Influence of NaFeEDTA fortification in soy milk on hemoglobin levels in male Sprague-Dawley rats
(Rattus norvegicus L.)

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Abstract. In this study, we investigated the influence of the fortification of soy milk with NaFeEDTA on blood hemoglobin levels in male Sprague Dawley rats (Rattus norvegicus L.). Using a completely randomized design, 25 rats were divided into five groups. A normal control group was administered standard feeding without the addition of soy milk and fortificant, whereas a treatment control group was administered soy milk without fortificant; three treatment groups were administered soy milk fortified with the following doses of NaFeEDTA: 2.7 mg Fe/kg bw (treatment group 1), 5.4 mg Fe/kg bw (treatment group 2), and 10.8 mg Fe/kg bw (treatment group 3). The five groups were consecutively treated for 21 days. Rat blood hemoglobin measurements were performed using a hematology analyzer. The results of one-way ANOVA and least significant difference tests demonstrated that after the 21-day consecutive treatment, there was a significant influence on the blood hemoglobin levels in all treatment groups when compared with treatment group 1, whereas treatment groups 2 and 3 exerted a significant influence on the blood hemoglobin levels when compared with the treatment control group. The highest increase in the blood hemoglobin levels was detected in treatment group 2, which was a 10.84 % increase from the normal control group and 9.28 % increase from the treatment control group.

Keywords: Fortification, NaFeEDTA, hemoglobin levels, male Sprague-Dawley rats, soy milk

1. Introduction
Iron, an essential micronutrient, is required in the physiological processes of living organisms [1]. Deficient iron intake causes iron deficiency anemia, possibly resulting in impaired physical and cognitive development in children and a decrease in work productivity of adults [2].

Iron can be obtained from animal and vegetable food sources [3]. Indonesians, particularly those living in rural areas, obtain most of their iron from vegetable sources [4]. One highly consumed vegetable in Indonesia is soy owing to its high nutritional content and inexpensiveness [5].
One of the soy-based products often consumed in Indonesia is soy milk because it is easily available, affordable, and rich in nutrients, including iron [6, 7]. Iron content in soy milk is almost equivalent to that in cow’s milk [8]. However, the rate of iron absorption from soy milk (2–20 %) is lower when compared with that from cow’s milk (15–35 %) [9] because soy contains phytic acid that inhibits iron absorption by binding to iron and forming an insoluble compound, leading to deficiency in iron absorption [10]. To overcome this problem, food fortification solutions need to be investigated to maximize iron absorption.

Fortification is the addition of micronutrients in the form of fortificants in commonly consumed food products within the community to improve the nutritional quality of the food [11]. One fortificant that is proven effective for iron fortification is NaFeEDTA, which was selected because of its high iron bioavailability, making it appropriate for use in foods with high levels of phytic acid, including soy [12].

Preliminary research into the influence of the addition of NaFeEDTA into soy milk on iron levels in the blood plasma of male Sprague-Dawley rats (Rattus norvegicus L.) has demonstrated an increase in the iron levels in the blood plasma of rats. Iron is essential in the formation of heme, which subsequently is necessary for the synthesis of hemoglobin [13]. Therefore, the addition of NaFeEDTA into soy milk is hypothesized to affect the hemoglobin levels; accordingly, the aim of our study was to understand the influence of NaFeEDTA in soy milk on the blood hemoglobin levels and to obtain the optimum dose for fortification.

2. Materials and method

2.1. Materials
Soybeans used in this study were procured from a grocery store in Depok, Indonesia, and NaFeEDTA was synthesized in the Inorganic Chemistry Laboratory within the Department of Chemistry, Universitas Indonesia. The pellet-form feed administered to the research rats were produced by CV PD Kasman, Jakarta, Indonesia.

2.2. Preparation of soy milk and fortified soy milk
The soy milk used in our study was freshly prepared daily. Soybeans were washed, soaked for 10–12 hours, ground in a blender, filtered, and blended and filtered again. The filtrate obtained was pasteurized at 62 °C–71 °C for approximately 30 min and cooled. Further, NaFeEDTA was added in the following concentrations: 20.3098, 40.6196 and 81.2392 mg/10 mL soy milk. The amount of NaFeEDTA added was determined using the following formula:

\[
\text{Amount of NaFeEDTA added (mg)} = \text{Iron dose in vivo} \times \frac{\text{Mw NaFeEDTA}}{\text{Aw Fe}} \quad (1)
\]

