The effects of the fortificant NaFeEDTA in tofu flour on red blood cell counts in male Sprague-Dawley rats (Rattus norvegicus L.)

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Abstract. The effects of the fortificant NaFeEDTA in tofu flour on red blood cell counts in male Sprague-Dawley rats (Rattus norvegicus L.) were examined. In total, 25 rats were divided into five groups as follows: normal control (NC), which was administered 0.5 % carboxymethyl cellulose (CMC); treatment control (TC), which was administered 0.5 % CMC and unfortified tofu flour; and three treatment groups, which were administered 0.5 % CMC and tofu flour containing 2.7 (T1), 5.4 (T2), or 10.8 mg Fe/kg bw NaFeEDTA (T3). All five groups were treated for 14 consecutive days. Red blood cell counts were measured using an automatic hematology analyzer (Nihon Kohden Celltac MEK-6450). One-way ANOVA revealed significant effects (P < 0.05) of NaFeEDTA on red blood cell counts in all treatment groups. A least significant difference test illustrated that the red blood cell count was significantly higher (P < 0.05) in the three treatment groups than in the NC and TC groups. The largest increase of the red blood cell count was detected in the T2 group at t14, and the change reflected increases of 22.26 % versus the NC group and 20.24 % versus the TC group.

Keywords: NaFeEDTA, fortificant effect, tofu, sprague-dawley rats, red blood cell

1. Introduction
Soy is a plant-based protein source that is consumed by the majority of Indonesians. Soy is a popular food because it is healthy, affordable, and easy to obtain. Tofu is a commonly consumed soy product that is rich in protein and iron [1], an essential mineral required for the proper functioning of the human body [2]. This mineral participates in the formation of red blood cells (erythropoiesis) and circulation of oxygen from the lungs to tissues throughout the body. Iron exists in four forms in the human body: hemoglobin, myoglobin, storage iron (ferritin and hemosiderin), and transport iron (transferrin). Transferrin generally transports iron to the bone marrow, where it is used for erythropoiesis as the constituent structure of heme (hemoglobin), which transports oxygen for metabolic processes [3].
Heme iron (obtained from animal-based foods) and nonheme iron (obtained from plant-based foods) are the two major types of iron. Heme iron has greater bioavailability than nonheme iron (25% vs 5%) [4, 5]. The bioavailability of nonheme iron is low because of the presence of inhibitors of iron absorption such as phytic acid in plant-based foods. Phytic acid forms a complex bond with iron or other minerals, making the latter insoluble and difficult to be absorbed by the intestines [6]. The consumption of soy-based foods is not sufficient to increase the availability and absorption of iron. Low iron absorption can result in iron deficiency and subsequently iron deficiency anemia.

One strategy for overcoming iron deficiency is iron fortification using micronutrients called fortificants. Food fortification is defined as the addition of one or more essential nutrients (vitamins and minerals) to food in order to prevent or correct a demonstrated deficiency in the population. The process requires chelators, most commonly sodium iron (Fe⁺) ethylenediaminetetraacetic acid (NaFeEDTA) [7, 8]. Sodium EDTA binds strongly to iron in acidic environments (e.g., stomach), thereby preventing iron from binding to inhibitors such as phytic acid, permitting optimal absorption [7, 9].

Based on these considerations, we examined the effects of NaFeEDTA-fortified tofu flour consumption on red blood cell counts in male Sprague-Dawley rats (Rattus norvegicus L.). We also aimed to determine the optimum concentration of NaFeEDTA required for increasing red blood cell numbers.

2. Materials and method

2.1. Materials
Soybeans and tofu flour were obtained from a supermarket in Jakarta, Indonesia. NaFeEDTA was synthesized by the Inorganic Chemistry Laboratory, Department of Chemistry, Universitas Indonesia, Depok.

2.2. Production of tofu flour and fortified tofu flour
Soybeans weighing 15 g were washed and soaked with water for 10–12 h. The soaked soybeans were then blended until smooth and filtered, after which the filtrate was boiled using a hot plate. Then, 1.1% vinegar was added to the filtrate with slow stirring until clumps were formed. The clumps were filtered, placed on an aluminum foil, and heated in an oven at 60–70 °C for 15 h. Once dry, the tofu was blended into flour. Next, 0.45 g samples were divided for each treatments. Tofu flour was fortified by adding 20.31, 40.62, or 81.24 mg of NaFeEDTA, as indicated by the following formula:

\[
\text{Fortificant addition amount (mg) = dose Fe } \times \frac{\text{Mw NaFeEDTA}}{\text{Ar Fe}}
\]  

