blood cell count in the normal control group was relatively stable throughout the study and remained within the normal range, making this data an appropriate reference for changes in red blood cell count in the treatment control and NaFeEDTA-fortified treatment groups (1, 2 and 3).

Red blood cell count increased slightly (2.07 %) in the treatment control group compared with the normal control group (table 2), possibly because their soy milk intake increased their overall iron intake. In addition, the soy milk also contained 18 μg of folic acid per 100 g [5], which also plays a role

| Repetition | Initial red blood cell count (t_0; × 10^6/μL) | Final red blood cell count (t_21; × 10^6/μL) |
|------------|---------------------------------------------|---------------------------------------------|
|            | NC  | TC  | T1  | T2  | T3  | NC  | TC  | T1  | T2  | T3  | NC  | TC  | T1  | T2  | T3  | NC  | TC  | T1  | T2  | T3  | Mean | SD |
| 1          | 7.29| 6.96| 7.39| 7.19| 7.18| 7.20| 7.56| 7.94| 8.69| 8.23|    |    |    |    |    |    |    |    |    |    |    |    |
| 2          | 7.15| 7.36| 7.21| 7.27| 6.97| 7.08| 7.48| 8.21| 8.81| 8.29|    |    |    |    |    |    |    |    |    |    |    |    |
| 3          | 7.34| 7.26| 7.34| 7.45| 7.42| 7.41| 7.29| 7.89| 8.79| 8.10|    |    |    |    |    |    |    |    |    |    |    |    |
| 4          | 7.26| 6.91| 7.48| 7.38| 7.31| 7.46| 7.45| 8.28| 8.54| 8.48|    |    |    |    |    |    |    |    |    |    |    |    |
| 5          | 7.06| 7.29| 7.22| 7.26| 7.46| 7.17| 7.28| 8.16| 8.62| 8.34|    |    |    |    |    |    |    |    |    |    |    |    |
| Mean       | 7.22| 7.16| 7.33| 7.31| 7.27| 7.26| 7.41| 8.10| 8.69| 8.29|    |    |    |    |    |    |    |    |    |    |    |    |
| SD         | 0.11| 0.21| 0.12| 0.10| 0.20| 0.16| 0.12| 0.17| 0.11| 0.14|    |    |    |    |    |    |    |    |    |    |    |    |

NC: Normal control group; TC: Treatment control group; T1: Treatment group 1; T2: Treatment group 2; T3: Treatment group 3
in the maturation of red blood cells [1]. However, this increase was not statistically significant (figure 1). This was likely a result of other compounds contained in the soy milk, such as phytic acid. Phytic acid compounds may act as iron ligands or binders, forming insoluble Fe-phytate complexes, thus reducing iron absorption [10].

Significant increases in the red blood cell count also occurred in all treatment groups (1, 2 and 3) as compared to the normal control and treatment control groups (table 2) (p ≤ 0.05). These increases in red blood cell count were due to the Fe and chelating agent EDTA in the NaFeEDTA fortificant; the EDTA compounds shield iron from inhibitor compounds (such as the phytic acid present in soy milk) by forming Fe(III)EDTA complexes [11].

Iron derived from soy milk or NaFeEDTA is of the nonheme type, which is a form of ferric ion (Fe³⁺), and must first be converted to the Fe²⁺ form by a ferrireductase to be absorbed by the intestine [12]. Iron in the Fe⁴⁺ form is readily attached to either inhibitor or facilitator compounds; if Fe³⁺ iron binds to an inhibitor compound, such as phytic acid, an insoluble Fe-phytate complex is formed, which prevents the iron from being absorbed by the intestine [13]. However, if the Fe³⁺ iron attaches to a facilitator compound, such as EDTA, a soluble Fe⁴⁺ iron complex is formed, which is easily reduced by ferooxidase to form Fe²⁺ [11]. The NaFeEDTA fortificant present in food is broken down in the stomach. Sodium is separated from Fe(III)EDTA complex, while the Fe(III)EDTA complex is maintained. The Fe(III)EDTA bonds become stronger and more stable in the acidic environment of the stomach, preventing the iron from binding inhibitor compounds and allowing it to be absorbed by the intestine [14].

Iron that has been absorbed by the intestine and stored in the form of ferritin then transported by ferroportin toward the basolateral gut, where it is converted back to Fe³⁺. The Fe³⁺ then binds apotransferrin, converting it to transferrin, which eventually circulates Fe³⁺ to the body tissues [15].

Iron in the form of transferrin is released into the bone marrow, which contains erythroid cells. Fe-transferrin has the ability to bind very strongly with receptors in the erythroid cell membranes, such as transferrin receptor 1 (TfR1), which allow it to enter the cells. Acidic conditions inside the erythroid cells cause the Fe-transferrin to dissociate into Fe³⁺ and apotransferin-TfR1. Apotransferin-TfR1 then

![Figure 1. Diagram of average initial (t₀) and final (t₂₁) red blood cell counts during the present study. Different letters indicate significant differences (p ≤ 0.05).](image)

| Normal control group | Treatment control group |
|----------------------|------------------------|
| Treatment control group | 2.07 %                 |
| Treatment group 1     | 11.57 %                |
| Treatment group 2     | 19.70 %                |
| Treatment group 3     | 14.19 %                |
|                       | 9.31 %                 |
|                       | 17.27 %                |
|                       | 11.88 %                |
returns to the cell membrane, and Fe$^{3+}$ is reduced to Fe$^{2+}$ by STEAP3 metalloreductase. Fe$^{2+}$ is then be carried by DMT1 toward the cytoplasm and directed to the mitochondria by Mfrn1, where it is used to synthesize heme [16]. Next, heme binds the alpha and beta polypeptide subunits to form hemoglobin [1].

The highest increase in the red blood cell count compared with the treatment control group, was observed in the treatment group 2. This may be because the dose of NaFeEDTA used in the treatment group 2 was optimum for minimizing the effects of phytic acid. In addition, EDTA in the treatment group 2 is thought to be more associated with the iron derived from soy milk than that in treatment group 1. Thus, maximum quantity of iron was available for erythropoiesis in the treatment group 2. The ability of EDTA to bind iron from soy milk in the gastrointestinal lumen and carry it into the enterocytes is termed the “shuttle effect” [11].

Although red blood cell counts in all the treatment groups increased compared with the control group, there was a decrease in the red blood cell counts in the treatment group 3 compared with treatment group 2; this may be because the dosage of NaFeEDTA caused iron levels in the body to rise above the optimal level. When iron deposits are adequate or high, the liver produces hepcidin, a protein that plays a role in the regulation of blood iron levels. Hepcidin has the opposite mechanism as that of ferroportin; it transports iron out of the enterocytes as well as stimulates ferroportin degradation, leading to increased iron storage, decreased iron absorption, and decreased iron levels in the blood [17]. The iron regulation in the treatment group 3 likely caused a decrease in red blood cell counts compared with the treatment group 2. Despite this decrease, the red blood cell count was still higher in this group than those in the control group and treatment group 1.

4. Conclusion
In conclusion, NaFeEDTA in fortified soy milk may affect the red blood cell count of male Sprague-Dawley rats. The optimum dose of NaFeEDTA to increase the red blood cell count in the present study was 5.4 mg Fe/kg bodyweight. This dose successfully increased the red blood cell count in the treatment group 2 by 19.70 % compared with the normal control group and by 17.27 % compared with the treatment control group.

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