Annotation:
Mw : relative molecular weight
Aw : relative atomic weight

2.3. Animal testing
Twenty-five male Sprague-Dawley rats aged 2–3 months were obtained from the Resource and Development Department of Health Agency, Jakarta, Indonesia. The rats were observed in five plastic enclosures (27 °C–28 °C with a 12:12 light:dark cycle) with each cage containing five rats, representing each treatment group. A normal control group was administered only standard feed and drink, whereas a treatment control group was administered standard feed and drink including unfortified soy milk; treatment groups 1, 2 and 3 were administered standard feed and drink of fortified soy milk containing NaFeEDTA at concentrations of 2.7, 5.4 and 10.8 mg Fe/kg bw, respectively. Standard Feed and drink were administered ad libitum. The treatments were consecutively maintained for 21 days with blood
sampling performed on days 0 and 21 of the treatment. The rats were weighed daily during the study period. The amount of soy milk administered was adjusted with the weight of the test rat and determined by the following formula.

\[
\text{Volume given (mL)} = \frac{\text{Test animal weight (g)}}{100 \, \text{g}} \times 1 \, \text{mL}
\]  

(2)

2.4. Measurement of hemoglobin levels
Up to 0.5 mL blood was collected from the orbital sinus and stored in 0.5 mL EDTA tubes. Then, the blood hemoglobin levels were measured using a hematology analyzer (Nihon Kohden Celtac α MEK-6450) provide by Primate Research Center (PSSP), IPB University.

2.5. Statistical analysis
Statistical analysis was performed using the Statistical Product and Service Solution for Windows, version 16. One-way ANOVA and least significant difference tests were performed to analyze the blood hemoglobin level differences between the treatment groups (p < 0.05).

3. Results and discussion

3.1. Initial blood hemoglobin levels (T0)
The results of the initial average hemoglobin measurements (T0) in the normal control group, treatment control group, treatment group 1, treatment group 2, and treatment group 3 were 13.58 ± 0.46, 13.36 ± 0.55, 13.34 ± 0.61, 13.18 ± 0.37 and 12.98 ± 0.50 g/dL, respectively. The results of the statistical tests (p < 0.05) indicate that these data were homogeneous and did not significantly differ between the treatment groups (figure 1).

3.2. Final blood hemoglobin levels (T21)
The results of the final average hemoglobin measurements (T21) in the normal control group, treatment control group, treatment group 1, treatment group 2, and treatment group 3 were 14.02 ± 0.34, 14.22 ± 0.41, 14.62 ± 0.41, 15.54 ± 0.39 and 14.88 ± 0.62 g/dL, respectively. The results of the statistical tests (p < 0.05) showed significant differences between the treatment groups (figure 1).

![Figure 1](image)

**Figure 1.** Bar chart of average initial (T0) and final (T21) hemoglobin levels. Different letters indicate the existence of a significant difference (p < 0.05).
3.3. Discussion

Based on our experiment, the treatment control group rats exhibited an increase in the blood hemoglobin levels of 1.43% compared with the normal control group (table 1). The increase in the blood hemoglobin level is hypothesized to be due to the increased iron content in their feeding with soy milk. Iron is required for the formation of heme, a constituent of hemoglobin [14]. Therefore, the levels of available iron in the body can influence the production of hemoglobin in the blood [15]. In addition, soy milk also contains protein, copper, vitamin B6, vitamin B12, and folic acid, the compounds required for the synthesis of hemoglobin in rats [16].

Notably, there was no statistically significant difference in terms of the blood hemoglobin levels between the treatment control and normal control groups (figure 1). We hypothesized that this was due to the presence of phytic acid in soy milk, which forms the iron(III)–phytic complex, inhibiting iron absorption and neutralizing the effect of the presence of iron in soy milk. Because the iron(III)-phytic complex is highly stable, it cannot be reduced to form iron(II), the only iron complex absorbable by the intestinal enterocyte receptors [17]. Considering that any added iron in food products will become unabsorbable in the presence of phytic acid, feeding with unfortified soy milk is insufficient to increase the blood hemoglobin levels.

Increased hemoglobin levels were observed in all treatment groups when compared with the normal control and treatment control groups (table 1). The increase in the blood hemoglobin levels is hypothesized to be due to the chelating ability of the EDTA in NaFeEDTA, which is capable of chelating iron(III) from NaFeEDTA and soy milk, forming the iron(III)–EDTA complex. Subsequently, it prevents the formation of bonds between iron(III) and phytic acid, inhibiting absorption of iron by the intestinal mucosa [18]. The use of NaFeEDTA as a fortificant optimizes the iron absorbed from the fortified soy milk.

NaFeEDTA consumed via soy milk is broken down in the stomach; while sodium (Na) is released, iron(III) remains bound to EDTA, forming an iron(III)–EDTA complex [19]. Iron(III) bound to EDTA prevents the binding of iron(III) with phytic acid present in soy milk because EDTA is able to mask iron(III) from phytic acid [17, 19].