Mw: Relative molecular mass; Ar: Relative atomic mass

2.3. Test animals
A total of 25 ~2-month-old male Sprague-Dawley rats, each weighing around 200 g, were obtained from the Agency for Health Care Research and Development in the Ministry of Health, Jakarta. Groups of five rats were kept in plastic cases (43 × 37 × 15 cm³) at 27–28 °C with a 12-h/12-h light/dark cycle. The rats were divided into five groups as follows: normal control (NC, 0.5% carboxymethyl cellulose [CMC]); treatment control (TC, 0.5% CMC + 0.45 g of tofu flour); treatment group 1 (T1, 0.5% CMC + 0.45 g of tofu flour 0.45 g + 2.7 mg Fe/kg bw NaFeEDTA); treatment group 2 (T2, 0.5% CMC + 0.45 g of tofu flour 0.45 g + 5.4 mg Fe/kg bw NaFeEDTA); and treatment group 3 (T3, 0.5% CMC + 0.45 g of tofu flour 0.45 g + 10.8 mg Fe/kg bw NaFeEDTA). Feed and water were provided ad libitum. Rats were weighed daily during the study period. Rats were treated for 14 consecutive days, and blood sampling was performed at the beginning (t₀) and end of the study (t₁₄). The amount of tofu flour administered to the animals was derived using the following formula:
2.4. Red blood cell count
Blood samples were obtained from the eye (retro orbital plexus) via venipuncture using a capillary pipe and collected into tubes containing 0.5 mL of EDTA K3. Red blood cell counts were measured using a hematology analyzer.

2.5. Statistical analysis
Statistical analysis was performed using Statistical Product and Service Solution for Windows version 23. Data were analyzed using the Levene test, Shapiro Wilk test, one-way ANOVA, and a multiple comparison test (least significant difference).

3. Results and discussion

3.1. Initial red blood cell count (t0)
The initial red blood cell counts in the five groups are shown in table 1. The initial counts in the NC, TC, T1, T2, and T3 groups were 7.31 ± 0.18, 7.39 ± 0.33, 7.48 ± 0.14, 7.41 ± 0.28 and 7.52 ± 0.11 (× 10⁶/μL), respectively (table 1). Statistical analysis revealed no significant differences in the initial cell counts among the groups (P < 0.05) (figure 1).

3.2. Final red blood cell count (t14)
The red blood cell counts at t14 in the NC, TC, T1, T2, and T3 groups were 7.36 ± 0.30, 7.49 ± 0.22, 8.22 ± 0.19, 9.01 ± 0.13, and 8.65 ± 0.18 (× 10⁶/μL), respectively (table 2). The study results illustrated that the mean red blood cell count at t14 was 1.76 % higher in the TC group than in the NC group (table 2). However, statistical analysis illustrated that this difference was not significant (figure 1). Meanwhile, the final red blood cell count was significantly higher in all three treatment groups than in the NC and TC groups.

Different letters indicate significant differences (P < 0.05). NC, normal control; TC, treatment control; T1, treatment group 1; T2, treatment group 2; T3, treatment group 3.

The initial red blood cell counts in all five groups were within the reference range for rats according to the research conducted by Linda et al. (4.5–7.7 [× 10⁶/μL]) [10]. Based on this range, red blood cell counts after 14 days of treatment were also within the reference range in the NC group, permitting comparisons with the remaining groups.

| Repetition | Initial red blood cell count (t0) (10⁶/μL) | Final red blood cell count (t14) (10⁶/μL) |
|------------|------------------------------------------|------------------------------------------|
|            | NC | TC | T1 | T2 | T3 | NC | TC | T1 | T2 | T3 |
| 1          | 7.50 | 7.54 | 7.65 | 7.73 | 7.34 | 7.29 | 7.51 | 8.34 | 8.87 | 8.74 |
| 2          | 7.15 | 7.65 | 7.46 | 7.19 | 7.67 | 7.10 | 7.21 | 8.27 | 8.91 | 8.45 |
| 3          | 7.41 | 7.26 | 7.56 | 7.08 | 7.56 | 7.14 | 7.33 | 8.45 | 8.97 | 8.90 |
| 4          | 7.07 | 7.66 | 7.47 | 7.38 | 7.53 | 7.45 | 7.74 | 8.04 | 9.07 | 8.51 |
| 5          | 7.43 | 6.86 | 7.26 | 7.68 | 7.54 | 7.85 | 7.66 | 7.98 | 9.21 | 8.66 |
| Mean       | 7.31 | 7.39 | 7.48 | 7.41 | 7.52 | 7.36 | 7.49 | 8.22 | 9.01 | 8.65 |
| SD         | 0.18 | 0.33 | 0.14 | 0.28 | 0.11 | 0.30 | 0.22 | 0.19 | 0.13 | 0.18 |

Oral volume (mL) = \( \frac{\text{test animal body weight (g)}}{100 \ g} \times 1 \ mL \) (2)
The increase in the red blood cell count after 14 days of treatment in the TC group was presumably due to the consumption of iron in the feed and the additional intake of tofu flour. According to the research by Rezita [11], the additional intake of tofu flour can increase blood plasma iron levels. As iron is an important component for hemoglobin synthesis, increased iron levels lead to increased red blood cell counts [12, 13]. Meanwhile, the lack of a significant difference in red blood cell counts between the TC and NC groups may be due to the presence of iron absorption inhibitors in tofu flour, namely phytic acid.

The finding of significant increases in red blood cell counts in the three treatment groups supports the role of NaFeEDTA as an iron chelator. Specifically, the masking ability of NaFeEDTA causes more iron to bind to EDTA than phytic acid. EDTA forms a complex bond with iron derived from both tofu flour and NaFeEDTA. This complex bond is water-soluble, reversible, and reinforced in acidic environments such as the stomach [2, 7, 14].

Iron derived from tofu flour and NaFeEDTA is nonheme iron in the form of ferric iron (Fe³⁺), whereas only ferrous iron (Fe²⁺) can be absorbed by intestinal enterocytes. Therefore, ferric iron must be converted to ferrous iron by ferric reductase in order to be absorbed by intestinal enterocytes as shown in figure 2. Meanwhile, not all iron in the body is absorbed by intestinal enterocytes, as a portion is used for other metabolic processes (figure 3). Specifically, some iron is stored as ferritin, whereas another portion is transported through ferroportin in the form of transferrin [2, 14].

In erythropoiesis, diferric transferrin (Fe⁴⁺-transferrin) is transported to the bone marrow, which consists of erythroid cells. Fe⁴⁺-transferrin binds to transferrin receptor 1. The entry of transferrin into erythroid cells leads to an acidic environment that stimulates the expression of divalent metal transporter 1 [15]. Iron then detaches from the apotransferrin (aTf) complex. Some of the transferrin is released back into blood plasma in the form of aTf. The rest is stored in the form of ferritin, whereas ferric iron is reduced to ferrous iron and then transported to the mitochondria. In the mitochondria, ferrous iron binds to protoporphyrin, forming the heme structure that subsequently binds to the globin protein to form hemoglobin [16, 17].

![Figure 1. Initial (t0) and final (t14) red blood cell counts in the study groups.](image)

| Table 2. Percentage increase of red blood cell counts at t14. |
| Treatment | NC (%) | TC (%) |
|-----------|--------|--------|
| TC        | 1.76   | -      |
| T1        | 11.68  | 9.74   |
| T2        | 22.42  | 20.30  |
| T3        | 17.53  | 15.49  |
The finding that the greatest increase in red blood cell counts occurred in the T2 group suggests that the effect of NaFeEDTA is not completely concentration-dependent. Presumably, the NaFeEDTA concentration of 5.4 mg Fe/kg bw is optimal for minimizing the effects of iron inhibitors. EDTA can optimize the absorption of iron via “shuttle effect” mechanism [18]. As EDTA can bind iron derived from both NaFeEDTA and tofu flour, the availability of iron for absorption by intestinal enterocytes is greatly increased. Therefore, the iron available for erythropoiesis is increasingly abundant.

Similarly, the amount of iron in rats in the T3 group may have exceeded the iron-storage capacity of the animals. If stored iron reaches its capacity (0.125 g in 200 g rats), then the body will stop absorbing iron and degrade red blood cells (figure 4a). Iron levels are regulated by hepcidin produced in hepatocytes. Excess iron in the body leads to increased hepcidin production. Hepcidin induces the degradation of ferroportin in enterocytes and lymph cells; hence, iron remains in the form of storage iron, and it is not transported to tissues. Such conditions stimulate increases in iron storage and decreases in iron absorption and the rate of red blood cell degradation (figure 4b). The absence of hepcidin leads to excess iron levels in blood plasma, which can be toxic [19, 20].

![Figure 2. The mechanism of iron absorption.](image1)

![Figure 3. Regulation of iron in erythroid cells.](image2)
Figure 4. Regulation of iron levels by hepcidin, (a) the body will stop absorbing iron and degrade red blood cells, (b) the rate of red blood cell degradation.

Free iron can damage cells, tissues, and organs such as bone marrow, the liver, and lymph nodes. Bone marrow plays an important role in erythropoiesis, and thus, damage to erythroid cells in bone marrow can disrupt erythropoiesis [3, 19, 21]. Therefore, as blood plasma iron levels were lower in the T3 group than in the T2 group, it is assumed that less iron was also available for erythropoiesis.

4. Conclusion
From the results of this research, it can be concluded that the addition of NaFeEDTA to tofu flour can increase red blood cells counts in male Sprague-Dawley rats. The optimal effect of NaFeEDTA was observed in the T2 group, in which red blood cell counts were increased by 22.26 % and 20.24 % versus the levels in the NC and TC groups.

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