Then, the iron(III) bound within the iron(III)–EDTA complex enters the duodenum and separates from EDTA due to an increase in pH that causes the bonds within the iron(III)–EDTA complex to weaken [20]. Further, iron(III) is reduced to iron(II) by the ferrireductase enzyme, which is absorbed by the enterocyte and made available in the blood [18]. After releasing from iron(III) in the duodenum, ± 5% of total EDTA is absorbed into the body and excreted in the urine [21]. Unabsorbed EDTA remains in the lumen of the gastrointestinal tract and is available to bind with any consumed iron and is carried into the enterocyte. This mechanism of EDTA is often termed as the “shuttle effect” and is hypothesized to increase the effectiveness of iron absorption in the body [22]. Next, iron is stored as ferritin or distributed to body tissues via blood circulation by transferrin [23]. Transferrin distributes iron to body tissues, including bone marrow, to be utilized in hemoglobin synthesis [24].

Transferrin carries iron(III) to erythroblast cells, which subsequently binds to transferrin receptors (TfR1) found in the erythroblast cell membrane. Further, transferrin, along with transferrin receptors, enters the cell via endocytosis. Then, iron(III) separates from transferrin due to a decrease in pH within the endosome, mediated by ATPases. Then, iron(III) is reduced to iron(II), catalyzed by the Steap3

| Table 1. Percentage increase of hemoglobin levels of inter-group treatment at T_{21}. |
|-------------------------------|-------------------------------|
|                               | Normal control group (%)      | Treatment control group (%) |
| Treatment control group       | 1.43                          | -                            |
| Treatment group 1             | 4.28                          | 2.81                         |
| Treatment group 2             | 10.84                         | 9.28                         |
| Treatment group 3             | 6.13                          | 4.64                         |
enzyme followed by iron(II) exiting the endosome, whereas transferrin is recycled back to the plasma membrane and released out of erythroblast cells to be reused to distribute iron. Iron(II) is taken by DMT-1 to facilitate passages via the outer membrane of the mitochondrion. After passing through the outer mitochondrial membrane, iron(II) detaches from DMT-1 and binds to mitoferin-1, which helps iron(II) pass across the inner mitochondrial membrane into the mitochondrial matrix. Iron(II) binds to protoporphyrin IX, catalyzed by the ferrochelatase enzyme, resulting in heme formation [13]. The formed heme binds to polypeptide to form hemoglobin [25]. The iron utilization scheme in heme synthesis is shown in figure 2 [26].

Despite an increase in the overall hemoglobin levels observed in the blood of the treatment groups compared with the normal control and treatment control groups, the increase observed in treatment group 1 was not significant ($p < 0.05$). We hypothesize that the NaFeEDTA dose was too low to meet the iron requirements of the test rats to increase hemoglobin synthesis. A low concentration of NaFeEDTA reduces the EDTA-mediated chelation of iron in soy milk, thereby allowing iron to bind to phytic acid and resulting in NaFeEDTA being the primary source of absorbed iron. Thus, we conclude that 2.7 mg Fe/kg bw of NaFeEDTA fortificant is insufficient to significantly increase the blood hemoglobin levels.

Treatment group 2 displayed the highest increase in hemoglobin when compared with the other two treatment groups. We hypothesize that this is because the dose of EDTA administered to this group was sufficient to chelate iron not only originating from NaFeEDTA but also from soy milk. After the release of iron(III), EDTA chelates iron from the consumed soy milk, thereby increasing the effectiveness of iron absorption in the body [21, 22]. In addition, it results in more optimal iron absorption in treatment group 2 than other treatment groups.

Because NaFeEDTA concentration increased from treatment group 2 to treatment group 3, we observed a significant decrease in the hemoglobin levels. We hypothesize that it is caused by a negative feedback mechanisms wherein heme inhibits the absorption of iron(III) from transferrin. Consequently, less iron is inserted into protoporphyrin IX by the ferrochelatase enzyme, and additional heme is not produced [27].

4. Conclusion
Based on the results of this investigation and previous reports, we conclude that the administration of NaFeEDTA in soy milk can increase the blood hemoglobin levels in male Sprague-Dawley rats. The highest observed increase in the hemoglobin levels occurred in treatment group 2 on the final day of the treatment (T21), which was a 10.84 % increase from the normal control group and 9.28 % from the treatment control group.
Acknowledgments
This work was financially supported by Universitas Indonesia under research grant PUPT Batch 2 (Penelitian Unggulan Perguruan Tinggi).

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